Chapter 1. Bone Embryology

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INTRODUCTION

The cells that make up the vertebrate skeleton are derived from three lineages. Neural crest cells give rise to the branchial arch derivatives of the craniofacial skeleton, paraxial mesoderm contributes to the craniofacial skeleton and forms most of the axial skeleton (through the sclerotome division of the somites), and cells in the lateral plate mesoderm contribute to the skeleton of the limbs. In areas where bones are formed, mesenchymal cells from these sources condense and form regions of high cell density that represent outlines of future skeletal elements. During this condensation process, important changes take place in the extracellular matrix between the cells allowing cells to establish contact with each other and activate signaling pathways that regulate cell differentiation. Classical experiments have shown that, by the time mesenchymal condensations appear, the cells within them have already acquired properties that give them positional identity. Mechanisms that ensure the development of a complex skeleton with elements of unique size, shape, and anatomical identity can therefore be traced back to molecular and cellular events in precondensed mesenchyme.

MEMBRANOUS AND ENDOCHONDRAL BONE FORMATION

As mesenchymal cells within condensations differentiate, they can follow one of two paths. They can either differentiate into bone-forming cells, osteoblasts, or they can differentiate into chondrocytes and secrete the characteristic extracellular matrix of hyaline cartilage. Differentiation into osteoblasts occurs in areas of membranous ossification, such as in the calvaria of the skull, the maxilla, and the mandible, and in the subperiosteal bone-forming layer of long bones. Differentiation into chondrocytes occurs in the remaining skeleton where cartilage models of the future bones are formed. These models, frequently described by their German term anlagen, are subsequently replaced by bone in a process called endochondral ossification.

The choice of mesenchymal cells to differentiate into osteoblasts or chondrocytes is regulated by what is known as canonical Wnt signaling. In this case, binding of members of the large family of extracellular Wnt cytokines to Frizzled receptors and Lrp 5/6 co-receptors on cells stimulates intracellular events that prevent proteolytic degradation of β-catenin in the cytoplasm. This stable β-catenin can enter the cell nucleus and control (by a process mediated by specific transcription factors) transcription of downstream target genes. In areas of membranous ossification, Wnt signaling (induced in part by signaling through another important cytokine, sonic hedgehog) results in high levels of β-catenin in mesenchymal cells.[1] This induces the expression of genes that are required for osteoblastic cell differentiation and inhibits transcription of genes needed for chondrocytic differentiation. One of the induced transcription factors, CBFA1/RUNX2,[2–4] in turn induces the expression of another transcription factor called osterix (OSX),[5] and these two factors are critical for the differentiation of mesenchymal cells to osteoblasts. The importance of β-catenin for membranous bone formation is dramatically shown by experiments in which the gene for β-catenin has been genetically inactivated in limb and head mesenchymal cells during early embryonic development.[6,7] In the absence of β-catenin, the mesenchymal cells differentiate into chondrocytes instead of osteoblasts!

The formation of membranous bones occurs within mesenchymal condensations that are rich in blood vessels. As the bones grow by appositional growth, sprouting of capillaries (angiogenesis) is an essential part of the process. Angiogenesis is controlled by both pro-angiogenic and anti-angiogenic factors. An important pro-angiogenic factor, vascular endothelial growth factor (VEGF-A), is expressed by vascular endothelial cells and osteoblasts, and its binding to receptors on both cell types results in a stimulation of angiogenesis that is coupled to stimulation of bone formation.[8]

The differentiation of chondrocytes and formation of cartilage anlagen occur in mesenchymal condensations with low levels of β-catenin (Fig. 1). This results in upregulated expression of the transcription factor SOX9 and other members of the SOX family.[9] As a consequence of the activities of these transcription factors, cells become large and round in the center of the mesenchymal condensations. They develop organelles for high level synthesis and secretion of proteins (endoplasmic reticulum and Golgi complex) and switch from production of an extracellular matrix containing collagens I and III to a matrix containing the cartilage-specific collagens II, IX, and XI. The cartilage anlagen grow by interstitial and appositional growth so that they over time come to resemble the future bones in shape and size. While they normally are replaced by bone through endochondral ossification, they can grow into shapes that resemble the final bones even in the absence of bone formation. This is dramatically seen in mice that carry two inactivated alleles of the gene for CBFA1/RUNX2.[3,4] As mentioned above, this factor is critical for osteoblastic differentiation,[2] and in mice without functional CBFA1/RUNX2, no osteoblasts are formed. In these mice, the cartilaginous anlagen are not ossified, yet they form a nearly complete "skeleton" of the correctly shaped elements, consisting entirely of cartilage. The patterning of the endochondral skeleton therefore depends on processes that regulate the spatial differentiation and growth of cartilage. In humans, heterozygosity for loss-of-function mutations in *CBFA1/RUNX2* is associated with cleidocranial dysplasia.[10] Affected individuals exhibit short stature, hypoplasia/aplasia of the clavicle, hypoplasia of the pelvis, delayed ossification and closure of cranial sutures, and defects in tooth eruption and tooth number (supernumerary teeth in the permanent dentition).

In contrast to membranous bones that form within vascularized regions of mesenchyme, cartilage anlagen of endochondral bones form within mesenchymal condensations from which blood vessels are excluded (Fig. 1). Experiments with forced overexpression of a pro-angiogenic factor such as VEGF-A within the avascular mesenchymal condensations of endochondral limb bones show that angiogenesis within these regions inhibits chondrocyte differentiation.[11] Cartilage anlagen are therefore avascular from the start and they continue to grow in the absence of blood vessels within the cartilage. Curiously, the chondrocytes in the anlagen express VEGF-A at a low level.[12,13] This level of expression is insufficient for stimulating ingrowth of capillaries from the tissue (perichon-

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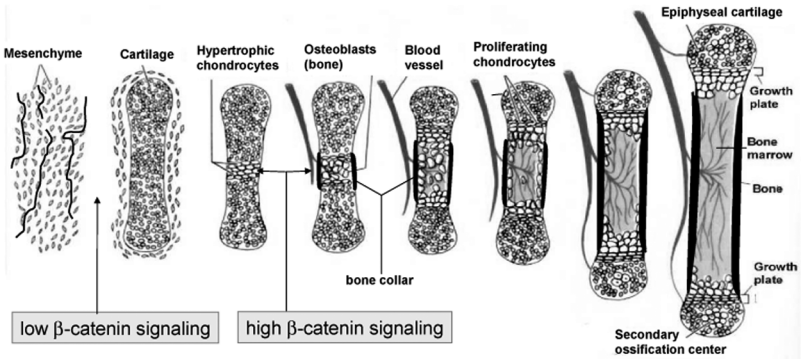


FIG. 1. Diagram showing the steps of endochondral ossification, from vascularized mesenchyme (left) to an endochondral bone with a primary ossification center (right). Cartilage models form within avascular regions of mesenchyme where β-catenin levels are low, and the cartilages become invaded by blood vessels only after hypertrophy of chondrocytes. VEGF-A, expressed at a high level by hypertrophic chondrocytes, plays an essential role in this invasion and the establishment of the primary ossification center. Chondrocyte hypertrophy and the formation of a perichondrial bone collar require high levels of β-catenin. A low-level expression of VEGF-A in the epiphyseal chondrocytes is required for their survival. Based on Horton WA 1990 The biology of bone growth. Growth Genet Horm **6:**1–3 with permission.[57]

drium) around the cartilage. However, it is essential for survival of the proliferating chondrocytes in the end regions (epiphyses) of the developing endochondral bones, and inactivation of VEGF-A expression in chondrocytes at an early developmental stage results in massive chondrocytic cell death in these epiphyseal regions.[13]

As development of endochondral bones proceeds, chondrocytes at the center of the avascular anlagen cease to proliferate and differentiate (in a process requiring Wnt signaling and upregulated levels of β-catenin in the cells) to hypertrophy. At the same time, mesenchymal progenitor cells in the perichondrium differentiate into osteoblasts and form a bone collar around the hypertrophic portion (diaphysis) of the cartilage anlage. The hypertrophic chondrocytes express high levels of the transcription factor CBFA1/RUNX2, and this results in upregulated expression of several genes that are not expressed by the smaller, proliferating chondrocytes in the epiphyseal regions. Among these genes is VEGF-A, which is expressed at high levels in hypertrophic chondrocytes, and connective tissue growth factor (Ctgf).[14,15] The two factors are important for invasion of blood vessels, osteoblastic progenitor cells, and cartilage/bone-resorbing cells from the perichondrium into the hypertrophic cartilage. This invasion results in formation of a primary ossification center, characterized by erosion of the hypertrophic cartilage and its replacement with bone marrow and trabecular bone. The primary ossification center does not form when VEGF-A expression is inactivated, indicating that VEGF-A is crucial for this critical step in endochondral ossification.[13]

REGULATION OF MESENCHYMAL CONDENSATION AND CHONDROCYTE DIFFERENTIATION IN THE AXIAL SKELETON

During the fourth week of human development, cells of the paraxial mesoderm condense to the segmented structures called somites on either side of the neural tube and the notochord (Fig. 2). Within the somites, cells undergo transition from a mesenchymal to an epithelial phenotype, and this is followed by transition back to mesenchyme and migration of cells in the most ventral region of the somites into an area surrounding the notochord (Fig. 2). These cells form the sclerotomes.

Sclerotome cells differentiate into chondrocytes that form the anlagen of vertebral bodies. The notochord subsequently disappears within the vertebral bodies, but remains as the nucleus pulposus within intervertebral discs. The neural arches of the vertebrae are formed by sclerotome cells that migrate dorsally around the neural tube, whereas sclerotome cells that migrate laterally form the rib anlagen in the thoracic region. In the anterior body wall, mesenchymal cell condensations on each side of the midline form the sternal anlagen. Cells within these condensations form two cartilaginous bars that later fuse into the sternum. Condensations above the sternum give rise to the manubrium and pairs of lateral condensations form the anterior cartilaginous portions of the ribs that are connected with the sternum.

The condensation, segmentation, and mesenchymal–epithelial transformation of paraxial mesoderm cells to somites is controlled in part by signaling between neighboring cells involving the cell surface receptor Notch1 and ligands that bind to it.[16,17] Notch1 is a large transmembrane protein with several repeated amino acid sequence domains. The ligands that bind to Notch1 and activate signaling pathways that are downstream of Notch1 are themselves transmembrane molecules with signaling potential.[18] The interactions between Notch1 and its ligands therefore require cell–cell contact. In mice carrying inactivated alleles for a mouse homolog of the *Drosophila* Notch ligand Delta[19] and in mice with inactivated *Notch1* genes,[5] early defects in the condensation and patterning of somites lead to defects in the vertebral column. Also, genes that control the expression of Notch1 or are controlled by Notch1 have been shown to be important for early stages of vertebral column development.[20,21] In humans, loss-of-function mutations in the Notch ligand δ-like 3 (DLL3) cause the recessive form of spondylocostal dysostosis, characterized by short stature, vertebral abnormalities in the form of semivertebrae, and deletions and fusions of ribs.[22] Mutations resulting in haploinsufficiency of the Notch ligand Jagged 1 (JAG1) are associated with Alagille syndrome.[23,24] Affected individuals have abnormally shaped vertebral bodies (butterfly vertebrae) and abnormalities of the liver, heart, eye, and facial structures.

Differentiation of cells within sclerotomes to cartilage-producing chondrocytes is also under complex regulation. A master regulator of sclerotome cell differentiation and therefore of vertebral column formation is the secreted cytokine sonic hedgehog.[25] Sonic hedgehog is produced by the notochord as a protein that undergoes proteolytic self-cleavage and gets a cholesterol residue attached before it becomes an active cytokine.[26,27] That its signaling activity is absolutely required for sclerotome differentiation is evident from the phenotype of mice that carry inactivated sonic hedgehog alleles.[28] Such mice develop without a vertebral column and the posterior portions of the ribs. However, they do form a sternum and the anterior portions of ribs, the shoulder, and pelvic girdles, showing that the anlagen for those portions of the skeleton are not controlled by sonic hedgehog. Another important regulator is the transcription factor PAX1, which is, at least in part, induced by sonic hedgehog. A mutation in *PAX1* in humans causes a neural tube defect,[29] and mutations in mice lead to defective sclerotome differentiation and abnormalities in the vertebral column.[30]

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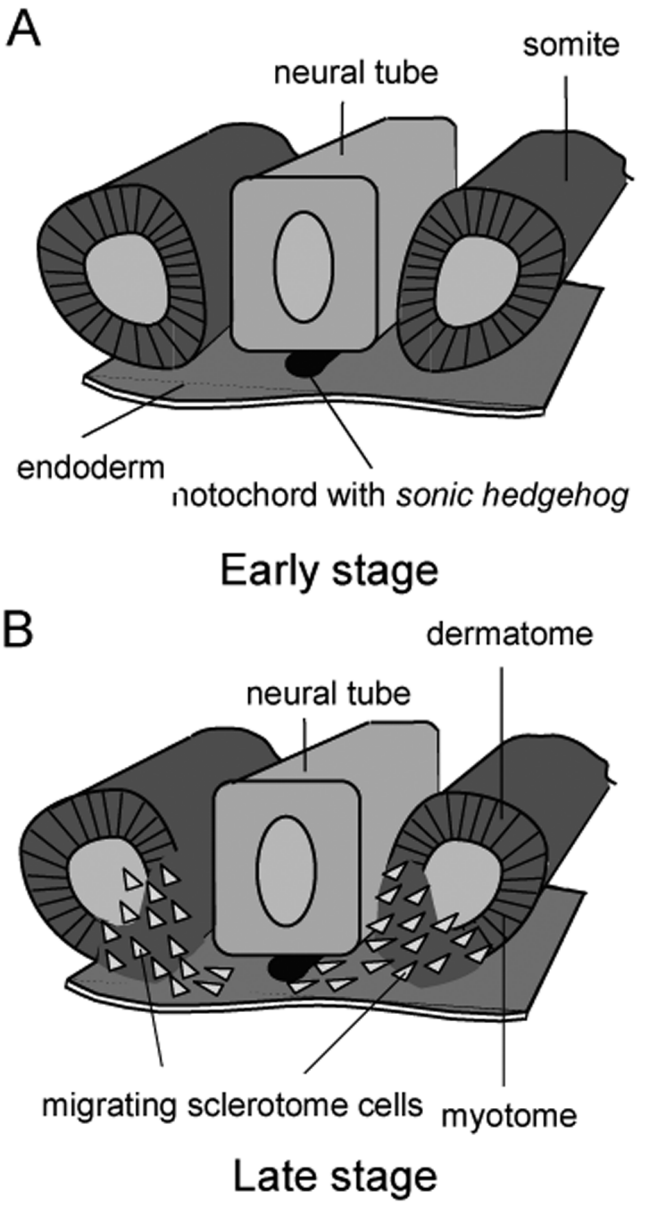


FIG. 2. Diagrams showing differentiation of somites in the trunk region of the embryo. (A) The ectoderm has been removed to reveal the somites adjacent to the neural tube. At this stage, the cells of the somites have an epithelial organization. (B) Later, cells from the medial region of the somites migrate toward the notochord and form the sclerotomes. The remainder differentiate into dermatome (skin) and myotome (striated muscle). Modified from Hogan B, Beddington R, Costantini F, Lacy E 1994 Manipulating the Mouse Embryo—A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, p.77 with permission.

SKELETAL MORPHOGENESIS IN THE LIMB

Skeletal development in the limb starts with formation of limb buds, outgrowths of the lateral body wall, appearing early in the second month of human development as a result of proliferation of mesenchymal cells from the lateral plate mesoderm. A group of tall, specialized epithelial cells, called the apical ectodermal ridge (AER), caps the limb bud. As the limb bud grows and cartilage anlagen of future bones develop within it, the developing limb is patterned along three axes: a proximal to distal axis, a dorsal to ventral axis, and a posterior to anterior axis.

Patterning along the proximal to distal axis is largely controlled by factors produced by the AER.[31] These include fibroblast growth factors that are important for stimulating proliferation and patterning of the underlying mesenchyme.[32,33] They are therefore essential for normal limb outgrowth. As the limb grows out, mesenchymal cells condense in the center to form the cartilage anlagen of the limb bones. The anlagen develop in a proximal to distal direction, and their development can be described as a series of bifurcations and segmentations that follow an axis along the humerus, through the ulna and the distal carpal (or tarsal in the foot) anlagen (Fig. 3).[34]

In the lower arm (leg) and hands (feet), the patterning of the anlagen from one side to the other is regulated by a cascade of signaling molecules and transcription factors. The most important of these include sonic hedgehog, secreted by a small group of cells (called the zone of polarizing activity) located at the posterior (ulnar) aspect of the developing limb bud, homeobox transcription factors, and members of the TGF-β superfamily of signaling molecules.[35] How all these and many other molecules are working together as an orchestrated system to generate the normal limb skeletal pattern is not known in detail, but recent studies are rapidly filling in the missing pieces.

Mutations in many of these genes result in striking abnormalities in the limb skeleton. For example, mutations involving expansions of a polyalanine stretch in the transcription factor *HOXD13* cause polysyndactyly (extra fingers) in humans[36]; other polysyndactyly syndromes have been shown to be caused by mutations in an intracellular, downstream target of sonic hedgehog signaling, a transcription factor called *GLI3*.[37,38] Mutations in a TGF-β homolog (GDF5/CDMP1) cause abnormal shortening of distal limb bones, perhaps as a result of a defect in formation of joints between bones.[39] Mutations in *Noggin*, an extracellular molecule that binds and inactivates GDF5/CDMP1 and other members of the TGF-β superfamily of molecules, are associated with proximal symphalangism, characterized by fusion of proximal joints between digits and early onset conductive deafness, and multiple dysostoses syndrome, including fusion of multiple joints in hands and feet, elbows, and hips and of intervertebral joints.[40]

Several studies also provide insights into mechanisms that ensure patterning along the dorsal–ventral axis. As a consequence of such studies, the basis for the Nail-patella syndrome in humans, characterized by dysplasia of nails and absent or hypoplastic patellae, has been found to be mutations in a transcription factor called *LMX1B*.[41]

Molecules and signaling pathways controlling the formation of digits in hands and feet are rapidly being identified from studies of human brachydactylies, a group of disorders characterized by shortening of fingers and toes. Several different clinical subtypes of brachydactyly are recognized; heterozygous mutations in the cytokine Indian hedgehog, receptor tyrosine kinase ROR2, and GDF5/CDMP1 have been described in subtypes A1, B, and C, respectively.[39,42,43]

CHONDROCYTE PROLIFERATION AND DIFFERENTIATION IN GROWTH PLATES

During endochondral ossification, cartilage anlagen are replaced by bone marrow and bone. In this process, epiphyseal growth plates are formed. A sequence of chondrocyte proliferation, differentiation to hypertrophy, and cell death (apoptosis) within the growth plate results in longitudinal bone growth and thus the final steps in bone morphogenesis. Proliferation and differentiation of growth plate chondrocytes are controlled in several ways. An important brake on chondrocyte proliferation is local signaling through fibroblast growth factor (FGF) (most likely

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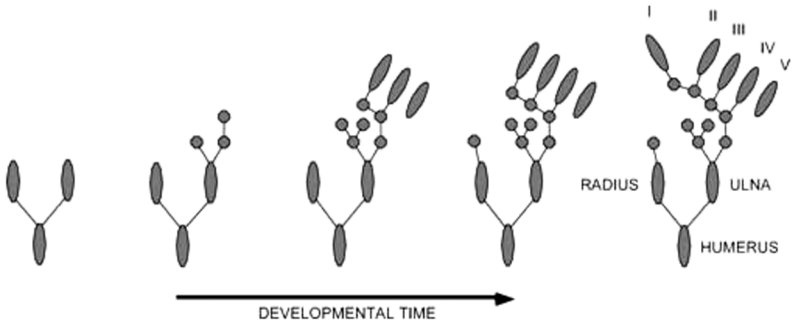


FIG. 3. Diagram showing branching and segmentation of mesenchymal condensations during limb development. As the limb bud grows (indicated by the horizontal arrow from left to right), the condensations develop in a distinct sequence to form the cartilage anlagen of the future bones (humerus, radius, ulna, carpal bones, and metacarpal bones for the five fingers [I–V]). Based on Shubin and Alberch 1986.[34]

Fgf18[44]) interaction with the cell surface tyrosine kinase receptor FGFR3. Activating mutations in *FGFR3* cause decreased bone growth in achondroplasia and hypochondroplasia in humans.[45,46] The cytokine PTH-related peptide (PTHrP) has an inhibitory effect on chondrocyte differentiation to hypertrophy,[47] and both activating and loss-of-function mutations in the PTHrP receptor (a cell surface G-protein–coupled receptor) cause decreased bone growth and distinct forms of dwarfism because of either inhibition of chondrocyte hypertrophy (Jansen's metaphyseal chondrodysplasia) or accelerated chondrocyte hypertrophy (Blomstrand's chondrodysplasia) in growth plates.[48,49] Signaling from the PTHrP receptor involves an inhibitory effect on the expression of a secreted signaling molecule called Indian hedgehog.[47] Indian hedgehog is homologous to sonic hedgehog and acts on cells through the same type of receptor as sonic hedgehog. Indian hedgehog induces expression of PTHrP so the two molecules seem to be involved in a self-regulating feedback loop within growth plates.[47] In addition, Indian hedgehog is a positive regulator of chondrocyte proliferation and induces (directly or indirectly) osteoblastic differentiation in the perichondrium (the bone collar) during endochondral ossification.[50]

As discussed above, chondrocyte hypertrophy leads to the synthesis of an extracellular matrix that is significantly different from that of the rest of growth plate cartilage. This hypertrophic matrix is readily degraded, and the high level expression of VEGF-A by the hypertrophic chondrocytes stimulates continued ingrowth of blood vessels, osteoclasts, and osteoblasts from the underlying bone marrow.[8,14,51] A member of a distinct subfamily of collagenous molecules, collagen X, is expressed at high levels by hypertrophic chondrocytes.[52] Because this collagen is not expressed by any other cell type, it is routinely used as a specific marker for hypertrophic chondrocytes. Genetic analyses of collagen X in mice show that this extracellular matrix component is required for normal growth plate function and longitudinal bone growth.[53,54] It may also be important for normal differentiation of hematopoietic progenitor cells in the adjacent bone marrow.[55] Mutations in collagen X in humans cause a distinct form of dwarfism, Schmid type metaphyseal chondrodysplasia.[56]

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Chapter 2. Anatomy and Functions of the Adult Skeleton

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INTRODUCTION

The adult human skeleton is comprised of 213 bones, each of which is sculpted by a process called modeling, and each is constantly renewed by a process termed remodeling. Depending on its location, each bone supports one or more specific functions, including structural support and movement, protection of vital organs, and maintenance of mineral homeostasis. Remodeling plays a key role in the latter and also provides a mechanism for preservation of bone strength by replacing old fatigued bone by new mechanically sound bone. Remodeling is achieved by the sequential action of osteoclasts and osteoblasts and the process is regulated by osteocytes. The rate of remodeling and balance between resorption and formation in each remodeling unit differs depending on anatomical location and also as a function of age and in disease states. Knowledge of the fundamental principles of remodeling provides an excellent framework for understanding age-related changes in bone structure and geometry, as well as the pathogenesis of metabolic bone diseases and the effects of drugs used to treat them.

MACROSCOPIC ANATOMY

Anatomists distinguish between two main types of bones: flat bones, such as the skull, mandible, and scapula, and long bones such as the femur, tibia, and radius. A process called membranous bone formation forms flat bones and long bones develop by a combination of a process called endochondral bone formation and membranous bone formation. These two modes of bone development are described elsewhere. Long bones consist of a hollow tube (shaft or diaphysis), which flairs at the ends to form the cone-shaped metaphyses, the regions below the growth plate, and the epiphyses, the regions above the growth plate (Fig. 1). The shaft is comprised primarily of cortical bone, whereas the metaphysis and epiphysis contain cancellous bone that is surrounded by a shell of cortical bone. Approximately 80% of the adult skeleton is composed of cortical bone and 20% is cancellous, but the relative proportions of the two types of bone vary considerably among different skeletal sites. For example the ratio of cancellous:cortical bone is estimated to be 75:25 in human vertebrae, 50:50 in the femoral head, and 95:5 in the diaphysis of the radius (Fig. 2).

Bones have an outer fibrous sheath called the periosteum, and the inner surface, which is in direct contact with the marrow, is referred to as the endosteum. The periosteum is an envelope of fibrous connective tissue that covers all bone surfaces except at the joints where the bone is lined by articular cartilage. The periosteum contains the blood vessels that nourish the bone, as well as nerve endings and osteoblasts and osteoclasts. The periosteum is anchored to the bone by strong, collagenous fibers called Sharpeys' fibers that penetrate into the bone tissue. The endosteum is a membranous sheath that also contains blood vessels, osteoblasts and osteoclasts. In addition to lining the marrow cavity, the endosteum also envelopes the surface of cancellous bone and lines the blood vessel canals (Volkman's canals) that run through the bone.

The author has reported no conflicts of interest.

CORTICAL AND TRABECULAR BONE STRUCTURE AND FUNCTIONS

At a macroscopic level, cortical bone appears dense and solid, whereas cancellous bone is a honeycomb-like network of interconnected trabecular plates and bars surrounding bone marrow (Fig. 1). At the microscopic level, both cortical and cancellous are composed of basic structural units or osteons (Fig. 3). In cortical bone, the osteons are commonly referred to as Haversian systems after Clopton Havers, the 17th century English anatomist who first observed the canals in the center of the structures. Haversian systems are cylindrical in shape and form an anatomizing network like the branches of a tree.[1] Their walls are formed by concentric sheaths or lamellae, reminiscent of a tree trunk in cross-section. Trabecular osteons, also referred to as packets, are saucer-shaped, ~35 mm in thickness, and are also composed of stacks of lamellae.

When it comes to function, the skeleton can be considered as a prototype for multitasking. The long bones serve as levers for the muscles, supporting locomotion and all other forms of motion. The flat bones, such as the ribs and those of the skull, serve as armor for the vital organs that they surround. It is common knowledge that the skeleton serves as the body's main repository for calcium and plays a key role in homeostatic regulation of serum calcium concentration. It is less well known that the skeleton plays a similar role with regard to acid–base balance.[2] Bone also serves as a rich source of growth factors and cytokines, a prime example of which is the finding that osteoblasts produce a number of factors that are important for the differentiation and survival of hematopoietic stem cells.[3]

From a functional standpoint, cancellous bone is generally considered to be more metabolically active than is cortical bone, which is viewed largely in its mechanical role. However, this is probably a misconception and may also be species-, and situation-dependent. For example, medullary bone in birds, which is cancellous in structure, serves as a labile source of calcium that is mobilized during eggshell calcification.[4] On the other hand, during antler formation in deer, it is the cortical bone that supplies the necessary extra calcium.[5] Increased demands for calcium during pregnancy and lactation in humans are met primarily from non–weight-bearing bone,[6] whereas bone is preferentially lost from cortical sites in primary hyperparathyroidism.[7]

MODELING

The term bone modeling describes the process whereby bones are shaped or reshaped by independent action of osteoblasts and osteoclasts. This occurs, for example, during growth or in the adult to change the shape of the bone in response to mechanical loads (mechanical adaptation). The radius in the playing arm of competitive, young tennis players has a thicker cortex and greater diameter than the contralateral radius as a result of modeling.[8] Bone modeling is distinguished from remodeling by the fact that bone formation is not tightly coupled to prior bone resorption. In adult humans, bone modeling occurs less frequently than bone remodeling, particularly in cancellous bone, but it does take place in normal subjects[9] and may be increased in hypoparathyroidism and renal bone disease.[10,11] Anabolic agents for the treatment of osteoporosis,

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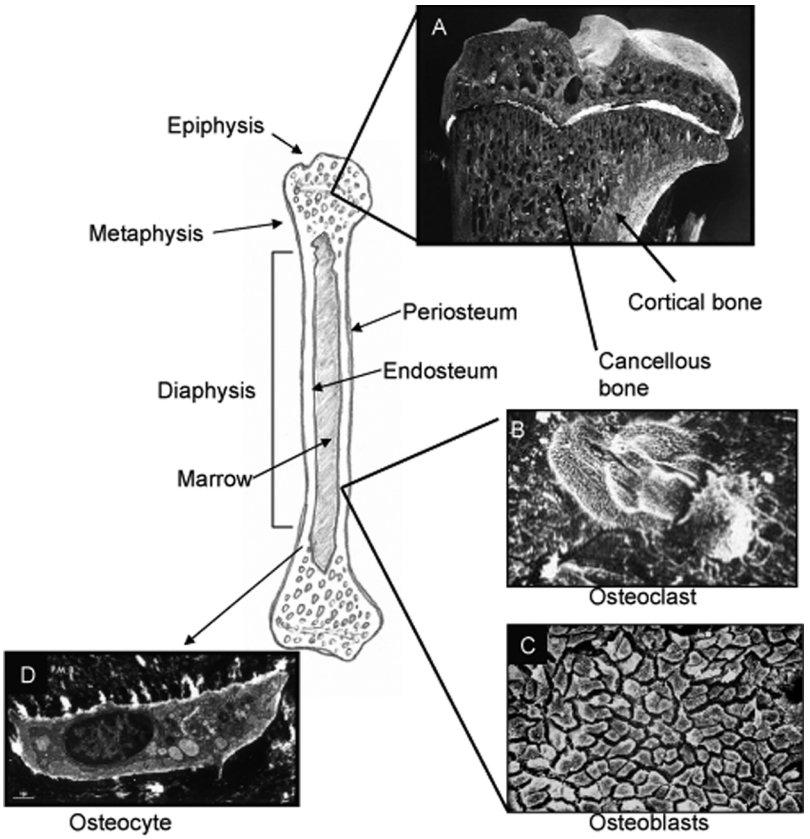


FIG. 1. (A) Scanning electron micrograph of a rat tibial growth plate. (B) Scanning electron micrograph of a human osteoclast cultured for 24 h on a bone slice (reproduced with permission from *J Bone Miner Res* 1989;**4:**259–268. (C) Scanning electron micrograph of osteoblasts on the endosteal surface of a rat long bone. (D) Transmission electron micrograph of a rat osteocyte.

such as PTH(1-34), are also capable of stimulating modeling.[12]

REMODELING

Half a century ago, Dr. A. M. Cooke wrote eloquently that "The skeleton, out of site and often out of mind, is a formidable mass of tissue occupying about 9% of the body by bulk and no less than 17% by weight. The stability and immutability of dry bones and their persistence for centuries, and even millions of years after the soft tissues have turned to dust, give us a false impression of bone during life. Its fixity after death is in sharp contrast to its ceaseless activity during life."[13] In the adult skeleton, the "ceaseless activity" largely refers to the process of bone remodeling. One of the more remarkable features of mammalian bone is its ability to constantly rejuvenate itself through the process of remodeling, which begins in utero and continues until death. Remodeling is accomplished by the collaborative and sequential efforts of a group of cells that are collectively termed the bone remodeling unit (BRU).[14–18] There are four distinct phases in the remodeling cycle: activa-

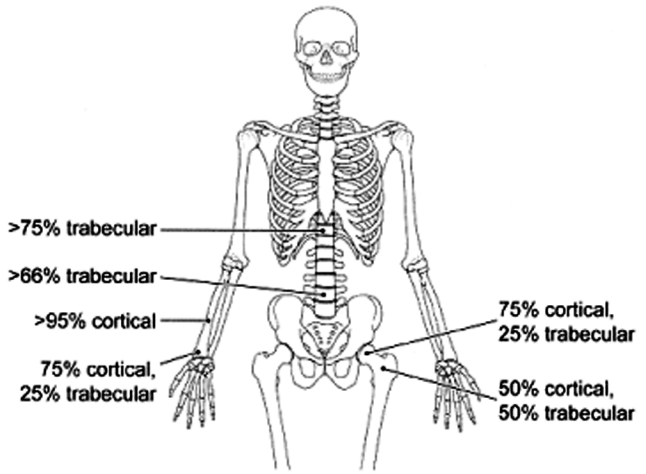


FIG. 2. Proportion of cancellous and cortical bone at different skeletal sites. Reproduced with permission from ASBMR Primer, 5th ed.

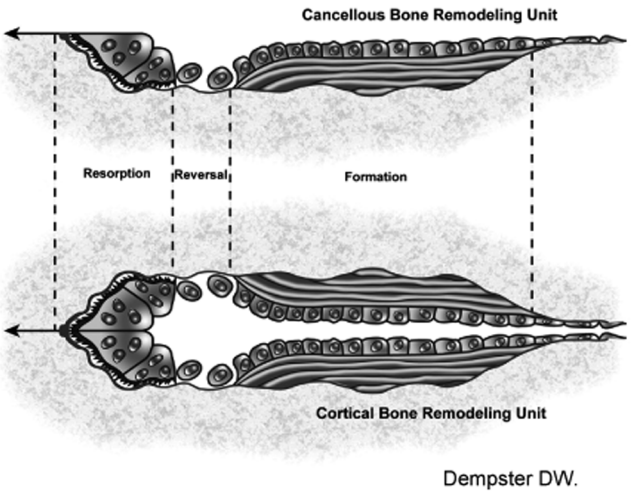


FIG. 3. Cross-sectional diagrams of BRUs in cancellous bone (top) and cortical bone (bottom). The arrow indicates the direction of movement through space. Note that the cancellous BRU is essentially one half of the cortical BRU.

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tion, resorption, reversal, and formation (Fig. 3). Activation describes the initiating event that transforms a previously quiescent bone surface into a remodeling one. Activation involves recruitment of mononucleated osteoclast precursors from cells in the monocyte–macrophage lineage in the circulation,[19] infiltration of the bone lining cell layer, and fusion of the mononuclear cells to form multinucleated preosteoclasts. The degree to which remodeling activation at a particular site on the bone surface is preselected and the extent to which it is random is unclear. There is some evidence that a proportion of the remodeling is targeted toward specific sites that need to be repaired (see below), but most remodeling is likely to be random.[20,21] The pre-osteoclasts affix themselves to the bone matrix through avid binding between integrin receptors in their membranes and RGD-containing peptides in the organic matrix, creating an annular sealing zone. In forming the seal, the osteoclast creates the bone-resorbing compartment, a unique microenvironment, between itself and the bone matrix. Osteoclast formation, activation, and activity of osteoclasts are all regulated by local cytokines such as RANKL, interleukins -1 and-6 (IL-1 and IL-6), and colony-stimulating factors (CSFs), and systemic hormones such as PTH, 1,25-dihydroxyvitamin D_3, and calcitonin.[19,22–25] During the resorption phase of the cycle, specific types of proton pumps in the osteoclast membrane transfer protons to the resorbing compartment lowering its pH considerably. pH values as low as 4.0 have been detected in this compartment.[26] Acidification of the resorbing compartment is accompanied by secretion of a number of lysosomal enzymes, such as TRACP and cathepsin K, as well as matrix metalloproteases including MMP-9 (collagenase) and gelatinase.[27] The acidic solution, containing enzymes that are most active at low pH, effectively dissolves and digests the mineral and organic phases of the matrix, creating saucer-shaped resorption cavities called Howship's lacunae on the surface of the cancellous bone and cylindrical tunnels within the cortex (Fig. 3). Initially, the resorption is accomplished by multinucleated osteoclasts, but there is both in vivo and in vitro evidence to suggest that excavation of the resorption cavity is completed by mononucleated cells.[28,29] The resorption phase ends with osteoclast apoptosis[30] and is followed by reversal. During reversal, the resorption lacuna is populated by mononuclear cells, including monocytes, osteocytes that have been liberated from the bone by osteoclasts, and pre-osteoblasts that are being recruited to begin the formation phase of the cycle.[31] It is during the reversal phase that all-important coupling signals are sent out to summon osteoblasts into the resorption cavities to replace the bone that has been removed. Without an efficient coupling mechanism, each remodeling transaction would result in net loss of bone. The exact nature of the coupling signal(s) is currently undefined, but there are a number of hypotheses. One attractive theory is that osteoclasts release growth factors from the bone matrix during the resorption phase and that these factors act as chemo-attractants for osteoblast precursors and stimulate osteoblast proliferation and differentiation. This hypothesis explains the correct spatial localization of the osteoblasts and recruitment of the requisite number of osteoblasts to replace the volume of bone that has been removed, which, in turn, will determine the amount of growth factor released. Bone matrix–derived growth factors, such as TGF-β, IGF-I and II, bone morphogenetic proteins (BMPs), platelet-derived growth factors (PDGF), and fibroblast growth factor (FGF) are all likely candidates for coupling factors.[32–36] TGF-β prolongs osteoblast life span in vitro by inhibiting apoptosis, and evidence that TGF-β plays an important role in bone remodeling comes from the demonstration that the concentration of TGF-β human bone is positively correlated with histomorphometric indices of bone resorption and bone formation and with serum levels of osteocalcin and bone-specific alkaline phosphatase.[37] In addition to stimulating formation during the reversal phase, it is likely that TGF-β released from bone also plays a role in shutting down resorption by inhibiting RANKL production by osteoblasts.[38] Another plausible mechanism for coupling of formation to resorption is that it is a strain-regulated phenomenon.[39] In this theory, it is suggested that, as bone remodeling units traverse through cortical bone, strain levels are reduced ahead of the osteoclasts and are increased behind them. Similarly, strain is thought to be higher at the base of Howship's lacunae in cancellous bone and reduced in the surrounding bone. It is theorized that this gradient of strain leads to sequential activation of osteoclasts and osteoblasts, with osteoclasts being activated in response to reduced strain and osteoblasts in response to increased strain. This hypothesis also has the potential to account for alignment of osteons to the dominant load direction.[40] Another recently proposed idea is that the osteoclast itself plays a role in coupling.[41,42]

Formation is a two-step process in which the osteoblasts initially synthesize the organic matrix and then regulate its mineralization. Once the collagenous organic matrix is secreted, the osteoblasts trigger mineralization by releasing small, membrane-bound vesicles, called matrix vesicles, that establish suitable conditions for initial mineral deposition by concentrating calcium and phosphate ions and enzymatically degrading inhibitors of mineralization, such as pyrophosphate and proteoglycans that are present in the extracellular matrix.[43] As bone formation continues, osteoblasts become entombed in the matrix as osteocytes. Although buried in the matrix, the osteocytes maintain intimate contact with one another, as well as with the cells on the bone surface, by means of gap junctions between the cytoplasmic processes that extend through small tubes in the matrix called canaliculae. Each osteocyte becomes part of a large, 3D, functional syncitium, which can "sense" a change in the mechanical properties of the surrounding bone and transmit this information to the cells on the surface to initiate or regulate bone remodeling when necessary.[44,45] It has therefore been suggested that, from this perspective, bone cells behave like a neuronal network.[46]

When the osteoblasts have completed their matrix-forming function, they endure a number of fates. Approximately 50–70% die by apoptosis, and the remainder are either incorporated into the matrix as osteocytes or remain on the surface as bone-lining cells. The bone-lining cells were once thought to serve primarily to regulate the flow of ions into and out of the bone extracellular fluid and, in so doing, constitute the anatomical basis of the blood–bone barrier. However, it has recently been shown that under certain circumstances, for example, stimulation by PTH or mechanical force, bone-lining cells can revert back to osteoblasts.[47,48] Another potentially important function of the lining cells is to create specialized compartments on the surface of trabecular bone in which bone remodeling occurs.[49]

The net result of each remodeling cycle is the production of a new osteon (Fig. 4). Note that the process of bone remodeling is essentially identical in cancellous and cortical bone. The BRUs in cancellous bone are equivalent to cortical BRUs split in half longitudinally[50] (Fig. 3). The difference between the volume of bone removed by the osteoclasts and that replaced by the osteoblasts is referred to as the "bone balance." BRUs on the periosteal surface of cortical bone produce a slightly positive bone balance so that, with aging, the periosteal circumference increases as the effect of the small positive balance in each BRU accumulates. Remodeling units on the endosteal surface of cortical bone are in negative balance so that the marrow cavity enlarges with age. Furthermore, the balance is more negative on the endosteal surface than it is on the peri-

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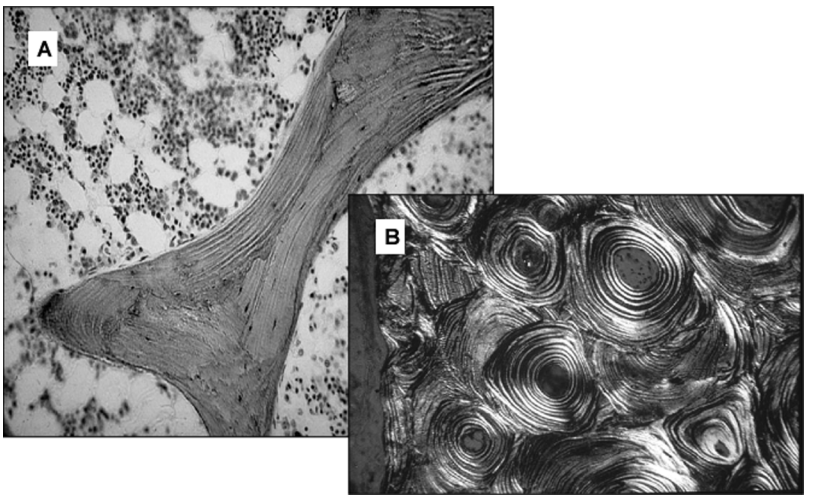


FIG. 4. Basic structural units in (A) cancellous bone and (B) cortical bone. Reproduced with permission from Dempster DW, Lindsay R 1993 Pathogenesis of osteoporosis. Lancet **341:**797–801 with permission from Elsevier.

osteal surface and, as a result, cortical thickness decreases with age. The bone balance on cancellous surfaces is also negative, resulting in a gradual thinning of the trabecular plates with the passage of time.[14]

FUNCTIONS OF BONE REMODELING

Two principal functions of bone remodeling are known, although it has been suggested that there must be other, as yet unknown functions or reasons why the human skeleton undergoes such extensive remodeling.[51] The primary functions of bone remodeling are presumed to be the preventive maintenance of mechanical strength by continuously replacing fatigued bone by new, mechanically sound bone, and, second, an important role in mineral homeostasis by providing access to the skeletal stores of calcium and phosphorus, as described above. While the turnover rate of 2–3% per year in cortical bone is consistent with maintenance of mechanical properties, the turnover rate in cancellous bone is much higher than would be required for this purpose, suggesting that this is driven more by its role in mineral metabolism.[18] In situations where calcium demand is high, activation of new remodeling units may be required, but it has been suggested that short-term mineral homeostasis may only require the presence of a minimum number of osteoclasts whose activity can be regulated. Continual remodeling activity therefore ensures the presence of this contingent of osteoclasts. Remodeling also ensures a constant continuous supply of new bone of low mineral density, which is needed for ionic exchange at quiescent surfaces.[18] The best evidence to date for a role of bone remodeling in the maintenance of the mechanical integrity of the skeleton has come from studies in dogs.[52,53] Long bones were loaded to induce fatigue damage and then studied histologically. A statistically significant association between microcracks and resorption spaces was observed, with up to six times as many cracks associated with resorption spaces as predicted by chance alone.[54] This could be explained by a propensity of microcracks to form close to resorption spaces. However, further experiments showed that the activation of remodeling was in response to the appearance of microcracks.[53] This was shown by loading the left forelimbs of dogs 8 days before death and loading the right forelimbs immediately before death. If cracks simply localize at sites of resorption, the number of cracks associated with resorption spaces would be expected to be identical in each limb. The results showed the same number of microcracks in each limb, but that the limb that was loaded a week before displayed more resorption spaces and a greater association between microcracks and resorption than the limb that was loaded immediately before death. The reasonable conclusion is that microcracks initiate resorption. One possible mechanism for this targeted remodeling is that microcracks cause debonding of cortical osteons.[55] As a result, there is a decrease in stress and strain in that region of the osteon, which is detected by the osteocytes and communicated to the surface lining cells. The lining cells trigger activation of a bone remodeling unit from the Haversian canal and this moves toward, and ultimately replaces the damaged area with a new osteon. Fatigue damage may also promote osteocyte apoptosis and it has been suggested that this is the initial trigger for targeted remodeling.[56–59]

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Chapter 3. Extracellular Matrix and Biomineralization of Bone

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INTRODUCTION

Bone composes the largest proportion of the body's connective tissue mass. Unlike most other connective tissue matrices, bone matrix is physiologically mineralized and is unique in that it is constantly regenerated throughout life as a consequence of bone turnover. Information on the gene and protein structure and potential function of bone extracellular matrix constituents has exploded during the last few decades. This information has been described in great detail elsewhere,[1,2] to which the reader is referred for specific references, which are too numerous to be listed adequately here. This chapter summarizes salient features of the classes of bone matrix proteins, and the tables list specific details for the individual components.

COLLAGEN

The basic building block of the bone matrix fiber network is type I collagen, which is a triple-helical molecule containing two identical α1(I) chains and a structurally similar, but genetically different, α2(I) chain[3] (Table 1). Collagen α chains are characterized by a Gly-X-Y repeating triplet (where X is usually proline, and Y is often hydroxyproline), and by several post-translational modifications including (1) hydroxylation of certain lysyl residues (2) glycosylation of lysyl or hydroxylysyl residues with glucose or galactose residues or both, and (3) formation of intra- and intermolecular covalent cross-links that differ from those found in soft connective tissues. Measurement of these bone-derived collagen cross-links in urine have proved to be good measures of bone resorption.[4] Bone matrix proper consists predominantly of type I collagen; however, trace amounts of type III, V, and FACIT collagens may be present during certain stages of bone formation and may contribute to the regulation of collagen fibril diameter (Table 1).

NONCOLLAGENOUS PROTEINS

Noncollagenous proteins (NCPs) compose 10–15% of the total bone protein content. Approximately one fourth of the total NCP content is exogenously derived. This fraction is largely composed of serum-derived proteins, such as albumin and α_2-HS-glycoprotein (Table 2), which are acidic in character and bind to bone matrix because of their affinity to hydroxyapatite. Although these proteins are not endogenously synthesized, they may exert effects on matrix mineralization, and α_2-HS-glycoprotein, which is the human analoga of fetuin, also may regulate bone cell proliferation. The remainder of this exogenous fraction is composed of growth factors and a large variety of other molecules present in trace amounts, which also may influence local bone cell activity.[2]

Bone-forming cells synthesize and secrete as many molecules of NCP as of collagen, on a mole-to-mole basis. These molecules can be broken down into four general (and sometimes overlapping) groups: (1) proteoglycans, (2) glycosylated proteins, (3) glycosylated proteins with potential cell-attachment activities, and (4) γ-carboxylated (gla) proteins. The physiological roles for individual bone protein constituents are not well defined; however, many seem to be multifunctional, participating not only in regulating the deposition and turnover of mineral, but also in the control of osteoblastic and osteoclastic metabolism.

PROTEOGLYCANS, LEUCINE-RICH REPEAT PROTEINS, AND HYALURONAN

Proteoglycans are macromolecules that contain acidic polysaccharide side chains (glycosaminoglycans) attached to a central core protein, and bone matrix contains several members of this family.[5] During initial stages of bone formation, the large chondroitin sulfate proteoglycan, versican, and the glycosaminoglycan, hyaluronan (which is not attached to a protein core), are produced and may delineate areas that will become bone (Table 3). With continued osteogenesis, versican is replaced by two small chondroitin sulfate proteoglycans, decorin and biglycan, composed of tandem repeats of a leucine-rich repeat sequence. These two proteoglycans belong to a family of small proteoglycans, known as SLRPs (small leucine-rich proteoglycans). Decorin has been implicated in the regulation of collagen fibrillogenesis and is distributed predominantly in the ECM space of connective tissues and in bone, whereas biglycan tends to be found in pericellular locales. Although their exact physiological functions are not known, they are assumed to be important for the integrity of most connective tissue matrices. Deletion of the biglycan gene in transgenic animals leads to a significant decrease in the development of trabecular bone, indicating that it is a positive regulator of bone formation.[6] One function might rise from the ability of decorin and biglycan to bind and modulate the activity of the TGF-β family in the extracellular space, thereby influencing cell proliferation and differentiation. Other SLRPs (which may or may not bear glycosaminoglycans), such as fibromodulin, osteoglycin, osteoadherin, lumican, and asporin are found in bone matrix but at lower levels than decorin and biglycan and may function in other aspects of bone metabolism[7] (Table 3).

GLYCOPROTEINS

One of the hallmarks of bone formation is the synthesis of high levels of alkaline phosphatase (Table 4). This enzyme, primarily bound to the cell surface through a phosphoinositol linkage, can be cleaved from the cell surface and may be found within mineralized matrix. The function of alkaline phosphatase in bone cell biology has been the matter of much speculation and remains undefined.[8] However deletion of the tissue nonspecific form of alkaline phosphatase results in an abnormal pattern of calcification, confirming the significance of this enzyme in the mineralization process.[9]

The most abundant NCP produced by bone cells is osteonectin, a phosphorylated glycoprotein accounting for ~2% of the total protein of developing bone in most animal species. Os-

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TABLE 1. CHARACTERISTICS OF COLLAGEN-RELATED GENES AND PROTEINS FOUND IN BONE MATRIX

Protein/gene	*Function*	*Disease/animal model*
Type I—17q21.23, 7q22.1	Most abundant protein in bone matrix (90% of organic matrix), serves as scaffolding, binds and orients other proteins that nucleate hydroxyapatite deposition	Osteogenesis imperfecta; oim mouse; mov 14 mouse; brtl knock-in mouse; bones mechanically weak; mineral crystals small; some mineral outside collagen
$[\alpha1(I)_2\alpha2(I)]$		
$[\alpha1(I)_3]$		
Type X—6q21	Present in hypertrophic cartilage but does not seem to regulate matrix mineralization	Human mutations—Schmid metaphyseal chondrodysplasia knockout mouse—no apparent skeletal phenotype
$[\alpha1(X)_3]$		
Type III—2q31	Present in bone in trace amounts, may regulate collagen fibril diameter, their paucity in bone may explain the large diameter size of bone collagen fibrils	Human mutations—different forms of Ehlers-Danlos syndrome
$[\alpha1(III)]_3$		
Type V—9q34.2-34.3; 2q24.3-31, 19q13.2		
$[\alpha1(V)_2\alpha2(V)]$		
$[\alpha1(V)\ \alpha2(V)\ \alpha3(V)]$		

teonectin is transiently produced in nonbone tissues that are rapidly proliferating, remodeling, or undergoing profound changes in tissue architecture and is found constitutively in certain types of epithelial cells, cells associated with the skeleton, and in platelets. Its function(s) in bone may be multiple, with potential association with osteoblast growth, proliferation, or both, as well as with matrix mineralization.[2,10] A transgenic mouse deficient in osteonectin exhibits a defect in bone formation.[11] Tetranectin deletion results in no abnormalities in bone formation, but by 6 months, mice lacking tetranectin develop a curvature of the spine that is not associated with muscle abnormalities, implying some role of this protein in stabilizing the vertebral bodies,[12] but its precise function is unknown. Bone cells produce another set of glycoproteins, tenascin-C and -W, which seem to regulate both the organization of the matrix and the activity of cells, although tenascin-C–deficient mice do not have a skeletal phenotype, perhaps because of compensation by tenascin-W.[13]

SMALL INTEGRIN-BINDING LIGAND, N-GLYCOSLATED PROTEIN AND OTHER GLYCOPROTEINS WITH CELL ATTACHMENT ACTIVITY

All connective tissue cells interact with their extracellular environment in response to stimuli that direct and/or coordinate specific cell functions, such as migration, proliferation, and differentiation. These particular interactions involve cell attachment through transient or stable focal adhesions to extracellular macromolecules, which are mediated by cell surface receptors (primarily integrins) that subsequently transduce intracellular signals. Bone cells synthesize at least nine proteins that may mediate cell attachment: members of the small integrin-binding ligand, N-glycoslated protein (SIBLING) family (osteopontin, bone sialoprotein, dentin matrix protein-1, MEPE; Table 5), type I collagen, fibronectin, thrombospondin(s) (predominantly TSP-2 with lower levels of TSP1,3,4 and COMP), vitronectin, fibrillin, BAG-75 (Table 6), and osteoadherin (which is also a proteoglycan).[1,2,5,14] Many of these proteins are phosphorylated and/or sulfated, and all contain RGD (Arg-Gly-Asn), the cell attachment consensus sequence that binds to the integrin class of cell surface molecules. However, in some cases, cell attachment seems to be independent of RGD, indicating the presence of other sequences or mechanisms of cell attachment. Both osteopontin and bone sialoprotein are known to anchor osteoclasts to bone, and in addition to supporting cell attachment, bind Ca^{2+} with extremely high affinity through polyacidic amino acid sequences. It is not immediately clear why there are such a plethora of RGD-containing proteins in bone; however, the pattern of expression varies from one RGD protein to another, as does the pattern of the different integrins that bind to these proteins. This variability indicates that cell–matrix interactions change as a function of maturational stage, suggesting that they also may play a role in osteoblastic maturation.[2,15]

γ-CARBOXYLIC ACID–CONTAINING PROTEINS

Three bone-matrix NCPs, matrix γ-carboxylic acid (gla) protein (MGP), osteocalcin (bone gla protein [BGP]), both of which are made endogenously, and protein S (made primarily in the liver but also made by osteogenic cells), are post-translationally modified by the action of vitamin K–dependent γ-carboxylases[2] (Table 7). The production of di-carboxylic

TABLE 2. GENE AND PROTEIN CHARACTERISTICS OF SERUM PROTEINS FOUND IN BONE MATRIX

Protein/gene	*Function*	*Disease/animal model*
Albumin—4q11-q13	Inhibits hydroxyapatite crystal growth	
69 kDa, non-glycosylated, one sulfhydryl, 17 disulfide bonds, high affinity hydrophobic binding pocket		
α2-HS glycoprotein—3q27	Promotes endocytosis, has opsonic properties, chemoattractant for monocytic cells, bovine analog (fetuin) is a growth factor	Knockout mouse—adult ectopic calcification
Precursor protein of cleaved to form A and B chains that are disulfide linked, ala-ala and pro-pro repeat sequences, N-linked oligosaccharides, cystatin-like domains		

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TABLE 3. GENE AND PROTEIN CHARACTERISTICS OF GLYCOSAMINOGLYCAN-CONTAINING MOLECULES, LEUCINE RICH REPEAT PROTEINS (LRRS), AND HYALURONAN

Protein/gene	*Function*	*Disease/animal model*
Aggrecan—15q26.1 ~2.5×10^6-kDa intact protein, ~180–370,000 kDa core, ~100 CS chains of 25 kDa, and some KS chains of similar size, G1, G2 and G3 globular domains with hyaluronan binding sites, EGF and CRP-like sequences	Matrix organization, retention of water and ions, resilience to mechanical forces	Brachymorphic mouse (mutation), accelerated growth plate calcification, nanomelic chick (mutation)—abnormal bone shape,
Versican (PG-100)—5q14.3 ~1×10^6-kDa intact protein, ~360-kDa core, ~12 CS chains of 45 kDa, G1 and G3 globular domains with hyaluronan binding sites, EGF and CRP-like sequences	May "capture" space that is destined to become bone	Heart defect mouse (insertion leading to null), homozygous embryonic lethal, appears to regulation pre-chondrogenic condensation
Decorin (class 1 SLRP)—12q21.3 ~130-kDa intact protein, ~38–45-kDa core with 10 leucine rich repeat sequences, 1 CS chain of 40 kDa	Binds to collagen and regulates fibril diameter, binds to TGF-β and may modulate activity, inhibits cell attachment to fibronectin	Knockout—no apparent skeletal phenotype, DCN/BGN double knockout—progeroid form of Ehler's-Danlos syndrome
Biglycan (class 1 SLRP)—Xq28 ~270-kDa intact protein, ~38–45-kDa core protein with 12 leucine rich repeat sequences, 2 CS chains of 40 kDa	Binds to collagen, may bind to TGF-β, pericellular environment, may be a genetic determinant of peak bone mass	Knockout mouse, Turners syndrome—osteopenia; thin bones, decreased mineral content, increased crystal size; short stature, thin bones; Kleinfelder's syndrome—excessive height
Asporin (class 1 SLRP)—9q22 67 kDa, most likely no GAG chains		
Fibromodulin (class 2 SLRP)—1q32 59-kDa intact protein, 42-kDa core protein, one N-linked KS chain	Binds to collagen, regulates fibril formation, binds to TGF-β	Fibromodulin/biglycan double knockout—joint laxity and formation of supernumery sesmoid bones
Osteoadherin/osteomodulin (class 2 SLRP)—9q22.3 85-kDa intact protein, 47-kDa core protein, RGD sequence	May mediate cell attachment	
Lumican (class 2 SLRP)—12q21.3-q22 70- to 80-kDa intact protein, 37-kDa core protein	Binds to collagen, may regulate fibril formation	Lumican/fibromodulin double knockout mouse—ectopic calcification
Osteoglycin/mimecan (class 3 SLRP)—9q22 299 aa precursor, 105 aa mature protein, no GAG in bone, keratan sulfate in other tissues	Binds to TGF-β	Knockout mouse—no apparent skeletal defect
Hyaluronan—multigene complex Glycosaminoglycan without a core protein, assembled by multiple proteins associated outside of the cell	May work with versican molecule to capture space destined to become bone	

glutamyl (gla) residues enhances calcium binding. MGP is found in many connective tissues, whereas osteocalcin is bone and dentin specifically. MGP-deficient mice develop calcification in extraskeletal sites such as in the aorta[16] and excessive calcification in skeletal sites. Osteocalcin-deficient mice have increased BMD compared with normal mice.[17] In human bone, osteocalcin is concentrated in osteocytes, and its release may be a signal in the bone turnover cascade. Osteocalcin measurements in serum have proved valuable as a marker of bone turnover in metabolic disease states.[4]

BONE MINERALIZATION

The composition of bone enables it to perform its unique mechanical, protective, and homeostatic functions. While this composition varies with age, anatomic location, diet, and health status, in general, mineral accounts for 50–70% of adult mammalian bone, the organic matrix for 20–40%, water for 5–10%, and lipids for <3%. The mineral, a calcium- and hydroxide-deficient analog of the geologic mineral, hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], provides mechanical rigidity and load bearing strength to the bone composite. In contrast to large geologic hydroxyapatite crystals, bone mineral crystals are extremely small (~200 Å in their largest dimension). Bone mineral contains numerous impurities (carbonate, magnesium, acid phosphate) and vacancies (missing OH^-), and is usually referred to as a poorly crystalline, carbonate-substituted apatite.[18] These small imperfect crystals are more soluble than geologic apatite enabling bone to act as a reservoir for calcium, phosphate, and magnesium ions.

The organic matrix, predominantly type I collagen, provides elasticity and flexibility to bone and also determines its structural organization. Both the collagen and the noncollagenous proteins associated with the collagen influence the way bone mineralization occurs and the way bone remodels. The cells that are responsible for bone formation, repair, and remodeling respond to hormonal, mechanical, and other extrinsic signals. Lipids, found in the membranes of these cells control the flux of ions and also are directly involved mineralization. Water, found within the cells and in the extracellular matrix, is important for maintenance of tissue properties and nutrition.

Bone mineral is initially deposited at discrete sites in the

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TABLE 4. GENE AND PROTEIN CHARACTERISTICS OF GLYCOPROTEINS IN BONE MATRIX

Protein/gene	*Function*	*Disease/animal models*
Alkaline Phosphatase (bone-liver-kidney isozyme)—1p36.1-p34 Two identical subunits of ~80 kDa, disulfide bonded, tissue specific post translational modifications	Potential Ca^{++} carrier, hydrolyzes inhibitors of mineral deposition such as pyrophosphates	Hypophosphatasia (decreased activity), TNAP knockout mouse—growth impaired; decreased mineralization
Osteonectin/SPARC—5q31.3-q32 ~35–45 kDa, intramolecular disulfide bonds, α helical amino terminus with multiple low affinity Ca^{++} binding sites, two EF hand high affinity Ca^{++} sites, ovomucoid homology, glycosylated, phosphorylated, tissue specific modifications	Regulates collagen fibrillogenesis, binds to growth factors, may influence cell cycle, positive regulator of bone formation	Knockout mouse—altered collagen cross-linking; decreased trabecular connectivity; decreased mineral content; increased crystal size
Tetranectin—3p22-p21.3 21-kDa protein composed of four identical subunits of 5.8 kDa, sequence homologies with asialoprotein receptor and G3 domain of aggrecan	Binds to plasminogen, may regulate matrix mineralization	Knockout mouse—no long bone phenotype, spinal deformity, increased mineralization in implant model
Tenascin-C—9q33 Hexameric structure, six identical chains of 320 kDa, cys rich, EGF-like repeats, FN type III repeats	Interferes with cell-FN interactions	Knockout mouse—no apparent skeletal phenotype
Tenascin-W Hexameric structure like tenascin C with 9 FN type III repeats	Expressed in same tissues as Tenascin C during bone development; may be involved in regulating osteogenesis	

collagenous matrix.[18] As bone matures, the mineral crystals become larger and more perfect (containing fewer impurities). The increase in crystal dimension is caused both by the actual addition of ions to the crystals (crystal growth) and by aggregation of the crystals.[19,20] The initial sites of mineralization are the "hole" zones between the collagen fibrils.[18] There is still debate as to whether bone mineral forms concurrently in the protected environment of membrane bound bodies known as extracellular matrix vesicles, as it does in calcifying cartilage and mineralizing turkey tendons.[2,21] Because the body fluids are undersaturated with respect to apatite (i.e., apatite will not precipitate spontaneously), the bone matrix must contain one or more components, which facilitate apatite deposition.

TABLE 5. GENE AND PROTEIN CHARACTERISTICS OF SIBLINGS IN BONE

Protein/gene	*Function*	*Disease/animal models*
Osteopontin—4q21 ~44–75 kDa, polyaspartyl stretches, no disulfide bonds, glycosylated, phosphorylated, RGD located 2/3 from the N-terminal	Binds to cells, inhibits mineralization in bone and soft tissues; inhibits remodeling; involved in tumorogenesis; inhibits nitric oxide synthase, may regulate resistance to viral infection	Knockout mouse—decreased crystal size; increased mineral content; not subject to osteoclast remodeling
Bone sialoprotein—4q21 ~46–75 kDa, polyglutamyl stretches, no disulfide bonds, 50% carbohydrate, tyrosine-sulfated, RGD near the C terminus	Binds to cells, initiates mineralization in vitro	Knockout mouse—no published data on phenotype.
DMP-1—4q21 513 amino acids predicted; acidic, RGD 2/3 from N-terminus	In vitro regulator of mineralization	Knockout mouse—craniofacial and growth plate abnormalities; decreased mineral content, increased crystal size
MEPE—4q21.1 525 amino acids predicted, ~48–57 kDa, acidic proteolysis resistant domain (ASARM)	Possible regulator of phosphate metabolism	Knockout mouse—increased bone formation, decrease in age-related trabecular bone loss, possible involvement in renal phosphate wasting diseases in humans
DSPP—4q21.3 Codes for both phosphophoryn (DPP) and dentin sialoprotein (DSP), DPP—85% acidic, phosphoserine-rich, DPP similar to BSP and OPN		Knockout mouse—dentin and enamel abnormalities—no obvious bone phenotype

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TABLE 6. GENE AND PROTEIN CHARACTERISTICS OF OTHER RGD-CONTAINING GLYCOPROTEINS

Protein/gene	*Function*	*Disease/animal models*
Thrombospondins (1-4, COMP)—15q15, 6q27, 1q21, 5q13, 19p13.1 ~450-kDa molecule, three identical disulfide linked subunits of ~150–180 kDa, homologies to fibrinogen, properdin, EGF, collagen, von Willebrand, *P. falciparum* and calmodulin, RGD at the C terminal globular domain	Cell attachment (but usually not spreading), binds to heparin, platelets, types I and V collagens, thrombin, fibrinogen, laminin, plasminogen and plasminogen activator inhibitor, histidine rich glycoprotein	TSP-2 knockout mouse—large collagen fibrils, thickened bones; spinal deformities
Fibronectin—2q34 ~400 kDa with two nonidentical subunits of ~200 kDa, composed of type I, II, and III repeats, RGD in the 11th type III repeat 2/3 from N terminus	Binds to cells, fibrin heparin, gelatin, collagen	Knockout mouse—lethal before skeletal development
Vitronectin—17q11 ~70 kDa, RGD close to N terminus, homology to somatomedin B, rich in cysteines, sulfated, phosphorylated	Cell attachment protein, binds to collagen, plasminogen and plasminogen activator inhibitor, and to heparin	
Fibrillin 1 and 2-15q21.1, 5q23-q31 350 kDa, EGF-like domains, RGD, cysteine motifs	Regulates elastic fiber formation	Fibrillin 1 mutations—Marfan's syndrome, Fibrillin 2 mutations—congenital contractural arachnodactyly

INITIAL MINERAL DEPOSITION

The membrane bound extracellular bodies released from chondrocytes (and osteoblasts) known as "extracellular matrix vesicles" can facilitate initial mineral deposition by accumulating calcium and phosphate ions in a protected environment. Additionally, they provide enzymes that can degrade inhibitors of mineralization (e.g., ATP, pyrophosphate, proteoglycans), which are found in the surrounding matrix. They also contain a "nucleational core," consisting of proteins and a complex of acidic phospholipids, calcium, and inorganic phosphate, which can induce apatite formation.[2,21] Because these matrix vesicles are not directly associated with the collagen fibrils, the question of how mineral crystals form at discrete sites on the collagen fibrils remains. It is thought that there may be some association of vesicle mineral with the mineral in the collagen matrix, or that the matrix vesicle mineral may serve as a source of calcium and phosphate ions to support initial collagen based mineralization.

In general, crystals form when the component ions of the crystal lattice, or clusters of those ions, come together with the proper orientation and with sufficient energy to generate the first stable crystal (critical nucleus). The formation of this miniature crystal, perhaps only one or two unit cells in size,[20] is the most energy-demanding step of crystallization. Nucleation is followed by the addition of ions and ion clusters to the critical nucleus as the crystal grows. Growth may occur in one or more dimensions, or additional crystals may start to form at "kink" sites on the crystal, in a fashion analogous to glycogen branching. This so-called "secondary nucleation" allows for exponential proliferation of crystals.

There is some debate as to whether noncrystalline calcium phosphate clusters (amorphous calcium phosphate) deposits before hydroxyapatite crystals, analogous to the way in which calcium carbonate in shells forms initially as an amorphous phase and is converted to crystalline forms.[22] However, to date, amorphous calcium phosphate has not been identified in embryonic bone or bone formed in cell culture, and it is currently believed that, because of the actions of anionic proteins, apatite crystals may form directly.

Macromolecules may facilitate the direct formation of the critical nucleus, sequester ions increasing the local concentrations, or bind one or more ions creating a structure on which "heterogenous nucleation" occurs. As crystals proliferate, the macromolecules may bind to crystal surfaces, blocking growth in one or more directions, thereby regulating the size, shape, or (if secondary nucleation is blocked) the number of crystals.

Several possible promoters (nucleators) of bone mineral formation have been identified based on solution studies and studies of animals in which the proteins have been ablated (knockouts), overexpressed (knock-ins), or mutated (transgenics) (see Tables 1–7 for specific proteins). Originally, collagen was thought to be the bone mineral nucleator, but later studies

TABLE 7. GENE AND PROTEIN CHARACTERISTICS OF GAMMA-CARBOXY GLUTAMIC ACID-CONTAINING PROTEINS IN BONE MATRIX

Protein/gene	*Function*	*Disease/animal model*
Matrix Gla Protein—12p13.1-p12.3 ~15 kDa, five gla residues, one disulfide bridge, phosphoserine residues	A negative regulator of mineralization	Knockout mouse—excessive cartilage calcification, osteochondral lesions
Osteocalcin—1q25-q31 ~5 kDa, one disulfide bridge, gla residues located in α helical region	Regulates activity of osteoclasts and their precursors, may mark the turning point between bone formation and resorption	Knockout mouse, osteopetrotic mouse (mutation)—thickened bones, decreased crystal size, increased mineral content
Protein S—3p11.2 ~72 kDa	Primarily a liver product, but may be made by osteogenic cells	Deficiency in human—osteopenia

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TABLE 8. TECHNIQUES FOR DETERMINING BONE MINERAL AND MATRIX PROPERTIES

Technique	*Information derived*	*Tissue type needed*	*Comments*
X-ray diffraction	Average mineral crystal size	Homogenized tissue	No information on matrix
Transmission electron microscopy	Crystal orientation Size of individual crystals	Small tissue segments	With immuno-gold labeling can localize matrix proteins
Backscatter electron imaging	High resolution distribution of mineral density	Thick tissue segments	No information on nature of crystals or matrix
Small angle scattering	Crystal size, morphology, and orientation; fibrous protein size	Thick tissue segments	No information on nature of crystals
Atomic force microscopy	Size of individual crystals Organization of matrix	Small tissue segments	With nano-indentation can measure elastic modulus
Mechanical testing	Ability of tissue to withstand torsion or compressive loads	Machined tissue segments or whole bones	
Dual photon absorptiometry	Two-dimensional tissue density	In situ measurements	DXA–BMD measurement
μCT	Three-dimensional density, tissue connectivity, bone architecture	Small tissue segments or small bones	Can be done with live small animals
Histomorphometry	Cellular activity	Stained tissue sections	Destructive technique
Infrared microscopy and imaging	Mineral composition, mineral and matrix properties (7-μm spatial resolution)	Embedded tissue sections	Nondestructive; can get information on cell activity, lipids, and sugars from same spectrum
Raman microscopy and imaging	Mineral composition, mineral and matrix properties (0.5-μm spatial resolution)	Wet, nonembedded tissue samples	Techniques being developed to allow penetration through skin (same as above)
Nuclear MRI	Composition of bone, mineral orientation	In situ measurements	P-imaging needed for crystal analysis; noninvasive

showed that removal of noncollagenous proteins from bone collagen matrices prevented these matrices from causing apatite formation.[23] Removal of protein phosphate from demineralized bones reduced their nucleational ability in a concentration-dependent fashion, implying that one of the bone mineral nucleators was a phosphoprotein.

As discussed above, the phosphoproteins of bone include collagen itself, the SIBLINGs (osteopontin, bone sialoprotein [BSP], matrix extracellular phosphoglycoprotein [MEPE], dentin matrix protein-1 [DMP-1]), osteonectin, and bone acidic glycoprotein-75 (BAG-75).[2] Of these, to date, only BSP and DMP1 have been shown to act as apatite nucleators.[1] Both DMP1 and BSP can also inhibit apatite proliferation and growth in solution. Table 8 lists the bone matrix proteins that have been shown to affect apatite formation and growth in cell-free solution or in cell cultures. As can be seen from Table 8, most of these macromolecules are anionic, and thus can bind Ca^{+2} in solution or on the apatite crystal surface.

Several enzymes that regulate phosphoprotein phosphorylation and dephosphorylation have also been associated with the mineralization process.[2] Of these, the phosphoprotein kinases, which regulate phosphoprotein phosphorylation, and alkaline phosphatase and other phosphoprotein phosphatases seem to be most important. Alkaline phosphatase hydrolyzes phosphate esters, increasing the local phosphate concentration, enhancing the rate and extent of mineralization. Blocking phosphoprotein phosphorylation in culture decreases rates of mineralization, and cells that lack alkaline phosphatase do not mineralize in certain culture systems unless transfected with that enzyme. Patients with hypophosphatasia, a deficiency of alkaline phosphatase, also show abnormal bone mineralization. Whether the function of alkaline phosphatase is simply to increase local phosphate concentrations; to remove phosphate containing inhibitors of apatite growth (such as ATP); or to modify phosphoproteins, thereby controlling their ability to act as nucleators, is still undetermined.

GROWTH, PROLIFERATION, AND MATURATION OF MINERAL CRYSTALS

The growth of bone mineral crystals is governed in part by the constraints of the collagen matrix on which the mineral is deposited. Non-collagenous proteins that bind to the mineral crystals can also regulate their sizes and shapes. These proteins are also important in recruiting bone resorbing cells (osteoclasts) to the apatite crystal surface.[2] As bone apatite crystals grow and mature, substances in addition to proteins and lipids can affect their fates. They may be introduced through the diet, given therapeutically, or may accumulate from dialysis fluids. For example, of the dietary cations, both Mg^{+2} and Sr^{+2} can be incorporated directly into the bone mineral substituting for Ca^{+2} in the crystal lattice, yielding mineral crystals that are smaller and less perfect than those formed in their absence. Cadmium (Cd^{2+}) a toxic pollutant, when ingested, has a similar effect on bone mineral. Carbonate ($CO_3^{=}$), part of the body fluids, is a common constituent impurity of bone mineral, substituting for both OH^- and PO_4^{-3}, as well as adsorbing on to the crystal surface. Citrate is another impurity that adsorbs onto the surface of bone mineral. With age the total amount of

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TABLE 9. EFFECT OF BONE MATRIX PROTEINS ON MINERALIZATION IN VITRO

Promote or support apatite formation	*Inhibits mineralization*	*Dual function (nucleate and inhibit)*	*No known effect on mineralization*
Type I collagen	Aggrecan	Biglycan	Decorin
Proteolipid (matrix vesicle nucleational core)	α2-HS glycoprotein	Osteonectin	BAG-75
	Matrix gla protein (MGP)	Fibronectin	Lumican
57K DMP1	Osteopontin	Bone sialoprotein	Tetranectin
	Osteocalcin	α2-HS glycoprotein	Osteoadherin
		DMP1	Thrombospondin
			Intact DMP1

carbonate increases, but the surface (labile) carbonate decreases with maturation of the mineral.[24]

While each of these impurities tend to make the crystals smaller, more imperfect, and more soluble, fluoride incorporation increases the size and therefore decreases the solubility of the apatite crystals. This in part was the basis for fluoride supplementation in osteoporosis therapy, because larger crystals are more resistant to osteoclastic turnover. Another type of antiresorptive agent used in osteoporosis, the bisphosphonates, can bind to the surface of apatite crystals, and thereby block dissolution, as well as altering the activity of bone resorbing cells. Thus, bisphosphonate-treated crystals are stabilized and not apparently altered in size.[25,26] Tetracycline and other fluorescent compounds used to measure bone formation rates are Ca-chelators, which bind with high affinity to the surface of the most recently formed mineral. Because the newest formed crystals are small, their surface to volume ratios are very high, and the amount of label bound is similarly high.

EFFECTS OF MINERAL ALTERATION ON BONE PROPERTIES

While the inclusion of "foreign" ions into the bone apatite lattice can influence the properties of bone, cellular activities can also influence mineral properties. For example, where mineral deposition is retarded (e.g., hypophosphatemic rickets), crystals tend to be larger than normal.[27] Crystals are smaller in osteopetrotic bone[28] where remodeling is impaired and the small crystals persist rather than being resorbed. In osteoporosis, where bone formation may be impaired and resorption accelerated, the larger crystals persist.[29] In skeletal fluorosis, and, in general, as bone fluoride content increases, mineral content is increased, and crystals are increased in length and decreased in width.[25]

The size and distribution of mineral crystals in the bone matrix influences bone mechanical properties.[26,30] Bone strength is dependent on bone architecture, as well as numerous other factors. The mechanical strength of bone is correlated with BMD,[31] a measurement that describes the amount of bone in a given area without providing information on architecture, mineral content, or crystal properties. However, there is not a 100% correlation between BMD and mechanical strength, and it is suggested that material properties (crystal size and composition, collagen maturity, etc.) may be important determinants.[26] It should be clear from the diseases mentioned above that if there are too few crystals or crystals are too small, the mechanical strength will be compromised. Similarly, if there are too many crystals or crystals are excessively large, as in the case of skeletal fluorosis, bones many become brittle (unable to bear load). Thus, there is an optimal crystal size distribution, as well as an optimal amount of mineral. There are multiple methods for characterizing the composition of bone mineral and matrix and these are listed in Table 8.

MINERAL FORMATION IN CELL CULTURE

Osteoblasts in vitro synthesize an extracellular matrix that mineralizes in the presence of an exogenous phosphate source. Cell culture studies for the most part have focused on the factors controlling osteoblast differentiation and proliferation and microarray analyses of expression of matrix proteins.[32,33] Only a few of these studies,[34–37] however, have shown that the mineral formed was bonelike apatite. Numerous methods can be used to prove that bonelike apatite is present. The least rigorous are the histochemical stains (von Kossa for phosphate or alizarin red for calcium). The von Kossa silver stain gives positive reactions with anions that complex silver and all phosphate-containing materials. The alizarin red stain, specific for calcium, similarly cannot distinguish calcium complexed to the organic matrix from calcium bound to phosphate. Even when these two stains are co-localized, this does not prove that apatite is present.[35] Similarly more sophisticated electron microscopic methods such as microprobe analyses, or chemical analyses of Ca and PO_4 contents, cannot conclusively establish the presence of apatite. Electron micrographs showing the presence of thin plates or needles associated with collagen fibrils provide more convincing evidence of the presence of apatite, but morphology can be confusing, and calcium phosphates can take on other shapes (apatite in Greek means "deceiving"). Thus, the electron micrographs must be accompanied by electron diffraction analyses that provide definite structure verification, or by other diffraction methods (X-ray, synchrotron, or neutron), which provide unambiguous proof that the mineral phase examined is apatite.[34–39] NMR and spectroscopic methods can also provide unambiguous identification of apatite. Infrared and Raman spectra also reveal the relative proportions and properties of the organic matrix, data not obtainable from diffraction methods.

Table 9 includes those bone matrix proteins that have been shown to affect mineral deposition in culture, as well as in solution, and Tables 1–7 list animal models and human diseases in which the absence, modification, or overexpression of these proteins affects bone mineral properties. It thus should be apparent that the bone cells, the bone matrix, and the extracellular environment can influence mineralization, and in turn, because the mineral properties affect mechanical strength, the properties of bone.

ACKNOWLEDGMENTS

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Chapter 4. Bone Formation: Maturation and Functional Activities of Osteoblast Lineage Cells

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INTRODUCTION

Bone is a dynamic connective tissue, comprising an exquisite assembly of functionally distinct cell populations that are required to support the structural, biochemical, and mechanical integrity of bone and its central role in mineral homeostasis. The responsiveness of bone to mechanical forces and metabolic regulatory signals that accommodate requirements for maintaining the organ and connective tissue functions of bone are operative throughout life. As such, bone tissue undergoes remodeling, a continual process of resorption and renewal. The principal cells that mediate the bone forming processes of the mammalian skeleton are: osteoprogenitor cells that contribute to maintaining the osteoblast population and bone mass; osteoblasts that synthesize the bone matrix on bone forming surfaces; osteocytes, organized throughout the mineralized bone matrix that support bone structure; and the protective bone surface lining cells (Fig. 1). The fidelity of bone tissue structure and metabolic functions necessitates exchange of regulatory signals among these cell populations.

It should be emphasized that within the osteoblast lineage, subpopulations of cells can respond selectively to physiologic signals. Studies show that osteoblasts from axial and appendicular bone exhibit different responses to hormonal, mechanical, and developmental cues. The extent to which this reflects the local, cellular and tissue environment or inherent properties of the cells selected at an early stage during osteoblast differentiation remains to be determined. Subtle yet important differences may reflect selective responses of osteoblasts that relate to either homeostatic functions or the establishment and maintenance of bone structure.

Bone formation involves osteoblast maturation that requires a spectrum of signaling proteins including morphogens, hormones, growth factors, cytokines, matrix proteins, transcription factors, and their co-regulatory proteins. They act coordinately to support the temporal expression (i.e., sequential activation, suppression, and modulation) of other genes that represent the phenotypic, structural, and functional properties of osteoblasts during the differentiation process. Several approaches are being used to identify and functionally characterize factors that play key roles in the growth, commitment, and differentiation of mesenchymal stem cells to the osteochondrogenic lineage and osteoblasts. These include expression studies in bone tissue by in situ analyses for mRNA and protein, characterization of gene abnormalities in human skeletal disorders, and functional studies with mice lacking the gene or transgenic mouse models expressing wildtype or mutant proteins to reveal skeletal defects. Lineage allocation and tracing strategies in mice and cell culture models augment these approaches. Examples of regulatory proteins characterized in vivo and in vitro for their effects on stem cells, committed progenitors, and differentiating osteoblasts are listed in Table 1. This chapter will present the basic concepts and requirements for osteoblast determination and differentiation from progenitor cells, the functional activities of bone-forming cells, and how these cells are regulated for the control of bone formation. Through an understanding of the molecular and cellular mechanisms supporting progression of osteoblast differentiation and functional activities of distinct cell populations, a basis can be provided for improved diagnosis of skeletal disease and treatment that is targeted to specific cells in bone tissue.

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OSTEOGENIC CELL POPULATIONS

Osteoprogenitor Cells: Origin and Regulation

By definition, stem cells are pluripotent and have self-renewal capacity, repopulating multiple cell lineages in a tissue environment. Osteoprogenitor cells will arise under the appropriate stimulus from stem cells present in many tissues in the adult organism. The bone marrow stroma contains cells with robust proliferative potential that will form single colonies (CFU-Fs) with the capacity to form bone, cartilage, adipocytes, and fibrous tissue when transplanted in vivo in diffusion chambers.[1–3] These CFU-Fs are now most commonly referred to as mesenchymal stem cells (MSCs) and are distinguished from the hematopoietic stem cell (HSC) lineage. It is estimated that only a low percentage (15%) of all CFU-F have stem cell-like properties or are bone colony–forming cells.[4,5] These data are consistent with the view that CFU-F belong to a lineage hierarchy in which only some of the cells are multipotential stem or primitive progenitors while others have more limited differentiation capacity (Fig. 2). Cells with features similar to adult bone marrow MSCs have also been isolated from adult peripheral blood,[6] fetal cord blood,[7] fetal liver, blood, and bone marrow,[8] and tooth pulp.[9] Muscle satellite cells have also recently been found to be multipotential, undergoing myogenic, osteogenic, and adipogenic differentiation in vitro.[10]

The potential for use of MSCs to treat genetic disorders and for tissue engineering for reconstruction of bone tissue has fueled attempts to identify markers for isolation and purification of the MSC populations, but this has been challenging. Immunoselection and flow cytometry procedures have been applied for enrichment of osteoprogenitor cells from whole marrow. Historically, the STRO-1 antibody was used to isolate CFU populations in human bone marrow.[11] Figure 1 lists other now frequently used antibodies, and the current view is that combinations of multiple positive markers and absence of HSC markers achieve better enrichment of MSCs than single antibody strategies. To date, no single specific marker has been identified for selecting all osteoprogenitor cells or for characterizing maintenance of their progenitor phenotype. A competent signature of markers may be maximally effective. However, primordial stem cells can be reliably identified by individual cell surface proteins. For MSC-based therapeutic applications, maintaining the undifferentiated stem cell like properties may be an important consideration for long-term engraftment expansion and repopulation of a tissue by these cells in vivo, as well as for maintained expression of transgenes.

Factors maintaining the undifferentiated state are beginning to be characterized and allow for approaches to expand a potential mesenchymal stem cell and osteoprogenitor population in vitro and in vivo (Table 1). Among these factors are those that promote the self-renewal or proliferation of MSCs (e.g., Sca-1,[12] IL-18[13]) and generally this continual cell cycling is inhibitory to terminal differentiation. Other factors such as osteoblast stimulat-

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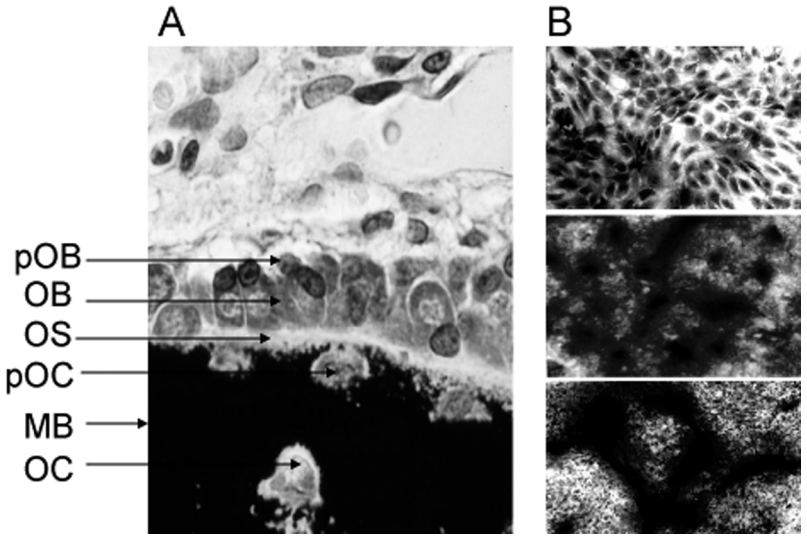


FIG. 1. Osteoblast differentiation (A) in vivo and (B) in vitro. (A) Cellular organization of bone formation. OP, osteoprogenitors; OB, osteoblasts; OS, osteoid; pre-OC, pre-osteocytes; MB, mineralized bone matrix; OC, osteocyte. (B) Recapitulation of osteoblast differentiation in cultured cells isolated from fetal rat calvarial bone. Proliferating osteoprogenitors (top), post-proliferative osteoblasts in nodules expressing alkaline phosphatase activity (middle); mineral deposition (von Kossa stain) of the ECM (bottom).

ing factor-1/pleiotrophin[14] are chemotactic for osteoprogenitors, stimulate colony formation, and increase the percent of alkaline phosphatase positive cells. IL-11, whose overexpression in mice causes thicker cortical bones, higher BMD, and greater bone mechanical strength, also increases the number of alkaline phosphatase–positive cells and colonies.[15] Some growth factors (e.g., IGF and connective tissue growth factor) and peptide hormones (e.g., PTH, growth hormone) display more widespread effects, stimulating proliferation of MSCs and osteoprogenitors but also promoting differentiation of osteoblasts. Growth factors, cytokines, and peptide hormones transduce their signals through nonreceptor kinases or membrane receptors that lead to phosphorylation of intracellular proteins through kinases. The contributions of specific kinases to cell responses are being dissected by in vitro studies with pathway inhibitors.

It should be noted that committed phenotypes from MSCs may dedifferentiate during proliferation and postmitotically assume a different phenotype dependent on the local cellular environment.[16] In this manner, pericytes can also be induced into the osteogenic lineage.[17] Mechanisms that directly inhibit the commitment of pluripotent MSCs to the osteoblast lineage also appear to be operative. This was observed on inactivation of Menin-1, the product of the *multiple endocrine neoplasia type-I* genes.[18] Such observations and the identification of the molecular pathways described above for proliferation and commitment have led to the concept of "plasticity" and transdifferentiation of stromal and other adult stem and precursor cell types.

Mechanisms Regulating Commitment to the Osteoblast Lineage

Commitment of MSCs to tissue-specific cell types is orchestrated by morphogens, developmental signaling pathways, and transcriptional regulators that serve as "master switches" (Table 1; Figs. 1 and 2). The canonical Wnt/β-catenin pathway provides early developmental cues that indirectly mediate the initial cascade of gene expression for skeletal development, bone formation, and osteoblast differentiation.[19,20] Specific Wnt pathway components with key roles in osteoblasts have recently been identified. Identification of the human mutation activating LRP5, a Wnt co-receptor protein, as a high bone mass trait gene has intensified research in this area.[21,22] Multiple skeletal defects are also manifested in mice with mutations in Wnts, the co-receptor LRP5, or the downstream transcriptional regulator β-catenin.[23–26] The data indicate that Wnts play a widespread role in skeletogenesis from embryonic skeletal patterning, through fetal skeletal development, and adult skeletal remodeling. In vitro experiments also implicate Wnt signaling in chondrogenesis and osteogenesis; however, there remain conflicting data on whether Wnts are uniformly stimulatory or inhibitory for osteoblast differentiation.[27] It seems most likely that this reflects diverse functions of Wnts at different stages of skeletogenesis, but at least part of their role may be to serve as a molecular switch between maintaining the self-renewal and stemness of MSCs and differentiation, as they do in the hematopoietic lineage.[28,29] There is also recent data to support a role for Wnt10b as a regulator of fate choice, i.e., a stimulator of osteoblastogenesis and inhibitor of adipogenesis from bipotential mesenchymal precursors in vitro and in mice.[30] Wnt10b shifts cell fate toward the osteoblast lineage by suppression of the adipogenic transcription factors C/EBPα and PPARγ and induction of the osteogenic transcription factors Runx2, Dlx5, Osterix, and Runx2.[31]

Bone morphogenetic proteins (members of the TGF-β family), BMP-2, BMP-4, and BMP-7 (also called OP-1) are potent inducers of osteogenesis in vivo and in vitro that function through a specific receptor complex BMPRIA/BMPRIB and intracellular receptor Smads (R-Smads) that heterodimerize with a DNA-binding Smad4 and/or other transcription factors to regulate gene expression.[32] BMP activity is regulated by inhibitory Smads and several antagonists including noggin and chordin that affect skeletal development.[33] Because of the early embryonic lethality of BMP null mutants, elucidation of their precise functions in osteoblastogenesis and bone formation is being approached through analysis of deficiencies and mutations of the receptors and intracellular SMAD signaling components.[34,35] Multiple BMP target genes, induced within a few hours of treatment include transcription factors essential for osteogenesis [e.g., runt homology domain (Runx) factors, homeodomain proteins (Msx2, Dlx3, Dlx2, Dlx5), and Osterix] that all promote commitment to the osteogenic lineage.[36,37] BMPs are currently being used clinically to augment fracture repair.[38]

In summary, the bone microenvironment supports a continual supply of osteoprogenitor cells by extracellular matrix accumulation of circulating and osteoblast synthesized cytokines, growth factors, non-collagenous proteins and other molecules that cooperatively function to recruit progenitor cells. The commitment and differentiation of these cells is induced by morphogenic/developmental proteins that signal to tran-

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TABLE 1. REGULATION OF OSTEOBLASTOGENESIS AND BONE FORMATION

Osteoprogenitor cell commitment and expansion (signaling proteins, growth factors)	
BMP 2/4/7	Chondro-osteoprogenitor cell specification
Indian hedgehog (Ihh)	Promotes osteogenic cell phenotype for bone collar formation
β catenin	Levels control canonical Wnt signaling for commitment to Chondrocytes (low β catenin) or osteoblasts (high β catenin)
IL-18	Stromal cell cytokine; interferon γ inducing factor that is mitogenic for chondrocytes and osteoblasts
Pleiotrophin	Osteoblast stimulating factor 1. Transgenic mice have increased bone formation
Biglycan and decorin	Modulates proliferation and survival of stromal cells
Menin	A Smad3 and Runx2 interacting protein required for commitment
Twist and dermo	HLH transcription factors support osteoprogenitor phenotype
Stromal-derived factor-1 (CXCL 12)	Promotes growth and survival of marrow stromal stem cells
Transcriptional regulators	
Homeodomain proteins	
Msx1/2	Control many aspects of craniofacial development and negative regulator of genes expressed in mature osteoblasts
Dlx3/5/6	Roles in limb development and positive regulator of osteoblast differentiation
SP factors	
Osterix	Essential for late stage of bone formation in vivo
SP-3	Contributes to ossification in vivo
Activating transcription factors	
ATF 2	Role in endochondral ossification
ATF 4	Maintains bone mass through regulation of osteoblast genes
Activating Protein1 (AP-1)	AP-1 factors respond to growth and mechanical stimuli
Cfos, Fra1, ΔFosB Jun B, Jun D	Interact as fos/jun heterodimers or jun homodimers at AP-1 sites in most osteoblast genes
Runt homology domain TFs	Essential for cell differentiation and organogenesis
Runx1 (Cbfa2/AML1)	Required for hematopoiesis; expressed in chondrocytes and osteoprogenitors
Runx2(Cbfa1/AML3)	Required for bone formation
Runx3(Cbfa3/AML2)	Required for gut and neural development; expressed in hypertrophic chondrocytes
NF-κB	Role in apoptosis of osteoblasts; negative regulator of osteoblast differentiation
LEF/TCF	Transduces the Wnt/B-catenin signal to target genes in the nucleus
Growth factors	
IGF-1/IGF-2	Anabolic regulators; IGF-1 required for anabolic effects of PTH
PDGF	Stimulates migration and proliferation; inhibits differentiation
EGF	Stimulates MSCs to differentiate
Endothelin	Peptide produced by endothelial cells; increases bone nodules in vitro
Hormones	
PTH	Numerous anabolic effects via regulation of many types of osteoblast genes
1,25(OH)2D3	Upregulates many osteoblast genes that are components of the ECM
Glucocorticoid	Promotes osteoprogenitor cell differentiation, but increases apoptosis of mature osteoblasts;
Sex Steroids	General anabolic effects on bone
Progesterone	Stimulates proliferation and formation of bone colonies in vitro
Estrogen	Increases IGF-1; stimulates osteoprogenitor proliferation and collagen synthesis
Androgen	Increases differentiation of post-proliferative osteoblasts and mineralization
Growth hormone	Stimulates proliferation and differentiation
Thyroid stimulating hormone	Decreases Wnt/Lrp5 signaling, and inhibits osteoblast differentiation and collagen synthesis
Signaling molecules	
Prostaglandin E2	Rapidly secreted in response to mechanical stimuli via EP4 receptor to increase BMP2 expression
Oncostatin M	Increases IGF-1 and alkaline phosphatase, promoting osteoprogenitor differentiation
Sclerostin	A BMP antagonist secreted by osteocytes to inhibit osteoblast differentiation and activity, but suppressed by intermittent PTH
Leptin	Dual activities: as a hypothalamic factor it is antiosteogenic in vivo, but with exogenous treatment of leptin deficient mice, chondrocytes and osteoblasts, anabolic effects are exhibited.
Adrenomedullin	A member of the calcitonin peptide family has mitogenic effects on osteoblasts
Pregnancy-associated plasma protein A	An IGFBP-4 protease and therefore regulates IGF-1 release as IGFBP4 is highly expressed in osteoblasts

scription factors for activation of osteogenic genes for synthesis of a bone matrix that provides additional extracellular matrix-mediated cues for osteoblast maturation.

Osteoblasts: Synthesize the Bone Matrix

Committed pre-osteoblasts are recognizable near the bone surface by their proximity to surface osteoblasts and by histochemical detection of alkaline phosphatase enzyme activity, one of the earliest markers of the osteoblast phenotype. The active mature osteoblast on the bone surface is distinguished by its morphological and ultrastructural properties, which are typical of a cell engaged in secretion of a connective tissue matrix, having a large nucleus, enlarged Golgi, and extensive endoplasmic reticulum. The osteoblast is highly enriched in alkaline phosphatase and vectorially secretes type I collagen and specialized bone matrix proteins as unmineralized osteoid toward the bone forming front. On quiescent bone surfaces, single layers of flattened osteoblasts or bone lining cells are observed. These inactive lining cells form either the endosteum separating bone from the marrow or they underlie the periosteum directly on the mineralized surfaces.

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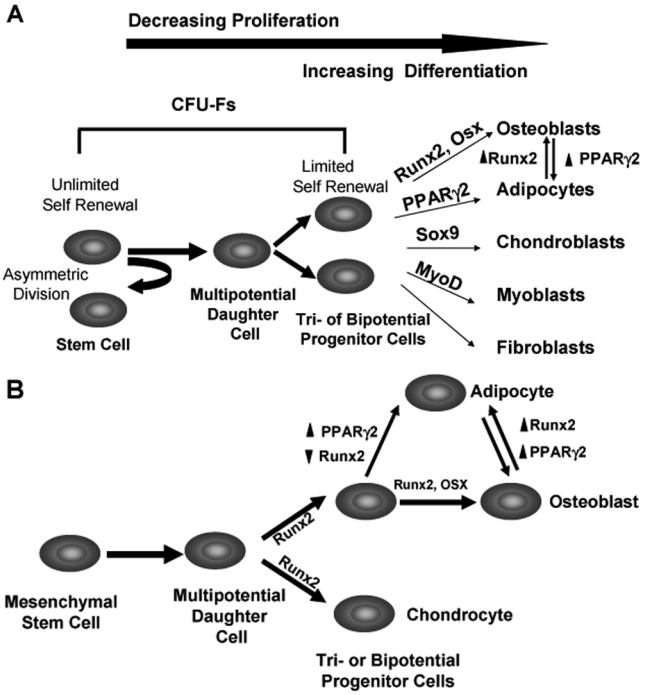


FIG. 2. Stem cell commitment to mesenchymal phenotypes. A schematic of stem cell commitment through either (A) a single-step process or (B) a multistep hierarchical process with increasing lineage restriction to various end-stage mesenchymal cell types. Stem cells have unlimited self-renewal through asymmetric divisions with daughter cells assuming properties of a pluripotent progenitor cell (CFU-Fs). Some of the known transcription factors playing key regulatory roles in the mesenchymal lineages are indicated. Also depicted is apparent plasticity between osteoblasts and adipocytes.

The osteoblasts and lining cells are in close contact with each other, joined by adherens junctions. Cadherins are calcium-dependent transmembrane proteins that are an integral part of the adherens junctions and, together with tight junctions and desmosomes, join cells by anchorage through their cytoskeleton. The changes in relative abundance of the different cadherins in mesenchymal cells seem to define their differentiation pathway.[39] Major cadherins expressed in osteoblasts include N-cadherin and cadherin-11, which are critical for embryonic limb development (Table 1). N-cadherin is important for cell–cell adhesion mediated by BMP2, and such cell–cell interactions promote differentiation of the early osteoprogenitor and osteoblast survival.[40]

Bone matrix synthesis by the osteoblast supports both cell–cell and cell–matrix interactions that are mediated by transmembrane proteins (e.g., Notch-1) and several classes of adhesion proteins. Integrins, which couple the extracellular matrix to the structural proteins of the cytoskeleton, mediate signals to modulate cell differentiation, cytoskeletal organization, cell adhesion, cell shape change, and cell spreading. The $\beta 1$ integrins play key roles in early bone development and during osteoblast differentiation by binding various collagens and inducing signals that lead to expression of osteoblast phenotypic genes. Recently, it was found that osteoblast differentiation is accelerated by interaction of the type I collagen matrix with $\alpha 1\beta 1$ or $\alpha 2\beta 1$ integrins, which activate the mitogen-activated protein kinase (MAPK) signaling pathway. This intracellular pathway is emerging as a key regulator of osteoblast differentiation.

The temporal expression of proteins involved in extracellular matrix biosynthesis and matrix mineralization provides a panel of osteoblast phenotypic markers that reflect stages of osteoblast differentiation (Fig. 3). Based on bone nodule formation in vitro, the process has been subdivided into three stages: (1) proliferation, (2) extracellular matrix development and maturation, and (3) mineralization, with characteristic changes in gene expression at each stage; some apoptosis can also be seen in mature nodules. While Fig. 3 presents only three major stages of maturation, additional stages of maturation are becoming apparent with newer methodologies.[41] In many studies, genes associated with cell growth and exit from the cell cycle [e.g., histones, cell cycle regulators (cyclins, cdk, and cdi), proto-oncogenes such as c-*fos* and c-*myc*] characterize the proliferative stage. Cell cycle inhibitors, such as p27 and p21, promote osteochondroprogenitor cell differentiation. Expression of the most frequently assayed osteoblast-associated genes type I collagen, alkaline phosphatase, osteopontin, osteocalcin, bone sialoprotein, and PTH/PTH-related protein (PTHrP) receptor (PTH1R) is asynchronously upregulated and/or downregulated as the progenitor cells differentiate and matrix matures and mineralizes. In general, alkaline phosphatase and PTH1R are early markers of osteoprogenitors that increase as osteoblasts mature and deposit matrix but decline again as osteoblasts transition to osteocytes, whereas osteocalcin is a late marker that is upregulated only in post-proliferative mature osteoblasts associated with mineralizing osteoid (Fig. 3).

When the pre-osteoblast ceases to proliferate, a key signaling event occurs for development of the large cuboidal differentiated osteoblast on the bone surface from the spindle shaped osteoprogenitor. The osteoblast is responsible for synthesis of the extracellular matrix (ECM) leading to a "matrix maturation" stage when induced expression of alkaline phosphatase and specialized bone proteins render the ECM competent for mineral deposition. The composition and organization of the bone ECM is critical for structural and functional fidelity of bone tissue for constituents of the ECM. Mineralization results in upregulated expression of several non-collagenous enriched proteins, thereby providing markers of the mature osteoblast (e.g., osteocalcin, osteopontin, bone sialoprotein). These calcium and phosphate binding proteins may function in regulating the ordered deposition of mineral, amount of the hydroxyapatite crystals, or crystal size. High-throughput screening analyses and microarray gene profiling approaches are identifying an increasing number of regulatory factors and signature genes for specific stages of osteoblast maturation.

The developmental sequence of gene expression in osteoblasts can be visualized at the single cell level in developing osteoblasts in vitro and in bone tissue by in situ methods.[41] It has been evident for some time that not all osteoblasts associated with bone nodules in vitro or in bone tissue are identical. Extensive diversity in expressed gene repertoires is not a consequence or an artifact of the in vitro environment as confirmed by analysis of osteoblastic cells in vivo where individual osteoblasts differentially express various osteoblast-associated molecules at both mRNA and protein levels. The observed differences in mRNA and protein expression repertoires in different osteoblasts may contribute to the heterogeneity in trabecular microarchitecture seen at different skeletal sites, to anatomic site-specific differences in disease manifestation such as seen in osteoporosis, and to regional variations in the ability of osteoblasts to respond to therapeutic agents.

Osteocytes: Mechanotransduction Properties Support Bone Structure and Function

The osteocyte is the terminal differentiation stage of the osteoblast and supports bone structure and metabolic functions. A distinguishing morphological feature of osteocytes is the location of each osteocyte in lacunae and the numerous cellular extensions of filapodia processes that lie in canaliculi. The canaliculi and

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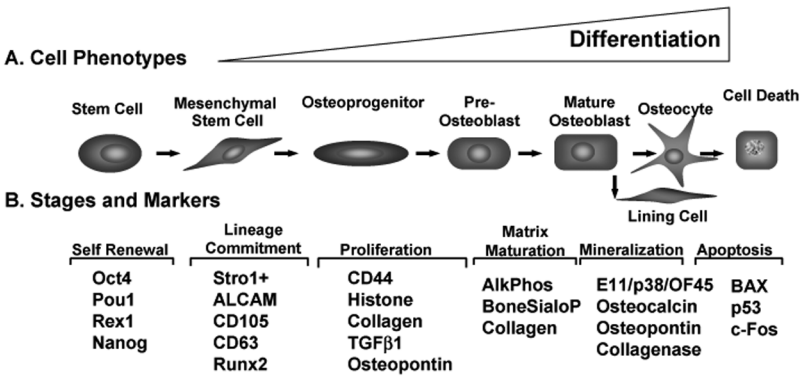


FIG. 3. Growth and differentiation of osteoblasts. Schematic illustration of (A) osteoblast lineage cells and (B) frequently used markers of the stages of maturation. Selected examples are shown.

cytoplasmic connections with adjacent cells help to ensure osteocyte viability as the mineralizing osteoid renders the ECM impermeable. Osteocytes isolated directly from bone tissue retain the filapodial processes in vitro.[42,43] In bone, osteocytes seem negative for alkaline phosphatase, but produce large amounts of osteocalcin, galectin-3, and CD44, a cell adhesion receptor for hyaluronate, and several bone matrix proteins that may be involved in cellular extensions.[43,44] Osteocytes have the capacity to synthesize certain matrix molecules to support cellular adhesive properties and regulate the deposition of mineral to maintain the necessary surrounding barrier of bone fluid in their lacunae and canalicular network for diffusion of physiological elements. They also function in osteolysis and contain lysosomal vacuoles and other features of phagocytic cells.

Osteocytes within the mineralized matrix are in direct communication with each other and surface osteoblasts through their cellular processes. They form a continuum by connection at the tip of their cell processes through gap junctions. Connexins are the integral membrane proteins that form the gap junctions between cells to allow direct communications through intercellular channels. Osteocytes are metabolically and electrically coupled through gap junction protein complexes principally comprised of Connexin 43. Gap junction formation is essential for osteocyte maturation, activity, and survival.[45–47] The primary function of the osteoblast (or lining cell)–osteocyte continuum is considered to be mechanosensory [i.e., to transduce stress signals (stretching, bending) to biological activity]. The flow of extracellular fluid in response to mechanical forces throughout the canaliculi induces a spectrum of cellular responses in osteocytes.[42] Rapid fluxes of bone calcium across these junctions are thought to facilitate transmission of information between osteoblasts on the bone surface and osteocytes within the structure of bone itself.[48,49] Signaling pathways mediating the mechanotransducing properties of osteocytes from the extracellular matrix through the cytoskeleton and finally to the nucleus to modulate gene transcription are beginning to be characterized. They involve prostaglandin E_2 and cyclooxygenase 2, kinases, Runx2, and NO-mediated responsiveness of the bone to systemic factors and mechanical forces.

Osteocytes can reside for long periods, even decades in humans, in healthy bone that is not turned over. In aging bone, empty lacunae are observed suggesting osteocytes may undergo apoptosis.[50] Disruption of intercellular communication systems, as gap junctions or cell–matrix interactions, will lead to apoptosis and nonviable bone that will be resorbed. Apoptosis or programmed cell death is important for skeletal development, as well as the growth and maintenance of bone tissue.[51] The timely apoptosis of mesenchymal cells is required for digit formation, and apoptosis of hypertrophic chondrocytes is necessary for endochondral bone formation, whereas osteoclast apoptosis is one control mechanism for the regulation of bone resorption. However, osteocyte apoptosis may be deleterious to bone structure. In vivo quantitation of apoptotic cells in the mouse and mouse models of accelerated aging and osteopenic bone induced by ovariectomy or glucocorticoid treatment confirm that programmed cell death contributes to active bone loss. Notably, bisphosphonates and estrogens, which inhibit bone resorption and are used to treat osteoporosis, have been found to inhibit osteoblast and osteocyte apoptosis.[50,52] Importantly mechanical loading of bone in a physiologic range in the rat reduces osteocyte apoptosis.[53] In summary, the organization of osteocytes in direct contact with osteoblasts or surface lining cells is consistent with the concept that bone cells support bone structure and responses to physiological signals and can communicate their responses for bone formation and resorption.

REGULATION OF OSTEOBLAST DIFFERENTIATION AND FUNCTIONAL ACTIVITIES

Transcriptional Mechanisms Control Osteoblastogenesis

Many classes of DNA binding proteins have been shown to regulate the transcription and required expression levels of bone phenotypic genes during the development of bone tissue. For example, steroid receptors, the homeodomain proteins, and other classes of transcription factors shown in Table 1 are among the early regulators of transcription in osteoprogenitors. Transcription factors further interact with co-regulatory proteins that can provide the necessary control for either activating or repressing gene expression dependent on the maturation stage of the osteoblasts or their physiologic responsive at a particular bone site.[54] Knowledge of these regulatory factors provides a basis for understanding bone related disorders. Examples include cleidocranial dysplasia resulting from mutations in Runx2,[55] Coffin-Lowry Syndrome related to ATF4 inactivation,[56,57] and the osteopenia associated with diabetes that results from inadequate expression of bone-related transcriptional regulators.[58] Transcription factors mediate physiologic control of gene expression by serving as scaffolds through protein–protein interactions for organization and assembly of regulatory complexes at strategic sites on target gene promoters. Specificity is determined by promoter context of the DNA sequence, structure of the gene, and temporal–spatial organization of the regulatory machinery within the nucleus.

The developmental expression patterns of transcription factors during osteoblast maturation reflect their roles as key determinants of osteoblast differentiation. The helix-loop-helix (HLH) proteins (Id, Twist, Dermo) are expressed at peak levels in proliferating progenitor cells and have been proposed to play a role in maintaining the osteoprogenitor population.[59,60] Because HLH proteins function as repressors of genes representing the mature osteoblast phenotype and are in effect negative regulators of

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osteoblast differentiation, they must be downregulated for osteoblast differentiation to proceed. Members of the family of activating proteins (APs) dimerize to form complexes at AP-1 recognition sites that occur in most genes. AP-1 factors are expressed primarily during growth (e.g., c-*fos*, c-*jun*), but also at later stages of osteoblast differentiation (e.g., junD, fra-2). The complexes either activate or repress gene transcription, thereby contributing to temporal expression of osteogenic genes. Homeodomain proteins are critical determinants of pattern formation and craniofacial and limb development.[27] These transcription factors become re-expressed during osteoblast differentiation (Table 1). Msx2 is expressed maximally in the mesenchymal/osteoprogenitor population and subsequently downregulated in the mature bone forming cells, whereas Dlx3 and Dlx5 expression are sustained in the post-proliferative osteoblast and increased during the mineralization stage.[61] Relatively few of the homeodomain proteins that have revealed phenotypes at the early stage of skeletal formation in null or transgenic mice[62] have been studied directly in osteoblasts.

Requirements for the runt homology domain factor Runx2 (Cbfa1/AML3/PEBPα1)[63] and the zinc finger protein Osterix (Table 1) in bone formation and osteoblast differentiation have been established by genetic studies.[37,64–68] Null mutations of either factor results in absence of a mineralized skeleton and perinatal lethality. Because Runx2 expression occurs earlier than Osterix (i.e., it is unaltered in Osterix$^{-/-}$ mice), these two factors may represent a temporal sequence of regulation essential for the early and final stages of osteoblast differentiation. Runx2 is a key regulator of cell growth[69,70] and commitment to the osteoblast lineage,[65,67,71] for maturation of chondrocytes to the hypertrophic stage, and mineralization of the cartilage,[72,73] endothelial cell migration,[74] and vascular invasion by directly upregulating vascular endothelial growth factor.[75] Runx2 target genes include those contributing not only to bone formation (osteocalcin, osteopontin, bone sialoprotein, galectin, TGFβ receptor I, and dentin sialophosphoprotein-1),[65,67,76–80] but also to bone turnover (collagenase 3, osteoprotegerin, and RANKL).[81–83] The two other Runx factors are expressed in the skeleton. Runx1 (AML1/Cbfa2), which is essential for hematopoiesis, is present in periosteum and perichondrium and seems to promote chondrogenesis.[84,85] Runx3 (AML2/Cbfa3) supports chondrocyte maturation.[73,86]

Studies on Runx2 have established new paradigms for understanding properties of a master gene regulatory switch for lineage determination[87] (Fig. 2). Runx2 remains associated with chromosomes during mitosis contributing to bone phenotype stability.[88] Runx2 binds to specific DNA sequence with a partner protein, CBFβ1, which is also required for normal bone formation.[89] Runx2 interacts with numerous co-regulatory proteins in subnuclear domains for gene activation and repression including factors that remodel chromatin [e.g., p300 and other factors with histone acetyl transferase activity (HAT) to activate gene transcription or attenuate levels of expression by recruiting histone deacetylase enzymes HDAC; see Table 1 for their effects on osteoblast differentiation]. Other co-regulatory proteins include the Wnt pathway regulator LEF-1,[90] AP-1 factors,[91,92] C/EBP,[93,94] the vitamin D receptor,[95] TGFβ/BMP-responsive SMAD proteins,[96,97] and the WW domain proteins.[98,99] Thus, Runx factors also integrate key signaling pathways for osteogenesis. A key property of Runx factors is its DNA binding domain and specific nuclear matrix signal that targets Runx2 to subnuclear domains, facilitating the formation of multimeric complexes with Runx2 partner proteins. Mutations in these functional domains and consequential loss of co-regulatory protein interactions result in absence of a mineralized skeleton in the mouse[68] and accounts for several of the cleidocranial dysplasia disorders (CCD) variants.[55,100]

The potency of transcription factors in lineage determination and differentiation is reflected by their ability to cause trans-differentiation of cells to other phenotypes when they are overexpressed or suppressed. For example, forced overexpression of peroxisome proliferation-activated receptor γ2 (PPARγ2) in osteoblasts will change their phenotype to adipocytes, whereas forced expression of Runx2 in adipocytes or other nonosseous cells results in acquisition of a differentiated osteoblast phenotype[101–103] (Fig. 2). It has been suggested that the inverse relationship sometimes seen between expression of the osteoblast and adipocytic phenotypes in marrow stroma (e.g., in osteoporosis) may reflect the ability of single or combinations of agents to alter the commitment or at least the differentiation pathway that multi or bipotential cells will transit. At some point in the future, transcription factors may be used to promote osteogenesis from stem cell populations.

Regulation of Osteoblast Maturation, Activity and Apoptosis

As already raised, steroid and polypeptide hormones, growth factors, and cytokines regulate not only the growth of osteoprogenitors and/or their progression to mature osteoblasts, but also regulate osteoblast activity and apoptosis. This regulation is often not at a single differentiation stage but occurs at multiple points in the maturational sequence and often with biphasic or opposite activities at different times or on different bone compartments. Thus, a single factor may increase osteoprogenitor proliferation but also stimulate or inhibit later differentiation stages sometimes in an anatomic site-specific manner. For example, PTH stimulates growth of osteoprogenitor populations, while also inhibiting osteoblast and osteocyte apoptosis.[50] However, in a study with overexpression of a constitutively active PTH1R, opposite effects were observed in trabecular and endosteal osteoblast populations (increased osteoblast activity) versus periosteal osteoblast populations (decreased activity),[104] reminiscent of the differential effects of PTH in trabecular versus cortical bone in primary hyperparathyroidism.[105] Many of these regulatory factors also act as local autocrine and paracrine regulators. Thus, PTHrP functions as a local cytokine-regulating cell growth for differentiation during development.[106] Numerous mouse genetic models have defined the role of this pathway, integrated with other developmental regulators, such as Indian hedgehog (Ihh), in the control of chondrocyte proliferation and maturation for endochondral bone formation and osteoblast differentiation.

Glucocorticoids promote the differentiation of human and rat marrow mesenchymal cells to osteoblasts in vitro as established by many studies. Recently, the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) regulating conversion of cortisone into active cortisol is controlled by osteoblast differentiation[107] and transgenic mice overexpressing 11β-HSD1 type 2 in osteoblasts has revealed an anabolic role for endogenous glucocorticoids in bone.[108] However, when used therapeutically, glucocorticoids can have negative effects on bone formation and can contribute to osteoporosis at least in part by inducing osteoblast and osteocyte apoptosis.[50,109] Vitamin D [1,25$(OH)_2D_3$] is a potent regulator of gene transcription, increasing or decreasing expression of numerous osteoblast phenotypic genes that are consistent with more differentiated osteoblasts, (e.g., enhanced osteocalcin synthesis).[110] The sex steroids have anabolic effects on bone and osteoblasts. Retinoic acid has a well-established role in skeletal development of the embryo; recently an orphan receptor for retinoic acid (ROR) has been recognized for contributing to bone formation in the postnatal mouse (Table 1).[111,112] Adrenomedullin increases cellular proliferation in cultured calvaria, suggesting stimulated osteoblast growth.[113] Leptin acts as a systemic factor that contributes to decreased bone mass in

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a null mouse model acting through the hypothalamus.[114] Leptin is secreted largely by adipose tissue, but also by human osteoblasts at the mineralization stage[115] and has been reported to have anabolic effects on osteoblasts and stromal cells promoting their differentiation in vitro,[115,116] but also inducing apoptosis of stromal cells.[117] Nonetheless, leptin reduced bone loss in ovariectomized rats,[118] and positive correlations between circulating leptin levels and BMD in postmenopausal and nonobese women have been reported.[119] It is thus apparent that leptin is an important regulator of bone cell function, but seems to control bone growth and osteoblast activities through several distinct mechanisms.

Cytokines of the LIF/IL-6 family, which signal through gp130, play a role not only in osteoclastogenesis, but also in bone formation. Lack of gp130 signaling alters osteoblast phenotype and activity,[120] and loss of SHP2/ras/MAPK activation by gp130 induces high bone turnover with increased formation and resorption.[121] Transgenic mice overexpressing the IL-11 cytokine exhibit higher BMD and greater bone mechanical strength, apparently as a result of the ability of IL-11 to act locally to stimulate the number of osteoprogenitors and bone formation by regulating transcription of BMP-2 target genes (e.g., *smad6*).[15] Overexpression of oncostatin M (OSM) in mouse knees induces, in addition to synovial inflammation, increased periosteal bone apposition,[122] which may be the in vivo correlate of the stimulatory effect of OSM seen on osteoprogenitors in rat calvarial-derived populations.[123] However, the signaling pathways induced and cellular mechanisms invoked to achieve these bone effects are complex. Overall, the data suggest that IL-11, OSM, LIF, and other family members, act differently not only at different differentiation stages but also differently on the periosteum/suture and bone tissue–derived cell populations compared with stromal/endosteal cells.[123]

The conflicting data on whether Wnt proteins are stimulatory or inhibitory for osteoblast differentiation is likely to reflect diverse functions of Wnts at different stages of skeletogenesis, and more data in different models will be required to expand our understanding of their complex role in osteogenesis. While canonical signaling promotes early stage osteogenesis (commitment to osteoblasts), recent data suggest that Lef1, a transcriptional regulator of the Wnt/β-catenin signaling cascade, promotes osteoprogenitor differentiation,[31] but delays osteoblast maturation and mineralization.[124] There is also accumulating evidence that Wnts regulate apoptosis of osteogenic cells. For example, Wnts prevent apoptosis of both uncommitted osteoprogenitors and differentiated osteoblasts by β-catenin–dependent as well as –independent signaling cascades involving Src/ERK and PI3K/Akt.[125] Thus, regulation of Wnt activity during osteoblast maturation is important. Secreted frizzled-related protein (sFRP)-1, an antagonist of canonical Wnt signaling, is a negative regulator of human osteoblast and osteocyte survival.[126]

A large number of in vitro and mouse experiments have shown a broad role of TGF-β1 throughout osteoblast differentiation and bone formation. Despite conflicting data and evidence for biphasic/concentration-dependent effects, it is generally accepted that TGF-β1 increases bone formation in vitro mainly by recruiting osteoprogenitors both by inducing chemotaxis and stimulating their proliferation, thus expanding the pool of committed osteoblasts. It also promotes the early stages of differentiation, while blocking later phases of differentiation, mineralization and osteoblast apoptosis.[127]

Among important concepts in regulation of osteoblast differentiation and activity is the idea that local or endogenous production of particular growth factors and cytokines undergoes changes required for osteoprogenitor differentiation to proceed and osteoblasts to modify their activity. For example, IGF-I expression is markedly increased during early osteoblast recruitment, but declines quite markedly as these cells undergo differentiation. Data from both IGF-I– or IGF-I receptor–deficient mice and mice with targeted overexpression of IGF-I in osteoblasts show that IGF-I is involved not only in embryonic bone development but also postnatal bone formation and remodeling.[128,129] Much remains to be clarified about the effects of IGF-I and IGF-II on osteoblast precursor proliferation and/or osteoblast activity, but it does seem that targeted overexpression of IGF-I in osteoblasts results in increased trabecular bone volume, increased osteoblast function with accelerated formation, and decreased mineralization lag time, but no change in the number of osteoblast precursors.[128] When overexpression is targeted to both pre-osteoblasts and osteoblasts, osteoblast number increased, cortical bone thickness increased, but trabecular bone volume decreased, possibly because of increased turnover. IGF-I also acts as a potent anti-apoptotic factor particularly for differentiated osteoblasts and osteocytes. Targeted deletion of the *type I IgfR* gene in mature osteoblasts results in a mineralization defect associated with lower BMD, but an increase in osteoblast precursor cells.[129]

Other Functional Activities of Osteoblast Lineage Cells

The functional properties and regulated activity of bone cells evolve during progression of osteoblast differentiation. As described above, they form the structural components of bone, producing a mineralized matrix and a cellular syncytium to respond to physiologic and mechanical demands on the skeleton. An equally important function of osteoblasts, as well as the pre-osteoblasts, which lie in close proximity, and osteocytes is their responsiveness to endocrine factors and the production of paracrine and autocrine factors for the recruitment of osteoprogenitors, the growth of pre-osteoblasts, and the regulation of osteoclastic resorption of the mineralized bone matrix.

The bone microenvironment seems to be essential for two components of osteoclastogenesis: (1) maturation and fusion of the mononuclear precursor to the multinucleated osteoclast and (2) activation and regulation of the activity of the functional osteoclast. The requirement for stromal osteoprogenitors or osteoblasts in mediating osteoclast differentiation is linked to the role of osteotrophic hormones and cytokines in regulating development of pre-osteoclasts and the multinucleated phenotype. Osteoblast lineage cells secrete osteoprotegerin, a soluble inhibitor of osteoclastogenesis and have receptors for RANKL, PTH, and $1,25(OH)_2D_3$, which will promote osteoclast activity and bone turnover. Osteoblasts are the major source of the colony-stimulating factor 1 (CSF1) and also secrete some cytokines, which participate in osteoclastogenesis. Prostaglandins produced by bone cells, a potent stimulator of bone formation, as well as resorption, exemplifies the complexity of cellular responses in bone. The regulation of bone resorption and coupling of osteoblast and osteoclast activities is detailed elsewhere.

The role of osteoblastic cells as supporting cells for the bone marrow and hematopoietic stem cells (HSCs) has also begun to receive new attention because of two recent papers in which novel transgenic mouse models, one using the conditional deletion of the *BMPR1A* gene[130] and the other using overexpression of an active PTH/PTHrP receptor (PPR) mutant within osteoblasts.[131] The studies show parallel, concordant increases in the generation of trabecular osteoblasts and the number of HSCs in vivo and in vitro with a physical proximity of HSCs to endosteal osteoblasts and interaction between them. These data support the notion that osteoblasts are a major, defining component of the HSC niche within the bone marrow.[130,131]

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Chapter 5. Osteoclast Biology and Bone Resorption

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CELL BIOLOGY OF THE OSTEOCLAST

Pathological bone loss, regardless of etiology, invariably represents an increase in the rate at which the skeleton is degraded by osteoclasts relative to its formation by osteoblasts. Thus, prevention of conditions such as osteoporosis requires an understanding of the molecular mechanisms of bone resorption.

The osteoclast, the exclusive bone resorptive cell (Fig. 1), is a member of the monocyte/macrophage family and a polykaryon that can be generated in vitro from mononuclear phagocyte precursors resident in a number of tissues.[1] There is, however, general agreement that the principal physiological osteoclast precursor is the bone marrow macrophage. Two cytokines are essential and sufficient for basal osteoclastogenesis, the first being RANKL[1,2] and the second being macrophage colony-stimulating factor (M-CSF), also designated CSF-1.[3] These two proteins, which exist as both membrane-bound and soluble forms, are produced by marrow stromal cells and their derivative osteoblasts, and thus physiological recruitment of osteoclasts from their mononuclear precursors requires the presence of these non-hematopoietic, bone-residing cells.[1] RANKL, a member of the TNF superfamily, is the key osteoclastogenic cytokine, because osteoclast formation requires its presence or its priming of precursor cells. M-CSF contributes to the proliferation, survival, and differentiation of osteoclast precursors, as well as the survival and cytoskeletal rearrangement required for efficient bone resorption (Fig. 2). The discovery of RANKL was preceded by identification of its physiological inhibitor osteoprotegerin (OPG), to which it binds with high affinity.[4] In contrast, M-CSF is a moiety long known to regulate the broader biology of myeloid cells, including osteoclasts[3] (see Fig. 2).

Our understanding of how osteoclasts resorb bone derives from two major sources: biochemical and genetic.[2] The unique osteoclastogenic properties of RANKL permit generation of pure populations of osteoclasts in culture and hence the performance of meaningful biochemical and molecular experiments that provide insights into the molecular mechanisms by which osteoclasts resorb bone. Further evidence has come from our capacity to generate mice lacking specific genes, plus the positional cloning of genetic abnormalities in people with abnormal osteoclast function. Key to the resorptive event is the capacity of the osteoclast to form a microenvironment between itself and the underlying bone matrix (Fig. 3A). This compartment, which is isolated from the general extracellular space, is acidified by an electrogenic proton pump (H^+-ATPase) and a Cl^- channel to a pH of ~4.5.[5] The acidified milieu mobilizes the mineralized component of bone, exposing its organic matrix, consisting largely of type 1 collagen that is subsequently degraded by the lysosomal enzyme, cathepsin K. The critical role that the proton pump, Cl^- channel, and cathepsin K play in osteoclast function is underscored by the fact that dysfunction of each results in human diseases of excess bone mass, namely osteopetrosis or pyknodysostosis.[2,5]

The above model of bone degradation clearly depends on physical intimacy between the osteoclast and bone matrix, a role provided by integrins. Integrins are $\alpha\beta$ heterodimers with long extracellular and single transmembrane domains.[6] In most instances, the integrin cytoplasmic region is relatively short, consisting of 40–70 amino acids. Integrins are the principal cell/matrix attachment molecules and they mediate osteoclast/bone recognition. Members of the $\beta1$ family of integrins, which recognize collagen, fibronectin, and laminin are present on osteoclasts, but $\alpha v\beta3$ is the principal integrin mediating bone resorption.[7] This heterodimer, like all members of the αv integrin family, recognizes the amino acid motif Arg-Gly-Asp (RGD), which is present in a variety of bone residing proteins such as osteopontin and bone sialoprotein. Thus, osteoclasts attach to and spread on these substrates in an RGD-dependent manner and, most importantly, competitive ligands arrest bone resorption in vivo. Definitive proof of the pivotal role that $\alpha v\beta3$ in the resorptive process came with the generation of $\beta3$ integrin knockout mouse, which develops a progressive increase in bone mass because of osteoclast dysfunction.[7] Based on a combination of these in vitro and in vivo observations, small molecule inhibitors of osteoclast function that target $\alpha v\beta3$ have been developed.[8]

Bone resorption also requires a polarization event in which the osteoclast delivers effector molecules like HCl and cathepsin K into the resorptive microenvironment. Osteoclasts are characterized by a unique cytoskeleton, which mediates the resorptive process. Specifically, when the cell contacts bone, it generates two polarized structures, which enable it to degrade skeletal tissue. In the first instance, a subset of acidified vesicles containing specific cargo, including cathepsin K and other matrix metallo-proteases, are transported, probably through microtubules, to the bone-apposed plasma membrane,[9] to which they fuse in a manner not currently understood. Insertion of these vesicles into the plasmalemma results in formation of a villous structure, unique to the osteoclast, the ruffled membrane. This resorptive organelle contains the abundant H^+ transporting machinery to create the acidified microenvironment, while the accompanying exocytosis serves as the means by which cathepsin K is secreted (Fig. 3B).

In addition to inducing ruffled membrane formation, contact with bone also prompts the osteoclast to polarize its fibrillar actin into a circular structure known as the "actin ring." A separate "sealing zone" surrounds and isolates the acidified resorptive microenvironment in the active cell, but its composition is almost completely unknown. The actin ring, like the ruffled membrane, is a hallmark of the degradative capacity of the osteoclast, because structural abnormalities of either occur in conditions of arrested resorption.[10] In most cells, such as fibroblasts, matrix attachment prompts formation of stable structures known as focal adhesions that contain both integrins and a host of signaling and cytoskeletal molecules, which mediate contact and formation of actin stress fibers. In keeping with the substitution of the actin ring for stress fibers in osteoclasts, these cells form podosomes instead of focal adhesions. Podosomes, which in resorbing osteoclasts are present in the actin ring, consist of an actin core surrounded by $\alpha v\beta3$ and associated cytoskeletal proteins.

The integrin $\beta3$ subunit knockout mouse serves as an important tool for determining the role of $\alpha v\beta3$ in the capacity of the osteoclast to resorb bone. Failure to express $\alpha v\beta3$ results in a dramatic osteoclast phenotype, particularly regarding the actin cytoskeleton. The $\beta3^{-/-}$ osteoclast forms abnormal ruffled membranes in vivo and, whether generated in vitro or directly isolated from bone, the mutant cells fail to spread when plated on immobilized RGD ligand or mineralized matrix in

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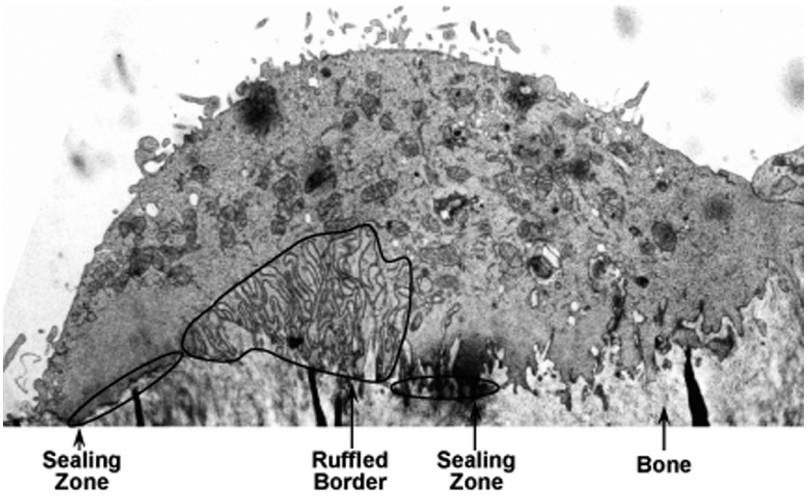


FIG. 1. The osteoclast as a resorptive cell. Transmission electron microscopy of a multinucleated primary rat osteoclast on bone. Note the extensive ruffled border, close apposition of the cell to bone, and the partially degraded matrix between the sealing zones. Courtesy of H. Zhao.

physiological amounts of RANKL and M-CSF. Confirming their attenuated resorptive activity, $\beta 3^{-/-}$ osteoclasts generate fewer and shallower resorptive lacunae on dentin slices than do their wildtype counterparts. In keeping with attenuated bone resorption, in vivo, $\beta 3^{-/-}$ mice are substantially hypocalcemic.[5]

Integrin Signaling

While integrins were viewed initially as merely cell attachment molecules, it is now apparent that their capacity to transmit signals to and from the cell interior is equally important, an event that requires that the integrin convert from a default low affinity state to one in which its capacity to bind matrix is significantly enhanced. The process, termed activation, arises

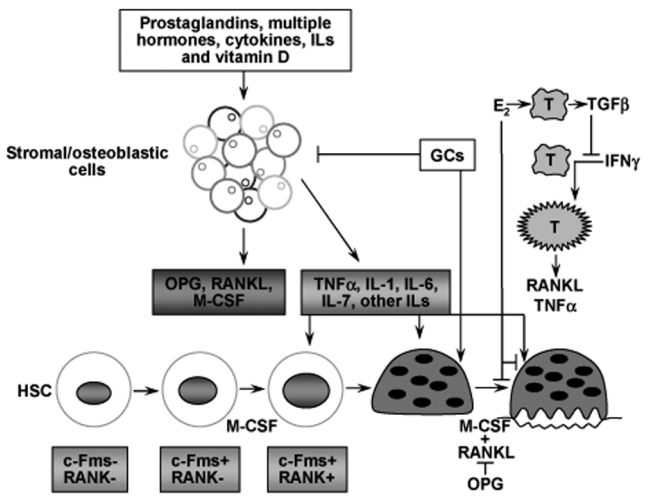


FIG. 2. Role of cytokines, hormones, steroids, and prostaglandins in osteoclast formation. Under the influence of other cytokines (data not shown), hematopoietic stem cell (HSCs) commit to the myeloid lineage, express c-Fms and RANK, the receptors for M-CSF and RANKL, respectively, and differentiate into osteoclasts. Mesenchymal cells in the marrow respond to a range of stimuli, secreting a mixture of pro- and anti-osteoclastogenic proteins, the latter primarily OPG. Glucocorticoids suppress bone resorption indirectly but possibly also target osteoclasts and/or their precursors. Estrogen, by a complex mechanism, inhibits activation of T cells, decreasing their secretion of RANKL and TNF-α; the sex steroid also inhibits osteoblast and osteoclast differentiation and lifespan. A key factor regulating bone resorption is the RANKL/OPG ratio.

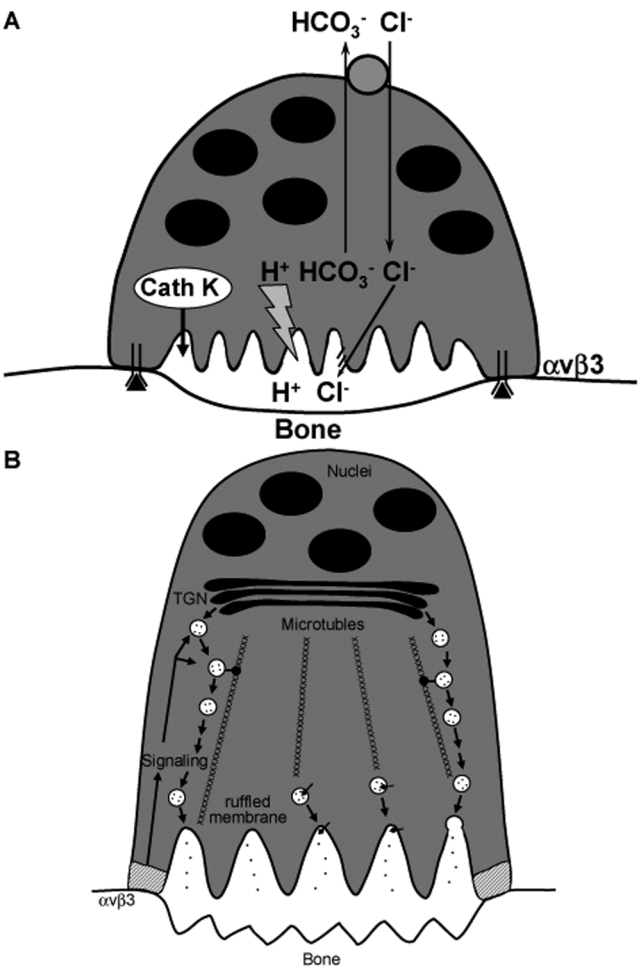


FIG. 3. Mechanism of osteoclastic bone resorption. (A) The osteoclast adheres to bone through the integrin $\alpha v\beta 3$, creating a sealing zone, into which is secreted hydrochloric acid and acidic proteases such as cathepsin K, MMP9, and MMP13. The acid is generated by the combined actions of a vacuolar H^+ ATPase, its coupled chloride channel and a basolateral chloride-bicarbonate exchanger. Carbonic anhydrase converts CO2 into H^+ and HCO_3^- (data not shown). (B) Integrin engagement results in signals that target acidifying vesicles (♦ = proton pump complex) containing specific cargo (black dots) to the bone-apposed face of the cell. Fusion of these vesicles with the plasma membrane generates a polarized cell capable of secreting the acid and proteases required for bone resorption.

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from either integrin ligation of their multivalent ligands or indirectly by growth factor signaling.[11]

$\alpha v\beta 3$ is absent from osteoclast precursors, but their differentiation under the action of RANKL results in marked upregulation of this heterodimer. The capacity of integrins to transmit intracellular signals to the cytoskeleton heightened interest in the cytoplasmic molecules mediating these events in osteoclasts and $\alpha v\beta 3$ signaling in this context is reasonably well understood. The initial signaling event involves the protooncogene c-src which, acting as a kinase and an adaptor protein, regulates formation of lamellipodia and disassembly of podosomes, indicating that c-src controls formation of resorptive organelles of the cell, such as the ruffled membrane, and also arrests migration on the bone surface. There is continuing debate surrounding the molecules which link c-src to the cytoskeleton, one proposal being that the focal adhesion kinase family member Pyk2, acting in concert with c-Cbl, a protooncogene and ubiquitin ligase.[12] A strong alternative candidate is Syk, a nonreceptor tyrosine kinase that is recruited to the active conformation of $\alpha v\beta 3$ in osteoclasts in a c-src–dependent manner, where it targets Vav3,[7] a member of the large family of guanine nucleotide exchange factors (GEFs) that convert Rho GTPases from their inactive GDP to their active GTP conformation.

Small GTPases

The Rho family of GTPases is central to remodeling of the actin cytoskeleton in many cell types,[13] and as such plays a central role in osteoclastic bone resorption. On attachment to bone, Rho and Rac bind GTP and translocate to the cytoskeleton. While both small GTPases impact the actin cytoskeleton, Rac and Rho exert distinctive effects. Rho signaling mediates formation of the actin ring and a constitutively active form of the GTPase stimulates podosome formation, osteoclast motility, and bone resorption, whereas dominant negative Rho arrests these events.[14] Rac stimulation in osteoclast precursors prompts appearance of lamellipodia, thus forming the migratory front of the cell to which $\alpha v\beta 3$ moves when activated.[15] In sum, it is likely that Rho's effect is principally on cell adhesion, whereas Rac mediates the cytoskeleton's migratory machinery. Importantly, absence of Vav3 blunts Rac but not Rho activity in the osteoclast.[16]

FACTORS REGULATING OSTEOCLAST FORMATION AND/OR FUNCTION

Proteins

In addition to the two key osteoclastogenic cytokines M-CSF and RANKL, a number of other proteins play important roles in osteoclast biology, either in physiological and/or pathophysiological circumstances.

As discussed earlier, osteoprotogerin, a high affinity ligand for RANKL that acts as a soluble inhibitor of RANKL, is secreted by cells of mesenchymal origin, both basally and in response to other regulatory signals, including cytokines and bone-targeting steroids.[4] Pro-inflammatory cytokines suppress OPG expression while simultaneously enhancing that of RANKL, with the net effect being a marked increase in osteoclast formation and function. Genetic deletion of OPG in both mouse and humans leads to profound osteopetrosis, whereas overexpression of the molecule under the control of a hepatic promoter results in severe osteoporosis. Together, these observation indicate that circulating OPG modulates the bone resorptive activity of RANKL and help to explain the increased bone loss in clinical situations accompanied by increased levels of TNF-α, interleukin (IL)-1, PTH, or PTH-related protein (PTHrP). Serum PTH levels are increased in hyperparathyroidism of whatever etiology, whereas PTHrP is secreted by bone-targeting lung and breast carcinoma.[17,18] TNF antibodies or a soluble TNF receptor-IgG fusion protein potently suppress the bone loss in disorders of inflammatory osteolysis such as rheumatoid arthritis.[19] The molecular basis of this observation seems to be that the inflammatory cytokine synergizes with RANKL in a unique manner, most likely because RANKL and TNF each activate a number of key downstream effector pathways, leading to nuclear localization of a range of osteoclastogenic transcription factors (see Fig. 6). Recent evidence suggests a new paradigm linking TNF, IL-1, and the natural secreted inhibitor for the latter cytokine, IL-1 receptor antagonist, which blocks IL-1 function. Specifically, it seems that, at least in murine osteoclasts and their precursors, many of the effects of TNF are mediated through its stimulation of IL-1, which in turn increases expression and secretion of IL-1ra, a set of events that represent a complex control pathway. The significance of IL receptor antagonist is shown by the fact an IgG fusion protein containing the active component of this molecule has been developed and enhances the ability of anti-TNF-α antibodies to decrease bone loss in rheumatoid arthritis.[20]

Elegant studies suggest that interferon gamma (IFN-γ) is an important suppressor of osteoclast formation and function.[21] Nevertheless, these findings seem to be in conflict with other in vivo observations, including the report that IFNγ treatment of children with osteopetrosis ameliorates the disease[22] and the fact that a number of in vivo studies indicate that IFN-γ stimulates bone resorption.[23] This conundrum highlights the importance of discriminating between in vitro culture experiments using single cytokines and results in vivo. Many additional studies have implicated a range of other cytokines in the regulation of the osteoclast. These include a range of interleukins, granulocyte macrophage-colony stimulating factor (GM-CSF), IFN-β, stromal-derived growth factor 1 (SDF-1), macrophage inflammatory protein 1α (MIPα), and monocyte chemotactic protein 1 (MCP-1)[24–27] but at this time the results are either contradictory, as for GM-CSF in the murine versus human systems, or lack direct proof in humans. Future studies are likely to clarify the currently confusing data set. Finally, interactions between immune receptors such as DAP12 and FcγR and their ligands on cells of the stromal and myeloid/lymphoid lineages are important for transmission of RANK-derived signals.[21]

Small Molecules

1,25-dihydroxyvitamin D has all the characteristics of a steroid hormone, including a high affinity nuclear receptor that binds as a heterodimer with the retinoid X receptor to regulate transcription of a set of specific target genes. The active form of vitamin D, generated by successive hydroxylation in the liver and kidney, is a well-established stimulator of bone resorption when present at supraphysiological levels. Studies over many years indicate that this steroid hormone increases mesenchymal cell transcription of the *RANKL* gene while diminishing that of OPG.[4] Separately, 1,25-dihydroxyvitamin D suppresses synthesis of the pro-osteoclastogenic hormone PTH[28] and enhances calcium uptake. Taken together, two latter effects would seem to be antiresorptive, but many studies in humans indicate the net osteolytic action resulting from high levels of the steroid hormone, suggesting that its ability to stimulate osteoclast function overrides any bone anabolic actions.

Loss of estrogen (E2), most often seen in the context of menopause, is a major reason for the development of signifi-

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cant bone loss in aging. Interestingly, it is now clear that estrogen is the main sex steroid regulating bone mass in both men and women.[29] The mechanisms by which estrogen mediates its osteolytic effects are still incompletely understood, but significant advances have been made over the last decade. The original hypothesis, now considered to only part of the explanation, is that decreased serum E2 led to increased production, by circulating macrophages, of osteoclastogenic cytokines such as IL-6, TNF, and IL-1. These molecules then act on stromal cells and osteoclast precursors to enhance bone resorption by regulating expression of pro-(RANKL, M-CSF) and anti-(OPG) osteoclastogenic cytokines (in the case of mesenchymal cells) and by synergizing with RANKL itself (in the case of myeloid osteoclast precursors; see Fig. 2). However, the realization that lymphocytes play a key role in mediating several aspects of bone biology has led to a growing realization that the cellular and molecular targets for E2 are more widespread than previously believed. A recent compelling model proposes that E2 impacts the resorptive component of bone turnover (the steroid has separate effects on osteoblasts), at least in part by modulating production by T cells of RANKL and TNF.[23] This effect is itself indirect, with E2 suppressing antigen presentation by dendritic cells and macrophages by enhancing expression by the same cells of TGF-β. Antigen presentation activates T cells, thereby enhancing their production of RANKL and TNF. As discussed previously, the first molecule is the key osteoclastogenic cytokine, whereas the second potentiates RANKL action. This newly discovered interface between T cells and bone resorption also clarifies aspects of inflammatory osteolysis. Finally, some studies indicate that E2 modulates signaling in pre-OCs, and that acting through reactive oxygen species, it increases the lifespan and/or function of mature osteoclasts.[30]

Both endogenous glucocortocoids and their synthetic analogs, which have been and continue to be a major mainstay of immunosuppressive therapy, are members of a third steroid hormone family having a major impact on bone biology.[31] One consequence of their chronic mode of administration is severe osteoporosis arising from decreased bone formation and resorption with the latter still (low turnover osteoporosis). The majority of the evidence focuses on the osteoblast as the prime target with the steroid increasing apoptosis of these bone-forming cells. However, numerous human studies document a rapid initial decrease in bone resorption, suggesting that the osteoclast and/or its precursors may also be targets. The molecular basis for this latter finding is unclear. However, because osteoblasts are a requisite part of the resorptive cycle, one consequence of their long-term diminution could be decreased osteoclast formation and/or function secondary to lower levels of RANKL and/or M-CSF production. Alternatively, glucocorticoids have been shown to decrease osteoclast apoptosis.[32]

A wide range of clinical information shows that excess prostaglandins stimulate bone loss, but once again, the cellular basis has not been established conclusively. Prostaglandins target stromal and osteoblastic cells, stimulating expression of RANKL and suppressing that of OPG.[33] This increase in the RANKL/OPG ratio, seen in a variety of human studies, is sufficient of itself to explain the clinical findings of increased osteoclastic activity. However, highlighting again the dilemma of interpreting in vitro studies, there have been a number of studies in which prostaglandins regulate osteoclastogenesis per se in murine cell culture.

Phosphoinositides play distinct and important roles in organization of the osteoclast cytoskeleton.[34] Binding of M-CSF or RANKL to their cognate receptors, c-Fms and RANK, or activation of $\alpha v\beta 3$, recruits phosphoinositol-3-kinase (PI3-K) to the plasma membrane, where it converts membrane-bound phosphatidylinositol-4,5-bisphosphate into phosphatidylinositol-3,4,5-trisphosphate (Fig. 4). The latter compound is recognized by specific motifs in a wide range of cytoskeletally active proteins,[35] and thus PI3-K plays a central role in organizing the cytoskeleton of the osteoclast, including its ruffled membrane. Akt is a downstream target of PI3-K and plays an important role in osteoclast function, particularly by mediating RANKL and/or M-CSF stimulated proliferation and/or survival.[34]

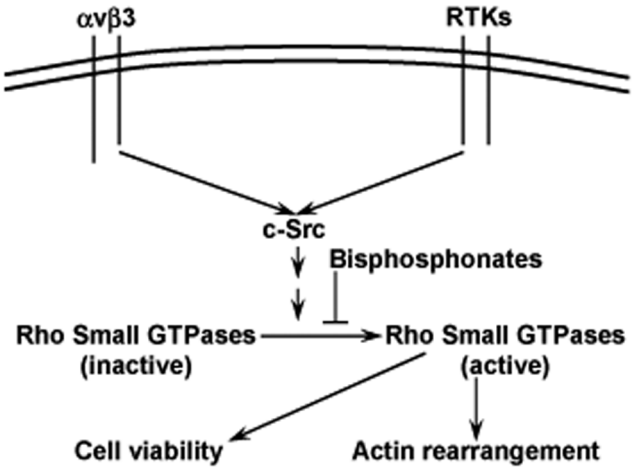


FIG. 4. Regulation and role of small GTPases in osteoclasts. Signals from $\alpha v\beta 3$ and/or receptor tyrosine kinases (RTKs) activate small GTPases of the Rho family in a c-src–dependent manner. Bisphosphonates, the potent antiresorptive drugs, block addition of hydrophobic moieties onto the GTPases, preventing their membrane targeting and activation. The active GTPases also regulate cell viability and thus bisphosphonates induce osteoclast death.[46]

Cell–Cell Interactions in Bone Marrow

Recent evidence indicates that a number of additional cell types are important for osteoclast biology in a range of situations (Fig. 5). First, as discussed previously, T cells play a key role in estrogen loss, but also are important in a range of inflammatory diseases, most notably rheumatoid arthritis[36] and periodontal disease.[37] Given the fact that both osteoclast precursors and the various lymphocyte subsets, such as T, B, and NK cells, arise from the same stem cell, it is not surprising that some of the same receptors and ligands that mediate the immune process also govern the maturation of osteoclast precursors and the capacity of the mature cell to degrade bone. This interface has given rise to the new discipline of osteoimmunology, which promises to provide important and exciting findings in the future.

Second, while it is well-established that mesenchymal cells are major mediators of cytokine and prostaglandin action on osteoclasts, it has become clear recently that cells of the same lineage, residing on cortical and trabecular bone, are the site of a hematopoietic stem cell (HSC) niche.[38] Specifically, HSCs reside close to osteoblasts as a result of multiple interactions involving receptors and ligands on both cells types.[26] Furthermore, the mesenchymally derived cells secrete both membrane-bound and soluble factors that contribute to survival and proliferation of multipotent osteoclast precursors, as well as molecules that influence osteoclast formation and function. Both committed osteoblasts and the numerous stromal cells in bone marrow produce a range of proteins both basally and in response to hormones and growth factors, resulting in modu-

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lation of the capacity of HSCs to become functional osteoclasts.

Third, cancer cells facilitate their infiltration into the marrow cavity by stimulating osteoclast formation and function. An initial stimulus is PTHrP generation by lung and breast cancer cells,[17,18,39] thus enhancing mesenchymal production of RANKL and M-CSF, while decreasing that of OPG. The resulting increase in matrix dissolution releases bone-residing cytokines and growth factors, which, feeding back on the cancer cells, increase their growth and/or survival. This loop has been termed "the vicious cycle."[18] Multiple myeloma seems to use a different but related strategy, namely secretion of MIPα and MCP-1, both of which are chemotactic and proliferative for osteoclast precursors.[40,41] The latter compound has been reported to be secreted by osteoclasts in response to RANKL and enhances osteoclast formation.[27] It seems likely further future studies experiments will uncover additional molecules mediating bone loss in metastatic disease.

Intracellular Signaling Pathways

The discussions above have not described in detail the intracellular signals by which osteoclasts are formed or those by which they degrade bone. The final major section of this review lays out the important pathways involved. Briefly, three major protein classes are involved: adaptors, kinases, and transcription factors (Fig. 6), with one significant exception, RANKL-induced release of Ca^{2+}, a pathway that activates the calmodulin-dependent phosphatase calcineurin. NFAT1c is a major substrate for this enzyme, resulting in its nuclear translocation and subsequent activation of osteoclast-specific genes. Importantly, the potent immunosuppressive drugs FK506 and cyclosporine inhibit calcineurin activity and therefore may target the osteoclast.[42]

The multiplicity of adaptors that link the various receptors to downstream signals precludes providing a meaningful summary, and so in Fig. 6 we summarize only the modulatory effects of kinases and transcription factors, which together regulate receptor-driven proliferation and/or survival of precursors. Proliferation is mediated by the surface receptors $\alpha v\beta 3$ and c-Fms,[7,43] re-organization of the cytoskeleton by $\alpha v\beta 3$, c-Fms, and RANK,[2,7] differentiation of mature osteoclasts from myeloid progenitors by c-Fms, RANK, TNFR1, and IL-1R1,[2,43,44] and their function by RANK, TNFR1, and IL-1R1.[44,45] Figure 6 and its legend provide details of the intracellular pathways involved. Not shown is the fact that multiple other cytokines and growth factors, targeting the same or other less prominent pathways or acting indirectly, probably contribute to overall control of bone resorption.[24]

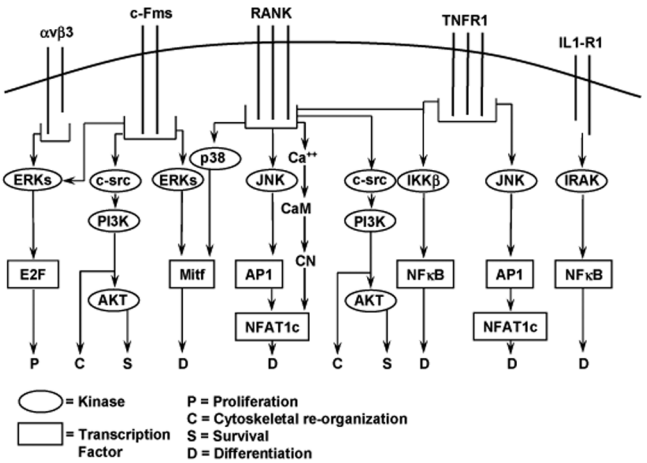


FIG. 6. Osteoclast signaling pathways. Summary of the major receptors, downstream kinases, and effector transcription factors that regulate osteoclast formation and function. Proliferation (P) of precursors is driven chiefly through ERKs and their downstream cyclin targets and E2F; maximal activation of this pathway requires combined signals from c-Fms and the integrin $\alpha v\beta 3$. As expected, given its rapid nature, regulation of the cytoskeleton (C) is independent of nuclear control but depends on a series of kinases and their cytoskeletal-regulating targets, whereas differentiation (D) is regulated largely by controlling gene expression. The calcium/calmodulin (CaM)/calcineurin (CN) axis enhances nuclear translocation of NFAT1c, the most distal transcription factor characterized to date. For further details consult the text or Refs. 2, 3, 7, 21, 34, 44, and 48–50.

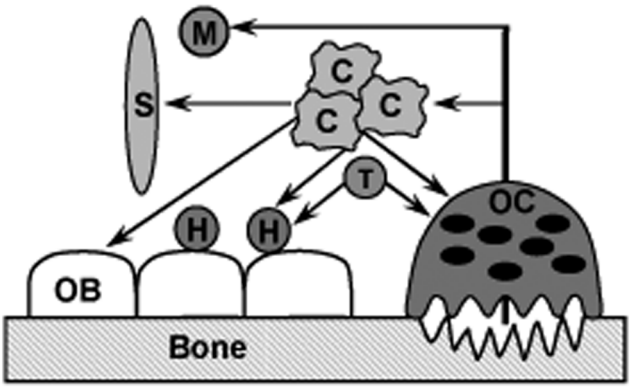


FIG. 5. Cell–cell interactions in bone marrow. Hematopoietic stem cells (H), the precursors of both T cells (T) and osteoclasts (OC), reside in a stem cell niche provided by osteoblasts (OB), which, together with stromal cells (S), derive from mesenchymal stem cells (M). Bone degradation results in release of matrix-associated growth factors (thick vertical line), which stimulate mesenchymal cells and thus bone formation. This "coupling" is an essential consequence of osteoclast activity.[47] After activation, T cells secrete molecules that stimulate osteoclastogenesis and function. Cancer cells (C) release cytokines that activate bone resorption; in turn, matrix-derived factors stimulate cancer cell proliferation, the so-called "vicious cycle."

Human Genetics

The text above might suggest that numerous mutations linked to the osteoclast are likely to have been discovered. In fact, few such genetic changes have been defined, with >50% of those reported being in the chloride channel that modulates acid secretion (Fig. 3). Sporadic reports relating to RANK are also known, while total loss of OPG in humans leads to devastating osteoporosis. For further details, see the Appendix titled "Summary of Gene Disorders of Serum Mineral Metabolism or Skeleton Formation."

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Chapter 6. Biomechanics and Mechanobiology of Bone

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INTRODUCTION

The primary responsibility of the skeleton is to provide structural support for the body. In this role, the skeleton is the basis of posture, opposes muscular contraction conveying locomotion, withstands the daily challenges of load bearing, and protects internal organs. The structural success of the skeleton, achieved through the interdependent contributions of the skeleton's morphology and mechanical properties, is determined both by genetic determinants of bone architecture and the tissue's sensitivity to mechanical signals; bone structure is enhanced by exercise or compromised by disuse. The quality of the tissue is as important to bone structure as quantity[(1)]; connectivity and orientation of trabeculae, as well as cortical bone's resistance to microdamage, helps define a bone's resistance to failure independent of a need to increase bone mass.

The success of the skeleton as a structure is jeopardized by genetic disorders such as osteogenesis imperfecta, metabolic diseases such as Paget's, or osteoporosis, the bone loss that parallels aging or menopause. Importantly, any decrease in the structural quality of the skeleton is accompanied by a proportional increase in skeletal fragility, whereas even subtle improvements in bone quality can result in significant reductions in fracture occurrence. Whereas the great majority of therapies for bone disease work through modulation (inhibition or promotion) of a specific bone cell function, the success of any particular intervention is evaluated as a biomechanical outcome—the ability to reduce the incidence of fractures. In summary, to appreciate the structural consequences of bone and mineral disorders and to understand how new interventions may ameliorate the impact of bone loss or reduced bone quality, it is essential to understand how the mechanical parameters responsible for the skeleton's structural success, how metabolic conditions and/or aging compromise these features, and how mechanical signals influence those cells responsible for achieving and maintaining the mechanical integrity of bone tissue. To appreciate the structural risks that accompany metabolic bone diseases and the manner in which the skeleton can adapt to alterations in its mechanical loading environment, it is important to review the biomechanics of bone, such as the principles of strain, stress, load, and failure and to consider the bone cell population that regulates and responds to mechanical challenges by modeling and remodeling a successful structure.

BIOMECHANICAL PRINCIPLES OF BONE

Strain

When a force is applied to any material, such as bone, it deforms. The amount of deformation in the material relative to its original length is the strain in the material. Strain is a dimensionless unit formally defined as the change in length divided by its original length ($\epsilon = \Delta L/L$). When a material is pulled, it gets longer (tensile strain), and when pushed together, the material shortens (compressive strain). Shear strain is the angle, measured in radians, through which a material has been deformed by forces acting parallel, rather than opposed, to the material. Shear strain arises when layers of a material slide against another, as might occur with torsion or bending. Peak compressive strains in bone during vigorous activity can reach as high as 3500 microstrain ($\mu\epsilon$) in compression (0.35% strain), 1000 $\mu\epsilon$ in tension, and 1500 $\mu\epsilon$ in shear (Fig. 1). Despite a wide range of activities, peak strains reached in the diaphyseal shafts of long bones are very similar across a spectrum of vertebrates.[(2)] In contrast, tensile strain in tendon might reach 25% during vigorous activity, while strain in ligaments approaches 5%.

Stress

To determine the material properties of bone (how stiff or strong it is), a machined sample is subjected to a known tensile or compressive force. Such controlled loading conditions aids in the direct comparison of the properties of one material (e.g., cortical bone from the femur of an 80-year-old woman) to another (e.g., cortical bone from a 20-year-old woman), or to facilitate comparisons between laboratories, and such tests indicate the magnitude of the force applied to a material of a defined cross-sectional area. The force per unit area is the stress (σ = Force/Area) and is reported in pascals, which are newtons per square meter (Nm^{-2}; where 1 N is the unit of force required to accelerate a mass of 1 kg 1 m/s/s). The compressive stress caused in the third metacarpal of a thoroughbred racehorse during a gallop[(3)] is on the order of 63,000,000 Pa, or 63 MPa, indicating that a force of 63,000,000N are effectively acting on 1 m^2 of bone.

Modulus

The degree to which a material deforms depends not only on the magnitude of the force and moments (turning, twisting, or rotational effect of a force is a moment, where the magnitude is the product of force times distance; M = Nm) applied to the structure, but also on the stiffness of the constituent materials. In the case of bone, stiffness is determined by the relative proportions of the hydroxyapatite crystals and the collagen fibers in the composite, as well as their organization and maturity. During the initial stages of a load:displacement test to define a bone's material properties, there is a linear increase in strain as the stress increases (Fig. 2), known as the elastic region. Should the load be removed during this phase of the test, the specimen will return to its original size and shape almost immediately, without incurring permanent damage. The linear relation between strain and stress is called Hooke's Law, which can be paraphrased as *ut tensio, sic vis*, which means, literally, *as the extension, so the force.*

The slope of the elastic region of the stress–strain curve reflects the stiffness of the material, otherwise known as the material's elastic modulus ($E = \sigma/\epsilon$). The stiffer the material, the steeper the slope of the line describing its stress–strain curve. A force of 100N pulling on a tendon will result in far greater strain (the tendon will be stretched more) than the same force pulling on a sample of bone of the same dimensions. While the stress (force per unit area) is identical, the modulus of bone is much higher than tendon (the slope of the stress–strain curve is much steeper), resulting in much less deformation in the bone tissue from the load.

A material like rubber, which has identical mechanical property values in all directions, is isotropic. A material like bone,

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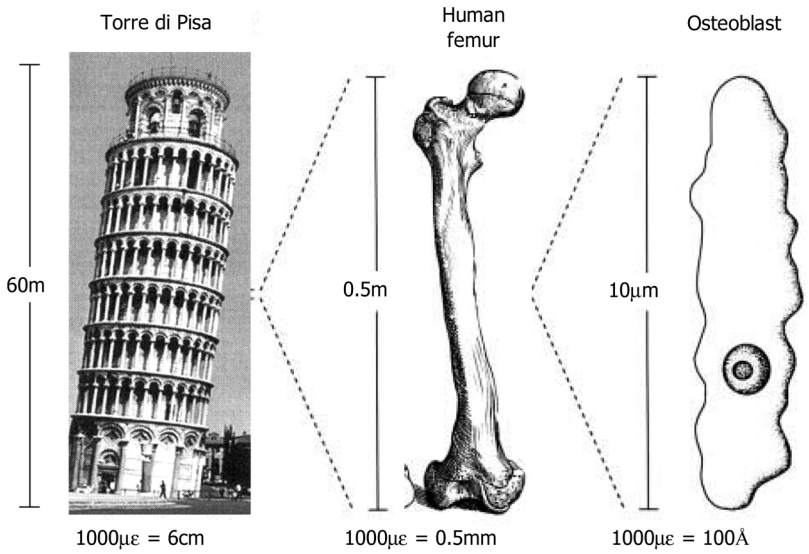


FIG. 1. Strain is defined as a (load-induced) change in length relative to the structure's original length; 1000 $\mu\epsilon$, or 0.1% strain, reflects the amount of strain experienced by bone tissue during an activity such as walking. For a structure such as the 60-m Leaning Tower of Pisa, 1000 $\mu\epsilon$ would represent a 6-cm change in length over the unloaded length of the entire structure. In a human femur, 1000 $\mu\epsilon$ would reflect a 0.5-mm change in the bone's original 500 mm length. At the level of a 10-mm bone-lining cell resting on the periosteum of that femur, the 1000 $\mu\epsilon$ induced by walking would relate to a 100-Å change in the cell's original length. The mechanisms responsible for perceiving and responding to such small biomechanical signals, whatever they may be, must be extremely sensitive.

which has distinct mechanical properties in different directions, is anisotropic. The modulus of mature cortical bone is on the order of 18 GPa in the longitudinal direction, 12 GPa in the transverse direction, and 3.3 GPa in shear, meaning it is stiffest in the longitudinal direction and relatively compliant in shear.[4] The material properties of cancellous bone are even more complex, because trabecular orientation, connectivity, and density greatly influence the stiffness, and depending on location can range from 0.1 to 3.5 GPa.[5] Reductions in the degree of mineralization (e.g., immature or woven bone), increases in porosity (e.g., osteoporosis), or damage (accumulation of microdamage) will greatly compromise the stiffness of the bone and thereby lower the elastic modulus. Under such conditions, a given load (walking up the stairs), inconsequential to the healthy skeleton, can be catastrophic to one of compromised structural quality.

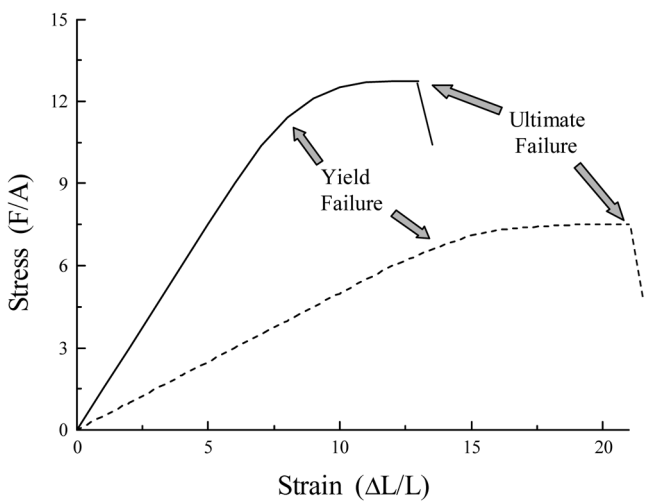


FIG. 2. The stress-strain curve of two different materials is given. In material A (solid line), the material is more stiff, as depicted by the steeper slope of the line ($E = \sigma/\epsilon$). Thus, for a given stress, there is less strain. However, the yield strain of material A is lower than that of material B (dashed line), as depicted by the point where the relationship between stress and strain is no longer linear (yield failure). As well, the ultimate strain of material A is lower than that of material B, as depicted by the point where there is catastrophic failure of the material, i.e., there is no longer any stress necessary to cause strain (ultimate failure). Material A is more brittle than material B. The area beneath the curves reflects the toughness of the materials.

Yield Failure

When the increase in strain is no longer linearly related to the applied stress, the elastic properties of the material have been superseded, and the material is unable to resume its original shape (Fig. 2). This indicates that the specimen has moved into the plastic region and that permanent damage has begun to accrue within the material. In terms of bone, yield failure first arises through ultrastructural microcracks within the hydroxyapatite and the disruption of the collagen fibrils. The yield strain of cortical bone in compression is on the order of 6800 $\mu\epsilon$, suggesting that a safety factor of 2 exists between peak strains caused by normal functional activity and the point where damage begins to accumulate.[6] The yield stress for cortical bone is ~130 MPa, which is where substantial cracking of the tissue would occur. The failure properties of bone experiencing tensile strain, defined predominantly by the collagen component of the material, is far lower than failure properties for compressive strain, where the mineral portion helps to support the load.

Ultimate Failure

As loading continues in the plastic region, the material will eventually experience ultimate failure, where the specimen fails catastrophically. The point at which the bone breaks can either be viewed as exceeding the ultimate strain (~15,000 $\mu\epsilon$ for cortical bone) or the ultimate stress (140 MPa in tension, 200 MPa in compression, and 65 MPa in shear). Because of this disparity, the nature of the failure test is very important to control, and factors that might contribute to fracture in humans are very important to consider (i.e., the contribution of bending and torsional moments during the impact of a fall). In considering a load test of bone, once in the plastic region of the material, the stress exceeds the ability of the bone to support it, and frank fracture occurs, reducing the load to zero.

Ductility

The ability of a material to withstand plastic deformation without rupture is a measure of the material's ductility, reflect-

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ing its ability to resist the propagation of cracks. A ductile material is one that can change form without breaking; a tendon is more ductile than bone because it can readily change shape and still retain its structure. A material that manages little postyield behavior before ultimate failure is considered brittle, such as would occur with glass or ceramic. While osteoporosis is often referred to as the brittle bone disease, in reality there is little experimental evidence that supports an actual reduction in the ductility of bone material examined from patients with this disease[7]; there is just less bone to resist a given force per unit area (stress during functional activity is just that much closer to yield stress). Perhaps a better example of brittle bone is provided by osteogenesis imperfecta, where a qualitative and/or quantitative deficiency in type I collagen in the material reduces its ductility,[8] condemning it to ultimate failure soon after the material has begun to yield.

Toughness

The toughness of a material is, in essence, its resistance to fracture when suddenly stressed: the ability of a femur to avoid fracture at impact, although some damage has been incurred. As revealed by the stress–strain curve (how a material of a given area deforms for a given load), the area under the curve reflects the amount of work, or energy per unit volume, possessed by the material at any given point on the curve, and a tough material can absorb a great deal of load before cracking (or failing). At ultimate failure, the area under the curve defines the energy required to break the object, or toughness. A major contributor to the toughness of cortical bone is its composite nature of haversian, circumferential, and interstitial lamellae. The analogy of a bundle of straws versus a plastic stick illustrates how the architecture of a composite anisotropic structure outperforms a single uniform isotropic material in resisting loads and avoiding yield and ultimate strain. The plastic stick breaks with relatively little bending because high strains are generated within the periphery of the material. A bundle of straws comprised of the same mass and subjected to the same bending conditions will continue to strain rather than break, as each independent element slips relative to adjacent bundles.

Bone, as an organ, has a requirement to be both stiff and tough. There is, however, an inevitable trade-off between these two attributes, because they must be attained through a balance between the resistance to crack propagation provided by collagen, cement lines, and lacunae, and the resistance to deformation provided by the mineral. Comparatively small changes in the mineral content of bone tissue can have significant effects on its properties as a material, as shown by Curry[9] in his determination of the mechanical properties of bones' with diverse functional responsibilities. By comparing the bovine femur, the deer antler, and the whale tympanic bulla, he showed that the mineral content changed to accommodate a specific functional responsibility. In the extreme, the mineral content ranged from 86% in the bulla, which requires high acoustic impedance, to 59% in the antler, which must resist high impact loads. The consequence of this high mineral content is revealed by comparing the relative toughness of these bones; the bulla is only 3% as tough as the antler.

Bone as a Composite Material

Bone's composite structure allows it to withstand compressive and tensile loads, as well as bending and torsional moments to such a degree that its overall strength approaches that of cast iron while remaining, essentially, as light as wood.[10] The inorganic phase of bone, with hydroxyapatite crystals arrayed in a protein matrix, provides the ability to resist compression. Individual calcium phosphate crystals of multiple sizes are imbedded in and around the fibrils of the collagen type I lattice.[11] Hydroxyapatite crystals, while effectively resisting compressive loads, have a poor ability to withstand tensile loads. As in concrete, a material that excels at resisting compression but is poor in resisting tension, tensile elements (e.g., steel reinforcing rods) are added to create a composite material that can cope with complex loading environments. In the case of bone, this tensile strength arises from collagen fibrils organized into lamellae.

The collagen orientation between adjacent lamellae can rotate by as much as 90°, permitting the tissue to resist forces and moments acting from several different directions, much like the added strength in plywood realized by the distinct orientation of the fibers in each specific ply.[12] While the ultrastructural organization of the skeleton is, to a certain extent, defined by the genome, the functional environment also contributes to the organization and distribution of lamellae as well the osteons that house them.[13] This directed deposition of collagen adds to the anisotropy of the bone. Given that >80% of functional strains are caused by bending (and thus a high percentage of strain is tensile), the structural quality of the bone may ultimately be determined by the quality of the collagen and the organization of the microarchitecture. Thus, the age-related deterioration of collagen would directly contribute to the declining material properties of the skeleton, just as a compromised inorganic phase of bone, which might occur under conditions such as rickets, may compromise the structural strength of bone.

Alterations in either the organic (e.g., collagen) or inorganic (e.g., hydroxyapatite) matrix components can bring about changes in bone strength. Mutations in the collagen gene give rise to several genetic skeletal problems, some of which increase fracture risk. In some forms of osteogenesis imperfecta, mutations in the primary structure of type I procollagen lead to brittle bone.[14] Another disorder of collagen resulting in excessively fragile bone is fibrogenesis imperfecta ossium[15]; a rare disease where remodeling results in a disorganized, collagen-deficient tissue. While the number of hydroxyapatite crystals contributes to the ability to resist compression, density is not everything. Fluoroapatite, which incorporates into the mineral phase of bone during fluoride poisoning, is denser than hydroxyapatite, but is brittle and shatters easily under load.[16]

Areal Properties

Areal properties, which define the overall mass and morphology of the structure, are as important in determining the ultimate success of the skeleton as the material properties. Size, density, girth, and architecture effectively describe areal properties at the gross level, such as can be seen in the 35% increase in cross-sectional area of the serving arm of the professional tennis player compared with the arm that simply throws the ball into the air.[17] Other more subtle properties are also key contributors to the structural efficacy of bone, including the long bone curvature, the geometry of the cross-sectional area, and the trabecular organization (e.g., connectivity and orientation).

Compressive loading of bone purely in the axial (longitudinal) direction results in very little strain for a given load; consider how strong a pencil is when you press straight down on the long axis of the shaft. At the same time, it is important to consider how easily the pencil is snapped when it is subject to bending. Consider now the neck of the femur while climbing a flight of stairs—the functional demands of the skeleton are very complex, and subject not only to axial conditions, but to bending and torsional moments. Recognizing the complex nature of the functional environment, it is clear that a strategy of

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minimal mass will not serve as a successful structure. Instead, the morphology of the bone must be designed to resist a wide assortment of loading conditions, perhaps to control and regulate the manner in which a bone is loaded, rather than necessarily to minimize the strain.[18]

Even the simplest of loading operations creates a complex strain and stress environment in any material, made even more complicated in isotropic materials such as bone. Axial loads applied to a slightly curved beam will cause tensile strain on the convex side and compressive strain on the concave side. The strains are greatest at these extremes, and decrease to zero in the middle of the beam. This area is called the neutral axis, where strain is essentially zero. The flexural rigidity of the material, *EI*, represents the amount of force per unit cross-sectional area required to deform the material a given amount, where *E* is elastic modulus (see above) and *I* is the second moment of area. The second moment, or moment of inertia, reflects the contribution of each bit of material to the stiffness, in each position in the cross-section of the beam ($I = \Sigma y^2 dA$, where y is the distance of each element of area A from the neutral axis). Therefore, the further the material is relative to the neutral axis, the better placed it will be to resist bending. This areal property is a powerful means of rapidly increasing flexural rigidity for a small investment of material. For example, in the elderly, it has been shown that very small increases in periosteal expansion that parallel aging will cause an increase in the second moment of inertia, making it more resistive to bending loads and may—to a certain degree—compensate for the inevitable loss of bone loss and thinning of the cortex.[19]

This brief overview of the mechanical principles of bone structure is intended to help define the architectural elements of a structure that determine the skeleton's ability to withstand loading. As important to the study of a bone quantity and quality, however, is to address the biological aspects of the bone tissue that allow it to perceive and respond to mechanical signals, permitting local adaptation to the functional loading environment to achieve an idealized structure for the demands placed on it. The next phase of this review will consider the bone cell population that coordinates a modeling/remodeling response to changes in its functional demands and the specific mechanical factors that are important in regulating this response.

MECHANOBIOLOGY OF BONE

Mechanically Responsive Bone Cells

The ability of bone cells to respond to mechanical signals, including stromal cells, osteoblasts, and osteocytes, have all been documented. However, even given the importance of bone cell sensitivity to mechanical signals, it is often difficult to isolate the critical cell responsible for orchestrating the response: for example, exposure to microgravity results in a decreased number of osteoblasts—but what cell senses and responds to the loss of gravity—the stromal cell, the differentiated osteoblast, or the interconnected osteocyte? Li et al.[20] found that marrow stromal cells change their proliferation rate and gene expression patterns in response to mechanical stimulation. With respect to osteoclast number: stromal cell expression of the osteoclastogenic factor, RANKL, is sensitive to mechanical force,[21] suggesting that the number of osteoclasts present is controlled through mechanical regulation sensed by stromal cells. As well, the osteoclast itself has been shown to respond to mechanical signals, adding another layer of control by which mechanical force may limit bone resorption.[22] Other cells present in bone, such as endothelial and smooth muscle cells in the penetrating vasculature, might also contribute to the skeleton's adaptive response to loading. After all, endothelial cells respond to shear stress and tensile strain generated by increased heart rate during exercise, by producing NO,[23] which has been shown to have pleomorphic effects on the skeleton.

Appreciation of the fact that >95% of the bone cells in the adult skeleton are osteocytes has stimulated consideration of their role in defining the mechanosensitivity of the skeleton.[24] The sequestered osteocyte, although losing size and organelles in differentiation from the osteoblast, adopts a new phenotype that allows connection to a dense network of other osteocytes and bone lining cells through cytoplasmic extensions that radiate outward from the central vascular canal. These interconnecting canaliculi may be pathways for chemical, electrical, and stress-generated fluid communication through the dense bone matrix. The antenna-like three-dimensional morphology of this osteocyte syncytium is—theoretically—ideally configured to perceive and perhaps even amplify biophysical stimuli.[25]

The volume of bone occupied by this syncytium is ~5% by the canaliculi network and 2% for the lacunae spaces. However, the connectivity of this network deteriorates markedly with age and may well contribute to the progressive loss of sensitivity of bone tissue to chemical and physical signals.[26] The surface area of the canalicular and system has been estimated to be at least 250 m^2/liter of calcified bone matrix and communicates with a submicroscopic, interfibrillar space representing 35,000 mm^2/mm^3. Thus, exchange of mineral, nutrients, and chemical and physical stimuli through this enormous network is both rapid and substantial and certainly essential to the homeostatic control of the tissue.

Mechanical Factors That Regulate the Bone Cell Response

Some component of the complex loading environment must be recognized by bone cell populations and transduced into cellular signals that regulate bone formation and resorption relative to the "form follows function" goals of the skeleton. However, which specific factors regulate the response? The loads that arise from functional activity generate strain in the bone tissue, pressure in the intramedullary cavity and within the cortices, transient pressure waves and shear forces through canaliculi, and even dynamic electric fields as interstitial fluid flows past charged bone crystals. What has been shown is that bone remodeling is sensitive to changes in strain magnitude,[27] the number of loading cycles,[28] the distribution of the loading,[29] and the rate of strain.[30] Importantly, the signal must be dynamic (time-varying) to influence the bone cell response; static loads are ignored by the skeleton, and the anabolic potential of a load regime increases when rest periods are inserted between mechanical events.[31] Even extremely low levels of bone strain, if induced at relatively high frequencies such as might arise through the spectral content of muscle contractibility,[32] have been shown to be anabolic to bone tissue,[33] enhancing not only the quantity of bone (bone mass) but quality of bone (bone strength; Fig. 3).

Whatever the mechanical factor, at the level of small volumes of tissue, all loads and bending moments resolve into strain and must be at least indirectly to deformation of the material. However, the strain levels that are actually "experienced" by bone cells in vivo is unclear and has even been proposed to be 10× that experienced by the matrix.[34] Furthermore, as bone cells are subject to flow of interstitial fluid through a pressure differential of the circulatory system as well as through applied mechanical loading,[35] they are subject to

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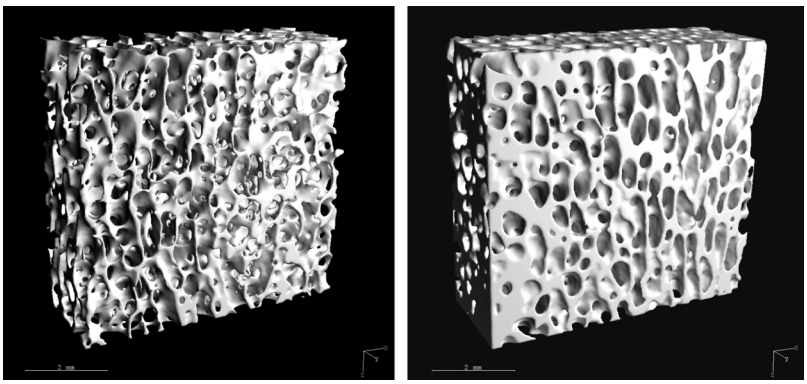


FIG. 3. μCT of the distal femur of adult (8 years old) sheep, comparing a control animal (left) to an animal subject to 20 minutes/day of 30 Hz (cycles per second) of a low-level (0.3g) mechanical vibration for 1 year.[39] The large increase in trabecular BMD results in enhanced bone strength, achieved with tissue strains three orders of magnitude below those that cause damage to the tissue. These data suggest that specific mechanical parameters may represent a non-pharmacologic basis for the treatment of osteoporosis.[40]

shear stresses and dynamic changes in pressure. In summary, the complex loading environment of the skeleton generates a diverse range of mechanical forces that are ultimately inseparable.

Using cultured osteocytes, Burger et al.[36] have shown osteocytes to be preferentially sensitive to shear strain, as opposed to hydrostatic pressure, suggesting that the osteocyte can differentiate between specific components of the mechanical milieu. Recent experiments even indicate that physiologic levels of strain actually reduce the rate of osteocyte apoptosis, suggesting matrix deformation is critical even to the survival of these cells.[37] However, it is also clear that too much strain will induce microdamage in the matrix and exacerbate the death of cells adjacent to the damaged matrix.[38] This "too much of a good thing" indicates a specific "window" where the strain signals would be beneficial to the viability of the cell population. Considering that factors other than matrix strain, per se, may be driving the adaptive response, recent work has shown that extremely low-level loading, generating strains less than 10 $\mu\epsilon$, can be anabolic to bone tissue if induced at a sufficiently high frequency.[39] Exploiting bone's sensitivity to physical signals, much in the same way as exercise can help modulate bone mass, these extremely low-level mechanical signals may provide a unique, non-pharmacologic means of preventing osteoporosis.[40] Thus, rather than deformation per se, these data support the notion that byproducts of the strain signal, such as shear stress arising from fluid flow or strain-generated potentials, may play an important role in regulating the biology of the adaptive response. However, how do the cells sense these mechanical signals?

Mechanoreceptors

Peak strains of 3000 $\mu\epsilon$ achieved during functional activity, once resolved to the level of the cell, imply deformations on the order of Angstroms (Å = 10^{-10} m, or one one-hundred millionth of a centimeter); certainly, whatever cellular system perceives these physical (e.g., mechanical, electrical) signals must be exquisitely sensitive. The ability of cells to interpret their physical environment requires that their mechanoreceptors must either be in contact with the outside, through the cell membrane and its attachment to substrate, or that the mechanoreceptor be able to sense changes in a loading-induced physical intermediary such as fluid shear on the apical membrane. While there are examples of channels that are regulated by movement of mechanosensory bristles or by tension waves,[41] a unified model of the most proximal events that lead to intracellular signal transduction in nonsensory tissues does not yet exist. Multiple candidates for various sensing paradigms have been suggested.

Alterations in ion channel activity in osteoblasts have been associated with bone cell activation, whether through alteration in conductance stimulated by PTH,[42] or by stretch/strain of the membrane or distortion of the cell.[43] Patch-clamp techniques have been used to show at least three classes of mechanosensitive ion channels in human osteoblasts.[44] Mechanically activated channels have been studied in limb bone cultures: gadolinium chloride, which blocks some stretch/shear-sensitive cation channels, was able to block load-related increases in prostacyclin (PGI)2 and NO.[45]

Membrane deformation and shear across the membrane, as well as pressure transients, are transmitted to the cytoskeleton and ultimately to the cell–matrix adhesion proteins that anchor the cell in place.[46] Integrins are membrane spanning proteins that couple the cell to its extracellular environment. A large number of additional adhesion-associated linker proteins with both structural and biochemical roles are potential molecular mechanotransducers. The architecture of the cytoskeleton itself, and its network of microfilaments and microtubules that link adhesion receptors to the cell nucleus, is such that it may also play a role in perceiving small deformations of the cell and directly informing the nucleus of mechanical challenges.[47] Rearrangement in proteins of the cell cytoskeleton can lead to adaptive responses.

Connexins are membrane-spanning proteins that form regulated channels that allow the direct exchange of small molecules with adjacent cells resulting in intercellular communication; an ideal feature considering that the loading of bone tissue may need to be spatially integrated across cell populations. Intercellular communication through gap junctions has been suggested to be the central feature of the osteocyte syncytium and critical to the transmission of information about the mechanical challenges at a given anatomic location and permit a sensing cell to control an effector at some distance removed from the mechanosensing event.[48]

Cells possess a complex organizational structure that supports compartmentalization of signals within an equally complex plasma membrane. While aspects of the plasma membrane can still be represented by the Singer-Nicholson fluid mosaic concept of a neutral lipid bilayer, this sandwich has been found to contain several phases of lipid including gel, liquid-ordered, and liquid-disordered states. The organized structure of so-called lipid rafts creates a complex association between the inner and outer membrane leaflets where transmembrane proteins are found. The organized membrane may have greater significance for mechanical response than for parsing signals arising from liganded receptors. In the vascular endothelium, for instance, increased flow causes the translocation of signaling molecules to special forms of lipid rafts, the caveolae; if caveolae are disassembled, both proximal and downstream

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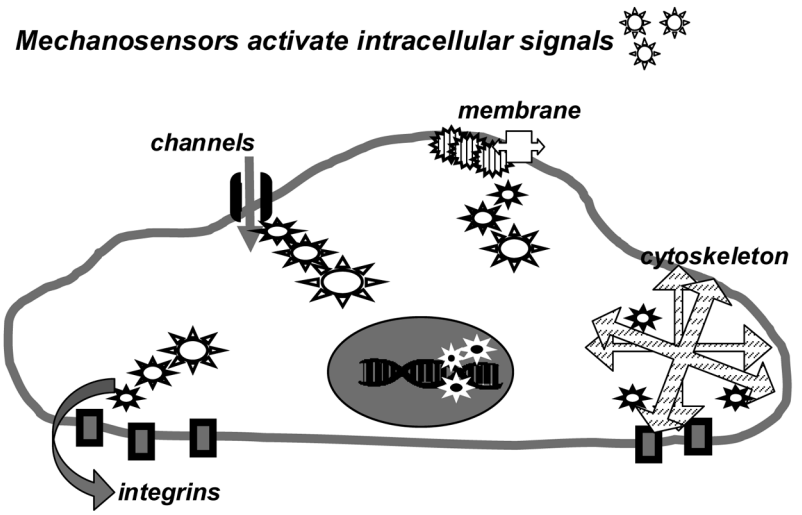


FIG. 4. Skeletal loading generates deformation of the hard tissue with strain across the cell's substrate, pressure in the intramedullary cavity and within the cortices with transient pressure waves, shear forces through canaliculi which cause drag over cells, and dynamic electric fields as interstitial fluid flows past charged bone crystals. Some composite of these physical signals interacts with the morphology of the cell through interactions such as potentiated by the membrane–matrix or membrane–nucleus structures or distortion of the membrane itself to regulate transcriptional activity of the cell.

signaling events, including activation of the mitogen-activated protein kinase (MAPK) pathway, are abrogated.[49]

With the multiplicity of mechanical signals presented to the cell, it is likely that no one mechanosensor or receptor mechanism is responsible for all of the integrated cell responses to the mechanical environment (Fig. 4). At the very least, these sensors are likely to interact with each other in integrating information that the cell receives through chemical and the mechanical signals.

Mechanical Signaling Networks

Because the distal responses to mechanical factors are similar to those elicited by ligand-receptor pairing, and result in changes in gene expression, mechanotransduction must eventually end up using similar intracellular signaling cascades. Mechanical forces have been shown to activate every type of signal transduction cascade, from increases in intracellular cAMP inositol 1,4,5 triphosphate (IP3), intracellular calcium, guanine regulatory proteins, and MAPK, detailed in a recent review.[50]

SUMMARY

An appreciation of both the biomechanical and mechanobiology of bone is critical to an improved understanding of the pathogenesis of metabolic bone disease, the potential to reduce risk factors for fracture through pharmacologic intervention, and the emerging possibility of controlling bone mass and morphology through mechanical signals. Indeed, not only does bone geometry and material properties between individuals, as defined by the genome, play a role in defining resistance to loading failures, there may be genome-specific sensitivities to mechanical loading, and thus predispose some individuals to high risk.[51] As important as inherent mechanical properties may be, it is also essential to appreciate that bone is extremely sensitive to its mechanical environment, and to a large extent, it is this functional milieu that defines skeletal morphology and ultrastructural organization postpartum. This "form follows function" aspect of skeletal tissue helps us to understand how mechanically based prophylaxes such as exercise serves as an anabolic agent to bone, and how disuse, cast immobilization, bed rest, and microgravity place the skeleton at severe risk of fracture.[52]

Clinicians, musculoskeletal scientists, and bioengineers must appreciate that the most devastating complication of bone disease is the structural collapse of the skeleton. There is every reason to believe that not only can an understanding of biomechanics help determine the pathogenesis of skeletal failure and mechanisms of treatment, but that biomechanical strategies can retard, prevent, or even reverse the structural demise of the skeleton. As importantly, scientists beginning to generate bone with molecular triggers will need to assure that the new bone is mechanically adequate to the functional demands placed on it. Skeletal science must strive to incorporate and integrate a multidisciplinary understanding of the biomechanical functions of the skeleton and consider them not only in terms of the engineering basis of the bone material, but the biological response of the bone tissue to the potent mechanical stimuli that arise from function.

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Chapter 7. Fracture Healing: The Biology of Bone Repair and Regeneration

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CELL BIOLOGY OF FRACTURE HEALING

Bone healing is unique in that, once a fracture is sustained, either by trauma or surgical osteotomy, the injured bone regenerates its original structural geometry and biomechanical integrity. The process of fracture healing is comprised of four phases: (1) inflammatory response; (2) mesenchymal stem cell recruitment and skeletal cell differentiation, leading to formation of cartilage and bone; (3) cartilage resorption and primary bone formation; and (4) secondary bone formation and remodeling in which the newly formed callus is shaped to restore the anatomical structure and support mechanical loads. It is generally accepted that the molecular mechanism(s) that control the processes of bone healing largely recapitulates those that

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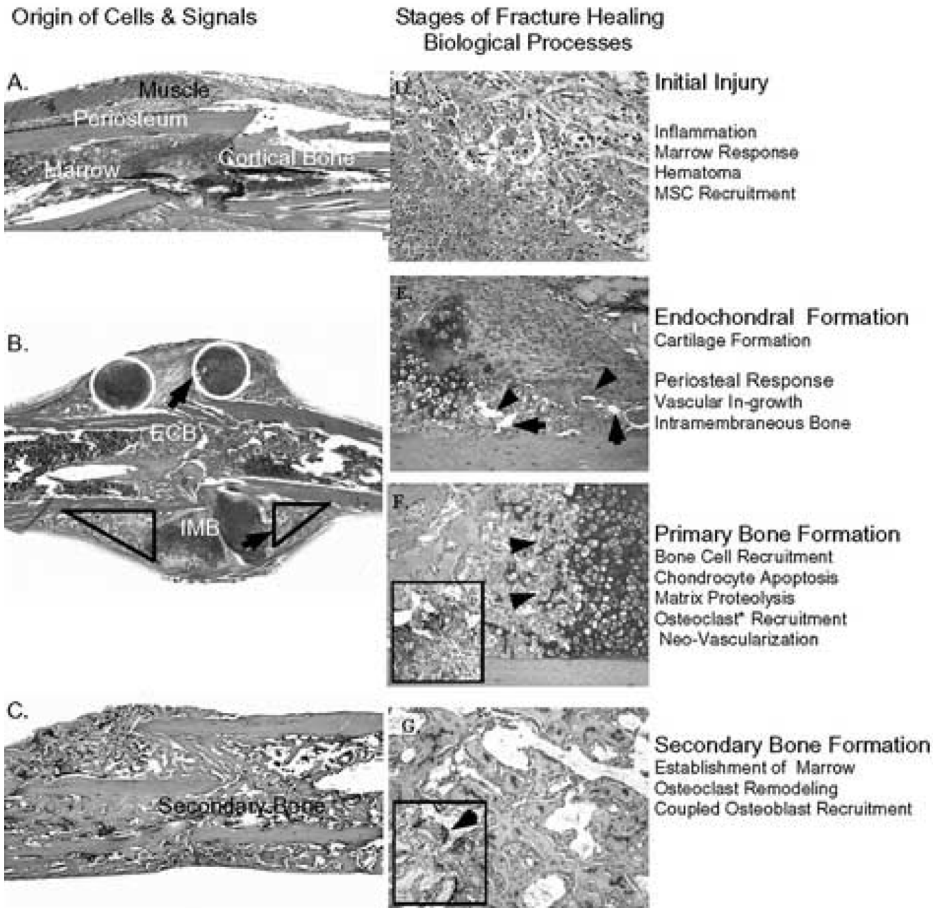


FIG. 1. Anatomic characterization of fracture repair. (A–C) Overview of the morphogenetic fields of tissue development and the proximate tissue interactions. (A) Histological section of the fracture site immediately after fracture. Potential tissue origins of mesenchymal stem cells and morphogenetic signals are denoted by the arrows and are denoted in the figure. (B) Histological section of the fracture site at 7 days after fracture. The two types of bone formation processes are denoted as endochondral bone formation (ECB) and intramembraneous bone formation (IMB). The two processes in a symmetrical manner around the fracture site. (C) Histological section of the fracture site at 28 days after fracture. Late stage of bone repair predominated by secondary bone formation and coupled remodeling. (D–G) Summary of the multiple stages of fracture healing. Histological sections are presented for each stage and a summary of the various processes that are associated with each stage. All histological specimens are from sagittal sections of mouse tibia transverse fractures and were stained with safranin O and fast green. Micrographic images are at ×200 magnification. (D) Section for the initial injury was taken from the fracture site 24 h after injury. (E) Section depicting the initial periosteal response and endochondral formation is from 7 days after injury. Arrows denote vascular in growth from the peripheral areas of the periosteum. (F) Section depicting the period of primary bone formation is from 14 days after injury. Arrows denote neovascular growth areas of the underlying new bone. Inset depicts ×400 images of an osteoclast (*chondroclast) resorbing an area of calcified cartilage. (G) Sections depicting the period of secondary bone formation are from 21 days after injury. Callus sites. Inset depicts ×400 images of an osteoclast resorbing an area of primary bone.

were operative during the embryological development of the skeleton[1] as described in earlier chapters.

Postnatal bone regeneration has unique attributes that distinguish it from prenatal skeletal development. First, inflammation and immune function play a substantial role in initiating bone healing. Second, because fracture involves disruption of a pre-existing skeletal unit, there is a structural context in which the morphogenetic factors function as defined by the pre-existing anatomy of the bone. Thus, the bone regenerates with the exact or near-exact geometric and structural attributes of the uninjured bone. Finally, the postnatal biomechanical environment is one of the most important epigenetic modifiers of tissue morphogenesis, and the rate of skeletal healing is greatly affected by the mechanical strain environment to which it is exposed. The primary factors affecting the rate of fracture repair are the underlying physiological and biomechanical conditions that either promote or retard the transition of the cells through each of the phases of healing. Therefore, a failure to transition through an individual phase of the healing process will lead to either delayed healing or nonunion.

The cell types and the structural arrangements of tissues that establish the morphogenetic fields during healing are summarized in Fig. 1. The fracture site sets up the overall spatial relationships of the morphogenetic fields during tissue regeneration. This is shown by the endochondral bone formation response involving two discrete circular-shaped centers of cartilage that form on both sides of the fracture and which taper proximally and distally along the cortices of the bone (best seen in the top middle microphotograph in A and B). Concurrently, a crescent-shaped region of intramembranous bone formation is initiated in the periosteal tissues at the proximal and distal ends of the area that taper inward toward the fracture, deep to the ring of cartilage tissue. Thus, two distinct types of bone formation processes contribute to bone healing: one around the central region of the fracture (endochondral) and one more peripheral and adja-

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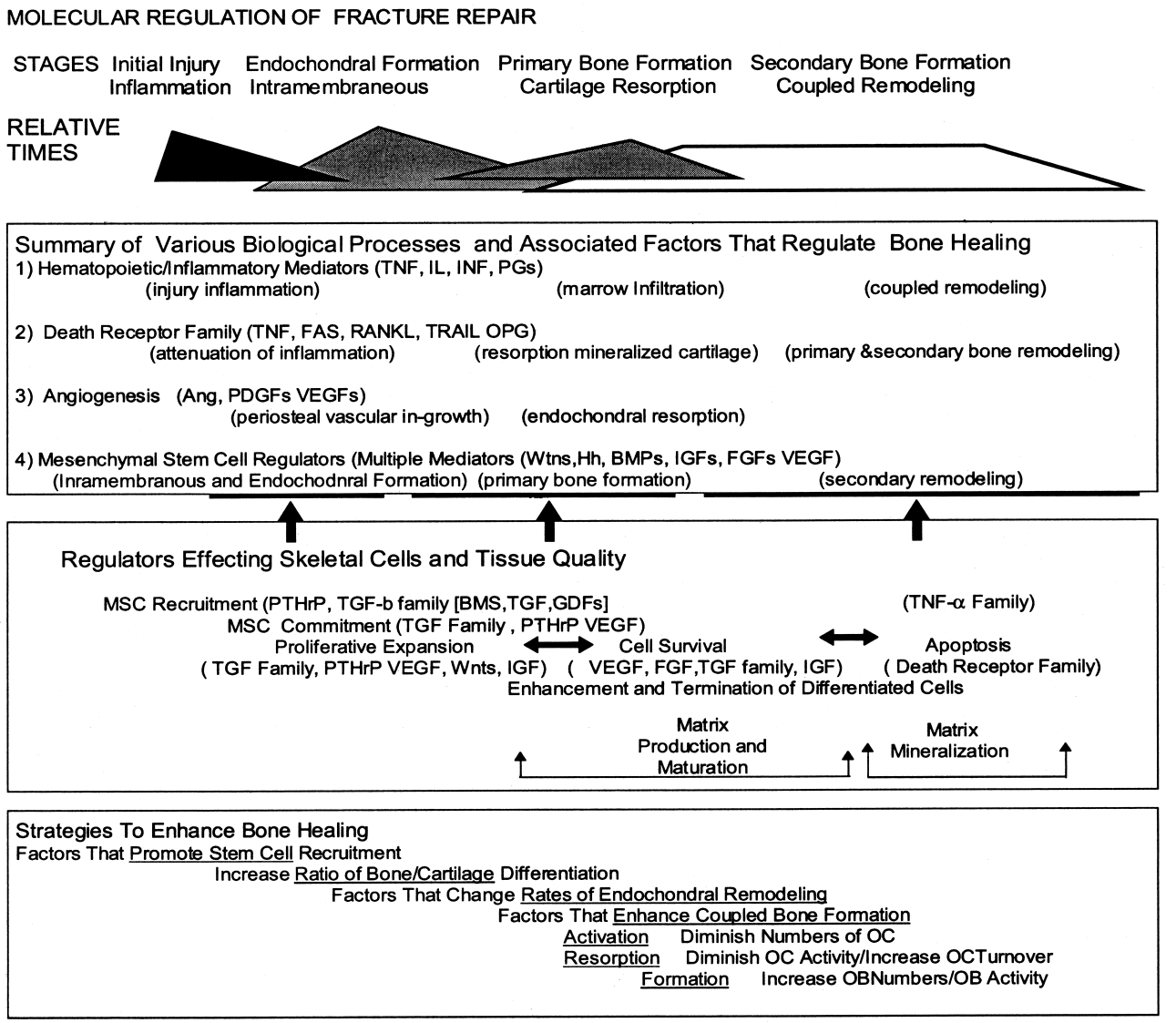


FIG. 2. Schematic summary of the stages of fracture healing and their associated molecular processes. (Top) The relative temporal and spatial scale of each of the stages of the fracture healing process are denoted by basic geometric shapes that connote both the relative intensity and time in which key stages of the healing of the molecular processes that define each of the stages. Time frames in each of the panels are matched for cross reference. (Middle) Biological processes and factors associated with each stage of bone healing. This panel denotes the basic biological processes that are occurring across the time-course and the types of individual molecular factors that regulate these processes are indicated. (Bottom) Major regulatory factors that affect skeletal cell formation and bone tissue quality. Three waves of MSC recruitment and differentiation are denoted by the corresponding lines and arrows. Each round of anabolic activity would be potentially regulated by similar temporal series of events, but the individual regulatory factors would be unique to that stage of bone healing.

cent to the cortices of bone (intramembranous). The instructive interactions initiated between cells during bone repair must occur among the external soft tissues adjacent to the fracture, the underlying cortical bone and marrow, and the developing endochondral and intramembranous tissues comprising the callus (Fig. 1A).

REGULATORY MECHANISMS OF BONE HEALING

A further understanding of the molecular mechanisms that regulate bone healing and regeneration are of immense clinical importance. The increasing interest in the use of pharmacologic modifiers of the healing processes makes elucidation of these mechanisms essential to establishing the safety of specific drugs in the context of clinical care. A schematic summary of the biological responses that contribute to bone healing, the types of factors that are involved, and the biological strategies that might be used to enhance bone healing are shown in Fig. 2. While these biological processes are presented to provide a logical framework within which to temporally and spatially relate the activities of the various factors, many of these processes overlap and are interrelated.

Hematopoietic/Inflammatory Mediators and the Role of Death Receptors During Bone Healing

Unlike the processes associated with embryologic development and postnatal growth that are regulated by ontogenetic, endocrinological, or neurological mechanisms, fracture healing after traumatic or surgical injury is initiated locally in response to regulatory mechanisms associated with inflammation and the innate immune response.[2] The hematopoietic/inflammatory

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and death receptor mediators are considered together because their activities are coordinated during repair. The collaboration of these factors' activities is also representative of three successive waves of tissue resorption/remodeling that occur during the time-course of bone healing.

Immediately after injury, there is a massive influx of macrophages and other cells associated with an innate immune response. During this period, the hematoma and necrotic tissues associated with bleeding and soft tissue injury begin to clear.[3] Descriptive studies examining the expression of various inflammatory factors have shown that prostaglandins (PGs), interleukin (IL-1), IL-6, IL-11, TNF-α, TNF-β, lymphotoxin-β (LT-β), macrophage-colony stimulating factor (M-CSF), RANKL, osteoprotegerin (OPG), and IFN-γ are all induced within the first 3 days after fracture.[2,4,5] While the importance of cytokines and small molecule mediators such as PGs are well known as they relate to immune cell function, they also play key roles in the initiation of the repair process.

One of the best studied examples showing the crucial role these factors play during bone repair concerns the role of cyclooxygenase-2 (COX-2). These studies are also illustrative of the intimate relationship between the hematopoietic elements and factors that regulate the cells responsible for skeletal tissue formation. In studies in rats comparing both a nonselective nonsteroidal antiinflammatory drug (NSAID) and a COX-2 selective drug, impaired fracture healing was observed with a greater effect seen for the COX-2 selective drug.[6] Moreover, in studies of mice homozygous for a null mutation in the *COX-2* gene, induced prostaglandin production was shown to be essential for the optimal mesenchymal cell differentiation during skeletal repair.[6,7] The histology of the fractures in the COX-2-null mice showed a persistence of undifferentiated mesenchyme and a marked reduction in osteoblastogenesis resulting in a high rate of nonunion. In a number of retrospective human studies examining the effects of NSAIDs in various clinical situations of bone healing, some negative effects were observed. Finally, it is interesting to note that recent studies have focused on the selective use of PG receptor agonists that have been used to specifically promote bone healing.[8]

At later stages of fracture healing, the major functions of the hematopoietic/inflammatory and death receptor mediators are associated with two periods of tissue remodeling. The first occurs at the end of the endochondral phase at which time mineralized cartilage is removed and primary bone formation takes place. The second occurs after woven bone has been laid down and remodeling is required to restore the injured bone to its original shape and structure. The role of TNF-α and its receptors has been the focus of a number of studies because this cytokine is both a major inflammatory mediator and has been implicated in bone remodeling. While it is acutely expressed at very high levels immediately after injury, its expression is largely absent during the initial periods of endochondral differentiation. However, when chondrocyte hypertrophy and mineralized cartilage resorption begins, its expression reemerges.[4] During this same period, the key regulators of osteoclastogenesis, RANKL, OPG, and M-CSI, increase in expression. Two of these, RANKL and OPG, are also members of the TNF-α superfamily. RANKL, OPG, and M-CSF become elevated.[4,5] Once mineralized cartilage resorption is completed and woven bone formation has occurred, a subsequent phase of bone resorption takes place in which IL-1 and IL-6 increase in their expression but OPG, M-CSF, and RANKL levels decline. Indeed the role of interactions of hematopoietic/lymphopoietic and osteogenic microenvironments in regulating the homeostatic balance of bone remodeling is emerging as a major area of research interest and changes in cytokines that alter lymphopoiesis have been shown to affect bone homeostasis as well as immune function.[9]

The death receptors (TNF superfamily of cytokines) are the central arbitrators of a tissue's microenvironment by promoting the survival of one population of cells, yet destining another to undergo apoptosis. After tissue injury, this family of cytokines plays many crucial roles in all aspects of bone healing. During the initial immune response induced by tissue injury, TNFR1 and Fas mediate activation-induced cell death of macrophages, T cells, and B cells and thereby regulate the timing of the innate and acquired immune responses.[10] The pathological manifestations of inappropriate control of the apoptotic processes and a failure to initiate programmed cell death in one or another population of immune cells may trigger autoimmune disease.[11]

The death receptor family has been shown to play a pivotal role in the regulation of many different types of developmental processes. During postnatal tissue repair, these cytokines may also replace the function of other morphogenetic factors that act during embryogenesis.[12] Alternatively, these factors may act to initiate postnatal repair or regenerative processes that are not operative during embryological development. It is also now well established that chondrocytes undergo apoptosis during normal endochondral development and during arthritic disease.[13] Currently three members of the TNF family of cytokines have been implicated, including Fas ligand, TNF-α and TRAIL.[14,15] Treatment of human articular chondrocytes with Fas ligand in vitro causes apoptosis and the Fas system has been shown to be present in both growth plate chondrocytes and during endochondral development during fracture healing.[14,16] In previous studies, chondrocytes in fracture callus[16] have been shown to express Fas, and articular chondrocytes in joints have been shown to undergo programmed cell death in response to TNF-α.[5,14] The relationship between the apoptotic process and the normal progression of endochondral development is observable in data that have now been gathered from many naturally or genetically engineered defects that affect growth cartilage development. The hallmark of almost all of these defects is either a foreshortening or expansion of the growth plates. One such example is the expansion of the growth plate in genetically engineered mice deficient in matrix metalloproteinase (MMP)-9.[17] It is interesting to note that such basic defects in mineralized cartilage removal as in seen in the absence of MMP-9 are also seen in during fracture healing. This show how processes that are operative during development do indeed recapitulate themselves during postnatal skeletal repair.[18]

Processes of Angiogenesis in Bone Repair

Vascularization of the growth plate is one of the underlying mechanisms that couples chondrogenesis and osteogenesis. As discussed above, essential components of the terminal stages of chondrogenesis are chondrocyte hypertrophy, apoptosis, and the recruitment and activation of chondroclasts that resorb the mineralized cartilage matrix allowing for the formation of primary bone by osteoblasts. Morphological evidence suggests that chondrocyte apoptosis occurs readily after the invasion of endothelial cells[13,19] and that chondrocyte death is induced by diffusible factors arising either from the vasculature or hematopoietic elements brought in during angiogenesis.[20]

The intimate relationship between the in growth of vascular tissues and the differentiation and development of skeletal tissues has made the role of angiogenic mediators a central focus of many studies of bone healing. Fracture healing, as well as any tissue repair process, results in increased blood flow to the surrounding tissues. Thus, it may be speculated that the initial interactions of the vascular elements and the initiation and propagation of the periosteal response are the primary driving mechanisms that facilitate intramembranous bone formation. It has also been suggested that periovascular mesen-

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chymal cells that exist in blood vessel walls may contribute to this process.[21] Functional demonstration of the importance of vascularization during fracture repair has been shown in several recent studies suggesting that treatment with broad spectrum angiogenic inhibitors completely prevents fracture healing and the formation of callus and periosteal woven bone.[22] In contrast, treatment of fractures with vascular endothelial growth factor (VEGF) improves bone healing, leading to a more rapid regain of mechanical strength and increased mineralization of the callus.[22]

While a large focus has been placed on the role of VEGF, angiogenesis seems to also be regulated by angiopoietin-dependent signaling. Interestingly, both Ang 1 and Ang 2 have been localized to bone cells during development,[23] distraction osteogenesis, and fracture healing.[24,25] In studies of murine fracture repair or distraction osteogenesis, Ang 1 and Ang 2, as well as their Tie receptors, were shown to be expressed throughout the healing time-course concurrent with both VEGF-A and VEGF-C. These regulators of angiogenesis showed expression throughout the chondrogenic phase of healing and reached maximal levels of expression during the late phases of endochondral remodeling and bone formation. Unlike the VEGF family that promotes new vessel formation through the stimulation of endothelial cell division, Ang 2 promotes blood vessel destabilization and regression in the absence of VEGF-A or basic fibroblast growth factor (FGF).[26] Because recent findings have suggested that Ang 2 in the presence of VEGF will promote new vessel formation both by inducing remodeling of capillary basal lamina and stimulating endothelial cell sprouting and migration,[26] the predominant expression of Ang2 during bone repair suggests a similar role in this process.

It is interesting to note that the angiogenic, hematopoietic, and death receptor mediators show collaborative interactions. Studies of fracture repair in mice deficient in TNF-α receptor–dependent signaling show greatly diminished vascular endothelial growth inhibitor (VEGI) expression. VEGI, in fact, is a death receptor ligand that interacts with death receptor 4 and is the primary regulator of the progression of vascularization. It plays a dual role in the maintenance of growth arrest of endothelial cells in G0/G1 interfaces while inducing apoptosis in any cells that enter the S phase.[27,28] Taken together, these results suggest that after injury, existent vessels are first dissociated into a pool of nondividing endothelial cells through the actions of Ang 2 and VEGI. At the time endochondral remodeling is initiated, VEGF levels rise and VEGI levels fall, stimulating cell division and allowing endothelial cells to contribute to neoangiogenic processes.

Regulators of Cell Physiology and Bone Quality

While the roles of these regulatory factors may influence several essential tissue repair processes, there is a group of factors that are specific in their actions to enhance cellular recruitment, proliferation, and differentiation (Fig. 2, middle). Indeed, they may be the most important anabolic factors in bone development because they ultimately determine the spatial organization of the skeleton and thereby control tissue structure and material properties.

The TGF-β superfamily of proteins, which includes TGF-β (1, 2, and 3), the bone morphogenetic family of factors (BMPS), and the growth and development factors (GDFs), seems to be involved in the overall recruitment and differentiation of mesenchymal stem cells. The PTH/PTH-related protein (PTHrP) and Hedgehog (Hh) families of factors primarily regulate committed chondrocyte differentiation and the progression of endochondral bone formation, although PTH, when administered intermittently, also seems to have an anabolic effect through its actions on coupled remodeling. The Wnt, FGF, and IGF families, each by different mechanisms, all primarily control the proliferative expansion of the different mesenchymal stem cell populations.

The BMP family is perhaps one of the most extensively studied groups of morphogenetic factors involved in bone healing. After Urist's landmark demonstration that the implantation of demineralized bone at extraskeletal sites induces de novo formation of cartilage and bone,[29] investigators have sought to understand the osteoinductive activity of demineralized bone matrix (DBM), and this culminated in the cloning and sequencing of individual BMPs.[30] The subsequent expression of BMPs in recombinant systems permitted their use in a variety of animal models and led to the demonstration of their abilities to enhance fracture healing and the repair of skeletal defects repair.[30–32] At this time, both rhBMP-7 (OP-1) and rhBMP-2 have been approved for use in patients to enhance bone repair in specific clinical settings.

Despite numerous studies showing that exogenous BMP may enhance fracture healing, our understanding of their role in the normal physiology of skeletal repair and regeneration remains incomplete. Several studies have attempted to define the role of endogenous BMPs in normal fracture healing. Using RT-PCR amplification, Nakase et al.[33] were the first to show the temporal and spatial distribution of BMP-4 during bone repair. Recently, Cho et al.[34] has shown that specific members of the TGF-β super family, including the BMPs, may act in combination to promote the various stages of intramembranous and endochondral bone formation observed during fracture healing. Taken together, these studies suggest that the coordinated expression of multiple BMPs and their receptors during fracture healing is important in both skeletal development and repair. Further study is needed to determine the critical roles of the individual BMPs within this coordinated network of fracture healing.

The roles of PTH and PTHrP as autocrine/parcrine regulators of endochondral bone formation during axial skeletal development may be of particular importance in bone healing.[35] Studies examining PTHrP during long bone growth and in various human pathologies have shown that the PTH/PTHrP receptor mediates the effects of Indian hedgehog and controls the progression of terminal hypertrophic chondrocyte differentiation.[36] These studies suggest that PTH signaling plays an essential role in a variety of mechanisms of bone formation including endochondral ossification.

Although the effects of PTH are usually associated with bone resorption, the response of osteoclasts to PTH are most likely mediated through osteoblastic activity, and the receptors for PTH are found in the cell membranes of osteoblasts.[37] Indeed, while continuous exposure to PTH leads to an increase in osteoclast number and activity, low intermittent doses stimulate osteoblasts and result in increased bone formation in humans.[38] Thus, the characteristic effects of this hormone on the skeleton, which are produced primarily by its amino terminal portion (1-34), are anabolic with respect to bone. These finding led to clinical trials showing that intermittent PTH treatment increases BMD and reduces the risk of vertebral and nonvertebral fractures in postmenopausal women.[39,40]

The approval of PTH(1-34) as an anabolic treatment for osteoporosis provided the major impetus to examine PTH in various models of bone healing. Several recent reports have examined effects on fracture healing with doses ranging from 10 to 200 μg/kg in rat models, and all showed significant increases in both mechanical and histological properties of the calluses.[41–43] In other studies, using models of impaired bone metabolism, PTH analogs were shown to reverse the inhibition of bone healing observed in ovariectomized rats[44] and rabbits treated with corticosteroids.[45] Finally, one report showed that PTH(1-34) increased bone in growth and pull-out strength in porous metallic implants.[46] The one drawback of most of these studies was that

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doses of this hormone were much higher than those which would be tolerated in humans. In one of the earliest studies using low intermittent daily subcutaneous injections of 10 μg/kg of recombinant human PTH(1-34) [rhPTH(1-34)] administered over a 28-day period of fracture healing, a larger cartilaginous callus was observed but no delay in chondrocyte differentiation was seen during fracture healing.[43] Such findings clearly suggested that these low doses could have a positive effect on fracture healing. Subsequently, studies using the recombinant human PTH [sqb]PTH(1-34); teriparatide; Forteo] at doses that were pharmakinetically comparable with those used in patients were carried out.[47] In this study, multiple time-points starting after a 21-day course of PTH treatment were assessed. Significantly improved healing as assessed by biomechanical testing and μCT was seen at the end of the endochondral period of repair. By later time-points, even the lowest dose treatment groups showed significant increases in BMC, BMD, and total osseous tissue volume. While dosing was discontinued on day 21, these data show that daily systemic administration of low-dose PTH(1-34) enhanced fracture healing by increasing BMD and BMC and produced a sustained anabolic effect throughout the remodeling phase of repair.

EFFECT OF TISSUE STRUCTURE AND FRACTURE SITE STABILITY

As noted above, one of the most unique features of bone healing is that is that it is a regenerative process such that the original geometry and internal architecture of the tissue is restored. It follows that there must be some underlying relationship among the gradients of morphogenesis that promote the developmental processes, the original anatomy of the tissue, and the biomechanical demands imposed on the injury repair mechanisms. One of the most obvious characteristics of the repair phenomenon is the resultant symmetry of bone reparative tissue around the fracture site. It may be speculated that the signals responsible arise either from the marrow or are released from the injured bone matrix. Indeed, how the injury itself influences these responses in the tissues may be of considerable relevance because the field of inflammatory signals is propagated from the point of origin of the injury.[4]

The role of the biomechanical environment in guiding the bone repair processes has received attention. Studies in which bending and shear loading were introduced at an osteotomy site were shown to selectively drive chondrogenesis versus osteogenesis.[48] Other studies have similarly shown that mechanical instability leads to persistence of cartilage tissue at the fracture site by upregulating molecular signals such as Indian hedgehog that control chondrogenesis.[49] It has also been shown that the time-course of healing and whether the injured bone goes on to union are also greatly affected by initial shear motion at the fracture site and the stability of fixation.[50] Finally, there are considerable data suggesting that some aspects of mechanical stimulation promote healing.[51]

The structural geometry of callus development might also be dependent on the muscular anatomy and vascularity of the tissues. The question of how the morphogenetic fields are established and how biomechanical factors drive both tissue differentiation and the geometry of the callus are of considerable importance in identifying the molecular signals induced by the fracture and relating them to the origins of skeletal stem cells that conduct the repair. The answers to these questions may help to determine how present and future therapeutic compounds can be introduced at the correct time and for the appropriate duration.

FUTURE PERSPECTIVES

It is now well established that multiple morphogenetic factors regulate skeletal development and the technological advances that have made these factors available for the treatment of fractures have already had important clinical impact. However, the use of some of these factors has had mixed success, and none have been shown to uniformly promote healing. Indeed, the major objective in the enhancement of fracture healing is a regain in biomechanical competency of the bone. This involves much more than simply promoting stem cell recruitment, proliferation, and differentiation. It also requires restoration of the appropriate skeletal geometry and tissue material properties. Hence, future directions involve identifying novel factors to promote repair and the appropriate times and durations in which to introduce them. Finally, while there has been considerable focus on the factors themselves, it is now clear that their activation and association with receptors and signal transduction pathways is key aspect to their success. Understanding and correcting the challenges of human host responsiveness to growth-promoting factor is perhaps the most important future direction of fracture healing research.

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