# Chapter 1
# Bone Structure, Development and Bone Biology

Fayez F. Safadi, Mary F. Barbe, Samir M. Abdelmagid, Mario C. Rico, Rulla A. Aswad, Judith Litvin, and Steven N. Popoff

**Keywords** Runx-2 (runt-related transcription factor 2) • cbfa-1 (core binding factor alpha1) • Pebp2aA (Polyoma enhancer binding protein 2aA) • Osterix • cleidocranial dysplasia • osteopontin • Leptin • osteoblast specific factor-1, N-syndecan • osteoblast/osteocyte factor-45 (OF45) • dentin matrix protein 1 • fibroblast growth factor 23 • sclerostin • Sclerosteosis • SOST gene • osteocyte • osteoclastogenesis • parathyroid hormone • 1, 25 dihydroxyvitamin $D_3$ • transforming growth factor alpha • epidermal growth factor • tartrate resistant acid phosphatase • osteoprotegerin • integrins • integral membrane proteins • fibronectin • collagen type I • bone sialoprotein II • osteopontin • suppressor of cytokine signaling-1 • osteoclast-associated receptor • apposition • growth plate • drosophila • sarcolemma • myofilaments • motor end plate • somites • skeletogenesis • osteoactivin • biglycan • decorin • calcitonin • calcitriol • bone morphogenetic proteins • connective tissue growth factor

## Introduction

The skeleton serves as an internal structural support system for vertebrates. It has mechanisms to grow and change in shape and size to suit varying stressors including the ability to resist the mechanical forces. In addition, bone is a major source of inorganic ions, and actively participates in the body's calcium/phosphate balance.

Bone tissue is continuously formed and remodeled throughout life. Initially, the bone achieves its increase in size and shape through *growth* (increase in size) and a complicated process known as *skeletal modeling*. In late childhood and adulthood there is continuous renewal of the skeleton via a process termed *remodeling*. Both modeling and remodeling *require* two separate processes namely bone resorption and bone formation to *occur simultaneously* to be effective. This requirement is known as "coupling".

**Overview** Bone forms the skeletal framework of all vertebrates. It is a composite tissue consisting of organic matrix, inorganic minerals, cells, and water. Bone is formed by the hardening of this matrix entrapping osteoblasts which then become osteocytes.

The inorganic portion of bone matrix is composed mainly of crystalline calcium phosphate salts, present in the form of hydroxylapatite. This allows bone to serve as a reservoir of calcium and phosphate that can be stored or mobilized in a controlled fashion. Bone also contains carbonate, fluoride, acid phosphate, magnesium, and citrate. Hydroxyapatite crystals also form in tissues that are not normally calcified, including in atherosclerotic plaque, in soft tissues of some patients with abnormally high circulating calcium or phosphate, and in articular cartilage of some patients with degenerative joint diseases. Crystals in these situations are often distinctly larger.

The organic component of bone matrix comprises 40% of the dry weight of bone. Most of the organic component is Type I collagen, which is synthesized intracellularly as tropocollagen and then exported as collagen fibrils. Pathological disorders of the bone matrix exist, such as *osteogenesis imperfecta*, a disorder caused by a defect in Type I collagen. This defect results in less organized bone with loss of normal osteon structure. With loss of normal osteons, which function to withstand deformation, the bone fails (fractures) with only minimal amounts of force. In addition to collagen, bone matrix is composed of proteoglycans, glycoproteins, phosopholipids and phosphoproteins, as well as various growth factors including osteocalcin, osteonectin, and bone sialoprotein.

Bones are fashioned in the form of a hollow tube or a bilaminar plate of bone, each commonly termed *compact bone*. Additionally, the architecture is strengthened by internal "struts" of *trabecular bone* that follow the lines of stress. Trabecular or cancellous bone is a metabolically active component of bone and has about nine times greater turnover than the outer compact bone. This kind of design is known in engineering terms as "composite" and allows bone to take

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advantage of the strength of components. This type of design also allows bone to resist mechanical compression and able to deform significantly before failing (i.e. breaking).

## Part 1 Bone Structure

## Macroscopic Features of Bone

At the gross level, bone can be broadly categorized into five types: long bones (femur, tibia, ulna and radius), short bones (carpal bones of the hand), flat bones (skull, sternum and scapula), irregular shaped bones (vertebra and ethmoid), and sesamoid bones (bones embedded in tendons). These bones form through different mechanisms during embryonic development. The long bones form by endochondral mechanisms, while the flat bones form by intramembranous mechanisms. These processes are discussed later in this chapter. Both long and flat bones are organized with a hard, but relatively thin, outer region composed of dense, *compact* bone called the *cortex or cortical bone*. Inside the cortex is the marrow cavity containing hematopoietic elements, fat and spicules of bone. The bone spicules are also referred to as *trabecular, spongy*, or *cancellous* bone (Fig. 1).

### *Types of Bones*

*Long-bones:* Macroscopic examination of long bone shows two extremities (*epiphysis*) and a cylindrical tube in the middle (*diaphysis*) and a transitional zone between them (*metaphysis*) (Fig. 2). In growing long bone, the epiphysis and the diaphysis originate from independent ossification centers and are separated by a layer of cartilage, termed the *epiphyseal* or *growth plate* (see more details later in this chapter).

*Short bones:* Carpal and tarsal bones of the hand and foot, respectively, are examples of short bones. These bones are typically cube-shaped, and have only a thin layer of compact bone surrounding a spongy (trabecular bone) interior.

*Flat-bones:* Excellent examples are the bones of the skull, which consist of inner and outer tables of compact bone with spongy (trabecular) bone (diploe) between them.

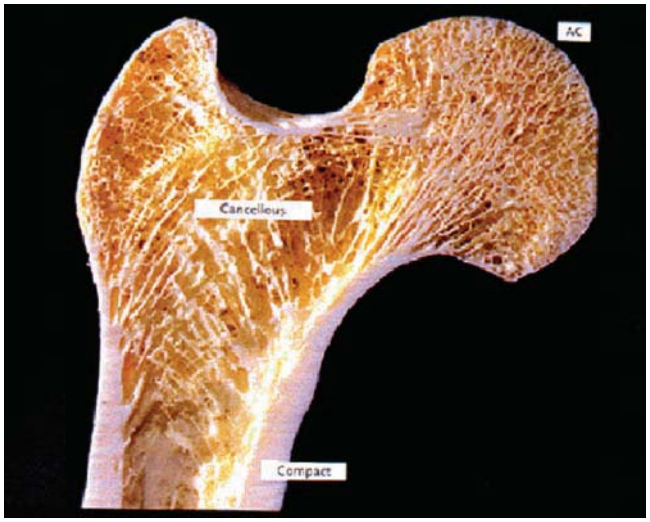


**Fig. 1** Adult long bone. Sagittal section through long bone showing the internal structure of the bone. Note the outer dense compact bone (also called cortical bone) and the inner cancellous bone filled with spicules (trabeculae), these latter small bundles of bone traverse the inner substances of bone and are usually interconnected with one another.

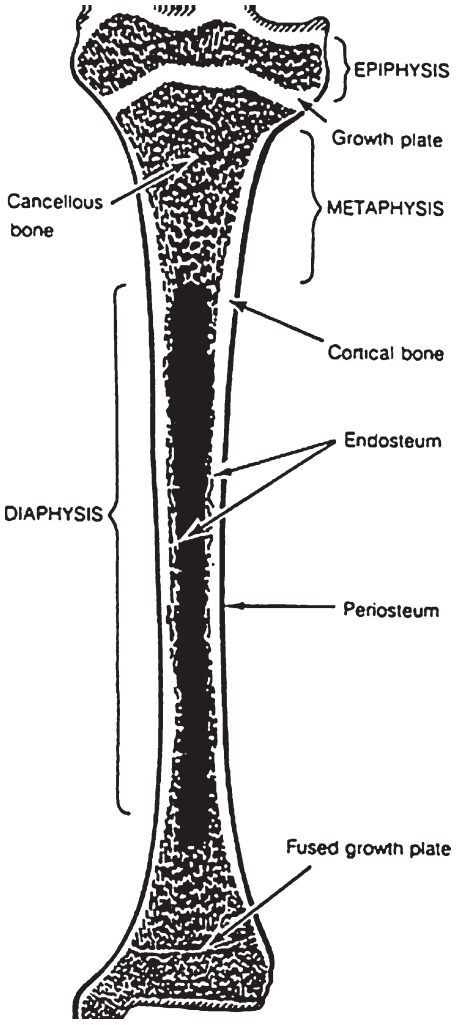


**Fig. 2** Schematic diagram of a tibia. The interior of a typical long bone showing middle diaphysis, a growing proximal end (epiphysis) with a still active epiphyseal growth plate and a distal end with the epiphysis fused to the metaphysis. The diaphysis (shaft) of a long bone contains a large marrow cavity surrounded by thick-walled tube of compact bone. A small amount of spongy bone lines the inner surface of the compact bone. The proximal and distal ends, or epiphyses, of the long bone consist of spongy bone with a thin outer shell of compact bone. The outer surface of the bone is covered by a fibrous layer of connective tissue called the periosteum.

*Irregular bones* consist of an outer thin layer of compact bone covering an inner region of spongy bone. The scapula is an example of an irregular bone.

*Sesamoid bones*: These bones are a subtype of short bones that are embedded in tendons. The patella and pisiform are examples of sesamoid bones.

## Individual Bone Structure

*Cortical (compact) bone* refers to the dense hard, calcified bone that forms the hard outer "shell" of bone that surrounds the marrow cavity (Figs. 1, 2). This type of bone has few gaps or spaces. In the adult, cortical bone (or the cortex) is composed of dense aggregations of lamellar type bone (see below). Compact bone also contains within it Haversian (2) and Volkmann canal systems for vascular supply (Fig. 3). Cortical bone is surrounded externally and internally by a *periosteum* and *endosteum*, respectively.

*Epiphysis:* This term refers to the end of a tubular bone, lying between the epiphyseal (growth) plate (in developing bone) and the articular cartilage (Fig. 2). In adults, the growth plate is absent. The place it is thought to have occupied is arbitrarily selected to define the portion referred to as the epiphysis. The epiphyses consist mostly of spongy bone inside a thin sheet of dense bone.

*Physis or Epiphyseal Plate:* This term refers to the growth plate in children (before cessation of growth). Injuries or other disruptions such as infections can seriously affect the subsequent size and shape of the bone. For example, increased vascularity around the epiphysis, occurring during the repair process after a fracture at this site, can cause elongation of the limb. Inflammatory destruction of the physis may cause shortening while its partial destruction may cause an angular deformity. Fractures of the physeal plate have been classified based on the amount and kind of disruption caused. This is the basis for the Salter-Harris classification *(3)*.

*Metaphysis:* This refers to the widened portion of bone occupying the area between the cylindrical diaphysis and the physis/epiphysis (Fig. 2). Remodeling and modeling defects in this region are frequent in conditions such as multiple osteochondromatosis. Several tumors have an epicenter in the metaphysis.

*Diaphysis (shaft):* This refers to the middle, cylindrical portion of a tubular bone (Fig. 2). There is a thick cortex surrounding a marrow space.

*Bone Marrow:* The medullary cavity is filled with varying proportions of hematopoietic marrow, fat and trabecular bone. The marrow is most prevalent in younger age groups and in the metaphyseal region of long bones. The diaphysis contains mainly fat in adults. In comparison to the appendicular (limb) skeleton, the axial skeleton has a greater proportion of bone marrow.

*Periosteum:* The periosteum is a thick fibrous membrane that covers the entire surface of a bone, with the exception of the articular cartilage. It is composed of an *outer fibrous* layer and an *inner cambium* (cellular) layer. The outer layer is a connective tissue layer containing fibroblasts as well as nerves and blood vessels supplying the underlying bone. The inner cambium layer contains osteoprogenitor cells capable of forming new bone, and is thus an osteogenic layer. When tendons insert into bone, the collagen fibers (*Sharpey's fibers*) pass through the periosteum and then into the bone lamellae. The Sharpey's fibers contribute to the appositional growth of bone (see intramembranous bone formation).

*Endosteum:* The endosteum is composed of a resting layer of marrow at its interface with bone. This is not a morphologically recognizable layer of tissue at the light or electron microscopic level. However, it is a convenient concept which exists to explain the functional changes seen in physiologic and pathologic alterations in bone.

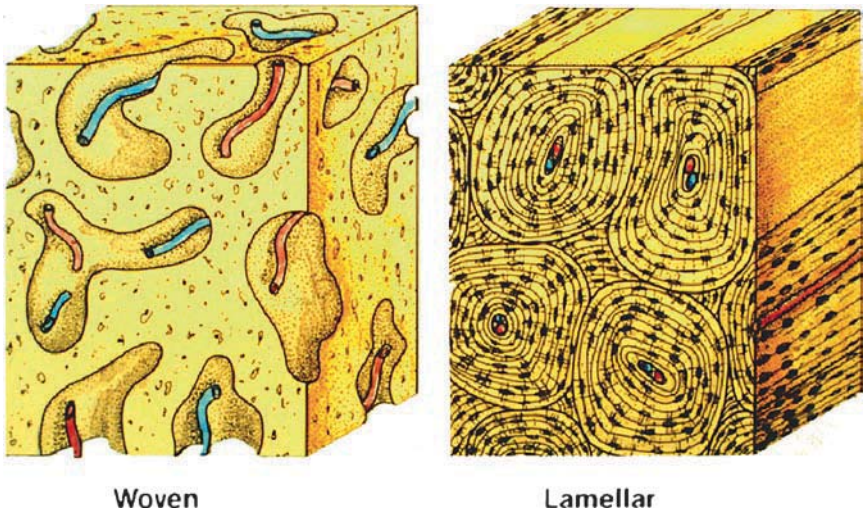


**Fig. 3** Diagram of immature and mature bone. Immature (woven) bone displays a disorganized lamellar appearance because of the interlacing arrangement of collagen fibers. The cells (osteoblasts and osteocytes) tend to be randomly arranged, whereas the cells in the mature bone are organized in circular fashion that reflects the lamellar structure of the Haversian system. Resorption canals in mature bone have their long axes in the same direction as the Haversian canals.

*Trabecular bone*, also called *spongy* or *cancellous* bone, consists of slender spicules and trabeculae of bone that are separated by marrow spaces. Trabecular bone fills the interior of long bones, the metaphyseal region of long bones, and epiphyseal ends of bones. Trabeculae form a network of rod- and plate-like elements that act as scaffolding for the marrow cavity, lighten bone, and allowing room for blood vessels and marrow. The spicules of trabeculae usually consist of several lamellae of bone tissue.

## Microscopic Features of Bone

Bone tissue can be classified based on collagen fiber arrangements into two different types: woven bone and lamellar bone. To repeat from above, bone is also classified into compact bone and trabecular bone.

*Woven Bone:* This form of bone consists of randomly oriented collagen fibers, with large numbers of osteoblasts and osteoprogenitor cells alongside (Fig. 3). Under polarized light, it has a haphazard structure which is in great contrast to lamellar bone (see below). Woven bone contains relatively more cells per unit area than mature bone. Although woven bone is the major bone type in the developing fetus, and lamellar (mature) bone is the major bone type in the adult, areas of immature bone are also present in adults, especially where bone is being remodeled. Areas of woven bone are also seen regularly in the alveolar socket of the adult oral cavity and where tendons insert into bones. Except for the above examples, woven bone is generally considered pathologic if seen in adults. It occurs in regions of rapid growth, such as in the growing skeleton especially in the embryo, fracture callus, fibrous dysplasia, areas of remodeling osteosarcoma, and several other tumors. The molecular signals that are required to trigger woven bone synthesis are thought to include platelet derived growth factor (PDGF A and B), insulin like growth factor (IGF I and II) and perhaps others. It is likely that a combination of growth factors may be required (for more details see growth factors in this chapter).

*Lamellar bone:* Lamellar bone is the mature form of adult bone. It is readily identified on polarized light microscopy as parallel lines of deposited bone (Fig.3). Studies have shown that lamellar bone has a well-organized arrangement of collagen fibers. Lamellar bone is formed when the rate of deposition is slow. In general, it is formed only on pre-existing bone, either woven or lamellar. The control mechanisms involved in the formation of lamellar bone are still under investigation.

Secondary organization is a hallmark of lamellar bone. In the cortex, the lamellae are arranged in *circumferential* as well as tubular arrangements (Figs. 3, 4). The tubular arrangement is called an *osteon* (Fig. 4). Under the microscope, these tubes can look like circles or parallel sheets depending on how they are sectioned during histologic preparation. The central part of the tube is the Haversian canal (Figs. 4, 5), which contains blood and lymphatic vessels and nerves. The osteons play an important role in the mechanical properties of cortical bone, since the long axis of an osteon is parallel to the long axis of a long bone. Each osteon acts as a fiber that resists failure (fracture) with deformation (stretch).

*Types of Lamellae:Outer circumferential lamellae* are several lamellae that lie next to the periosteum and are oriented parallel to it. *Inner circumferential lamellae* lie next to the endosteum (Fig. 5). The circumferential lamellar bone resists compressive forces. The *interstitial lamellae* are remnants of previous concentric lamellae (Fig. 5).

*Haversian systems (osteons) and Volkmann's canals:* These are cylindrical units of 5 to 15 concentric lamellae, which surround a central *Haversian canal*. Each lamella is several microns in thickness and its fibers run in a spiral fashion around the canal. The Haversian canal contains capillaries, venules, lymphatic vessels, and a loose connective tissue containing osteoprogenitor cells. Since the Haversian systems are arranged around branching blood vessels, it is easy to see how the Haversian systems comprise a *branching system* of cylinders that are oriented in the long axis of the bone. *Volkmann's canals* are vascular channels that connect Haversian canals to each other as well as connect the Haversian system with the blood vessels in the periosteum. Canaliculi containing the processes of osteocytes (see below) are largely arranged in a radial pattern with respect to the canal. The system of canaliculi that opens to the Haversian canal also serves for the passage of substances between the osteocytes and blood vessels.

### *Compact versus Trabecular Bone*

*Compact bone:* To summarize from above, compact bone usually consists of *concentric lamellae* arranged into Haversian systems (osteons), *interstitial lamellae* between the Haversian systems, and *inner and outer circumferential lamellae* (Figs 4 and 5). Located in spaces between these lamellar are bone cells called *osteocytes* (see below and Fig. 5). Because of this organization, compact mature bone is also called lamellar bone.

*Trabecular bone:*This type of bone refers to the spongy spicules of bone found within the marrow space and is also called spongy, cancellous or medullary bone (Fig. 1 and 6). Each spicule of trabecular bone is composed of several lamellae and is usually not more than 0.2-0.4 mm in thickness to allow for diffusion of nutrients to the osteons. If they were thicker, they would need osteonts in order to insure adequate vascular perfusion (Fig. 6). In trabecular bone the lamellae are normally arranged in a longitudinal fashion, and osteons are usually not formed.

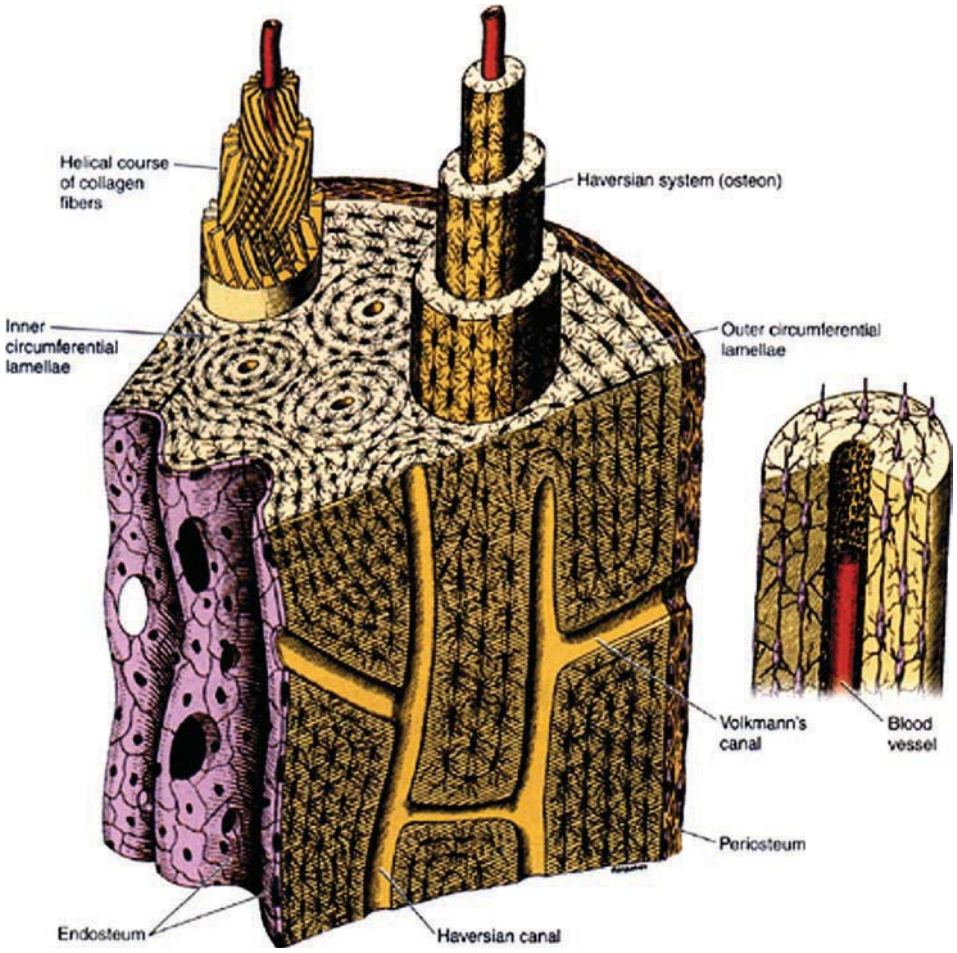


**Fig. 4** Schematic drawing of the cortical bone. Sectioned cortical bone showing the tubular and circumferential arrangement of osteon. In the center of each osteon is a canal, called the Haversian canal. Each Haversian canal contains blood vessels, nerve endings and lymphatic vessels.

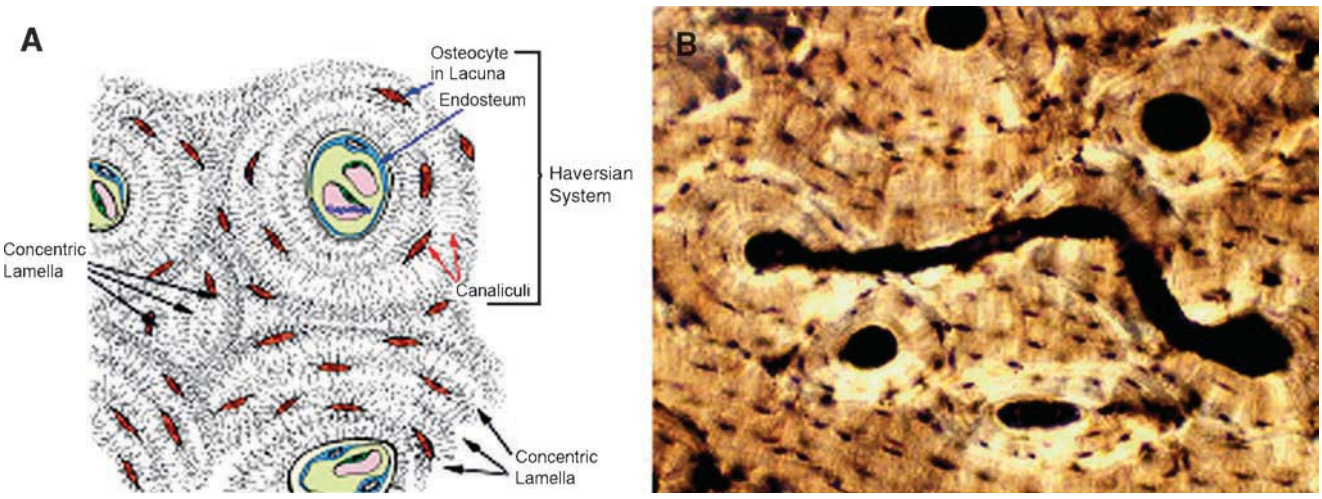


**Fig. 5** Cartoon and a microscopic photograph depicting the lammelar organization of bone. **A**. Cartoon representing a Haversian system (osteon) with interstitial lamellae, osteocytes and canaliculi (cellular processes). **B**. Histological representation of Volkmann's canal connecting Haversian canals of adjacent osteons.

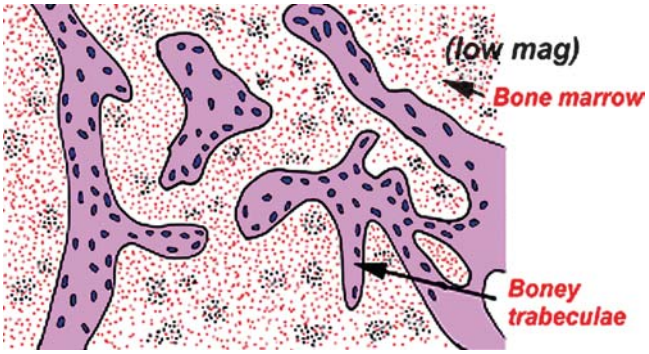


**Fig. 6** Cartoon of spongy bone. Spongy bone (also called trabecular bone) is composed of trabeculae surrounded by bone marrow. Each trabecula consists of lamellar bone, osteocytes and mineralized matrix. Bone marrow fills the space between trabeculae.

Separating the trabeculae from the marrow is an endosteum. Under electron microscopy, the endosteal layer has a rich supply of osteoclasts and osteoblasts. Trabecular bone is more metabolically active than compact bone. Consequently, metabolic bone studies are best carried out on this component of bone. Radiologic studies (such as dual and single energy computerized tomography (CT) scan methods as well as micro-CT developed for the study of osteoporosis) have devised methods to exclusively study the trabecular component and to exclude the cortex. Some amount of success has been achieved using these approaches with the ability of obtaining a "region of interest" by modern computer software. Microscopically this is done using bone histomorphometry with image analysis software such as Bioquant Osteo (www.bioquant.com), Osteomeasure (www.osteometrics.com), and SkyScan CTAn (www.skyscan.be).

### *Bone Matrix*

The organic component of bone makes up 40% of the dry weight and is composed of collagen, proteoglycans, glycoproteins, phosopholipids and phosphoproteins. The inorganic component makes up the remaining 60% of the dry weight of bone and is composed primarily of calcium hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$. At an ultrastructural level, bone is organized to maximally resist applied mechanical forces. Calcium hydroxyapatite crystals are arranged parallel to collagen fibers (59). This orientation maximizes the collagen's resistance to tensile (stretch) forces and the calcium hydroxyapatites resistance to compressive forces. Diseases characterized by abnormal bone collagen content result in weak bone matrix and lowered abilityof the bone to resist mechanical forces. A more detailed discussion on the bone matrix is given below in Part III, the section on Bone Biology.

### *Blood Supply of Bone*

Bone has a rich vascular supply. It receives 10-20% of the cardiac output. Blood supply varies with different types of bones. Blood vessels are especially rich in areas containing red bone marrow. The extent to which the periosteal and endosteal vascular supplies meet the metabolic needs of bone is controversial. Some authors feel that the periosteal and endosteal supplies are able to meet the needs of the outer and inner halves of the cortex. Others contend that the periosteal supply is able to meet only the ends of the outer third of the cortex. The question however is an important one, especially in situations of operative fracture repair and in the surgical technique of bone elongation called distraction osteogenesis.

## Vasculature and Nerve Supply in Long Bones

The diaphyseal nutrient artery is the most important supply of arterial blood to a long bone. One or two principal diaphyseal nutrient arteries first pass through the cortical bone obliquely. These arteries then divide into ascending and descending branches and supply the inner two thirds of the cortex and medullary cavity. There are also numerous metaphyseal and epiphyseal arteries supplying the ends of bones. They arise mainly from the arteries that supply the adjacent joints. They anastomose with the diaphyseal capillaries and terminate in bone marrow, cortical bone, trabecular bone, and articular cartilage. In growing bones, these arteries are separated by the epiphyseal cartilaginous plates. Finally, periosteal arterioles are vessels that supply the outer layer of cortical bone.

### *Arterial Supply of Large Irregular Bones, Short Bones, and Flat Bones*

These bones receive a superficial blood supply from the periosteum and frequently from large nutrient arteries that penetrate directly into the medullary bone. These two arterial systems anastomose freely.

### *Venous and Lymphatic Drainage of Bone*

Blood is drained from bone through veins that accompany the arteries and frequently leave through foramina near the articular ends of the bones. Lymph vessels are abundant in the periosteum.

### Nerve Supply of Bones

Nerves are most rich in the articular extremities of the long bones, vertebrae, and larger flat bones. Many nerve fibers accompany the nutrient blood vessels to the interior of the bones and to the perivascular spaces of the Haversian canals. Accompanying the arteries inside the bones are vasomotor nerves, which control vascular constriction and dilation. The periosteal nerves are sensory nerves, some of which are pain (nociceptive) fibers. Therefore, the periosteum is especially sensitive to tearing or tension. Nerve endings have also been demonstrated adjacent to bone trabeculae and in proximity to bone cells.

Bones are also innervated by sympathetic fibers. In the upper limb, the sympathetic fibers destined for bone originate from the sympathetic ganglion. In the lower limb, selective peripheral neuroctomy studies revealed that the sympathetic nerves, with reference to the tibia, descend in the sciatic nerve, and thereafter principally in the medial popliteal nerve, and enter bone alongside the nutrient vessels (4).

### Neurotransmitters and Bone

Neurotransmitters released by these nerve endings within bone are the subject of increasing interest in the field of bone biology since they appear to have a role in bone formation and remodeling. For example, mice homozygous for deletion of the dopamine transporter gene DAT (-/-) demonstrated reduced bone mass and strength. Cancellous bone volume in DAT (-/-) proximal tibial metaphysis was significantly decreased with reduced trabecular thickness. The ultimate bending load (femoral strength) for the DAT (-/-) mice was 30% lower than the wild-type mice. Thus, deletion of the DAT gene resulted in deficiencies in skeletal structure and integrity (5).

### Cannabinoids and Bone

Recent studies have shown that the endogenous cannabinoid system plays a role in regulating bone remodeling. The endogenous cannabinoids bind to and activate two G protein-coupled receptors, the predominantly central cannabinoid receptor type 1 (CB1) and peripheral cannabinoid receptor type 2 (CB2). Whereas CB1 mediates cannabinoid psychotropic and analgesic effects, CB2 has been implicated recently in the regulation of liver fibrosis and atherosclerosis. Genetically engineered mice null for CB2 receptors showed accelerated age-related trabecular bone loss and cortical expansion, although cortical thickness remains unaltered. These changes are reminiscent of human osteoporosis and may result from differential regulation of trabecular and cortical bone remodeling. The CB2 null mouse phenotype is also characterized by increased activity of trabecular osteoblasts (bone forming cells), increased osteoclast (bone resorbing cell) number, and a markedly decreased number of diaphyseal osteoblast precursors. CB2 is expressed in osteoblasts, osteocytes, and osteoclasts (6). A CB2-specific agonist enhances endocortical osteoblast number and activity and restrains trabecular osteoclastogenesis, apparently by inhibiting proliferation of osteoclast precursors and receptor activator of NFκB ligand expression in bone marrow-derived osteoblasts/stromal cells. This same agonist attenuates ovariectomy-induced bone loss in animals and markedly stimulates cortical thickness through the respective suppression of osteoclast number and stimulation of endocortical bone formation. These results demonstrate that the endocannabinoid system is essential for the maintenance of normal bone mass by osteoblastic and osteoclastic CB2 signaling. Hence, CB2 offers a molecular target for the diagnosis and treatment of osteoporosis, and other bone-loss associated diseases (6,7).

## Bone Cells

The cells important in bone biology are osteoblasts, osteocytes and osteoclasts. Osteoblasts are the primary cells responsible for bone formation (osteogenesis) and mineralization, while osteoclasts are primarily responsible for bone resorption. Osteoblasts and osteocytes are derived from mesenchymal stem cells, while osteoclasts are derived from hematopoeitic stem cells and are related to monocyte/macrophages.

### Osteoblasts

Osteoblasts originate from mesenchymal stem cells that have the potential to proliferate and the capacity to differentiate into several connective tissue cell types. These pluripotent mesenchymal cells can differentiate into osteoblasts, chondroblasts, bone marrow stromal cells, fibroblasts, muscle cells or adipocytes depending on the nature of the stimulus within their local microenvironment. Given the appropriate stimuli to differentiate into osteoblasts, they will first give rise to osteoprogenitor cells, cells still capable of proliferating yet committed to the osteoblast lineage. Osteoprogenitor cells can be found in the inner layer of the periosteum, the endosteum lining marrow cavities, osteonal (Haversian) canals, perforating (Volkmann's) canals, and in perivascular tissue adjacent to bone. Osteoprogenitor cells can also be found in

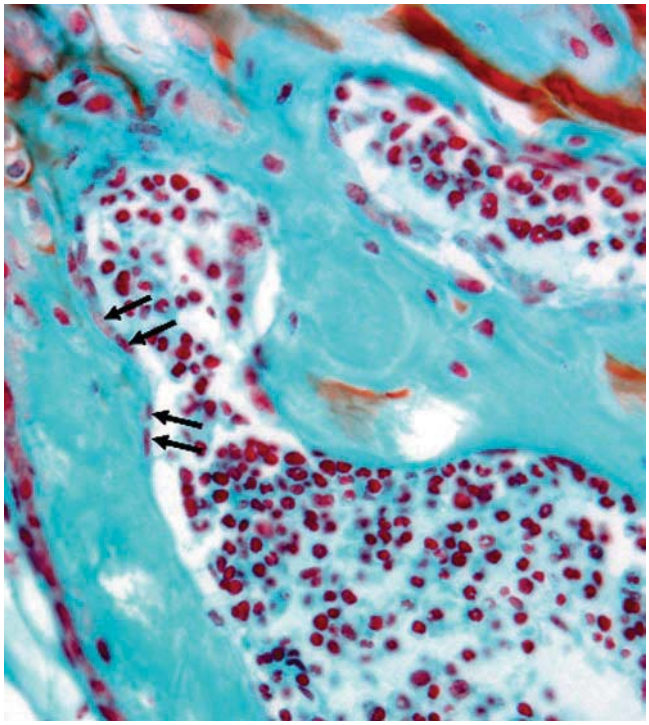

**Fig. 7** Bone lining cells. Histological microphotographs of femurs stained with Saffranin-O and counter stained with Goldner stain showing metaphyseal bone with bone lining cells (black arrows). These cells are inactive but can redifferentiate into active, bone forming osteoblasts in response to the appropriate stimuli.

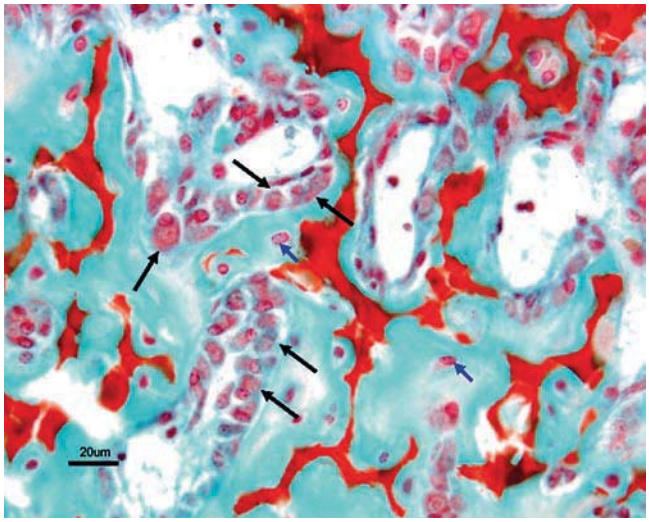


**Fig. 8** Osteoblasts lining trabecular bone surface. Photomicrographs of metaphyseal trabecular bone, showing active cuboidal osteoblasts lining the trabecular bone surfaces (black arrows). Note also some osteocytes encased within the bone matrix (blue arrows).

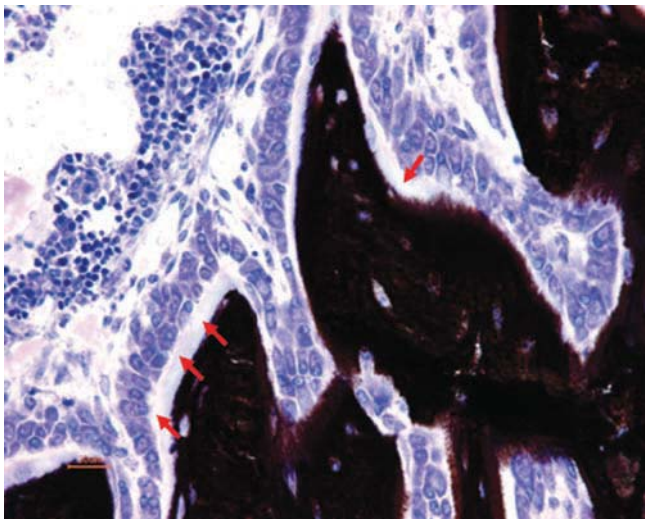

**Fig. 9** Osteoid synthesis by active osteoblasts. Photomicrographs of undecalcified metaphyseal trabecular bone stained with von Kossa (for mineral, black stain) and toluidine blue. Note the osteoid, unmineralized bone matrix (red arrows), compared to the mineralized matrix (black).

the bone marrow where they are indistinguishable from marrow stromal cells to which they are related.

Osteoblasts are generally cuboidal or columnar in shape, and are found lining bone surfaces at sites of active bone formation such as during bone development (see below) or fracture repair (Fig. 8). Osteoblasts are responsible for the production of type I collagen and the proteoglycans (glycosoaminoglycans) that largely comprise the organic component of bone matrix, also known as osteoid. Osteoid can be visualized using specific stains as shown in Fig. 9. Osteoblasts are also involved in the subsequent mineralization of osteoid via the liberation of matrix vesicles and the deposition of calcium and phosphate (8,9). Osteoblasts are joined by adherens type junctions, including desmosomes and tight junctions. Cadherins are transmembrane proteins that are integral to these adherens junctions and function to join cells through their cytoskeleton.

The phenotypic characteristics of osteoblasts depend on their stage of differentiation. Ultrastructurally, osteoblasts are typical protein producing cells with an extensive amount of rough endoplasmic reticulum, a large Golgi apparatus and numerous mitochondria. Alkaline phosphatase enzyme activity is one of the earliest markers of the osteoblast phenotype. In addition to the production of type I collagen and proteoglycans, osteoblasts also produce a variey of other non-collagenous proteins including osteocalcin, osteopontin, bone sialoprotein and osteonectin (see below). These proteins are also markers of the osteoblast phenotype and each has a unique temporal pattern of expression during osteoblast differentiation. Osteoblasts secrete a variety of cytokines and colony stimulating factors (CSF), such as interleukin-6, interleukin-11, granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF), and

thus play a role in myelopoesis. Osteoblasts also secrete numerous growth factors including transforming growth factor-beta (TGFβ), bone morphogenetic proteins (BMPs), platelet derived growth factors (PDGFs), and insulin-like growth factors (IGFs). Mature osteoblasts possess receptors for parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D, two hormones that play important roles in regulating bone metabolism and mineral homeostasis (see below). Osteoblasts also secrete receptor activator of nuclear factor kappa B (RANK) ligand, a protein that plays an essential role in the differentiation of osteoclasts (see below).

Constant mechanical stress is essential for the maintenance of bone mass and strength, which is achieved through the cooperative functions of osteoblasts, osteocytes and osteoclasts. Osteoblasts respond to mechanical stimuli to mediate changes in bone size and shape. This effect may be modulated by a piezo-electric effect of the calcium hydroxyapatite crystals (see section on mechanosensory systems and stretch studies).

Bone lining cells are flattened, squamous cells found lining bone surfaces in areas where there is no active bone formation. They are particularly prevalent in adult and aging bone where many of the bone surfaces are inactive. These cells can be thought of as quiescent osteoblasts and are similar to osteoprogenitor cells in that they can be reactivated to become functional osteoblasts under conditions that warrant active bone formation such as during remodeling, fracture repair and in certain types of bone pathology (Fig. 7).

## Regulation of Bone Formation and Osteoblast Differentiation

Osteoblast differentiation is regulated by numerous secreted growth factors including transforming growth factor-β (TGFβ), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and others (see matrix proteins of bone below) (10,11). Furthermore, various transcription factors also play important roles in osteoblast differentiation. Runx-2 (runt-related transcription factor 2)/cbfa-1 (core binding factor alpha 1) and osterix are essential transcription factors for osteoblast differentiation (12,14). Experimental animal models with targeted deletion of the Runx-2 gene demonstrated that mice develop to term but have a skeleton consisting exclusively of cartilage that does not ossify. There is no evidence of osteoblast differentiation or bone formation in these mice. In addition, Runx-2 null mice lack osteoclasts (see regulation of osteoclast differentiation). Although null mutations for Runx-2 have not been identified in humans, mutations in the Runx-2 gene cause a disease known as cleidocranial dysplasia (CCD) (12,14). CCD is characterized by hypoplastic clavicles and delayed ossification of cranial sutures. Runx-2 target genes include osteocalcin, bone sialoprotein, osteopontin and collagen α1 (15,16). Osterix is another transcription factor that was more recently shown to play a role in osteoblast differentiation presumably by acting downstream of Runx-2 (13).

Leptin is a peptide synthesized by adipocytes with binding affinity to its receptor in the hypothalamus. This protein regulates bone formation via a central mechanism. Mice deficient for leptin or its receptor have considerably higher bone mass than normal wild-type mice. It has been shown that patients with generalized lipodystophy (absence of adipocytes and white fat) exhibit osteosclerosis and accelerated bone growth (17). Although details of the leptin-hypothalamic control mechanism are not fully understood, additional experimentation on the role of the central nervous system and the regulation peripheral leptin production will enhance our understanding of how the leptin-hypothalmic axis regulates bone metabolism.

Other transcription factors, secreted proteins and receptors have been reported to have significant effects on osteoblast differentiation and bone formation. Many of these effects were identified or confirmed in studies involving genetically modified animals (for list of these animal models refer to Table 1).

Due largely to the mineralized matrix of bone, it has proven difficult to study osteoblasts experimentally in vivo. Many studies of osteoblast differentiation and function utilize organ or cell culture systems. Some studies employ primary osteoblast cultures in which osteoblasts are enzymatically digested from the endocranial surface of neonatal rodent (rat and mouse) calvaria. Primary cultures of human osteoblasts are generally established by enzymatic digestion of osteoblasts from the surfaces of cancellous bone samples. When cultured under conditions that favor the osteogenic lineage (i.e. ascorbic acid and β-glycerophosphate supplemented media), many of the cells in these primary cultures differentiate into mature osteoblasts capable of producing a mineralized bone matrix. One major drawback to these cultures is the heterogeneity of cells that can be isolated from calvaria or cancellous bone resulting in significant contamination of non-osteogenic cells as well as heterogeneity in the stage of differentiation of the osteogenic cells. To eliminate the potential heterogeneity of cell types inherent in primary culture systems, some investigators have utilized osteosarcoma cell lines. However, transformed cells may not behave the same as non-neoplastic cells prompting questions about the physiological relevance of data generated using transformed osteoblast cell lines. Another approach has been to establish permanent cell lines from primary osteoblast cultures such as the widely used MC3T3-E1 cell line derived from mouse calvaria. There have also been many studies of osteogenic differentiation from less differentiated (osteoprogenitor) stromal or

**Table 1** Skeletal phenotype of selected genetically engineered mice

| Gene Name | Skeletal phenotypes of the null, mutant and transgenic animals |
|---|---|
| *Runt-Related Transcription Factor 2; Runx2, Cbfa1* | **Null mice:** Complete lack of ossification of the skeleton. Mice died just after birth (14) and showed marked changes in tooth morphogenesis (312). Transgenic mice: Normal skeleton at birth but developed an osteopenic phenotype thereafter (313). |
| *Secreted Phosphoprotein 1; Spp1, Osteopontin* | **Null mice:** Normal and viable with altered wound healing (314). These animals also showed resistance to ovariectomy-induced bone resorption (315). These knockout animals demonstrated an increase in ectopic calcification especially in the medial layer of the arteries (316). |
| *Gamma-Carboxyglutamic Acid Protein, Bone; Osteocalcin* | **Null mice:** Develop higher bone mass and bones of improved functional quality. Histomorphometric studies showed an increase in bone formation without impairing bone resorption (317). |
| *Secreted Protein, Acidic, Cysteine-Rich; Osteonectin* | **Null mice:** After six months of age, the animals developed severe osteopenia, cataracts, rupture of the lens capsule and accelerated closure of dermal wounds (318). These animals also have greater deposits of subcutaneous adipose tissue (319). |
| *Connective Tissue Growth Factor; CTGF* | **Transgenic mice:** Just a few months after birth the animals showed dwarfism, decreased bone density, alterations in endochondral ossification and affected fertility (202).<br>**Null Mice:** CTGF deficiency leads to skeletal dysmorphisms as a result of impaired chondrocyte proliferation and extracellular matrix composition within the hypertrophic zone (204). |
| *Thrombospondin I; Tsp1* | **Null mice:** Abnormal curvature of the spine, lung abnormalities and increase in the number of circulating leukocytes (320). |
| *Thrombospondin II; Tsp2* | **Null mice:** Increase cortical bone thickness and density and abnormal long bleeding times (321). |
| *Thrombospondin III; Tsp3* | **Null mice:** Young adult TSP3-null mice are heavier than controls, and analyses of the geometric and biomechanical properties of long bones show increases in the moments of inertia, endocortical and periosteal radii, and failure load (322). |
| *Transcription Factor Sp7; Osterix* | **Null mice:** Absent bone formation and no deposition of bone matrix in these animals (13). |
| *Parathyroid Hormone; PTH* | **Null mice:** These animals showed reduced cartilage matrix mineralization and decreased metaphyseal osteoblasts and trabecular bone (323). |
| *Parathyroid Hormone-Like Hormone; PTHrP* | **Trangenic mice:** Chondrocyte-specific overexpression of PTHrP causes a profound delay in the developmental program of chondrocyte differentiation and endochondral ossification (324). |
| *Sry-Box 9; Sox9* | **Tissue specific null mice:** Inactivation of Sox9 in limb buds before mesenchymal condensations resulted in a complete absence of both cartilage and bone, but markers for the different axes of limb development showed a normal pattern of expression (325). |
| *Vitamin D Receptor; VDR* | **Null mice:** After weaning, animals showed severe impairment of bone formation such as the phenotype observed in vitamin D-dependent rickets type II. Animals also exhibit alopecia, hypocalcemia and infertility. Animals died within 15 weeks after birth (326). |
| *Peroxisome Proliferator-Activated Receptor-Gamma; PPARg* | **Heterozygous mice:** PPARgamma-deficient mice exhibited high bone mass with increased osteoblastogenesis, but normal osteoblast and osteoclast functions. The osteogenic effect of PPARgamma haploinsufficiency became prominent with aging but was not changed upon ovariectomy (327). |
| *V-Src Avian Sarcoma (Schmidt-Ruppin A-2) Viral Oncogene; Src* | **Null mice:** These animals demonstrated severe osteoclast dysfunction resulting in osteopetrosis. Animals survived for only a few weeks (328) |
| *Serum Response Factor; Srf, c-Fos* | **Null mice:** Embryos failed to gastrulate and died due to cardiac insufficiency during chamber maturation (329). Mice lacking Fos (encoding c-Fos) develop osteopetrosis due to an early differentiation block in the osteoclast lineage (330). |
| *Spleen Focus Forming Virus Proviral Integration Oncogene; Spi1, PU.1* | **Null mice:** Exhibit the classic hallmarks of osteopetrosis, a family of sclerotic bone diseases. Animals were rescued by marrow transplantation, with complete restoration of osteoclast and macrophage differentiation, verifying that the PU.1 is intrinsic to haematopoietic cells (331). |
| *Dickkopf; Dkk1* | **Heterogygous mice:** Progressive Dkk1 reduction increases trabecular and cortical bone mass and even a 25% reduction in Dkk1 expression could produce significant increases in trabecular bone volume fraction. Thus Dkk1 is a negative regulator of normal bone formation in vivo (332). |

mesenchymal cells (isolation of primary cells and established cell lines have been employed for these types of cultures). These types of cultures have been particularly useful to study osteogenic commitment and early differentiation.

As opposed to cell culture studies, bone organ cultures utilizing whole calvaria obtained from fetal rats or mice have been used to study bone responses to exogenous factors such as vitamin D and cortisol. These organ cultures allow one to examine the effects of systemic (hormones) and locally-produced factors on the multitude of cell types within the context of their normal microenvironment. However, the major drawback is that it is often not possible to attribute primary versus secondary effects due to the different cell types that coexist in these organ cultures. From this description of cell/organ culture systems employed to study osteoblast differentiation and bone formation, it is

clear that each particular culture model has certain advantages as well as limitations compared to the others. It is particularly important to be aware of the limitations when choosing a culture model for an experimental approach, and also when interpreting and extrapolating data generated using these culture models.

The osteoblast cell culture systems are widely used to study the effects of growth factors, secreted proteins, extracellular matrix components and transcription factors on differentiation and function (reviewed in Aubin and Triffit, 2002 (18) (Fig. 10). Many of the osteoblast culture models have been well characterized identifying the temporal sequence of gene/protein expression associated with osteoblast differentiation. In general, after the cells are plated in osteogenic medium, there are three stages of differentiation; proliferation, matrix production/maturation, and mineralization. The proliferative phase involves the expression of cell cycle and histone genes. This is followed by expression of genes associated with the formation of bone matrix such as type I collagen and alkaline phosphatase. In the final stage, genes associated with mineralization, such as osteocalcin and bone sialoprotein, are expressed at the highest levels.

## *Osteocytes*

In contrast to surface cells, such as osteoblasts, osteocytes are bone cells that live within the substance of bone. These cells comprise 90%–95% of all bone cells. They are derived from osteoblasts (Fig. 10) that became trapped and surrounded by bone matrix which they themselves produced.

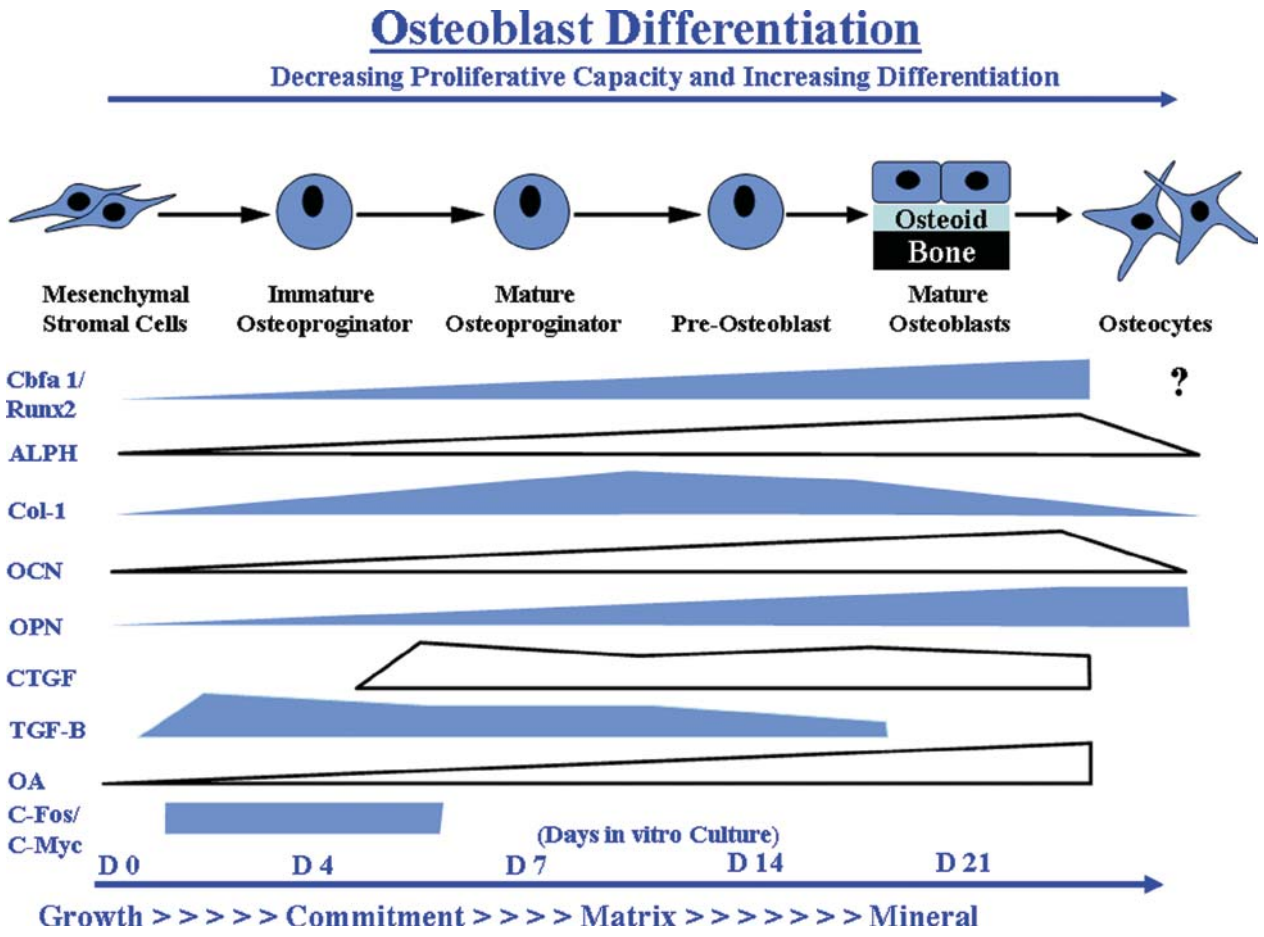


**Fig. 10** Temporal pattern of expression of markers during osteoblast differentiation in culture. As osteoblasts proliferate and differentiate from mesenchymal stem cells they express/produce various proteins including growth factors, transcription factors and extracellular matrix (ECM) proteins, each of which has a distinct temporal pattern of expression. Runx2 (Cbfa1), c-fos and c-myc are transcription factors; ALPH, alkaline phosphatase; Col-1, Collagen type I; OCN, osteocalcin; OPN, osteopontin; CTGF, connective tissue growth factor; OA, Osteoactivin; TGF-β, transforming growth factor-β.

The spaces which they occupy are known as lacunae. They are said to be involved in cell signaling and maintaining the viability of bone matrix. The processes of osteocytes communicate with each other and with osteoblasts through a network of canaliculi. The processes of adjacent osteocytes are joined together by gap junctions thereby allowing this vast network of cells within the bone matrix to communicate with one another and with cells outside of the bone matrix. It is also believed that osteocytes are important in the translation of mechanical loads to cellular events such as bone formation and remodeling.

Osteocytes have the ability to stimulate osteoblasts and the accompanying matrix by expressing osteoblast specific factor-1 (OSF-1) (19). OSF-1 accumulates on bone surfaces near the osteocytes and binds to its receptor, N-syndecan (20) located on osteoblast progenitor cells. Dentin matrix protein 1 (termed *DMP1*) is highly expressed in osteocytes and, when deleted in mice, results in a hypomineralized bone phenotype. A recent study investigated the potential role of this gene to direct skeletal mineralization as well as to regulate phosphate ($P_i$) homeostasis. Both *Dmp1*-null mice and individuals with a newly identified disorder, autosomal recessive hypophosphatemic rickets, manifest rickets and osteomalacia with isolated renal phosphate-wasting associated with elevated fibroblast growth factor 23 (FGF23) levels and normocalciuria (21,22). Mutational analyses revealed that an autosomal recessive hypophosphatemic rickets family carried a mutation affecting the *DMP1* start codon, while a second family carried a 7-base pair deletion disrupting the highly conserved C terminus of DMP1. Studies using the *Dmp1*-null mice demonstrated that the absence of DMP1 resulted in defective osteocyte maturation and increased fibroblast growth factor 23 (FGF23) expression, leading to pathological changes in bone mineralization. These findings suggest a bone-renal axis that is central to guiding proper bone mineral metabolism (21,22).

Osteocyte-derived signals have remained largely enigmatic, but it was recently reported that human osteocytes secrete sclerostin, an inhibitor of bone formation. Sclerosteosis, a skeletal disorder characterized by high bone mass due to increased osteoblast activity, is caused by a loss of the SOST gene product, sclerostin (23). Osteocytes possess receptors for parathyroid hormone (PTH), a known regulator of mineral ion homeostasis (24). Osteocytes also express molecules typically associated with nerve cells, such as NMDA receptors, which are involved with glutamate neurotransmission.

Finally, osteocytes act as mechanosensory cells. Their cell body and processes are surrounded by a thin layer of unmineralized matrix, which allows a loading-derived flow of interstitial fluid over the osteocyte surface. Their mechanosensory receptor ability has been demonstrated in studies examining loading-facilitation of macromolecule diffusion (25).

## *Osteoclasts*

Osteoclasts are multinucleated cells generally containing 3-25 nuclei per cell. Osteoclasts are related to the monocyte/macrophage lineage, with both cell types being derived from hematopoietic progenitor cells. Although the lineages of osteoclasts and osteoblasts are independent of one another, the genesis of osteoclasts requires the presence of osteoblasts along with a variety of hematopoetic cytokines, such as interleukins 1, 3, 6, and 11, tumor necrosis factor (TNF), colony stimulating factors (CSF), stem cell factor and others (26).

Colony stimulating factors are required for the proliferation and differentiation of osteoclast progenitor cells. After the osteoclast is formed, other cytokines are required for their activation and bone resorption. Parathyroid hormone (PTH), 1,25 dihydroxyvitamin $D_3$, transforming growth factor alpha (TGFα), and epidermal growth factor (EGF) act to stimulate osteoclastogenesis, whereas calcitonin inhibits the formation of osteoclasts (26).

Osteoclasts are the primary bone resorbing cells. Osteoclasts are found at sites where resorption is taking place, or if active resorption has already occurred, within "eaten out" pits or cavities known as **Howship's lacunae** (Fig. 11). Osteoclasts can also tunnel through cortical bone creating channels. Osteoclasts are highly polarized cells and the nuclei congregate away from the resorbing bone surface. The cell surface in direct apposition to the bone has an extensive infolding of the plasma membrane called the ruffled border. When the cell is in the resting state or in certain pathological conditions in which the osteoclast is dysfunctional, the ruffled border disappears or is absent (28). There is a three dimensional ring-like area of the cell membrane around the perimeter of the ruffled border called the clear zone or sealing zone. This zone contains abundant microfilaments (actin filaments) but lacks other organelles. It is here in the clear

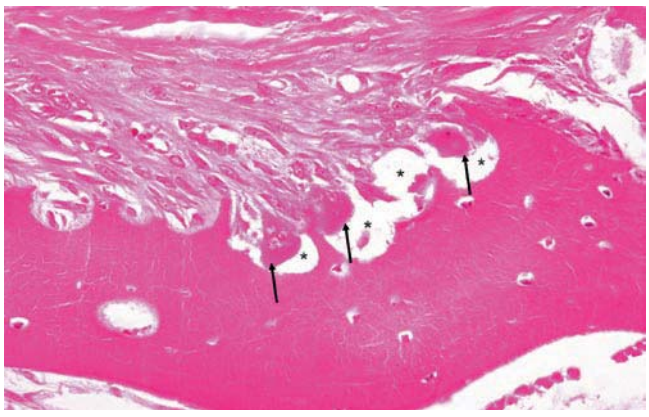

**Fig. 11** Osteoclasts in actively growing bone. Osteoclasts are multinucleated cells (arrows) associated with a shallow pits or concavities (Howship's lacunae, *) along the bone surface.

zone that the osteoclasts attach to the bone matrix, a process involving the participation of actin filaments and the $\alpha_v\beta_3$ integrin. The actin reorganization or actin ring within the clear zone can be visualized microscopically by staining for actin filaments (28,29). The area of cytoplasm between the nuclei and the ruffled border is rich in carbonic anhydrase and in tartrate resistant acid phosphatase (TRAP) (27). By electron microscopy, there is also an abundance of mitochondria, lysosomes, vesicles and free ribosomes in the cytoplasm.

## Molecular Mechanism of Osteoclast-mediated Bone Resorption

The mechanism of bone resorption is complex and involves an initial trigger by osteoblasts (see below). Once osteoclasts have been formed, bone resorption requires the secretion of hydrogen ions by an ATP driven proton pump in the ruffled border. This results in acidification of the extracellular space between the bone surface and the ruffled border which is sealed off from the general extracellular compartment by the clear zone forming a tight seal around the perimeter of the ruffled border region. The enzyme carbonic anhydrase II is essential in the generation of hydrogen ions. Simultaneously, acid hydrolases are released from lysosomes into the acidified extracellular space thus creating an extracellular lysosome. Osteoclasts can move over the bone surface, creating many resorption pits in their path. These pits are easily visualized by scanning electron microscopy, and correspond to the Howship's lacunae in routine sections. It is believed that osteoclasts must be stationary for resorption to occur, and therefore they do not resorb bone during the motile phase. Instead they cycle between motility and resorption, moving about, attaching to and resorbing bone, then releasing and moving to another region for resorption.

A combination of the acid created by the hydrogen ions and the proteolytic enzymes released from the lysosomes provide optimal conditions for the resorption of bone and degradation of collagen. This environment also results in the release and activation of certain growth factors and cytokines such as TNF-α and TGF-β. It is possible that oxygen-derived free radicals are also important in this process.

Osteoclastic stimulation may also be influenced by interactions of the integral membrane proteins (integrins) present on the osteoclast cell membrane and proteins in the bone matrix that contain the amino acids RGD (arganine-glycine-aspargine). These bone matrix proteins include fibronectin, collagen type I, bone sialoprotein II and osteopontin, all of which bind integrins and initiate out-side-in signaling pathways that can regulate the bone resorption process. Much of the data regarding factors that are important for the development, differentiation and function of osteoclasts have come from genetically modified animal models (see Table 1.1).

Osteoclasts have calcitonin, but not PTH or Vitamin D receptors. They are stimulated by IL-6 (perhaps in combination with IL-1, IL-3 and IL-11) and RANK-ligand. These cytokines are produced locally by cells of the osteoblast lineage under the influence of PTH, Vitamin $D_3$, TGF-β, IL-1 and TNF-α. It is interesting to note, that giant cell tumor (osteoclastoma) cells respond to IL-6 (30, 31). Anti IL-6 antibodies have been shown to inhibit osteoclastic activity in these cells. However, in the physiologic state, the evidence for the action of IL-6 in osteoclastogensis is lacking. Perhaps threshold levels or additional cytokines play a role (32, also reviewed in 26).

## Regulation of Osteoclast Differentiation

Macrophage colony-stimulating factor (M-CSF) is a secreted protein that is produced by osteoblasts and bone marrow stromal cells. M-CSF is required for the proliferation and survival of osteoclast precursors, cells of the monocyte-macrophage lineage (Fig. 12). Both osteoblasts and stromal cells produce RANK-ligand that has a high affinity for binding to the RANK receptor on osteoclast precursors. Treatment of osteoblasts or stromal cells with PTH, vitamin D, PGE2, or IL-11 stimulates the expression of RANK-ligand mRNA (Fig. 12)(33).

The interaction between RANK (on osteoclast precursors) and RANK-ligand (on osteoblasts and stromal cells) requires cell-cell contact for further maturation of osteoclast precursors (34–36). Osteoblasts also secrete osteoprotegerin (OPG), a member of the TNF-α receptor superfamily (37). This protein lacks the transmembrane domain and is presented as a secreted form. OPG is a soluble protein and acts as a decoy receptor that binds RANK-ligand and prevents RANK/RANK-ligand interaction. Through this mechanism, OPG can modulate the process of osteoclastogenesis. Macrophage-colony stimulating factor (M-CSF) is also essential for osteoclastogenesis. M-CSF induces cells of the monocyte/macrophage lineage to proliferate and become osteoclast precursors. RANK-ligand stimulates M-CSF-induced cells to differentiate into functional osteoclasts. Animal models of genetically modified RANK, RANK-ligand and OPG have been generated to understand the role of these proteins in osteoblast biology (see below). Mouse knockouts for RANK and RANK-ligand share similar phenotypes (38–40). Both models develop severe osteopetrosis, a disease associated with the absence of osteoclasts and failure of tooth eruption. Transgenic mice that over-express OPG in the liver also resulted in severe osteopetrosis. Therefore, the RANK/RANK-ligand/OPG axis appears to play a key physiological role in osteoclast differentiation and function. Clinically,

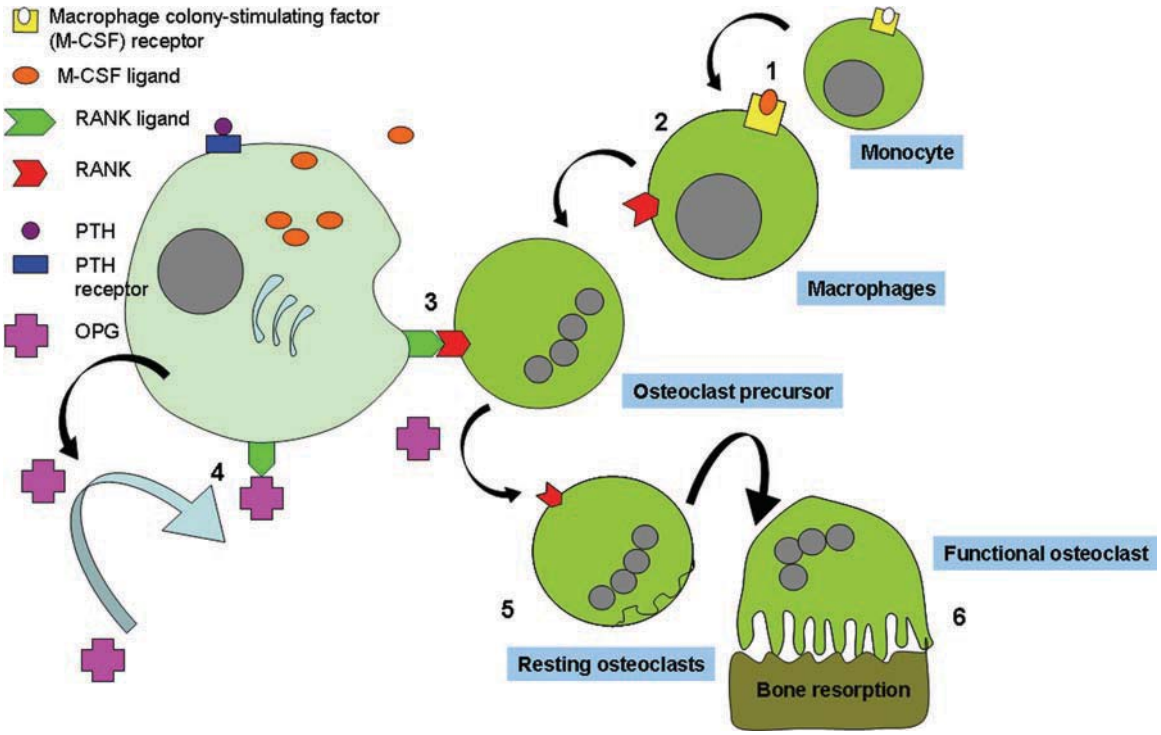


**Fig. 12** Regulation of osteoclast differentiation. 1. Monocytes derived from vessels in the bone marrow, reach an area of bone formation or remodeling. Monocytes express M-CSF receptor on their cell surface. 2. Monocytes differentiate into macrophages. M-CSF binds to M-CSF receptor and induces the expression of RANK. 3. RANK ligand (RANKL) is produced by stromal cells/osteoblasts and binds to the receptor, RANK, on mononuclear osteoclast progenitors (bone marrow macrophages). 4. OPG, a decoy receptor that binds to RANKL and inhibits osteoclast differentiation. 5. Following the interaction between RANK and RANKL, mononuclear osteoclast progenitors fuse to form a resting (non functional) multinucleated osteoclast that uncouples from the osteoblast. 6. Multiple factors including transcription factors, hormones, locally produced cytokines/growth factors and matrix proteins, mediate the activation (bone resorption) of osteoclasts.

it has been reported that two mutations of heterozygous insertion were detected in the first exon of RANK in families with familial expansile osteolysis or familial Paget's disease of bone (41).

## Signaling Pathway of RANK

The cytoplasmic tail of RANK interacts with TNF receptor-associated factor (TRAF) TRAF family members. TRAF2-mediated signals appear important for inducing osteoclast differentiation, and TRAF6-mediated signals are indispensable for osteoclast activation. Activation of NF-κB, JNK and ERK pathways, all induced by RANK–ligand in osteoclast precursors and mature osteoclasts, appear to be involved in the differentiation and function of these cells (42,43). OPG strongly blocks osteoclastic bone resorption *in vivo*, suggesting that inhibiting the RANK-ligand/RANK interaction or RANK-mediated signals are promising targets to prevent increased bone resorption in metabolic bone diseases such as rheumatoid arthritis, periodontitis and osteoporosis. Studies have also shown that TNF-α and IL-1 can substitute for RANK-ligand in inducing osteoclastogenesis *in vitro*. These studies suggest that signals other than these induced by RANK may also play important roles in osteoclastic bone resorption under pathological conditions.

## Positive Regulation of Osteoclastogenesis

Suppressor of cytokine signaling-1 (SOCS-1) is an inhibitor of cytokine signaling and may play a positive role in the regulation of T-cell-mediated osteoclastogenesis by counteracting inhibitory cytokines such as interferon-gamma (IFN- γ) (44). It has been reported that (SOCS)-1-deficient osteoclast precursor cells are more susceptible to the inhibitory effects of IFN-γ on osteoclastogenesis when compared to wild-type cells (44). SOCS-1 has been shown to be induced by RANK-ligand stimulation during osteoclastogenesis (45), indicating that these precursor cells are resistant to IFN-γ-mediated inhibition if they are first stimulated by RANK-ligand. It is likely that the fate of osteoclast precursor cells is determined not only by the balance of cytokines, including IFN-γ and RANKL (46), but also by the cytokine first encountered (47).

### Negative Regulation of Osteoclastogenesis by Interferon-β

Using a genomewide screening of RANK-ligand-inducible genes, several interferon (IFN)-α/β-inducible genes were identified in RANKL-stimulated osteoclast precursor cells. The bone phenotype of mice deficient in a subunit of the interferon- α/β receptor, IFN- α receptor type I (IFN-α-R1), was analyzed (48). These mice exhibited marked osteopenia accompanied by enhanced osteoclastogenesis in vivo. Detailed molecular analyses showed that RANK-ligand induces interferon-β in osteoclast precursor cells, and IFN-β inhibits the expression of c-Fos, an essential transcription factor for osteoclastogenesis. RANK-ligand-mediated induction of interferon- β is dependent on c-Fos, constituting a negative feedback loop in which RANK-ligand-induced c-Fos induces its own inhibitor. Thus, although type I interferons were originally characterized as critical antiviral factors, these studies situate the interferon system in a novel context and provide a compelling example of osteoimmunologic regulation (49).

### Transcriptional Regulation of Osteoclastogenesis

Genomewide screening of RANK-ligand-inducible genes identified NFATc1 (nuclear factor of activated T-cells) to be the most highly induced transcription factor in osteoclast precursor cells. NFATc1 binds to the Nfatc1 promoter and induces itself (50). This strategy is often observed in hematologic cells that undergo irreversible differentiation (51). $Nfatc1^{-/-}$ embryonic stem cells cannot differentiate into osteoclasts in vitro and overexpression of NFATc1 induces osteoclastogenesis. These results suggest that NFATc1 is an essential regulator of osteoclastogenesis (52), but it has proven difficult to show that this transcription factor is indispensable for osteoclast differentiation in vivo due to the embryonic lethality of $Nfatc1^{-/-}$ mice.

### Immunoreceptors in Osteoclastogenesis

The close relationship between bone and immune system extends beyond the cytokines and transcription factors they share. Activation and nuclear localization of NFAT are dependent on its dephosphorylation by the phosphatase calcineurin, which is activated by calcium ($Ca^{2+}$) signaling. $Ca^{2+}$ oscillation is observed during osteoclastogenesis, and the calcineurin inhibitors cyclosporine A and FK506 strongly inhibit osteoclastogenesis (52). It is not clear, though, how $Ca^{2+}$ signaling is activated during osteoclastogenesis. DNAX-activating protein 12 (DAP12) is an adaptor molecule that associates with immunoglobulin-like receptors and harbors an immunoreceptor tyrosine-based activation motif (ITAM), which is known to be crucial for the activation of $Ca^{2+}$ signaling in the immune cells. The osteopetrotic phenotype in DAP12-deficient mice made evident the importance of ITAM for osteoclastogenesis (53). Mice deficient in both DAP12 and the Fc receptor common γ subunit (FcRγ) have been shown to exhibit severe osteopetrosis, indicating that immunoglobulin-like receptors also provide important signals for osteoclastogenesis in addition to the RANK and M-CSF receptors (54,55). However, immunoreceptor signaling alone cannot induce osteoclastogenesis, suggesting that these receptors provide co-stimulatory signals for RANKL. FcRγ-associating receptors include osteoclast-associated receptor (OSCAR) and paired immunoglobulin-like receptor-A, while DAP12-associating receptors include triggering receptor expressed on myeloid cells (TREM-2) and signal-regulatory protein (SIRP-β) (54). The ligands for these immunoreceptors on osteoclasts are yet to be identified. OSCAR expression is upregulated significantly during osteoclast differentiation and its induction is mediated by NFATc1 (56, 57). Thus, OSCAR-NFATc1 constitutes a positive feedback loop in osteoclast precursor cells.

### Physiological versus Pathological Bone Resorption

Under physiological conditions, osteoclast formation requires cell-to-cell contact with osteoclast/stromal cells which express RANK-ligand as a membrane-bound factor in response to a number of factors that have been shown to stimulate bone resorption. In contrast, under pathological conditions that stimulate bone resorption, such as in rheumatoid arthritis, macrophages and/or T cells secret inflammatory cytokines such as TNF-α and IL-1. These cytokines act directly on osteoclast progenitors and mature osteoclasts without cell-to-cell contact. This paradigm is characterized by the uncoupling of bone resorption and bone formation.

### Model Systems Used to Study Osteoclasts

Given the technological challenges associated with isolating sufficient numbers purified osteoclasts, it has been difficult to study these cells experimentally. The models developed to study osteoclasts in culture are more cumbersome than those for osteoblasts. The recent discovery of the role for RANK-ligand and M-CSF in osteoclastogenesis has allowed investigators to differentiate large numbers of osteoclasts from bone marrow progenitors or splenic macrophages (58) in the presence of recombinant forms of these essential factors. Large numbers of osteoclasts can also be generated from the

RAW cell line, a macrophage cell line that has the ability to differentiate into osteoclasts when treated with RANK-ligand and M-CSF. Another alternative is the osteoclast-osteoblast co-culture system in which primary osteoblasts are cultured with bone marrow hematopoietic cells containing osteoclast progenitors (monocytes/macrophages). This culture system requires both vitamin D and $PGE_2$(26).

Osteopetrosis (see section on metabolic bone disease) in humans and animals has been useful in providing models to study osteoclast development and function. One form of the disease is associated with carbonic anhydrase II deficiency. Osteoclasts are present but functionally incompetent in CAII deficiency, and children afflicted with this disease also suffer from renal tubular acidosis. In the *op/op* osteopetrotic mouse and the *tl/tl* toothless rat models, there are abnormalities in the coding region of the CSF-1 gene resulting in the production of trauncated and functionally incompetent CSF-1 protein. In these forms of osteopetrosis, osteoclasts are absent or greatly reduced in number, and treatment with exogenous CSF-1 results in normal osteoclastogenesis and correction of the disease. There are other mouse models in which targeted disruption of certain proto-oncogenes, such as src and fos, causes osteopetrosis (for a more comprehensive list, see Table 1).

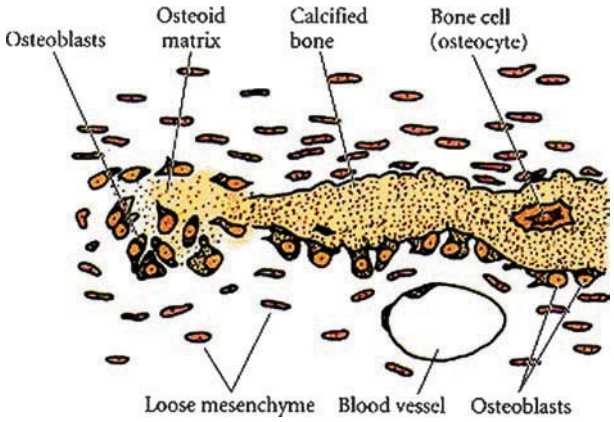


**Fig. 13** Intramembranous Ossification. Mesenchymal stem cells condense to produce osteoblasts, which deposit osteoid and mineralize the bone matrix. These osteoblasts line the surfaces of the developing bone and continue to produce bone matrix by apposition. Osteoblasts that become trapped within the bone matrix become osteocytes. There is no cartilage that precedes the formation of bone in this type of bone formation. Permission granted from Sinauer Associates (Gilbert, 6th edition, 1997).

## Part 2 Bone Development

The bones of the axial and appendicular skeleton are formed by one of two processes, intramembranous or endochondral bone formation. The primary difference between these two processes is the absence or presence of a cartilaginous intermediary. In intramembranous bone formation, bone is formed in the absence of a cartilage model while in endochondral bone formation, a cartilage model is first formed and then replaced by bone tissue.

## Intramembranous Bone Formation (Membranous)

The flat bones of the skull and face are formed by intramembranous ossification (Fig. 13). Osteoprogenitor cells (which will give rise to osteoblasts and osteocytes) are present within the mesenchyme. These cells aggregate at the sites where new bone is to be formed and differentiate into osteoblasts that actively synthesize new bone matrix. Growth of intramembranous bones occurs by *apposition* (deposition upon prior bone) of osteoblasts lining the surfaces of the growing bones. Ossification centers develop within the bone and enhance the rates of mineralization. As the growth rate slows down, the bones take on a *lamellar* character. In the adult, Haversian remodeling occurs.

Chondroid bone has also been described within the skull, occurring in association with suture closure. This type of bone has a histological intermediate between cartilage and bone, containing both types I and II collagen. Chondroid bone forms a scaffold upon which lamellar bone is deposited. It is not replaced by bone as occurs in the endochondral model (see below).

There are many factors that play potential roles regulating bone formation, and their functions and interactions are complex. For instance, core binding factor-alpha1 (cbfa-1) is responsible for osteoblast differentiation, and binds the osteocalcin promoter, resulting in osteocalcin expression. Mutation of cbfa-1 causes cleidocranial dysplasia, a disorder in which there is delayed ossification of cranial sutures.

## Endochondral Bone Formation (Cartilage Model)

Endochondral ossification involves the formation of cartilage tissue from aggregated mesenchymal cells and the subsequent replacement of this cartilage tissue by bone tissue (60). All of the skeletal components of the vertebral column, the pelvis, and the appendicular skeleton (limbs) develop via endochondoral ossification. The process of endochondral ossification is divided into five stages (Fig. 14). ***First***, the mesenchymal stem cells are committed to become cartilage cells through expression of two transcription factors,

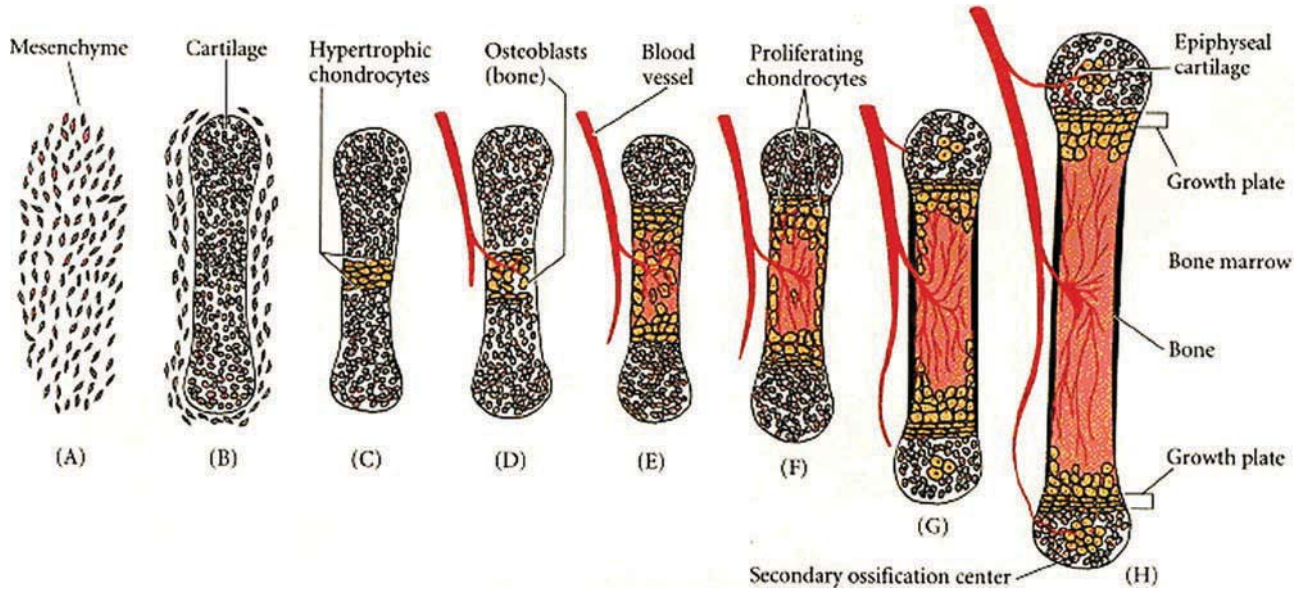


**Fig. 14** Endochondral Ossification. (A, B) Long bones such as humerus, femur and tibiae develop through endochondral ossification where the mesenchymal stem cells condense and differentiate into chondrocytes to form the cartilaginous model of the bone. (C) Chondrocytes in the center of the shaft undergo hypertrophy and apoptosis and their death allows blood vessels to enter that region (primary ossification center). (D, E) Blood vessels bring in osteoblasts, which lay down new bone matrix on the remants of the calcified cartilage (scaffold). (F-H) Secondary ossification centers also form as blood vessels enter near the ends of the bone. Bone formation and growth in length continue at the growth (epiphyseal) plates with ordered arrays of resting, proliferating, hypertrophic (mineralizing) chondrocytes. Permission granted from Sinauer Associates (Gilbert, 6th edition, 1997).

Pax1 and Scleraxis. These factors are thought to activate cartilage-specific genes (61–63). During the ***second*** phase of endochondral ossification, the committed mesenchymal stem cells condense into compact nodules and differentiate into chondrocytes. N-cadherin appears to be crucial for the initiation and maintenance of these condensations (64,65). In humans, the SOX9 gene (sex reversal Y-related high-mobility group box protein) and chondrocyte commitment, that encodes a DNA-binding protein, is expressed in the precartilaginous condensations. Mutation of the SOX9 gene alters skeletal development and results in deformities of most of bones of the body. Infants with specific mutations of the SOX9 gene die from respiratory failure due to poorly formed tracheal and rib cartilages (66). During the ***third*** phase of endochondral ossification, chondrocytes proliferate rapidly to form the cartilage model that will eventually be replaced by bone tissue. As they divide, chondrocytes secrete a cartilage-specific extracellular matrix. In the ***fourth*** phase, the chondrocytes stop dividing and become hypertrophic. Hypertrophic chondrocytes have increased production of collagen type X and fibronectin, thus altering the remaining cartilage matrix so that it can be mineralized by calcium carbonate. Finally, in the ***fifth*** phase, blood vessels begin the invasion of the cartilage model. The hypertrophic chondrocytes undergo apoptosis and the spaces are invaded by ingrowing blood vessels. As the cartilage cells die, osteoprogenitor cells differentiate into osteoblasts and begin to lay down bone matrix on the partially-degraded, mineralized cartilage remnants (67,68). The site at the center of the cartilaginous model where ossification first occurs is known as the ***primary center of ossification*** (the eventual diaphysis of the long bone). Eventually, all the cartilage is replaced by bone so the cartilage tissue serves as an intervening model for the bone that will eventually replace it. In experimental models, the precise sequence of events has been worked out reasonably well (for a detailed review see 85).

In the long bones of mammals, this process of endochondral ossification spreads along the vertical axis of the developing bone in both directions from the primary ossification center (Fig. 14). As the bones grow in length, **secondary centers of ossification** form at the ends of each bone. Once these secondary ossification centers form, there remains an area of cartilage between the primary and secondary ossification centers. The secondary ossification center becomes the epiphysis and the primary center becomes the diaphysis. The intervening cartilage is the epiphyseal growth plate and it is here that continued growth in length occurs at both ends of the developing bone. The epiphyseal plates contain three regions: a region of chondrocyte proliferation, a region of chondrocyte maturation, and a region of hypertrophic chondrocytes (reviewed in 69). A complex series of events occur in the growth plate as the resting chondrocytes

proliferate, mature and become oriented in columns. As the cells hypertrophy at the expense of the intervening cartilaginous matrix, the cartilage matrix becomes calcified. It is these calcified cartilage remnants that then serve as a scaffold for the deposition of bone matrix by osteoblasts. The spaces left behind by the apopototic hypertrophic chondrocytes are invaded by blood vessels, a critical event in the formation of new bone tissue. Abnormalities in chondrocyte function can disrupt this sequence and produce abnormally short and misshapen bones. One illustration of this is achondroplasia, a condition causing short stature and thought to be caused by a mutation in fibroblast growth factor (FGF). Without the normal cellular processing of the signal from the fibroblast growth factor, the chondrocytes do not proliferate normally (70). This results in a disorganized and malfunctioning growth plate.

## Molecular Regulation of Growth Plate (Chondrocytes)

Chondrocytes of the growth plate behave very differently than chondrocytes of articular cartilage. Chondrocytes within different parts of the growth plate are markedly different from each other. Investigators have been studying the factors that are important in each region. Indian hedgehog, is a factor that plays a role in the normal differentiation of growth plate chondrocytes. The study of factors that activate Indian hedgehog (Ihh) and other associated factors are advancing our understanding of the molecular biology of the growth plate and its disorders (69).

There are systemic and local mediators that regulate growth plate chondrocyte proliferation and differentiation. Systemic factors include insulin growth factor-1 (IGF-1) (71), growth hormone (72), thyroid hormone (73), estrogens (74), vitamin D (75) and glucocorticoids (76). All of these factors have been reported to have an effect on the linear growth of bone both prenatally and postnatally. Other factors act locally to regulate growth plate chondrocytes, include TGF-β, PTHrP (78), Ihh (79) and FGF-receptor type 3 (FGFR3) (80,81). It has been reported that TGF-β has the ability to inhibit chondrocyte proliferation, hypertrophic differentiation and matrix mineralization (82).

Control of the growth plate is also an area of intense research. Indian hedgehog (Ihh), a protein of the same family as sonic hedgehog, regulates the rate of hypertrophic chondrocyte differentiation. It is produced by the pre-hypertrophic chondrocytes and induces the expression of parathyroid hormone related protein (PTHrP) in the perichondrium, which blocks chondrocyte differentiation. The Ihh/PTHrP axis acts as a negative feedback loop modulating chondrocyte differentiation. One can imagine that abnormalities in this control loop can alter growth plate function or lead to inhibition of chondrocyte proliferation (83).

The overlapping expression of FGFR3 and PTH-receptor-1 (PTHR1) in the growth plate suggested that these signaling pathways interact. Genetic inactivation of either PTHrP or the PTHR1 in mice resulted in a marked decrease in the size of the proliferative zone of the growth plate, a phenotype that is seen with the constitutive activation of the FGFR3 signaling. Other *in vivo* studies have shown that FGFR3 signaling can repress Ihh and PTHR1 expression in the growth plate (84). These studies suggest a link between Ihh/PTHrP signaling and the FGFR3 pathway in the growth plate.

## Limb Development

The development of the human limb begins at day 24 of gestation and is orchestrated by the expression of a sequence of genes expressed in specific regions of the developing limb. Three zones typify the growing limb bud: a thickened ectoderm located distally at the periphery, called the apical ectodermal ridge (AER), proliferating mesoderm just deep to the AER called the progress zone (PZ); and a zone of polarizing activity (ZPA) located posterior to the PZ (86).

The AER is responsible for general limb and bone development. Grafting the AER from one animal to the PZ of another, patterns the growing limb after the donor AER. In contrasts, the ZPA orients the limb in an anterior-posterior direction. Grafting the ZPA to the anterior border of a developing limb, results in a duplicated limb in opposite orientation. The AER maintains continuous limb bud outgrowth along the proximal-distal (P-D) axis (shoulders to digits). Concomitant to its elongation along the P-D axis, the limb becomes flattened along the dorso-ventral (D-V) axis (back of hand to palm) and is asymmetric along the antero-posterior (A-P) axis (thumb to little finger). Differentiation of mesenchymal stem cells becomes morphologically apparent as these cells condense to form the primordia of individual skeletal elements. The most proximal elements (stylopod) begin to differentiate first, followed by the progressive differentiation of more distal structures (zeugopod and autopod) (87, 88).

Limb patterning also plays a role in how bones develop (89). Genes such as sonic hedgehog, and the homeobox genes, organize segmentation, anterior-posterior, medial, lateral and longitudional limb patterning during fetal development. Abnormalities in patterning genes may lead to extra or deficient digits, short or long limbs, or congenital amputations. The growth plate is altered in many of these malformations, and thus, the patterning genes likely modify factors involved in chondrocyte function and differentiation in the growth plate.

### *Molecular Biology of Limb Formation*

As the limb bud grows, the proximal-distal, dorso-ventral and antero-posterior axes are apparent and development is mediated by multiple signaling molecules. Members of the fibroblast growth factor (FGF) family produced by AER cells are required for P-D outgrowth. FGF signals are responsible for keeping the underlying undifferentiated mesenchymal cells in the progress zone, in an undifferentiated, rapidly proliferating stage. At least five FGFs (FGF 2, FGF 4, FGF 8-10) and two FGF-receptors (FGFR1 and FGFR2) are expressed during limb bud initiation. FGF 2, 4, 8, 9 and FGFR 2 are found in the ectoderm and AER while FGF 10 and FGFR 1 are present in the underlying mesenchyme (90,91). Sonic hedgehog (Shh) is an important molecule in the ZPA, and is responsible for duplication of limb structures as demonstrated in grafting experiments. FGF maintains *Shh* expression in the ZPA and FGF 4 is largely responsible for maintenance of its expression as the limb elongates (92). Select FGFs in conjunction with *Shh* regulates expression of the bone morphogenetic (BMP2 and 7) and Hox genes, mostly Hoxd-12 and Hoxd-13. These genes are members of the *Hoxd* complex and are expressed in the distal wrist (Hoxd 12), within the hand and fingers (Hoxd 12 and 13). These genes regulate proximal-distal differentiation of limb segments and mutation of Hoxd-13 in the human transforms metacarpals to carpals and metatarsals to tarsals. On other hand, overexpression of Hoxd13 in chick limb bud in vivo resulted in the transcriptional repression in the proximal part of the limb of *Meis*, the vertebrate ortholog of an homeo-box containing gene in *drosophila* called *homothorax* (hth) that is required for proximal leg development (92).

Wnt proteins regulate many events in limb development, from patterning to controlling cell proliferation, differentiation and survival. The Wnt family signals through ten different transmembrane frizzled receptors. These receptors also function together with the LDL-related protein receptor (LRP). Two LRP receptors bind Wnt, LRP 5 and 6.

Wnt signals through three pathways, the β-catenin pathway, the JNK (planer polarity) pathway and the Wnt/$Ca^+$ pathway (93). The β-catenin pathway is also called the canonical Wnt signaling pathway and is well characterized (94,95). Wnt signaling is antagonized by different factors, such as members of the TGFβ and FGF families, frizzled related proteins (Sfprs), cerberus, dickkopfs (DDKs) and members of the CCN family of proteins (93). Wnt proteins are expressed in the AER which controls limb outgrowth (97) and the dorsal ectoderm which controls dorso-ventral patterning. Wnts are also expressed in the AER and in lateral plate mesoderm where their overexpression leads to ectopic limb formation (96). Mutation in Wnt proteins and their downstream signaling molecules has been linked to human limb malformations. Wnt3a and 7a regulate the expression of Csa1, mutated in human pre-axial polydactyly (98). Spontaneous, naturally occurring mutations in Wnt7a has also been associated with postaxial hemimelia characterized by duplication of the sesamoid bones and foot pads (93). It is evident that Wnt and associated signaling pathways play a major role in limb development and pathogenesis of limb deformities and our knowledge of the Wnt signaling pathways and their role in limb development will enhance our understanding of the genetic basis of limb deformities.

## Skeletal Muscle

### *Type(s) of Muscle*

Muscle is characterized as either striated or non-striated. The term striated originated from the appearance of the ordered structure of this muscle type under the microscope. As opposed to this, non-striated muscle does not show the same regimented pattern of order. Striated muscle includes two types of muscle: skeletal and cardiac. Smooth muscle is classified as non-striated. Here we will focus on only one type of striated muscle: skeletal muscle. In longitudinal section, these cells appear tubular with multiple nuclei/cell located at the periphery of cells. In cross-section the cells are polygonal with the nuceli located at the periphery. They are characterized as striated, voluntary muscles with the ability to undergo strong; quick contractions.

### *Organization of Skeletal Muscle at the Light and Electron Microscope Level*

A muscle cell or fiber is surrounded by a plasma membrane referred to as the sarcolemma. In normal muscle, an important component of the sarcolemma is dystrophin, where it forms a complex with the sarcolemmal cytoskeletal network. Dystrophin is absent in patients with muscular dystrophy. Muscular dystrophy is a general term used to describe a group of inherited myogenic disorders. The cytoplasm is referred to as the sarcoplasm. The sarcoplasm is filled with filamentous myofibrils which run parallel to the long axis of the cell. The myofibrils are composed of myofilaments which are made up of polymers of primarily myosin and actin, proteins responsible for contraction. A view of a myofilament shows thick filaments made up of myosin and thin filaments of actin. The filaments interdigitate and have the appearance of light and dark bands. The light bands are comprised of actin and are termed the I band (isotropic). The dark bands are made up of myosin and the overlapping region of actin and are called A bands (anisotropic). These alternating light

and dark bands give skeletal (and cardiac) muscle their striated appearance. The actin filaments are attached to Z lines. The actin filaments extend on either side of the Z line (disc) to interdigitate with the myosin molecules. The Z disc is composed of a variety of proteins which anchor the actin filaments to the Z line and which extend from one myofibril to another in cross-section. Alpha-actinin is one of those proteins involved in anchoring actin to the Z disc. Another more recently identified giant (300kD) protein 'titin' spans the distance between the Z disc and the M line of the sarcomere. It is involved in providing elasticity to the sarcomere and a main contributor to 'passive tension' (passive or resting tension is that which is present in a muscle even before contraction is initiated and is a result of the elastic forces in the muscle). Although not discussed here many other proteins are part of the contractile apparatus including C protein, Myomesin, and Nebulin. The region between Z lines is called a sarcomere, a unit repeated throughout the structure of the myofibril. The A band is bissected by the M line. This is the region where lateral connections are made between the thick filaments. The main component of the M line is creatine kinase, an enzyme that catalyzes the transfer of a phosphate group from phospho-creatine (storage form of high energy phosphate) to ADP. This provides the energy in the form of ATP necessary for muscle contraction.

## Characteristics of the Contractile Filaments

The myosin thick filament is made up of many myosin molecules. The tail region is made up of two polymers of myosin heavy chain molecules twisted together as a double helix forming the body of the thick filament. At the end of the tail is found two pairs (four) of myosin light chain molecules which form the head region of the myosin polymer. The head region has an ATPase, an enzyme essential to the production of energy for contraction. The head region and a portion of the helix of the myosin molecule (which makes up the arm: can move away or towards the helix) extends outward to form cross bridges that make contact with the thin actin filaments as described in the "sliding filament theory". These cross bridges that extend outward can bend and have hinges at two points: one, where the head attaches to the arm and the second, where the arm attached to the axial body of the myosin molecule. These hinges facilitate movement. Cross bridges project around the entire thick.

The actin filament is a double stranded helix of F-actin protein. Polymerized G- actin molecules make up the F-actin filament. Each G-actin molecule has a myosin head binding site. The thin actin filament also contains two strands of the tropomyosin molecule which cover the myosin interactive sites on actin, preventing interaction of actin and myosin. Also, along the tropomyosin molecules is found the globular troponin complex. Troponin I has a strong affinity for actin, Troponin T for tropomyosin and Troponin C for calcium ions.

## Innervation of Muscle

Myelinated motor nerves which originate from spinal cord neurons give rise to several terminal branches which make contact with multiple individual muscle cells. At the site of innervation the nerve terminal sits in a trough *(motor end plate)* at the surface of the muscle cell. The space between the axon and the muscle is the *synaptic cleft.* An action potential at the neuromuscular junction (or motor end plate) causes the release of acetylcholine from the axon which binds to receptors on the highly folded sarcolemma *(junctional folds).* In patients with myasthenia gravis, acetylcholine receptors on the sarcolemma are blocked by antibodies generated as an autoimmune response. The muscle cell cannot respond to the nerve stimulus resulting in muscle weakness. After acetylcholine binds to its receptor, the sarcolemma becomes permeable to sodium ions resulting in membrane depolarization. Depolarization is propagated along the muscle cell into the cell via the *transverse tubule system.* This is a network of tubules arising as invaginations of the sarcolemma and surrounds the A-I interface of each sarcomere in each myofibril. These tubules associate with two *terminal cisternae* (expanded regions of the SR) of the sarcoplasmic reticulum and together are known as *triads.* At the triad the depolarization signal is transmitted to the SR resulting in the release of stored calcium ions and initiating contraction. Calcium is released near the thick and thin filaments. When depolarization ends; calcium reenters the SR and contraction ceases.

## Mechanism of Contraction

The actin filament is inhibited from interacting with the myosin cross bridges by the troponin-tropomyosin complex. Before contraction can take place this inhibitory interaction must be removed. It is the introduction of large amounts of calcium ions that changes this inhibitory effect. Calcium ions released from the sarcoplasmic reticulum combine with troponin C which changes the configuration of the troponin complex and moves the tropomyosin molecules further into the grooves between the actin filaments. This makes the active sites on the actin filament available for interaction with the cross bridges of myosin and is the binding step in the contraction process. Once the cross bridges are attracted

to the active site on actin the process of contraction begins. The hypothesized mechanism is referred to as the "walk-along" theory of contraction.

Remember that the head of the myosin molecule binds ATP. ATPase activity in the head cleaves the ATP but the ADP remains attached. In this conformation the head is extended towards the actin filament. When the inhibitory effect of the tropomyosin-troponin complex is lifted, the myosin head binds to the actin molecule. This causes a conformational change in the myosin head causing it to tilt towards the arm and this process causes a power stroke. The energy for this is already stored in the head from the cleavage of ATP. ADP and Pi is released allowing for a new ATP to bind to its site on the myosin head. This binding causes the myosin to detach from the actin and return to its original perpendicular position, the new ATP is cleaved and the myosin forms a new bond further down the actin filament to again perform a power stroke and consequently move another step. These steps occur again and again as the actin filament pulls the Z disc towards the ends of the myosin filament. Remember that contraction is not a result of shortening of individual filaments but an increase in overlap between the actin and myosin filaments. Therefore, as sarcomere length decreases, tension in the muscle and strength of contraction increase.

## The Neuromuscular Spindle

The sensors that keep the central nervous system informed of the state of contraction and position of voluntary muscle are referred to as neuromuscular spindles (NMS). This structure consists of modified muscle fibers encased in a fluid filled sheath of connective tissue. The fibers comprising the neuromuscular spindle are referred to as intrafusal fibers. The fibers we have discussed earlier (making up the bulk of skeletal muscle) are extrafusal fibers. Intrafusal fibers come in two varieties: nuclear bag fibers (nuclei accumulated in the midregion) and nuclear chain fibers (nuclei arranged in a central chain). Nuclear bag fibers extend beyond the confines of the connective tissue sheath and are attached to the extrafusal fibers (i.e. skeletal muscle proper)

Innervation is a very complex subject. Anterior horn cells may be either large alpha motor neurons that give rise to motor nerve fibers that innervate extrafusal fibers or even smaller gamma motor neurons that give rise to fibers that innervate the small intrafusal fibers. Afferent sensory fibers transmit information from the NMS to cell bodies in the sensory posterior horn of the spinal cord. Therefore, these encapsulated proprioceptors are a mechanism by which the CNS remains informed as to the state of your muscles allowing the CNS to better coordinate the function of voluntary muscle.

## Repair of Skeletal Muscle

Satellite cells are stem cells found between the plasma membrane and the basal lamina of muscle fibers. These cells can differentiate to form myotubes. If damage includes disruption of the basal lamina satellite cells are not responsible for the repair that results. Instead fibroblast repair results in scar tissue.

## The Origins of Skeletal Muscle

The development and maturation of skeletal muscle has been studied since the early 1900's and much is know compared to either cardiac or smooth muscle. It is fashioned during embryogenesis from the mesoderm which is generated after the process of gastrulation. On either side of the neural tube, that lies dorsal to the notochord, extends a band of mesoderm called the paraxial mesoderm (Fig. 15). This band of mesoderm separates into blocks referred to as somites. This separation and the subsequent development of somites proceeds in a rostral to caudal direction, with the rostral somites budding off and developing before those located caudal. The number of somites is often used as an indicator of the stage of embryonic development, i.e. how far development has progressed. For example, a chicken embryo is defined as being at stage 11 (40–45 hours of incubation after laying) by the presence of thirteen pairs of somites (99,100).

Somites are transient structures that supply cells which populate the vertebrae and ribs, the dermis of the dorsal skin, the skeletal muscles of the back, body wall and limbs (101,102). They form as mesenchymal structures but convert to an epithelial block with tall columnar cells arranged

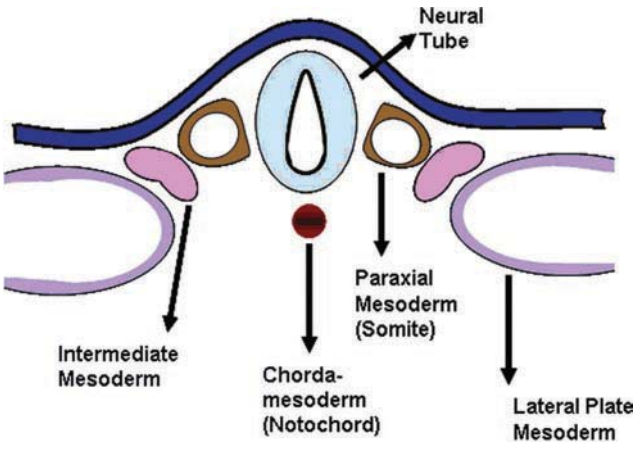


**Fig. 15** Diagram of cross-sectional area of a vertebrate embryo that shows organization of mesoderm tissue. Somites which comprise the paraxial mesoderm generate skeletal muscle, the focus of this section in the chapter. Drawing by James O.H. Montgomery

around a central cavity, a process referred to as epithelialization. This process is accompanied by changes in cell-cell and cell-matrix interactions and the signals regulating the segmentation and epithelialization of somites has been studied extensively (103–105). Although all somites look very much alike they form different structures along the anterior-posterior axis and this specification is dictated by the expression of Hox genes (106,107).

Somites contribute cells to different structures and the commitment of cells to their ultimate fate takes place during the early stages of somite development. As the somite matures its various regions become destined (committed) to form certain cell types. Cells in the ventral/medial region, those which are furthest from the back and closest to the neural tube change their shape, become mesenchymal and migrate away to form the sclerotome. These cells ultimately form the chondrocytes of the vertebrae and the ribs. The remaining epithelial somite becomes organized into three regions. The region closest to, and furthest from, the neural tube (epaxial and hypaxial myotome, respectively) is the myotome. The cells in the myotome proliferate and delaminate to produce a lower layer of myoblasts, precursor cells committed to the skeletal muscle lineage. This double layered structure is referred to as the dermamyotome. Myoblasts formed from the region of the dermamyotome closest to the neural tubed will form the muscles of the back (epaxial muscles), whereas the myoblasts in the region farthest from the neural tube will form the muscles of the body wall, limbs and tongue (hypaxial muscles). The dermatome located in the central region of the dermamyotome will form the dermis of the skin of the back (Fig. 16). Signals that direct the formation of the sclerotome, dermatome and myotome are well understood. Briefly, formation of the sclerotome from the ventral-medial cells of the somite is directed by Sonic Hedgehog secreted from the notochord and the floor plate of the neural tube. The sclerotomal cells which become chondrocytes of the vertebrae and ribs themselves express Pax 1, a transcription factor necessary for the formation of cartilage (108,111). The dermatome forms under the direction of two factors secreted by the neural tube: Neurotropin 3 and Wnt 1 (112,113). The epaxial portion of the myotome is induced by factors from the neural tube (Wnt 1 & 3a and Sonic hedgehog) whereas the hypaxial portion is specified by proteins from the epidermis (Wnt) and from the lateral plate mesoderm (bone morphogenetic protein 4). It should be noted that in this brief discussion of muscle, the inhibitory or negative signals are not discussed. Nonetheless their role is vital to normal somite development (114,116). After formation of the double layered dermamyotme, a subset of cells located in the central region of the dermamyotome, identified as being Pax 3 and 7 positive, were found to proliferate and give rise to skeletal muscle cells upon activation of muscle specific transcription factors. Later in development, these cells make

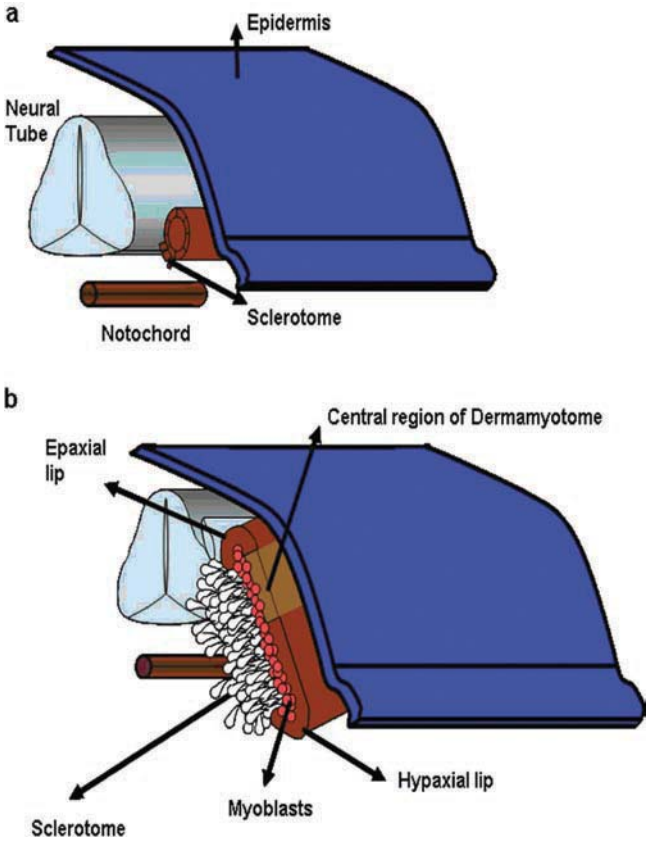


**Fig. 16** Maturation of the somite in vertebrate embryos. (**a**) In the 3 day old chicken embryo cells detach from the ventral-lateral somite and begin to migrate away to form the sclerotome. (**b**) In the late 4 day old chicken embryo a layer of myoblasts (muscle precursor cells) are formed beneath the overlying dermatome. Drawing by James O.H. Montgomery. Adapted after Developmental Biology, 7th, edition, Scott F. Gilbert

a major contribution to the embryonic and fetal muscle mass. These cells also give rise to dermal cells. In addition, the dermamyotome gives rise to endothelial and smooth muscle cells of the dorsal aorta.

## *Techniques used to Study Skeletal Muscle Development*

The development and maturation of skeletal muscle is most often studied in vertebrate animals: amphibians, chicken, quail and mice, but the avian model system is most commonly used. This is attributed to the ease with which avian embryos can be manipulated and dissected, cultured either in vitro or *in ovo* during embryonic development observed under a dissecting scope or sectioned and then analyzed. Chicken-quail chimeras have been used to trace the origin of muscles from specific somites. A natural marker that differentiates quail nucleoli from chicken is used to identify tissues

of chicken versus quail origin. Consequently, when quail somites are transplanted into recipient chicken embryos and the embryos studied after a period of incubation, the muscles derived from the quail somite can be easily identified and studied. This same model has been used to study Notch signaling and somite formation (101,117). Genetic manipulations to determine the function of a protein by knock-out or transgenic methods are most often performed in mice (118,119).

### *Development of Skeletal Muscle*

Signals that induce the formation of muscle differ from the epaxial and hypaxial regions of the dermamyotome. In the hypaxial portion, factors from surrounding tissues induce expression of the transcription factor Pax 3, which in turn induces the expression of MyoD. However, in the epaxial portion MyoD is induced by the Myf5 protein. Both MyoD and Myf5 are basic helix-loop-helix (bHLH) transcription factors that belong to the myogenic bHLH family of myogenic regulatory factors (MRFs). These transcription factors bind to similar sites (-CANNTG-) to turn on transcription of muscle-specific genes.

Cells in the epaxial and hypaxial regions of the dermamyotome that produce MyoD and Myf5 are myoblasts, cells committed to the myogenic lineage. How these cells progress from stellate-appearing single cells to tube-like myofibers in mature skeletal muscle has been studied in culture as well as in chimeric mice. Myoblasts proliferate when cultured in media containing serum. As serum is withdrawn the cells withdraw from the cell cycle and fuse to ultimately form multinucleated myofibers or mature skeletal muscle cells. Once growth factors, specifically fibroblast growth factor, are withdrawn from the culture media (by lowering the serum concentration) the myoblasts begin to produce fibronectin and interact with this extracellular matrix protein through the integrin receptor $\alpha_5\beta_1$. The myoblasts then line up, a process mediated by plasma membrane proteins such as CAMs and cadherins and the cells fuse. Fusion of myoblasts to form myotubes also referred to as myofibers is calcium mediated process and appears to require metalloproteinases, enzymes involved in remodeling of the extracellular matrix. Just prior to fusion the cells expresses another MRF, myogenin. Myogenin mediates differentiation of these cells. Differentiation is the process whereby cells become specialized; in this case they express muscle-specific contractile proteins and acquire the ability to contract. In vivo, the force of contraction of skeletal muscle is transmitted via tendons to bones, allowing these structures to move. During adult muscle regeneration, satellite cells (adult skeletal muscle stem cells) that are present under the basal lamina become activated, proliferate to generate additional satellite cells and differentiate into muscle fibers. As during embryonic development, both Pax and MRFs are involved in this process (109,111,116).

## Part 3 Bone Biology

The structure and function of bone can best be understood with an insight into the composition of the bone matrix, its mineral, its cells, the mechanism of turnover and remodeling and the unique responses that the mechanical environment evokes in bone. These studies are forming the basis of a rapidly evolving field of bone biology.

## Collagen

### *Classification of Collagens*

Connective tissues contain varying amounts of collagen, elastin (a related fibrous protein), glycosaminoglycans and proteoglycans. Of these, collagen is the most abundant. The details of collagen synthesis and function have been extensively reviewed in several specialized texts, and only some of the relevant aspects will be discussed.

Collagens are a class of proteins with common features such as a unique triple helix composed of three component polypeptide alpha chains. However, there are several subtypes (types I to XIII) . Each of these are a product of a different gene and differ from each other in their biochemical structure. Several different types of *fibrillar, basement membrane-associated, fiber-associated, and short chain* collagens are recognized. Type I collagen is the most abundant type of collagen in most connective tissues.

### *Type I Collagen, The Primary Component of Bone*

Type I collagen is a fibrillar type collagen and is found in bone, skin, meniscus, tendon, ligaments, annulus fibrosis and joint capsules. About 90% of bone matrix is composed of type I collagen. There are several subtypes of type I collagens. The bone type I collagen appears to have predominantly galactosyl-hydroxylysine as opposed to glucosyl-galactosyl-hydroxylysine, the predominant amino-acid configuration found in the skin. Hydroxylation and glycosylation are post-translational modifications of collagen and are specific to bone. These modifications, partially explain why mineralization only occurs in bone and not in other sites (120).

The basic structure of type I collagen is composed of a repeating tripeptide sequence that form a left handed helix.

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

## Other Collagen Types

Types II, III, V and XI are also fibrillar type collagens. Type II collagen is located mainly in articular cartilage, fibrocartilage, the vitreous humor of the eye and the nucleus pulposus of the intervertebral disk. The other fibrillar collagens are the "minor" collagens. Type III is present in large blood vessels (30%), and in other tissues in association with Type I. Type III collagen is also present in tissues undergoing repair. Type V collagen is present in large blood vessels (5%), cornea, bone, and a few other connective tisues, while Type XI is present only in articular cartilage, comprising 5-20% of articular cartilage.

Basement membrane-associated and fiber-associated type collagens include Types IV, VII, IX and XII. Type IV collagen is the prototype and major component of the *basement membrane* (95%). Type VII forms the anchoring filament in epithelial basement membranes, while Type IX comprises 5-20% of cartilage. Small amounts of Type XII collagen are associated with Type I. The least understood class of collagens is the *short chain* collagens and are comprised of the Types VI, VIII and X. Short chain collagens may function in association with other collagens and have a role in cartilage physiology.

## Collagen Synthesis and Cross-linking

Collagen synthesis is under the control of over 20 genes (120). The biosynthesis of collagen, its secretion and aggregation is a complicated process, and has been the subject of several reviews (121,122). Aspects directly applicable to musculoskeletal pathology will be mentioned in other chapters of this book.

The promoters for synthesis of many of the collagen chains have been identified. Many growth factors and hormones also exert their effect on collagen synthesis at the transcriptional level. Collagen mRNAs usually contain a large number of introns. Once the precursor mRNA is transcribed, the introns are removed and the mRNA is transported from the nucleus to the cytoplasm for translation. At this point, additional translational control can be exercised. The N and C propeptidases (see later) are thought to act at this level. Like many other proteins, a precursor form (procollagen) is first synthesized, with peptide extensions at each end. It is at this point that the α chain of collagen is formed and is transfered into the endoplasmic reticulum. At this stage of synthesis, several amino acids are modified posttranslationally (such as hydroxylation of proline residues and lysine residues, forming hydroxyproline and hydroxylysine, respectively), the addition of sugars (such as glucose and galactose to the hydroxylysines), and the formation of hydroxylysine and lysine aldehydes. Co-factors for these processes include atmospheric oxygen, ascorbic acid, ferrous ion and several required enzymes. Once the translation is complete, the triple helix forms, from the C terminal end, and intra- and inter- molecular disulfide bonds are formed. The completed procollagen is then secreted via vesicles into the extracellular space. Glycosylation may be important to facilitate this final step.

As stated earlier, the fundamental units of collagen fibrils are three polypetide chains arranged in a helical fashion. The polypeptide chains aggregate in units of threes to form *tropocollagen*. The tropocollagen, in turn, aggregates in a staggered fashion in a collagen microfibril. Collagen fibers are made up of several of these microfibrils. The process of collagen fibril formation is not fully understood. Removal of the N and C propeptides may be important. After their removal, the molecules aggregate in a head-to-tail fashion with a characteristic stagger, resulting in a 64 nm banding pattern seen under the electron microscope. The ultrastructurally dark area between two tropocollagen molecules is termed a "hole". It measures about 41 nm and is the site where mineralization is thought to first occur (121).

The collagenous scaffolding is stabilized by cross linking and perhaps by interaction with proteoglycans. The process of cross-linking is important for stabilization and structural integrity. The first step is the enzymatic production of aldehydes by the removal of terminal amino groups of lysyl or hydroxylysyl groups of tropocollagen (123). These then can either condense with a lysyl or hydroxyl group to form a cross link and produce a Schiff base, or condense with a similar aldehyde in an aldol reaction (a stronger bond). The amino-oxidase enzyme that catalyzes the aldehyde formation is susceptible to blockage by nitriles. Nitriles are alkyl cyanide substances involved in the disorder called Lathyrism. Lathyrism is characterized by spinal deformities, demineralized bone, dislocations, aortic aneurysm, and various nervous system manifestations.

Cross-linking of collagen occurs in the extra-cellular space. Collagen molecules are cleaved in this space at both the N- and the C- terminal ends by specific peptidases. Cross-linking then occurs and the collagens are packed into a one-quarter stagger array. Specific interactions also occur among collagen and other extracellular macromolecules such as fibronectin, osteonectin and the proteoglycans.

Extensive "cross-linking" between α component chains results in a rigid, brittle character to the connective tissue. This type of cross-linking is found in aging individuals. Defects in the process of forming cross links can render the collagen susceptible to collagenases (discussed more below). Penicillamine prevents collagen cross-linking; and is administered to patients with scleroderma, a disorder of excessive collagen deposition. Genetic defects in collagen can also result in several lethal and non-lethal conditions. Examples include Ehlers-Danlos syndrome (loose joints, characterized by a Gly to Serine change) or Osteogenesis imperfecta (brittle bones, characterized by a Gly to Cystine change). Osteogenesis imperfecta (see section on metabolic diseases) is a heritable disorder of Type I collagen. It is due to a variety of point mutations in either the pro-α1 or pro-α2 collagen chains. Over 100 point mutations have been found in probands with osteogenesis imperfecta. In a few cases, the mutations cause a decrease in the synthesis of pro-α1 and pro-α2 chains. In the majority of cases, however, there is a production of structurally abnormal collagen chains (124). A mouse model has also been developed which demonstrates the relationship between abnormal collagen genes and osteoporosis in heterozygous animals. In homozygous animals, osteogenesis imperfecta develops (125,126).

Collagenases are enzymes that catalyze the hydrolysis of collagen. Several collagenases have been isolated, purified and synthesized. Collagenase levels are increased in rheumatoid arthritis nodules and in synovial fluid from patients with rheumatoid and septic arthritis. Colchicine and heparin increase collagenase synthesis. Collagenases and several other enzymes, such as cathepsin B, are capable of degrading collagen and have been implicated in the pathogenesis of collagen-vascular diseases.

Urinary excretion of hydroxyproline (found exclusively in collagen) and other products of collagen degradation (cross-linked products such as pyridinoline and deoxypyridinoline), act as markers of collagen breakdown. The level of collagen degradation byproducts in urine or serum reflect the amounts of bone turnover (see section on the laboratory in orthopaedic practice).

## Non Collagenous Matrix Proteins

### *Calcium Binding Proteins (The Glyco- and Phosphoproteins)*

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

#### Osteopontin

The amino acid sequence has been determined, and its gene localized to chromosome 4. Osteopontin is a sialated and highly phosphorylated phosphoprotein, which exists in multiple forms, due to both alternate splicing as well as post-translational variations in the degree of phosphorylation. This protein is transcriptionally regulated by substances such as 1,25 dihydroxyvitamin D, TGF-β, dexamethasone and parathyroid hormone, at least in experimental models.

Osteopontin contains a GRGDS cell attachment sequence similar to binding proteins such as fibronectin (see below). Osteopontin in particular is important in that it has been shown to bind to the integrin receptor on osteoclasts. This binding leads to the activation of the phospholipase C pathway in osteoclasts, and a resultant increase in intracellular calcium. This process may involve the src tyrosine kinase.

Immunolocalization of osteopontin reveals high amounts in the extracellular matrix of developing intramembranous and endochondral bones. Its localization within cells reveals a broad pattern, including osteoblasts, osteocytes, osteoclasts, precursor cells, chondrocytes and fibroblasts. In situ hybridization studies have also shown the presence of mRNA in mononuclear marrow cells, proximal convoluted tubules of the kidney, neuronal cells within the brain and inner ear as well as (murine) placenta (127,128).

#### Bone Sialoprotein-II (BSP II)

The gene for this sialoprotein has been localized to chromosome 4. Northern blot studies have suggested that BSP II is fairly bone specific. Fetal bone studies have indicated that the initial translation product may differ significantly from the mature form. Like BSP I, this protein has cell attachment properties due to its RGD sequence. However, osteopontin is more active than BSP II in this regard, and maintains cell attachment for more prolonged periods (129).

#### Bone Acidic Glycoprotein (BAG -75)

This protein binds to the small bone proteoglycans. There is cross-reaction of antibodies with osteopontin in some species; however there are significant differences between this protein and BSP I at the N-terminal end. Complete characterization of this protein is not clearly understood (130,131).

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

These proteins have a role in regulating the extracellular calcium hydroxyapatite formation and mineralization. Examples include phosphorylated glycoproteins like *osteonectin*. This protein also called secreted protein, acidic, rich in cysteine (SPARC), culture shock protein or basement membrane-40 (BM-40). Osteonectin binds to $Ca^{2+}$, collagen type I, hydroxyapatite and thrombospondin. It promotes and initiates crystal growth. The gene for osteonectin has been localized to chromosome 5. Several tissues express osteonectin, however, its concentration is extremely high in bone (up to 10,000 times that of other connective tissues). In fact, in bone it may be the most abundant non-collagenous protein. The concentration of osteonectin in bone increases with maturity. Other tissues /cells having osteonectin include skin fibroblasts, tendon cells (but not tendon matrix) and odontoblasts. Interestingly, when osteonectin was activated by the use of blocking antibodies during tadpole development, there was a disruption of somite formation and malformation in the head and trunk (132). Mice lacking osteonectin develop severe cataracts (133) and low turnover osteopenia. *In vitro* studies of osteonectin-null osteoblastic cells showed that osteonectin supports osteoblast formation, maturation and survival (134). Osteonectin also plays role in cell attachment, migration, proliferation and differentiation.

## *Mineralization Proteins: Gamma-carboxyglutamic acid proteins ("Gla" proteins)*

Osteocalcin (also called bone Gla protein) is an example of this group. Osteocalcin contains three γ-carboxyglutamic acid (Gla) residues. It comprises about 20% of the non collagenous proteins in human bone. There is also a matrix Gla protein found in bone, cartilage, lung, heart and kidney (135).

Osteocalcin is made by osteoblasts and odontoblasts in response to 1,25 dihydroxyvitamin $D_3$. It is secreted into the osteoid after the initiation of mineralization. The bone localization of osteocalcin has been confirmed by several different methods including Northern blotting, immunohistochemistry and electron microscopy. It therefore serves as marker for mineralized tissue. In fact, both osteocalcin and alkaline phosphatase are valuable markers in the repertoire of the surgical and clinical pathologist. Osteocalcin serves as a marker of increased bone turnover, in particular of enhanced osteoblastic activity. Serum osteocalcin levels do not always correspond well with the levels of serum alkaline phosphatase, suggesting that these two markers may be synthesized by osteoblasts at different stages of development. These substances can be used for following the progress of patients with osteosarcoma and may be can be used as a marker for recurrences or metastases in this situation (135).

The role of osteocalcin in the body is unclear, but it may function in regulating mineralization and remodeling. It may also act as a chemoattractant for osteoclast progenitors (also see section on mineralization). Its secretion is under the control of many factors including Vitamin D, TGF-β, PTH and others. Serum levels reflect bone turnover. Osteocalcin has an affinity for $Ca^{2+}$ that is dependent on the presence of Gla residues and an intact disulfide bond. It therefore may have a role in the regulation of crystal growth and recruitment of osteoclasts. Developmentally, low levels are found in the early stages of bone development while maximal levels are reached at maturity. The entire primary structure of osteocalcin has been determined (amino acid sequencing, cDNA clone sequencing, etc.) and the gene is localized to chromosome 1 in humans. The promoter region has a TATA box and a CCAAT sequence. There is a NF1 site, and a binding site for two other nuclear factors AP1 and AP2. There is a cAMP responsive region as well as a 1,25-dihydroxyvitamin $D_3$ enhancer element. Genetic studies showed that osteocalcin acts as an inhibitor of osteoblast function. Osteocalcin knockout mice were reported to have increased bone mineral density compared to normal controls, but the changes in mineral properties that occur with age were not observed in osteocalcin deficient mice compared to age-matched normal control mice (136,137). Collectively, the published literature provides evidence that osteocalcin is required to stimulate bone mineral maturation.

## *Adhesion Proteins (Osteopontin, Fibronectin, Sialoproteins and Thrombospondin)*

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

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

### Fibronectin (FN)

FN is a multifunctional glycoprotein present in the extracellular matrix as an insoluble component or in circulating plasma as a soluble protein. FN mediates the adhesion, migration, differentiation, and proliferation of cells and has been implicated in wound healing and embryonic development

(138). FN is one of the most prevalent and versatile of the extracellular matrix proteins. Disruption of the FN gene in mice results in an embryonic lethality, confirming the importance of FN in embryonic development (139,140). The molecule is a dimer, its subunits being held together by two disulfide bonds. The subunits contain binding domains for fibrin, heparin, bacteria, gelatin, collagen, other extacellular matrix proteins, DNA and cell surfaces. The primary sequence of fibronectin has been determined, and the gene localized to chromosome 7. Fibronectin is characterized by several repeat sequences, for fibrin, collagen and integrin receptor binding. The latter is composed of the Gly-Arg-Gly-Asp-Ser cell attachment consensus sequence known as the GRGDS sequence.

There is heterogeneity associated with fibronectin mRNA, both dependent on origin (plasma versus tissue) and on stage of development (fetal versus adult). This results from alternative splicing of the primary transcript. This may allow the cell to utilize the form more suited to its needs. FNs exhibit molecular heterogeneity arising from alternative splicing of the primary transcript at three distinct regions termed EDA, EDB, and IIICS (141–144). Alternative splicing at the EDA and EDB regions is regulated in a tissue specific and developmental stage-dependent manner. Despite accumulating evidence for the regulated expression of EDA- and/or EDB-containing FNs *in vivo*, the biological functions of these isoforms are poorly understood. Recent studies have shown that the EDA segment regulates the binding affinity of FNs for integrin $\alpha 5\beta 1$ and thereby stimulates integrin-mediated signal transduction and subsequent cell cycle progression. Unlike the EDA segment, the EDB segment does not enhance FN binding to integrin $\alpha 5\beta 1$ (145,146).

Fibronectin is synthesized during bone development. During embryonic development, fibronectin is present at high levels during mesenchymal condensations and plays a crucial role in the overt differentiation of these cells into chondrocytes (65). It is also present around osteoblasts during osteogenesis. Osteoblasts can utilize fibronectin as a cell attachment protein. The synthesis of fibronectin from osteoblasts is probably under the control of TGF-$\beta$.

Mice deficient for the EDB domain of FN were apparently normal and fertile, although the fibroblasts obtained from the homozygous mice exhibited reduced potential for cell growth and FN matrix assembly *in vitro* (147). Skeletal characterization of EDB null mice revealed no changes in any cartilage elements of skeletal development when compared to the wild type mice.

#### Thrombospondin (TSP)

This is a 450 kilo Dalton trimeric glycoprotein. It is composed of identical subunits that are disulfide-bonded to each other. It is the predominant protein of the $\alpha$ granules of platelets, but is synthesized in several connective tissues. Like fibronectin, there are "domains" for binding to a host of connective tissues and serum proteins. The molecule also binds $Ca^{2+}$ to hydroxyapatite and to osteonectin. Thrombospondin and osteonectin co-localize in the $\alpha$ granules of platelets, where they bind to one another.

The structure of thrombospondin reveals a homology to fibrinogen with binding sites to collagen, thrombin, fibrinogen, laminin, plasminogen activator and plasminogen. There are areas with homology to $\alpha$ (1) chains of types I and III collagen, von Willebrand factor and epidermal growth factor. There is a region for activating platelet aggregation, as well as sequences with homology to calmodulin and paralbumin. In addition there is an RGD sequence in the middle of a $Ca^{2+}$ binding region. Thrombospondin is distributed in a variety of tissues, including the dermo-epidermal junction of skin, in small blood vessels, surrounding skeletal muscle and beneath glandular epithelium.

Temporally, there is an orderly increase in amounts during organogenesis, followed by a reduction as differentiation proceeds. There is evidence to suggest that TGF-$\beta$ may be involved in the modulation of thrombospondin biosynthesis. The proposed functions of this molecule include mediation of platelet aggregation, organization of the extracellular matrix (by its multiple binding sites) and action as an autocrine growth factor (148).

TSP is expressed by bone cells such as osteoblasts as well as chondrocytes and this protein is usually deposited into the matrix and regulates other extracellular matrix proteins. There are different types of TSP including TSP1, 2, 3 and 4, some of which have common physiological roles while others do not. Genetically targeted mouse models have been used to define the physiological role of TSPs in bone and other tissues (149). Mice lacking TSP1 exhibit curvature of the spine and minor abnormalities in trabecular bone (150). TSP2-null mice display increased endocortical, but not periosteal, bone formation rates, compared to wild-type, normal mice, as a result of a larger pool of marrow osteoprogenitor cells (151). From the above information it is evident that the role of TSPs in bone is varied and is largely context-dependent.

### *Other Proteins, Cytokines and Growth Factors*

Osteoblast cell culture studies have revealed the presence of several bio-products. Plasminogen activator and its inhibitor have been identified. Collagenase and tissue inhibitor of metalloproteinase (TIMP) have been isolated from such experiments. The extrapolation of these results across species and to *in vivo* situations should be treated with caution. Several plasma products including albumin and $\alpha$ 2 HS-glycoprotein

can bind to bone. There is evidence in the literature suggesting a role for plasminogen activators in bone remodeling. Plasminogen activators tPA and uPA are involved in tissue remodeling and bone metabolism. Mice lacking tPA and uPA show increased bone formation and bone mass associated with increase osteoblast function and delay in extracellular matrix degradation (152).

## Connective Tissue Growth Factor

Connective Tissue Growth Factor (CTGF) is a cysteine-rich protein first discovered by Bradham and colleagues (153) while screening a human umbilical vein endothelial cell cDNA expression library using a polyclonal anti-PDGF antibody. At about the same time, two independent groups isolated mouse CTGF (Fisp 12/βIG-M2) from serum-stimulated NIH-3T3 cells and TGF-β -stimulated mouse AKR-2B cells using differential cloning techniques (154,155). Since that time CTGF has been isolated, cloned and sequenced in other species including the cow, pig (156), frog (157), and most recently in the rat (158). The CTGF gene belongs to a larger CCN gene family that also includes Cyr61/CEF10 and *nov.* Cyr61 and CEF10 were isolated by differential cloning techniques from mouse and avian fibroblasts, respectively, and *nov* was identified from myeloblastosis-associated virus-induced avian nephroblastomas (159). More recent additions to this protein family include ELM-1 (WISP-1) (160,161), WISP-3 (161) and COP-1 (WISP-2) (161), bringing the total to six distinct members. With the exception of *nov*, CTGF family members are immediate early growth-responsive genes that regulate the proliferation and differentiation of various connective tissue cell types (159,162). Most of the functional information on CCN proteins has emerged within the last 5 years, including the identification of receptors (i.e. integrins) and the elucidation of potential mechanisms of action, the field is poised for major advances in understanding the activities and functions of these proteins. For reviews of CTGF and the CCN family see 163–166.

All members of the CTGF gene family share 30-50% amino acid sequence identity overall, possess a secretory signal peptide at the N terminus, and contain 38 cysteine residues that are largely conserved (165,166). The CCN proteins are organized into four discrete and conserved structural domains, each encoded by a separate exon. Domain I shares significant sequence homology with the N-terminal region of the insulin-like growth factor binding proteins (IGFBPs) (167), although only low levels of IGF binding activity have been demonstrated for CTGF (168). Since the affinity of CTGF for IGF is much lower than that of the IGFBPs, the physiological significance of this binding is unclear. Domain II includes a von Willebrand factor type C repeat followed by a variable region that is highly charged and devoid of cysteine residues. This variable region may serve as a hinge connecting the N- and C-terminal halves of the protein. The central hinge region located between domains II and III of CTGF and other CCN family members is highly susceptible to enzymatic cleavage with additional sites of proteolysis between other domains (169). Domain III contains a region that is homologous to the thrombospondin type I repeat and may be involved in binding to the extracellular matrix via sulfated glycoconjugates (170). Domain IV is the C-terminal (CT) module resembling the CT domains of several other extracellular proteins believed to mediate protein-protein interaction or dimerization (171). Within this domain are six cysteines forming a motif called a cysteine knot. Cysteine knots are also found in other growth factors (TGF-β, PDGF and NGF) and are involved in their dimerization.

### CTGF Effects on Cellular Functions and Role in Biological and Pathological Processes

In general, CTGF (as with most other members of the CCN family) is a secreted, extracellular matrix-associated protein that regulates a diversity of cellular functions including adhesion, proliferation, migration, differentiation, matrix production, and survival. *In vivo*, CTGF mRNA is expressed in many tissues with highest levels in the kidney and brain (155,165). In bone, CTGF has been reported to be expressed in normal rat bone and overexpressed in osteopetrotic bone (158). To date, CTGF mRNA expression and protein production has been demonstrated in endothelial cells, fibroblasts (169,172) and chondrocytes (173). It is believed that CTGF acts as an autocrine or paracrine regulator of various cellular processes with its specific effects being target cell-dependent (169). Although CTGF is mitogenic for various cell types, it also promotes the differentiation of fibroblasts and chondrocytes in culture (173–175). CTGF has been shown to up-regulate the expression and production of extracellular matrix (ECM) proteins, such as type I collagen and fibronectin in fibroblasts (174–175) and osteoblasts (176), and collagen types II and X and aggrecan in chondrocytes (177) Since secreted CTGF is an ECM-associated heparin-binding protein, it is able to mediate cell-matrix interactions (178). CTGF also stimulates the migration/chemotaxis of fibroblasts, mesenchymal stem cells, endothelial cells and vascular smooth muscle cells (179,180). CTGF has also been shown to enhance cell survival or block apoptosis under conditions where cell adhesion is prevented (179,181) but induces apoptosis in vascular smooth muscle cells (182,183).

In addition to the cellular activities discussed above, CTGF has been implicated in more complex biological processes including embryonic development, angiogenesis,

endochondral ossification and wound healing. Based on the angiogenic activity of CTGF, it has been proposed that CTGF is involved in neovascularization of the mineralized hypertrophic cartilage during endochondral ossification (184,186) CTGF expression is induced during cutaneous wound healing. Its effects on fibroblast chemotaxis, extracellular matrix production and angiogenesis suggest that it contributes to wound repair (174,185,187). It has been postulated that CTGF family members play a role in various pathological processes including tumorigenesis such as in cartilaginous tumors (188), atherosclerosis, and various fibrotic diseases (189). It is interesting to note that several different mutations of WISP3, another CCN family member, have been associated with the autosomal recessive disorder progressive pseudorheumatoid dysplasia in which patients experience continued cartilage loss and destructive bone changes around synovial joints (190). This is the first study establishing a definitive link between a CCN family member and the pathogenesis of a disorder affecting bone and cartilage.

### CTGF and Skeletogenesis–CTGF and MSC Condensation

During embryonic development there two types of mesenchymal skeletal condensations, pre-cartilaginous condensations that develop into primary cartilage (endochondral ossification during limb development), and pre-osseous condensations that develop into membranous bones (65, 191, 192). During endochondral ossification, mesenchymal stem cells (MSC) aggregate and undergo condensation and subsequent chondrogenic differentiation to form a cartilaginous core (193). This cartilaginous core is then shaped to become the cartilaginous anlage of the future skeleton (192). The cellular condensation process is dependent on signals initiated by cell-matrix and cell-cell adhesion, and these signals are modified by a cell's response to growth and differentiation factors in the extracellular milieu. The hallmarks of cellular condensation include changes in cell adhesion and cytoskeletal architecture (194). The roles of adhesion molecules including N-cadherin, neural cell adhesion molecule (N-CAM), syndecans, and ECM proteins (fibronectin), and other signaling molecules, such as focal adhesion kinase (FAK), paxillin and Wnt have been reported in mesenchymal condensations (65,195). Perturbation of the functions of these molecules leads to disruption in MSC condensation and inhibition of normal cartilage formation (194). It has been reported that CTGF mRNA expression in the newly forming cartilage is high during the initial stage of condensation (193), and deceases as the chondrocytes mature (193,196) Studies by others has shown that CTGF mRNA is expressed strongly in mesenchymal condensations during Meckel's cartilage development, decreases in newly differentiated chondrocytes, and surges again in hypertrophic chondrocytes (193,197). In mice at embryonic stage E10.5, the CTGF protein is highly expressed in mesenchymal condensations of the developing vertebral column and is associated with strong expression of the condensation-matrix protein, fibronectin. In a model of fracture repair, CTGF mRNA and protein are expressed early in the developing fracture callus suggesting that CTGF plays a role in tissue repair. Primary high-density chick and murine limb bud micromass cultures are ideal methods of analyzing *in vitro* the process of mesenchymal condensation (198). Micromass cultures of the mouse cell line, C3H10T1/2, treated with TGF-β results in the formation of a three-dimensional spheroid structure (mesenchymal condensation) (191) Another study showed that Cyr-61, a closely related member of the CCN family, is expressed during mesenchymal condensation *in vivo*. Treatment of mesenchymal cells with recombinant Cyr-61 (199) or rCTGF induced mesenchymal condensation. CTGF is highly expressed during *in vitro* mesenchymal condensation, and that its expression is associated with the expression of condensation-related ECM proteins including fibronectin and N-cadherin. Together, these data suggest that CTGF plays an important role in mesenchymal condensation.

### CTGF and Chondrogenesis

During development and in newly formed cartilage, CTGF expression is localized in mesenchymal condensation and disappears in mature cartilage. This coincides with the strong proliferative effects of CTGF on chondrocytes (193) and the down regulation of the expression of this factor during chondrocyte re-differentiation (200) Whole mount *in situ* hybridization of CTGF mRNA in E17 mouse embryos showed that CTGF is selectively expressed in the hypertrophic, but not, proliferative chondrocytes, and in cells of the zone of calcifying cartilage (193) which correspond to the region of chondrocyte cell death. CTGF has been shown to induce apoptosis in a number of cell types including smooth muscle cells, mesangial cells and mammary cells (193). CTGF has also been shown to stimulate chondrocyte proliferation, and and late differentiation associated with increased expression of type X collagen (201). Important functional insights may also be gained from developmental approaches such as targeted gene disruption or cell/tissue-specific overexpression. Transgenic mice that over-expressed CTGF under the control of type XI collagen promoter were generated and their embryonic and neonatal growth was normal (202,203). However, they showed dwarfism within a few months of birth associated with decreased bone mineral density. These results suggest that CTGF over-expression in the growth plate can affect the process of endochondral ossification. The CTGF null mouse has provided great insight into the role of CTGF during chondrogenesis and

osteogenesis (204). These mice have an obvious skeletal phenotype involving both cartilage and bone. CTGF -/- mice die shortly after birth due to respiratory distress and cyanosis caused by severe malformation of the rib cage. CTGF deficiency resulted in impairment of chondrocyte proliferation and ECM composition within the hypertrophic zone, including aggrecan and link protein, suggesting a role of CTGF in acquisition of tensile strength in cartilage (204). It has been shown that there is an inverse relationship between CTGF and Sox-9 expression in MSC. TGF-β treatment inhibits Sox-9 expression, but this effect is completely abolished when the cells are transfected with CTGF siRNA. High levels of CTGF correlate with low levels of Sox-9 and vice versa, suggesting that CTGF is a negative regulator of early chondrocyte commitment/differentiation from MSC.

## CTGF and Osteogenesis

Using the *osteopetrotic* (*op*) rat as a model to examine differential gene expression in bone from normal and osteopetrotic rats, CTGF mRNA and protein expression was discovered in bone (158). *In situ* hybridization and immunohistochemical analyses demonstrated that CTGF mRNA and protein are localized in osteoblasts lining metaphyseal trabeculae. Examination of CTGF expression in the fracture callus of a fracture repair model demonstrated that CTGF was primarily localized mesenchymal cells and in osteoblasts lining active, osteogenic surfaces. In primary osteoblast cultures, CTGF mRNA levels demonstrated a bimodal pattern of expression, being high during the peak of the proliferative period, abating as the cells became confluent, and increasing to peak levels and remaining high during mineralization (205). This pattern suggests that CTGF may play a role in osteoblast proliferation and differentiation. Treatment of primary osteoblast cultures with anti-CTGF neutralizing antibody caused a dose-dependent inhibition of nodule formation and mineralization. Treatment of primary osteoblast cultures with rCTGF caused an increase in cell proliferation, alkaline phosphatase activity and calcium deposition, thereby establishing a functional connection between CTGF and osteoblast differentiation (205). *In vivo* delivery of rCTGF into the femoral marrow cavity induced osteogenesis that was associated with increased angiogenesis. These studies clearly showed that CTGF is important for osteoblast development and function *in vitro* and *in vivo*. Studies of CTGF null mice showed an impairment of endochondral ossification associated with decreased vascular endothelial growth factor (VGEF) in the ossification zone of the growth plates. CTGF null mice have decreased bone mineral density and osteopenia (204). Another group of investigators identified and cloned a CTGF-like cDNA from human osteoblasts encoding a protein of 250 amino acids (26 kDa) and sharing approximately 60% homology with other members of the CCN protein family (206). This CTGF-like protein is secreted in primary human osteoblast cultures and it promotes osteoblast adhesion. It was recently shown that CYR61, another member of the CCN family that is closely related to CTGF, is produced and secreted in cultures of human osteoblasts, suggesting that it may also function as an extracellular signaling molecule in bone (207). Furthermore, CYR61 has been shown to be up-regulated during fracture repair particularly in proliferating chondrocytes and osteoprogenitor cells suggesting that it may serve as an important regulator of fracture healing (208).

## Mechanisms of Action of CTGF in Skeletogenesis

The mechanisms of action of CTGF involved in mesenchymal stem cells, chondrocytes, osteoblast differentiation and bone formation are not well understood. One recent study reported synergistic or antagonistic roles for CTGF with TGF-β1 or BMP-4, respectively (209). In this study, CTGF enhanced TGF-β1 signaling through receptor and cell-surface binding, Smad-2 phosphorylation, activation of gene expression and cell differentiation. Since TGF-β1 has been shown to have a proliferative effect on osteoblasts (210) and to stimulate CTGF gene expression, it is possible that CTGF may mediate some of TGF-β1 effects on osteoblasts. This same study also demonstrated that CTGF binds directly to BMP-4 through its chordin-like domain, and that CTGF inhibited alkaline phosphatase activity induced by BMP-4 in C3H10T1/2 mesenchymal cells and Smad1 phosphorylation in human 293T cells. Another study showed that CTGF inhibits Wnt-3a and BMP-9-induced osteogenic differentiation of MSC, suggesting that CTGF may act as a negative regulator of early osteogenic differentiation of MSC (211). The inhibitory effect of CTGF in osteogenic differentiation of MSC is interesting.

In chondrocytes, another group has shown that CTGF mediates its effect on chondrocyte proliferation via the ERK pathway and on chondrocyte maturation via the p38 MAK kinase pathway (201) In separate studies by Nishida and colleagues (212,213), they identified a 280-kDa binding protein complex for CTGF on chondrocytic and osteoblastic cells lines, but the nature of the protein(s) contained in this binding complex has not been determined. CTGF has also been shown to bind low-density lipoprotein receptor-related protein (Lrp), such as Lrp5 and 6 and antagonizes Wnt signaling (214). However, the downstream signal transduction pathways in response to this interaction are not fully understood. Clearly, the association between CTGF and MSC is an interesting one that requires further investigation. For other cytokines and growth factors please see section on Regulation of Bone and Cartilage.

## Osteoactivin

### Identification and Characterization of Osteoactivin (OA) in Bone

The initial identification of OA came from studies using an animal model of osteopetrosis (*op*) in rats. This model was used to examine the differential gene expression in bone from normal rats and *op* mutant osteopetrotic rats. Osteopetrosis is a sclerosing bone disease characterized by generalized increase in the bone mass and has severe skeletal phenotype resulting from abnormalities during development (215). Using the technique of mRNA differential display, a novel cDNA that is highly up-regulated in *op* compared to normal bone was identified.

The over-expression of osteoactivin in *op* mutant bone compared to normal one was confirmed by Northern blot analysis and found to be increased 4 fold in *op* versus normal long bones and calvaria (216). Subsequent cloning and sequencing of the full-length OA cDNA revealed sequence homology with the previously reported human NMB (217), DC-HIL (dendritic cell heparan sulfate proteoglycan integrin dependent ligand) (218), HGFIN (hematopoietic growth factor inducible neurokinin) (219) and PMEL17 (melanocyte specific gene) (220).

Osteoactivin has an open reading frame of 1716 bp with a short 5'- untranslated region, encoding a protein sequence of 572 amino acids (a.a.) with a native M.W of 65 kDa. The first 22 a.a. represent a potential signal peptide, so OA is potentially a secreted protein (216). Bioinformatic analysis of the full length OA a.a. sequence revealed a potential polycystic kidney domain and an RGD integrin recognition site at position 556 that constitutes a potential site for cell attachment. OA protein sequence analysis revealed 11 N-linked and 19 O-linked potential glycosylation sites suggesting that OA is a highly glycosylated protein. A potential transmembrane hydrophobic sequence has been identified in OA protein sequence from a.a. 499 to a.a. 522 suggesting that OA may have a transmembrane isoform.

### Characterization of OA Expression in Primary Osteoblast Cultures

OA expression in primary osteoblast cultures showed that the level of OA increased markedly as the cells differentiate with maximal expression during matrix maturation and mineralization as correlated with the early differentiation marker, alkaline phosphatase and the late differentiation markers, osteopontin and osteocalcin (216). By RT-PCR analysis, OA showed the highest level in normal bone (long bones and calveria) and primary osteoblast cell cultures compared to much lower expression levels in brain, heart and skeletal muscles (216). The high level of OA expression during osteoblast terminal differentiation in culture and the preferential expression in bones compared to other tissues are consistent with a gene that has a functional role in osteoblast cell biology.

OA is highly expressed in various malignant tumors such as in glioma (221) and hepatocellular carcinoma (222). It has been shown that over-expression of OA in glioma cell lines (223), as well as in hepatoma cell lines (222), permits tumor invasiveness. OA also has been found to function as an activator of fibroblasts infiltrated into denervated skeletal muscles (224). Treating mouse NIH-3T3 fibroblast cell cultures with recombinant mouse osteoactivin increased the amounts of fibroblast markers, MMP-3 and MMP-9, suggesting that OA has a pathophysiological role in skeletal muscles atrophied by degeneration (224).

## Enzyme: Alkaline Phosphatase

Although not generally thought of as a matrix component, this enzyme is an osteoblast product (such as osteocalcin and osteonectin) and would best be discussed here. There are three related isozymes that are tissue related and are associated with three separate genes (reviewed in 225). These are the placental, intestinal and tissue nonspecific forms. The last is seen at high levels in a variety of tissues such as bone, liver, kidney and skin. All three isozymes require $Zn^{2+}$ and $Mg^{2+}$ and catalyze the hydrolysis of monoester phosphates (such as pyridoxal-5'-phosphate) at a pH between 8 and 10 (alkaline pH). The isozyme important in bone is the tissue non-specific form.

Although the tissue non-specific isozyme is associated with a single gene (on chromosome 1), there are electrophoretic and other subtle differences with the other isozymes from different tissues. These differences are probably due to altered glycosylation. The enzyme is attached to the cell membrane via phosphotidyl inositol and can be removed from the cell surface by phospholipase C. This may be a mechanism whereby it enters the serum. The enzyme can be demonstrated by a variety of histochemical and immunohistochemical methods. It has been seen that the bone form of the enzyme can be localized to osteoblasts and young osteocytes, but mature osteocytes are consistently negative for its presence. Vitamin D and thyroxin increase the biosynthesis of alkaline phosphatase. Glucocorticoids and parathyroid hormone inhibit it. The expression of alkaline phosphatase is cell cycle dependent with its activity increasing through the $G_1$ and S phases, and decreasing in $G_2$ and M phases.

The role on alkaline phosphatase in biomineralization is still speculative. It has been found in matrix vesicles, but its exact role is unclear. Serum alkaline phosphatase activity is increased in growing children, pregnancy, healing fractures, Paget's disease, rickets, osteomalacia, hyperparathyroidism, bone-forming tumors and certain skeletal metastases. It is reduced in hypophosphatasia.

### The Hydrated (Muco)Polysaccharide Gels

The functional units of bone extracellular matrix "gels" are the Glycosaminoglycans (GAGs). Of the several GAGs, four main groups are present. Hyaluronic acid, chondroitin and dermatan sulfates, heparan sulfate and heparin, and keratan sulfate. The majority are aggregated to a protein core to form a proteoglycan. Prior to release from the synthesizing cell, GAGs (except hyaluronic acid) are sulfated. This step imparts a net negative charge on these molecules. In turn, this charge serves two functions. First, it keeps the molecules extended, increasing the volume to weight ratio. Second, by attracting osmotically charged cations, it attracts water. This mechanism creates a gel with very high swelling pressures and tremendous resistance to compression.

The highly viscous hyaluronic acid is a major constituent of synovial fluid, where it helps in lubrication. Additionally, it may impede bacteria, by its physical and chemical properties. Its synthesis is thought to involve a pathway located in the cell membrane, thus hyaluronic acid is not sulfated. The remaining GAGs (mentioned above) are synthesized within the Golgi.

The kind of GAG present within bone is different than those in cartilage. Bone has longer chains, and is rich in chondroitin-4-sulfate. Cartilage on the other hand contains chondroitin-6-sulfate. Developing bone has been shown to contain a large chondroitin sulfate proteoglycan and a small chondroitin sulfate proteoglycan.

Further characterization of the small proteoglycan in turn reveals two proteoglycans (PG I or biglycan and PG II or decorin). Biglycan is rich in Leu, whereas Decorin is rich in Glu/Gln. Each is demonstrable by non-cross reacting antibodies. In bone, both biglycan and decorin can be immunolocalized within the matrix and cells of new bone formation. In mature bone, biglycan (but not decorin) is detected in lacunae and canaliculi.

Both biglycan and decorin have central proteins of 45 kilo Daltons. Biglycan contains two chondroitin sulfates, whereas decorin contins a single chondroitin sulfate which occurs in a periodic manner within collagen fibrils. In fact decorin was so named because of its peculiar localization around collagen ("decoration" of collagen fibers). For more details on biglycan and decorin refer to Table 2.

| Biglycan | Decorin |
|---|---|
| The gene for biglycan is located on chromosome X, the mRNA contains sites of homology to two morphogenic proteins (Toll and Chaoptin) as well as von Willebrand factor. | The mRNA for decorin contains a sequence of epidermal growth factor. |
| Rate of synthesis is faster, but reduced by vitamin D | Slow synthesis |
| Rate of degradation slows with age | Remains the same with age |
| Mouse null for biglycan has increased osteoclast differentiation due to defective osteoblasts (225) and hypomineralization of dentin (227). | Mouse null for decorin has hypomineralization of dentin (227). |

The rates of synthesis as well as degradation are faster for biglycan as compared to decorin. The rates for degradation of biglycan however, significantly slow down with advancing age. This leads to a relative accumulation of biglycan. Whether this reflects an added need for biglycan is speculative. Additionally, the rate of synthesis of biglycan is significantly reduced by 1,25 dihydroxyvitamin D, whereas for decorin it is unaffected.

The role of compressive forces and its role in proteoglycan removal prior to mineralization are controversial. Similarly, their role in mineralization and in the developing bone is unclear. There is a probable role in "space capture", hydroxyapatite precipitation and the development of collagen scaffolding.

## Mineralization

Calcium hydroxyapatite is the main form of mineral in the human. In invertebrates the mineral most prevalent is calcium carbonate, whereas in plants it is the oxalate. The body's "biologic" hydroxyapatite crystal however differs from the form found in igneous rocks in its crystallographic structure and its size.

Calcification in the body occurs in several different situations. Firstly, there is the physiologic process of cartilage and osteoid mineralization. Secondly, there is the pathologic extracellular or intracellular "dystrophic" calcification, which occurs in association with tissue damage. Thirdly, there is a "metastatic" calcification, which occurs in association with altered serum levels of calcium and phosphate, and finally, there are the diseases of abnormal crystal deposition.

Most cases of calcium deposition within the soft tissues are caused by calcium hydroxyapatite - trauma, fat necrosis, scleroderma, hyperparathryroidism, familial hyperphosphatemia, sarcoidosis, myeloma, metastases, and others. These encompass both dystrophic and metastatic calcification. Punctate or linear calcification seen radiographically along

menisci, articular cartilage or intervertebral disks is generally due to calcium pyrophosphate deposition (CPPD disease). This deposition is rarely massive and simulates tophaceous deposits. In such cases the term tophaceous pseudogout may be appropriate (228). In the latter, tissue processing may remove the crystals making the diagnosis difficult. Additionally, areas of chondroid metaplasia and chondrocyte cellular atypia may raise the possibility of chondrosarcoma. The identification of areas of granulomatous inflammation may be the only clue to the real nature of the process.

Calcification of osteoid (bone mineralization) differs from soft-tissue calcification in being an orderly process. It is distributed within the hole-zones of collagen molecules. This method does not disrupt the spatial organization of collagen. The process is tightly regulated, but poorly understood. The mineral is initially deposited as amorphous calcium phosphate. The initial solid phase is formed at neutral pH. This phase is randomly and poorly oriented. Subsequently a series of solid phase transformations occur that lead to crystalline hydroxyapatite as the final stable solid phase. The initiation of mineralization is probably caused by heterogeneous nucleation, the active binding of calcium, phosphate and calcium phosphate complexes at the nucleation site in the matrix rather than by simple precipitation. In mature bone, it is possible that crystalline calcium carbonate containing hydroxyapatite is deposited rather than an amorphous calcium phosphate or hydroxyapatite.

## The Process of Mineralization

The process of mineralization is complicated and has not been satisfactorily elucidated. It is probably under genetic control, and since the physiologic state of extracellular fluids is supersaturated with respect to octacalcium phosphate, there are probably crystal inhibitors present. These include substances such as pyrophosphate and serum proteins. A local increase in concentration far beyond supersaturation is required to overcome the energy of the reaction of crystal formation. Additionally a local environment containing phosphatases and proteases to remove the inhibitors is essential.

A candidate for such a local environment is the "matrix vesicle". These are membrane bound cell free structures derived from chondrocytes and osteoblasts. Matrix vesicles are eccentrically placed within these cells. They are likely to be important in calcified cartilage and woven bone, but less so in mature lamellar bone. Extrusion of the matrix vesicle occurs at cell surfaces, next to mineralizing bone. These structures are rich in alkaline phosphatases and in ATPases. They presumably function by concentrating calcium and phosphate, and also by removing the inhibitors of calcification.

It must be emphasized that mineralization is also under hormonal control. Vitamin D plays an important role. Other exogenous factors that affect mineralization include aluminum intoxication, fluoride intoxication (osteofluorosis), and phosphate deficiency. The mechanisms of action of aluminum and fluoride are unclear. Mineralization is thus more than just a straight-forward physico-chemical process.

Hydroxyapatite crystal formation follows two phases: nucleation and propagation (multiplicative proliferation). The mechanisms operative to achieve this are thought to include: *matrix vesicle mediated* and *collagen mediated* hydroxyapatite precipitation. There are at least two hypotheses for mineralization. The first (229,230) gives primacy to matrix vesicles. The second (231) gives primacy to factors within the matrix. Each has been under investigation since the 1960s.

## Hypothesized Mechanisms of Mineralization in Tissues

### Mineralization in Cartilage

Mineralization occurs within "matrix vesicles" derived from chondrocytes. These are structures 2-4 μm in size, where hydroxyapatite is deposited. They are derived from the plasma membrane of cells. The membrane retains its phosphatases, which serves to raise the phosphate level. The lipid component of the membrane helps to concentrate calcium. This leads to intravesicular crystal formation. Subsequently, part of or the whole crystal is extruded. This leads to crystal propagation. This phase of crystal growth is physico-chemical and matrix factors play an important part. These matrix factors include collagen, carboxyglutamate proteins (Gla proteins), phosphoproteins, glycoproteins such as chondrocalcin, calcium-acidic phopholipid-phosphate complexes and proteolipids. Inhibitors of calcification include proteoglycans, magnesium, pyrophosphate, ATP, ADP and several synthetic compounds such as diphosphonates.

### Mineralization in Woven bone

It occurs both within matrix vesicles and within the "holes" of the fibers of Type I collagen. Rapid mineralization occurs since the matrix vesicles act as a nidus for the larger deposits in the collagen fibers. This works well for the bone to act as repair or reactive bone, but the organizational architecture is haphazard. The bone has a lower strength and is less rigid. It is likely that a phosphoprotein is involved in modulating this process. The rapid mineralization is reflected on the presence of only a thin zone of osteoid separating the osteoblast from

the mineralization front. Mineralization is seen to occur within 24–72 hours of matrix deposition.

## Mineralization in Lamellar bone

Mineral is deposited in a regular fashion within the collagen fibers, initiating from the "hole" region (see section on collagen structure). It takes up to 10 days for this slow mineralization to occur, consequently the osteoid zone separating the osteoblast and the mineralization front is wider in lamellar bone. This collagen mediated mineralization is thought to be a "heterogeneous" nucleation - and non-collagenus proteins may play a role in mediating the process.

## Surgical Pathology of Mineralization

It is difficult to visualize the site of mineralization in routine decalcified sections. Increased basophilia in the cartilage or woven bone lends suspicion for its location. However, it is reasonably straight forward to be able to do so in undecalcified sections. This is especially true in the case of lamellar bone mineralization. Fluorescent microscopy following a flurochrome (such as a tetracycline) administered 48 hours prior to the study aids the delineation. Osteoid mineralization commences 7-9 days following a resorptive front, and takes up to 200 days to complete.

## Mineral Deposits

Pathologic mineralization can be of several different types (dystrophic, metastatic or crystal deposition). Metastatic calcification (associated with increased serum calcium) is often intracellular. The increased $Ca^{2+}$ of the tissue fluid is initially taken up by mitochondria in the cytosol in an attempt to preserve the cytoplasmic homeostasis. When the mechanism is "overwhelmed" the calcium precipitates within the cell. With intra-cellular dystrophic calcification the process may be similar. The plasma membrane associated $Ca^{2+}$ pump is thought to be destroyed as a result of tissue damage. In this situation, there are increased cytosolic levels of calcium and a similar process follows. Extracellular dystrophic calcification is associated with a different mechanism. It is postulated, in this situation, that tissue damage leads to extrusion of matrix vesicles, which then cause calcium precipitation outside the cell. Crystal deposition disease (chondrocalcinosis or pseudogout) is the most difficult to explain. The role of matrix vesicles is un-established. An imbalance between the production of intrasynovial inorganic pyrophosphate and its removal by joint phosphatases may be more important.

Recent studies by Xiao et al. using proteomic analysis of matrix vesicles of mineralizing osteoblasts showed these vesicles produce different proteins. Some of these are previously known proteins such as annexins and peptidases, while others are novel proteins including a variety of enzymes, osteoblast-specific factors, ion channels, and signal transduction molecules, such as 14-3-3 family members and Rab-related proteins. These studies suggest that these proteins play a role in osteoblast matrix mineralization (232).

Factors Important in Mineralization

| | |
|---|---|
| **Collagen** | Collagen provides oriented support for the newly formed crystals. The post-translational changes in collagen type I make it possible for diffusion of large hydrated ions such as calcium phosphate into the fibril. Collagen also has sites that may initiate crystal precipitation. Also, there are high energy phosphate bonds (obtained from molecules such as ATP) which facilitate the formation of solid phase from solution. Collagen however is unable to initiate mineralization. |
| **Calcium binding proteins** | Phosphoproteins and sialoproteins in the bone matrix may bind calcium to promote crystal deposition and growth, thus acting as nucleators. Crystal growth could then depend upon the conformational change in these proteins after deposition. The initiation of mineralization is coincident with the deploymerization of proteoglycan molecules. Proteoglycans may inhibit calcification by a number of mechanisms including shielding of collagen, chemical interaction with collagen side chains, sequestering calcium or phosphate ions or occupying critical space in the molecule. Different phosphoproteins have varying importance in mineralization. |
| **Pyrophosphate** | This is a naturally occurring inhibitor of calcification. It has a short half-life due to its rapid degradation by pyrophosphatases. Pyrophosphates are present in body fluids and increase the stability of the solution phase of calcium phosphate. Diphosphonates are pyrophosphate analogs, and are powerful inhibitors of calcification in large doses. |
| **Bone Gla proteins** | Osteocalcin is a highly conserved protein which is abundant in the bone matrix. Because of the Gla residues, it is able to bind calcium. Its role in mineralization is controversial and there is debate over whether it promotes or inhibits mineralization (233). Depletion of osteocalcin from newly formed bone by warfarin treatment results in no impairment of mineralization (234). (see also osteocalcin described above). |
| **Lipids and proteolipids** | Within bone there are acid phospholipids that form complexes with calcium phosphate and could thereby influence mineralization. These substances have the capacity to bind to calcium. |

Factors Important in Mineralization

| | |
|---|---|
| **Alkaline phosphatase** | This is an ectoenzyme produced by osteoblasts and is likely to be involved with the mineralization process. Patients with decreased amounts of enzyme (hypophosphatasia) have impaired mineralization (see rachitic syndromes in metabolic bone disease). Bone alkaline phosphatase is present in high concentrations in matrix vesicles, but its precise role in mineralization is unclear. Alkaline phosphatase may be involved in the degradation of inorganic pyrophosphate, thus providing a sufficient level of organic phosphate for mineralization to proceed. |

## Remodeling of Bone

Bone undergoes remodeling throughout life. This involves the coupling of resorption of existing bone and the formation of new bone. Thus the entire bony skeleton is "renewed" on a continuous basis. This mechanism is important, because of the cyclical loading and torsional stresses that the skeleton undergoes. In the absence of renewal, bone would exceed its tolerance limits within a short period of time.

Bone turnover or remodeling is thought to occur in discrete foci or packets scattered throughout the skeleton. Each packet takes 3-4 months to complete. Such foci have been termed bone remodeling units or BRUs by Frost, who described the process in 1964. About 25 percent of the metabolically active trabecular bone and about 3 percent of the cortical bone completely renews itself each year (32,235). The amount of bone added in each remodeling cycle, however, reduces slightly with age. This is probably due to a decreased number of osteoblasts. This has been suggested as a possible mechanism for age related (but not post-menopausal) osteoporosis.

In the adult bone, remodeling involves activation, resorption and formation at endosteal and periosteal surfaces and within Haversian systems. Remodeling at the endosteal and periosteal surfaces would result in alterations in the thickness and width of tubular bones. Conditions such as acromegaly, osteopetrosis and hypo- or hyperthyroidism alter trabecular and cortical bone mass.

Bone formation during a remodeling process requires a prior resorption. Resorption takes approximately 10 days. The resorption is carried out by a "cutting cone" of osteoclasts. The trigger for resorption includes the stimulatory cytokines IL-1 and IL-6 produced by osteoblasts, as well as the modulation of the integrin-RGD sequence interaction and other factors such as transcription factors and membrane proteins.

The defect created after resorption, is filled in by fibrovascular tissue. The vessel component is especially important. The formation of the Haversian and the Volkmann systems are thought to be created by these mechanisms. In addition, the fibrovascular core contains pericytes, loose connective tissue, macrophages, mesenchymal stem cells and undifferentiated osteoprogenitor cells. The outer edge of the osteon (where resorption ends and bone formation first starts) is marked by an intensely basophilic line - the "cement" or the "reversal" line. This area is poor in collagen and mineral, and has a high content of sulfur.

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

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

The linking or "coupling" of bone resorption and bone formation is complex and difficult to explain. There is emerging evidence however to suggest that "osteoclastogenic" cytokines such as IL-6, IL-1 and IL-11 as well as "osteoblastogenic" cytokines such as leukemia inhibitory factor may be stimulated together by the same signal transduction pathway. Glycoprotein 130 is a molecule present in this pathway, and is involved in the transduction of the signal delivered by each of these cytokines. Sex steroids inhibit, whereas parathyroid hormone and vitamin D increase glycoprotein 130 in experimental models (32,235). This type of model would conveniently explain bone formation-resorption coupling as well as the various acknowledged effects of these hormones on bone turnover.

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

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

compensatory and relatively balanced increase in bone formation, due to the coupling of these events.

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

## Regulation of Bone by Endocrine and Paracrine Factors

### *Endocrine Control*

Endocrine control of the bony skeleton is multifarious and includes the need to maintain a balance between bone formation and loss, maintenance of homeostasis in calcium and phosphate levels in the body, and maintenance of a reservoir of phosphate required for generating energy. The major players in the endocrine system that participate in this regulation include parathyroid hormone, PTH-related peptide, calcitonin, vitamins A and D, estrogens, androgens and growth hormone.

#### Parathyroid Hormone

Parathyroid hormone (PTH) viewed as catabolic for bone is synthesized in the parathyroid gland from a biosynthetic precursor pro-PTH. PTH, a single chain polypeptide (84 amino acids referred to as PTH 1-84) impacts bone, intestine and kidney function. PTH mediates bone loss in older animals in its role to maintain calcium homeostasis and is required in fetal and neonatal animals for normal trabecular bone formation. In response to a decrease in serum calcium, PTH is released from the parathyroid gland. It targets the kidney to reduce calcium excretion, inhibits phosphate resorption and stimulates 1, 25 - dihydroxy vitamin D production which in turn targets the gastrointestinal tract to increase dietary absorption of calcium resulting in suppression of PTH. In addition, both PTH and 1, 25 $(OH)_2$-vitamin D are able to bind to osteoblasts and through RANK and RANKL increase osteoclastic activity which results in calcium and phosphate release from the bony skeleton returning serum calcium levels to normal by an increase in bone resorption. Receptors for PTH are found on pre-osteoblasts, osteoblasts and chondrocytes. They are not, however present on osteoclasts supporting the notion that the action of PTH on osteoclasts is osteoblast-dependent and mediated via substances such as IL-1, IL-6 and prostaglandins of the E series. The net result is osteoclast activation and initiation of bone resorption leading to calcium release from bone.

Evidence suggests that in certain situations PTH stimulates bone formation. When administered continuously, it increases osteoclastic resorption and suppresses bone formation. When administered in low doses, intermittently, it stimulates bone formation without resorption. This anabolic effect, like the resorptive effect is probably indirect, and mediated via IGF-1, TGFβ, etc. High serum PTH levels, maintained for even a few hours, initiates osteoclast formation resulting in bone resorption that overrides the effects of activating genes that direct bone formation. Identification of PTH-related protein (PTHrP) expression early in the osteoblast progenitor cells, its action through the PTH 1 receptor (PTH1R) on mature osteoblasts, and the observation that PTHrP+/- mice are osteoporotic, raise the possibility that PTHrP is a crucial paracrine regulator of bone formation.

#### Calcitonin

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

The physiological role of calcitonin remains controversial. Calcitonin and alpha-calcitonin gene-related peptide (alphaCGRP)-deficient mice exhibit high bone mass mediated by increased bone formation with normal bone resorption. The absence of significant changes in bone mineral density caused by a decline or overproduction of calcitonin in humans questions the physiological relevance of calcitonin as an inhibitor of bone resorption. A recent study on the age-dependent bone phenotype in two mouse models, one lacking calcitonin and alphaCGRP (Calca-/-), the other lacking alphaCGRP (alphaCGRP-/-) reported osteopenia at all ages in AlphaCGRP-/- mice. However, the Calca-/- -mice displayed increased bone turnover with age and at 12 months of age a

significant increase in bone formation and resorption. These data suggest that calcitonin may have dual actions, in bone formation and resorption, which may explain, at least in part, why alterations of calcitonin serum levels in humans do not result in major changes in bone mineral density (236). In addition, calcitonin has a role in the therapy of hypercalcemia of malignancy, in Paget's disease and in osteoporosis. Osteoclasts from Paget's patients are hyper-responsive to calcitonin, for longer periods of time than control cells although the molecular mechanism(s) for this hyper-responsivity is unknown (233).

## Vitamin D

Ergosterol and 7-dehydrocholesterol are the precursors for vitamin D, best labeled as a hormone and vitamin. These compounds are stored in the skin, transported in the body via an alpha-globulin binding protein/vitamin D binding protein (DBP) and become activated by ultraviolet light. Findings procured from gene targeting experiments in mice suggest that DBP possibly maintains stable serum stores of vitamin D metabolites and modulates the rate of its bioavailability, activation, and end-organ responsiveness. These properties may have evolved to stabilize and maintain serum levels of vitamin D in environments with variable vitamin D availability (238).

Activation of ergosterol and 7-dehydrocholesterol in turn generates calciferol and cholecalciferol. These substances are hydroxylated in the liver to yield 25-hydroxy-vitamin D in the presence of magnesium, and then are converted in the proximal tubule of the kidney to generate metabolites of 25-hydroxy-vitamin D. The most active form of vitamin D is 1,25-dihydroxyvitamin D. This hormone is key to the control of calcium metabolism in the gut, proximal tubule in the kidney and bone. 1,25-dihydroxyvitamin D production is regulated by calcium and PTH . It stimulates calcium binding protein, affects osteocalcin production, osteoclastic resorption, monocytic maturation, myelocytic differentiation, skin growth and insulin secretion. Lack of vitamin D results in impaired mineralization of newly formed bone which results in rickets in children, and osteomalacia in adults. These conditions are typified by an increase in proteinaceous bone matrix which does not mineralize. An excess of vitamin D leads to an increase in bone resorption and hypercalcemia.

Vitamin D acts via vitamin D receptors, and receptor sites of 1,25-dihydroxyvitamin D have been identified on several cell types. The vitamin D receptor is a transcription factor which forms homo- or heterodimers with members of the steroid hormone receptor superfamily (most notably the retinoic acid receptor RXR). Errors in genes that code for these nuclear receptors are reported in several forms of rickets. It is also suggested that postmenopausal osteoporosis may be genetically predetermined by polymorphisms present on the vitamin D receptor gene (237).

The vitamin D receptor type II (VDR-II) null mouse suggests a role for vitamin D in bone metabolism. These mice are phenotypically normal at birth, survive to 6 months of age, develop hypocalcemia at 21 days of age, at which time their parathyroid hormone (PTH) levels begin to rise. They also develop hyperparathyroidism accompanied by an increase in the size of the parathyroid gland with a concomitant increase in PTH mRNA levels. This phenotype is also associated with rickets and osteomalacia as early as day 15, and there is an expansion in the zone of hypertrophic chondrocytes in the growth plate. Interestingly the VDR-II knockout mouse also develops progressive alopecia at 4 weeks of age (239). Studies using primary calvarial cultures revealed that ablation of VDR-enhanced osteoblast differentiation was associated with an increase in alkaline phosphatase activity, as well as an early sustained increase in formation of mineralized matrix. The expression of bone sialoprotein, a marker for mineralization, was also increased in VDR null osteoblasts. These studies demonstrate that VDR attenuates osteoblast differentiation in vitro, and that other endocrine and paracrine factors may modulate the effect of VDR on osteoblast differentiation in vivo (240).

Evidence suggests that marrow mononuclear cells and monocytes fuse to form osteoclasts on exposure to vitamin D (233). Vitamin D receptors are not present on mature osteoclasts, thus osteoblasts are needed to mediate the effects of vitamin D to induce bone resorption and PTH may act synergistically with vitamin D to mediate this activity. In addition, it is likely that IL-1 and IL-2 play an intermediary role in bone resorption mediated by vitamin D.

## Calcitriol and Osteogenesis

Calcitriol (1$\alpha$, 25$(OH)_2$ $D_3$), the active form of vitamin D3, is synthesized from 25-hydroxyvitamin $D_3$ by the action of 1$\alpha$ hydroxylase which is present predominantly in the kidney (247). Mutations in the human1$\alpha$ hydroxylase gene cause pseudo-vitamin D deficiency rickets (248). Targeted ablation of the 1$\alpha$ hydroxylase gene in a mouse model leads to development of retarded growth, and skeletal abnormalities characteristic of rickets (249). Calcitriol resorbs bone by stimulating the formation of osteoclasts. Receptors for 1$\alpha$, 25$(OH)_2$ $D_3$ are found on osteoblasts and osteoprogenitor cells but not osteoclasts (241). Stimulation of osteoclast formation requires cell-cell contact between osteoblasts and osteoclast precursor cells, and involves the upregulation of the osteoclast-differentiating factor, RANK ligand in

osteoblasts, and downregulatation of OPG expression, an osteoclastogenesis inhibitory factor that works as a decoy receptor for RANK (242). Through stimulation of osteoclast formation, 1α, $25(OH)_2$ $D_3$ is believed to mediate bone resorption and remodeling. In addition, 1α, $25(OH)_2$ $D_3$ has been shown to inhibit osteoblast proliferation and stimulate apoptosis through induction of tumor necrosis factor alpha (243).

I*n vitro* studies demonstrate that vitamin $D_3$ stimulates osteoblast differentiation through induction of osteocalcin and alkaline phosphatase expression (both markers of mature osteoblasts) (244). These findings are supported by studies showing that the $Ca^{+2}$ binding proteins osteocalcin and osteopontin secreted by osteoblasts during differentiation, are upregulated by 1α, $25(OH)_2$ $D_3$ through its response element on the osteocalcin and osteopontin promoter (245). Moreover, 1α, $25(OH)_2$ $D_3$ stimulates osteoblast differentiation by the release of alkaline phosphatase (ALP) through the ERK-MAPK signaling pathway. Treatment of primary osteoblast cultures with an ERK inhibitor resulted in reduced 1α, $25(OH)_2$ $D_3$ induction of ALP, which confirms that 1α, $25(OH)_2$ $D_3$ stimulates ERK expression in primary human osteoblasts (246).

### Vitamin A

Retinoids, in excess, decrease the formation of bone and cartilage matrix, whereas a deficiency has the opposite effect. Several years ago, it was discovered that an imbalance of vitamin A during embryonic development had dramatic teratogenic effects. These effects have since been attributed to vitamin A's most active metabolite, retinoic acid (RA), which itself profoundly influences the development of multiple organs including the skeleton. After decades of study, researchers are still uncovering the molecular basis whereby retinoids regulate skeletal development. Retinoid signaling involves several components, from the enzymes that control the synthesis and degradation of RA, to the cytoplasmic RA-binding proteins, and the nuclear receptors that modulate gene transcription. As new functions for each component continue to be discovered, their developmental roles appear increasingly complex and each has been implicated in skeletal development. Moreover, retinoid signaling comes into play at distinct stages throughout the developmental sequence of skeletogenesis, highlighting a fundamental role for this pathway in forming the adult skeleton. Consistent with these roles, manipulation of the retinoid signaling pathway significantly affects the expression of the skeletogenic master regulatory factors, Sox9 and Cbfa1. In addition to the fact that we now have a greater understanding of the retinoid signaling pathway on a molecular level, we are able to place retinoid signaling within the context of other factors that regulate skeletogenesis. Here we review these recent advances and describe our current understanding of how retinoid signaling functions to coordinate skeletal development. We also discuss future directions and clinical implications in this field.

Retinoic acid (RA) is an endogenous metabolite of vitamin A that acts as potent regulator of osteoblast growth and differentiation of (250). The actions of RA are mediated by nuclear receptors that belong to the steroid hormone receptor superfamily (251). Changes in levels of RA during skeletal development result in severe abnormalities in the appendicular and craniofacial skeleton (252–254). Several studies have investigated the effects of RA on osteoblasts *in vitro*. Low doses of RA (0.01 μM) resulted in a increased levels of osteopontin and osteocalcin mRNA in fetal rat calvarial osteoblasts (255). Similarly, treatment of clonal pre-osteoblasts with pharmacologic doses (1 μM) of RA have shown an increase in osteopontin transcript levels and enhancement in nuclear processing of primary mRNA transcripts (256). While these reports suggest a direct relationship between RA level and osteoblast differentiation, other studies have demonstrated a decrease in alkaline phosphatase activity with both low and high-doses of RA, and decreases in osteocalcin transcription at higher doses (255,257). Thus, the actual effect that RA has on osteoblast differentiation and matrix mineralization remains to be determined.

An extensive literature on the role of steroid hormones (estrogens and androgens), and growth hormone reviews their impact on musculoskeletal development and disease and is not covered in the introductory chapter. In brief, in experimental situations, reduced estrogen leads to bone loss. This may be a direct effect on osteoblasts and possibly osteoclasts, and may be mediated via PTH and calcitonin. Androgens are reported to maintain bone mass via receptors on osteoblasts and the effect of growth hormone on bone is primarily mediated via insulin-like-growth factor (IGF). There may however also be a direct effect through growth hormone receptors on osteoblasts and chondrocytes.

## *Growth Factors*

### Transforming Growth Factor-Beta

Initially isolated from "transformed" neoplastic cells in tissue culture studies. Two "factors" were isolated and named TGFα and-β. TGFα is not found in bone and is now called epidermal growth factor. A number of similar compounds also exist (the TGFβ supergene family) including bone morphogenetic proteins (BMP). There are four known receptors for TGFβ. Additionally, there are cross effects from the stimulation of similar receptors. The net effect is to increase DNA

at low concentrations, enhance the synthesis of type I collagen and non collagenous proteins (fibronectin, proteoglycans etc.), and reduce the activity of alkaline phosphatase. There is less information on the effect on osteoclasts. There may be stimulation at low and inhibition at high concentrations. The latter effect is in association with the production of prostaglandins. TGFβ is said to have a prominent role in soft tissue healing, in a cascade fashion. It is released from the degranulation of platelets as well as from macrophages. It may help in the deactivation of the production of hydrogen peroxide, inhibit proteolytic enzymes and upregulate the integrin receptors for extracellular matrix proteins allowing the production of abundant granulation tissue (258,259). The current hypothesis is that TGFβ induces bone formation during remodeling. Additionally, high amounts are seen in tissues undergoing endochondral ossification. Experimental evidence suggests that TGFβ plays a positive role in intramembranous and endochondral bone formation as well as fracture and wound healing in experimental animals (258,259). The action of TGFβ in bone induction may however be only in conjunction with other factors such as the BMPs.

### Role of TGFβ-1 in Osteoblast Development *in Vitro*

It is well established that the members of the TGFβ superfamily play a crucial role in bone development, remodeling, and disease. However, the various TGFβ members have contradictory functions that have been documented *in vitro* and *in vivo* models. For example, knockout of TGFβ-2 has been shown to result in bone defects, indicating a positive role for these molecules in bone development (260). However, transgenic mice over-expressing TGFβ-2 under the control of an osteocalcin promoter displayed an osteoporosis-like phenotype (261). On the other hand, TGFβ-1 has been demonstrated to either stimulate or inhibit bone formation *in vivo*, and to differentially modulate distinct osteoblast markers *in vitro*. It has been suggested that TGFβ-1 enhances the proliferation and early differentiation of osteoblasts *in vitro*, which is characterized by a high rate of collagen synthesis, but impairs their terminal differentiation based on osteocalcin production (a differentiation marker) and mineralization of culture matrix (262). The TGFβ-1 signaling pathway begins by the binding of TGFβ to TGFβ specific type I and type II receptors leading to the phosphorylation of Smads 2 and 3, complex formation with Smad 4, translocation of Smad 2/3/4 to the nucleus, and transcriptional activation of specific target genes (263).

TGFβ-1 enhances intracellular $Ca^{+2}$ transport. This is crucial for osteoblast adhesion and early development in culture, since $Ca^{+2}$ enhances expression of α5 integrin, which is important in the formation of focal contact adhesions and cytoskeletal reorganization. These early events are necessary for osteoblast adhesion. Thus, they determine the fate of the osteoblast cell and ultimately affect bone function (264).

TGFβ-1 abrogates the steady-state levels of mRNA for lysyl hydroxylase in human osteoblast-like cells *in vitro* thus inhibiting the matrix maturation by affecting the degree of lysyl hydroxylation in newly synthesized collagen. The mRNA for lysyl hydroxylase was reduced by one-third under the influence of TGFβ-1. However, the mRNAs for both procollagen I alpha-chains were stimulated by TGFβ-1. Thus, TGFβ-1 increases collagen production and decreases its maturation (265). TGFβ-1 also stimulates osteoblast proliferation indirectly through inhibition of p57 cyclin-dependent kinase inhibitory protein (CKIs), a negative regulator of the cell cycle acting through the ubiquitin-proteasome pathway in newly proliferating osteoblast cells (266).

Nishimori and his colleagues, (267) found when the constitutively active form of the TGFβ-1 type I receptor was ectopically expressed in osteoblast cells, the p57 that had been accumulated by serum starvation and causing the cell-cycle arrest was rapidly degraded in a manner analogous to TGF-β1 stimulation. Moreover, Smad2 or Smad3 binding to Smad4 enhanced the proteolytic pathway of p57. All of the pathways mediated by TGFβ-1 growth factor suggest its important role in osteoblast proliferation but not terminal differentiation.

Studies on TGFβ-1 null mice have shown that growth plates, alkaline phosphatase (ALP) activity and collagen maturity were reduced in the tibiae at all ages compared to age-matched wild-type (WT) control animals using Fourier transform-infrared imaging (FTIRI) and immunohistochemistry (268). Also analysis of proximal tibial metaphyses showed significant decreases in the bone mineral content of the TGFβ-1 null mice compared to TGFβ-1 wild-type (WT) control animals. However, no significant differences were observed in bone mineral density (BMD) between the groups of mice. Histomorphometry revealed that the width of the tibial growth plate and the longitudinal growth rate were significantly decreased in the TGFβ-1 null mice, resulting in shorter tibia (269).

### Bone Morphogenetic Proteins (BMP)

A bone inducing principal was first postulated in 1952 by Marshall Urist et al. (270) Since then, at least ten proteins with this property have been extracted from *demineralized bone*, the amino acid sequence has been characterized and synthesized by recombinant DNA technology (271–276). These have been named bone morphogenetic proteins 1-10. BMP 3 is also called osteogenin, BMP 4 is also called BMP 2B, BMP 6 is also Vgr-1 and BMP 7 is known as osteogenic protein-1. This clash of terminology is due to the reclassification after characterization. These BMPs can be thought of as three separate families. One consisting of BMP 2 and 4,

the other of BMPs 5, 6 and 7 and the last consisting of BMP 3. These divisions are on basis of homology of structures. The issue has become complicated by the finding that these proteins (mostly members of the TGFβ super gene family) are found in several tissue types other than bone (277). In fact, the developing embryo has areas such as the apical epidermal ridge, which exhibit this property of bone induction, possibly due to such factors being elaborated.

Urist's work had suggested that demineralized bone matrix contains biologic signals to induce endochondral bone formation when implanted in soft tissues (osteoinduction). The relative osteoinductive contribution of bone cells as opposed to matrix in demineralized bone is debated. In the 1970's Japanese workers identified bone inducing activity in certain osteosarcoma cell lines. The molecule involved in this bone induction was later characterized as BMP 4. Certain human osteosarcoma cell lines such as the Saos-2 have also shown to produce several BMPs and TGFβ. Current recombinant technology however, has allowed the synthesis of these proteins from cDNA, obviating the need of large amounts of demineralized bone or neoplastic cell lines. Most of the BMPs (except for BMP 1) are basic proteins of 15 kDa, existing as dimers and belonging to the TGFβ superfamily. Disulfide bonds link these dimers. BMP-1 has recently been shown to be a protease with procollagen as its substrate (275–276). The synthesis of most BMPs has been performed by Wozney et al. at the Genetics Institute (Cambridge, Massachusetts). Their approach has been to isolate and sequence the cDNA for each BMP using a cDNA library obtained from the U-20S human osteosarcoma line. Following cloning, functional regions of the BMP sequences were transfected into a second mammalian line (Chinese hamster ovary) for expression and secretion of the mature BMP molecules. These were then isolated and purified by a chromatographic method developed by the Genetics Institute and Genentech (Cambridge, Massachusetts). Osteoinductive activity was tested using a bioassay employing rats.

Purified BMPs have been used to promote bone repair. Several trials have shown their efficacy in experimental models (278–280). Mixtures of BMPs have also been used, and shown to be more effective than comparable doses of single homodimeric BMP (281).

### Bone Morphogenetic Proteins and Osteogenesis

Bone morphogenetic proteins (BMPs) are osteotrophic factors as well as members of the TGFβ superfamily. The activity of BMPs was first identified in the 1960s (282), but the proteins responsible for bone induction remained unknown until the purification and sequence of bovine BMP-3 (osteogenin) and cloning of human BMP-2 and BMP-4 in the late 1980s (274,283,284).

BMP-2 induces gene expression and synthesis of osteoblast differentiation markers, alkaline phosphatase and osteocalcin, in pluripotent and preosteoblast cells. BMP-2 exposure for a short duration is sufficient to induce cell differentiation (285). Functions of bone morphogenetic proteins, such as BMP-2, are initiated by signaling through specific type I and type II serine/ threonine kinase receptors. It was previously reported that BMP receptor type IB (BMPR-IB) plays an essential and specific role in osteoblast commitment and differentiation (285). Smad1, 5, and 8 are substrates for BMP receptor I (BMPR-I) and mediators of the BMP signals that inhibit myogenic differentiation and induce osteoblast differentiation, in the mesenchymal C2C12 cell line (286). Studies from transgenic and knockout mice and from animals and humans with naturally occurring mutations in BMPs and related genes have shown that BMP signaling plays critical roles in heart, neural and cartilage development. BMPs also play an important role in postnatal bone formation (287).

BMP-2 is known to induce osteoblast differentiation by inducing Runx2. a global regulator for osteogenesis. Runx2 co-operates with BMP-2-induced Smad proteins to stimulate osteoblast differentiation (288). BMP-2 receptor activated Smad proteins induce Runx2; however, Smad does not directly induce Runx2 expression. The mitogen-activated protein kinase/p38 (MAPK/p38) cascade is also involved in the induction of Runx2 by BMP-2 (289). In addition, BMP-2 induces osteoblast differentiation through activation of an endogenous β-catenin signaling pathway thus implicating β-catenin in early steps of BMP-2 mediated osteoblast differentiation (290). In support, ectopic expression of stabilized β-catenin in the murine embryonic mesenchymal C3H10T1/2 cell line or activation of endogenous β-catenin signaling with lithium chloride induced expression of alkaline phosphatase mRNA and protein (an early osteoblast differentiation marker). However, unlike BMP-2 protein, stabilized β-catenin does not induce osteocalcin gene expression (a late osteoblast differentiation marker) (291).

## Insulin like Growth Factors: IGF I and II

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

### Other Growth Factors

Growth factors discussed earlier include Epithelial Growth Factor, Acid and Basic Fibroblast Growth Factors and Platelet Derived Growth Factors A and B (PDGFA and PDGFB). PDGFs, in particular, are potent mitogens of osteoblasts in vitro and have a chemotaxic effect on them. PDGFs are thought to be particularly important in bone remodeling. They are heterodimers of A and B chains, and function via specific receptors. Mutations in fibroblast growth factors are thought to play a role in certain kinds of skeletal deformities, including achondroplasia, Apert's syndrome, Cruzon syndrome, Pfeiffer syndrome, and Jackson-Weiss syndrome.

### Cytokines: Prostaglandins and Interleukins

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

*Interleukin 6 (IL-6)*: This cytokine is produced by many cell types, including osteoblasts and bone marrow stromal cells. Bone cells produce IL-6 in response to PTH, Vitamin $D_3$, TGFβ, IL-1 and TNFα, to name a few. Human osteoclastoma cells respond to this cytokine; however, it is still unclear whether normal mature osteoclasts respond to IL-6. It is known though that IL-6 has a pathogenetic role in diseases such as multiple myeloma, Paget's disease, rheumatoid arthritis and Gorham's disease (vanishing bone disease). Experimentally, estrogens and androgens inhibit the production of IL-6 by osteoblasts. Additionally, there is evidence to suggest that osteoclastic activity may be inhibited by anti-IL-6 antibodies (32).

## Mechanosensory Systems and Stretch Studies (Wolff's Law)

Wolff's Law - Every change in *form and function* of bones, is followed by changes in *the internal architecture and external conformation,* in strict accordance with mathematical laws (Julius Wolff, 1882).

Wolff's law has been confirmed by experimental studies. However, only recently have studies been performed to investigate the basis of this law at *a molecular level*. It is clear that mechanical forces effect skeleton morphology. For example, individuals who lift weights tend to develop bigger and stronger bones. If use of a limb is stopped, it undergoes "disuse" osteoporosis. Children with malunited limb fractures frequently remodel into almost normal appearing bones. If, though, they are unable to bear weight or use the limb (such as with poliomyelitis), then the limbs stay malunited. Paraplegics or quadriplegics with a spastic form of paresis often have exuberant callus formation. In contrast, patients with flaccid paresis fail to develop such an exaggerated response. Weightlessness in space causes rapid decrease in bone mass reflecting the need for constant force in maintaining skeletal bone.

All these examples illustrate the close link of mechanical forces with skeletal response and bone formation. What is still under investigation, however, is how these mechanical forces are translated into cellular events. It is likely, that signaling mechanisms, such as electricity or chemical messengers, such as certain cytokines, mediate these responses.

Computer controlled membranes holding tissue cultures of osteoblasts and fibroblasts have been used to alter the amounts of "stretch" provided to the cells. These studies have suggested that there is an altered metabolism and DNA synthesis under conditions of load. It has been suggested (292–294) that there may be two components to this system:

*The Cell Network*: This consists of osteocytes and their processes in communication with surface cells. Stretch sensitive ion channels are thought to exist on osteoblasts and fibroblasts.

*The Mineralized Matrix*: Stream generated potentials are created when fluid flowing through the matrix carries along a species of ion (in the presence of another species attached to the matrix).

These two mechanisms may be responsible for the signal for altered cellular metabolism observed. A "piezo-electric" effect as a result of compression of the hydroxyapatite crystal is also theoretically possible, but unlikely to be responsible for the coupling of mechanical-electric phenomena in bone.

The mechanism by which strain induces osteoblast proliferation in strain studies has thought to be mediated by the inositol 1,4,5 triphosphate system (295–296). Inhibition of phospholipase C (by neomycin) blocks inositol triphosphate production and subsequent proliferation. Additional signaling pathways (such as by cyclic AMP) may co-exist.

There is a hypothesis that cells maintain a basal equilibrium stress state that is a function of the number and quality of focal adhesions, the polymerization of the cytoskeleton and the amount of extrinsic applied mechanical deformation (297). A load stimulus detected by a mechano-electrochemical sensory system (including stretch sensitive ion-channels, integrin cytoskeletal machinery, and load-conformational sensitive receptor tyrosine kinase) activates G proteins, induces second messengers, and activates another kinase cascade to allow a respose.

It is also possible that integrins serve as important components of the mechanical sensory system. The RGD sequence of matrix proteins undergoes conformational changes with tension. This allows matrix tension to be "communicated" to bone cells. Signaling molecules such as nitric oxide (NO) have also been postulated in this process (298). Some studies have suggested that molecules such as osteopontin might be intermediary in this signalling process. For example, the binding of the $\alpha_v\beta_3$integrin with the RGD sequence of osteopontin might trigger osteoclastic resorption (299).

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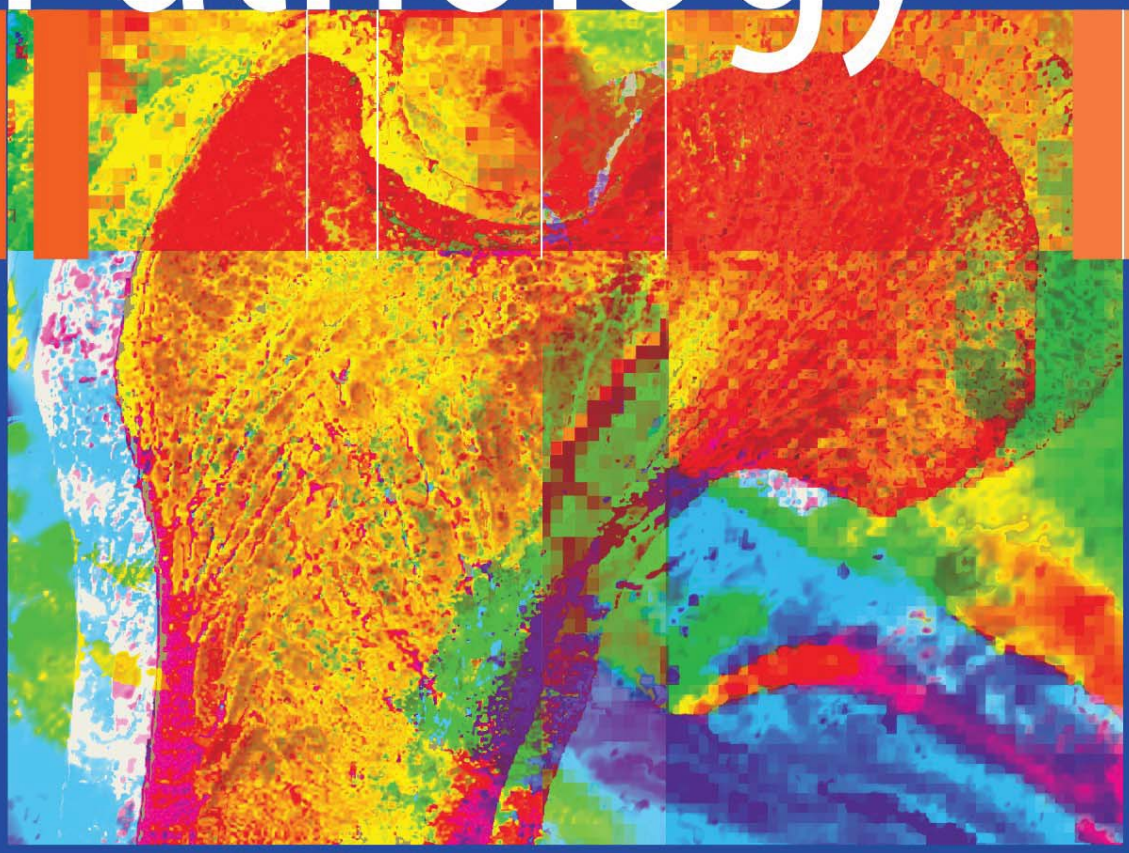

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*Editor*
Jasvir S. Khurana
Temple University Hospital
3401 North Broad Street
Philadelphia PA 19140
USA
jkhurana@temple.edu

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