

Osteogenesis: The Development of Bones

Three distinct lineages generate the skeleton. The paraxial mesoderm generates the vertebral and craniofacial bones, the lateral plate mesoderm generates the limb skeleton, and the cranial neural crest gives rise to some of the craniofacial bones and cartilage. There are two major modes of bone formation, or osteogenesis, and both involve the transformation of preexisting mesenchymal tissue into bone tissue. The direct conversion of mesenchyme into bone is called intramembranous ossification. In other cases, the mesenchymal cells differentiate into cartilage, which is later replaced by bone in a process called endochondral ossification.

Endochondral ossification

Endochondral ossification involves the formation of cartilage tissue from aggregated mesenchymal cells and the subsequent replacement of cartilage tissue by bone (Horton 1990). This type of bone formation is characteristic of the vertebrae, ribs, and limbs. The vertebrae and ribs form from the somites, while the limb bones form from the lateral plate mesoderm (see Chapter 19). Endochondral ossification can be divided into five stages: commitment, compaction, proliferation, growth, and finally, chondrocyte death and the generation of new bone.

Phases 1 and 2: commitment and compaction. First, the mesenchymal cells commit to becoming cartilage (Figure 1A). This commitment is stimulated by Sonic hedgehog, which induces nearby sclerotome cells to express the Pax1 transcription factor (Johnson et al. 1994; Teissier-Lavigne 1994). Pax1 initiates a cascade that is dependent on external paracrine factors and internal transcription factors.

During the second phase of endochondral ossification, the committed mesenchyme cells condense into compact nodules (Figure 1B). These inner cells become committed to generating cartilage, and the outer cells become committed to becoming bone. BMPs appear to be critical in this stage. They are responsible for inducing the expression of the adhesion molecules N-cadherin and N-CAM and the transcription factor Sox9. N-cadherin appears to be important in initiating these condensations, and N-CAM critical for maintaining them (Oberlender and Tuan 1994; Hall and Miyake 1995). Sox9 activates other transcription factors as well as a suite of genes, including those encoding collagen 2 (type II collagen) and aggrecan, which are required in cartilage function. In humans, mutations of the *SOX9* gene cause campotomic dysplasia, a rare disorder of skeletal development that results in deformities of most of the bones of the body. Most affected babies die from respiratory failure due to poorly formed tracheal and rib cartilage (Wright et al. 1995).

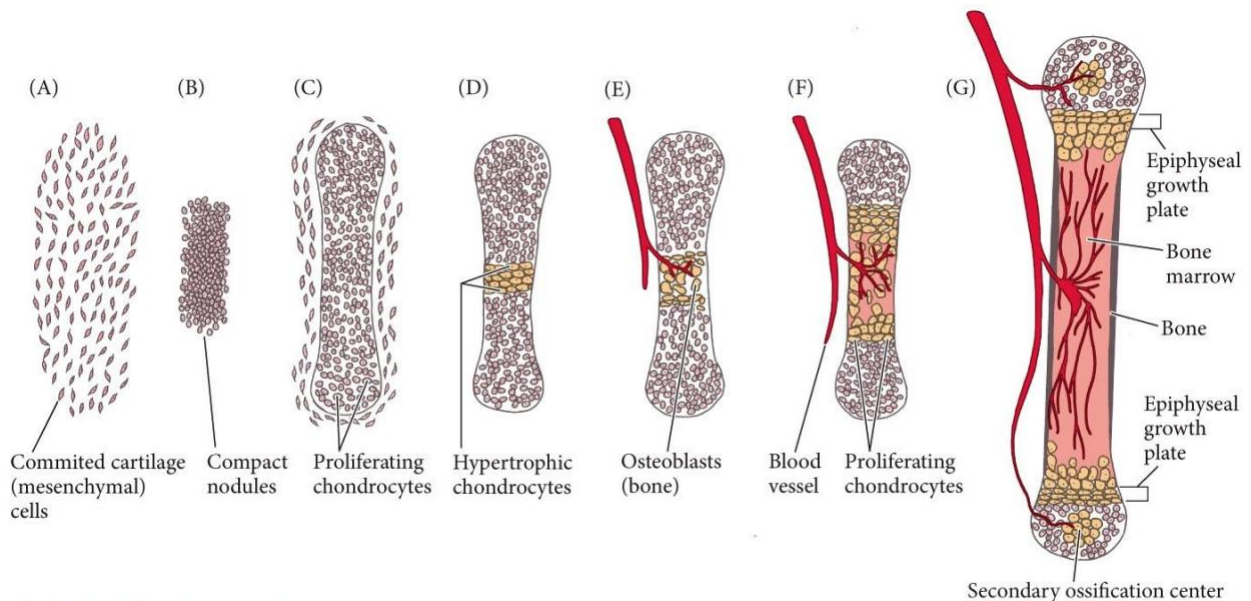
Phases 3 and 4: proliferation and growth. During the third phase of endochondral ossification, the chondrocytes proliferate rapidly to form a cartilaginous model for the bone (Figure 1C). As they divide, the chondrocytes secrete a cartilage-specific extracellular matrix. The outermost cells become the perichondrium that ensheaths the cartilage.

In the fourth phase, the chondrocytes stop dividing and increase their volume dramatically, becoming hypertrophic chondrocytes (Figure 1D and Figure 2). This step appears to be mediated by the transcription factor Runx2 (also called CBF α 1), which is necessary for the development of both intramembranous and endochondral bone. *Runx2* expression is regulated by histone deacetylase-4 (HDAC4), a chromatin-restructuring enzyme that is expressed solely in the prehypertrophic cartilage. If HDAC4 is overexpressed in the cartilaginous ribs or limbs, ossification is seriously delayed; if the

Hdac4 gene is knocked out of the mouse genome, the limbs and ribs ossify prematurely (Vega et al. 2004). Hypertrophic cartilage is exceptionally important in regulating the final size of the long bone. Indeed, the greatest contribution to the growth rate in mammals is the relative size of the hypertrophic cartilage (Cooper et al. 2013). The swelling of this cartilage determines the elongation rate of each skeletal element and is responsible for the differences in the growth rates between different skeletal elements both within an organism (hands vs. legs, for instance) and between related organisms (the legs of a mouse vs. the legs of a jerboa).

These large chondrocytes alter the matrix they produce (by adding collagen X and more fibronectin) to enable it to become mineralized (calcified) by calcium phosphate. These hypertrophic cartilage cells also secrete two factors that will be critical for the transformation of cartilage into bone. First, they secrete the angiogenesis factor VEGF (vascular endothelial growth factor), which can transform mesodermal mesenchyme cells into blood vessels (see Chapter 20; Gerber et al. 1999; Haigh et al. 2000). Second, they secrete Indian hedgehog, a member of the Hedgehog family and a close cousin of Sonic hedgehog, which activates *Runx2* transcription in the perichondrial cells surrounding the cartilage primordium. This step initiates the differentiation of those cells into bone-forming osteoblasts. Mice lacking the *Indian hedgehog* gene completely lack the osteoblasts of the endochondral skeleton (trunk and limbs), although the osteoblasts formed in the head and face by intramembranous ossification form normally (St-Jacques et al. 1999).

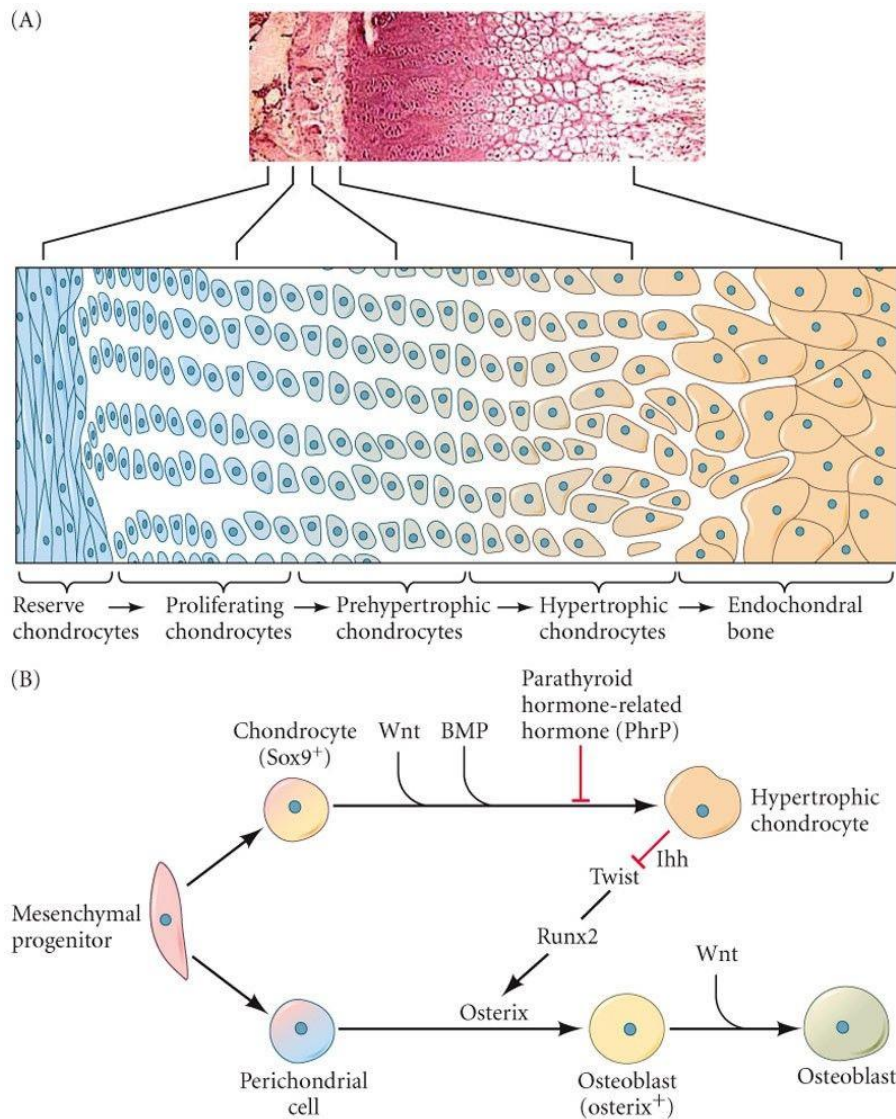
Phase 5: chondrocyte death and bone cell generation. In the fifth phase, the hypertrophic chondrocytes die by apoptosis (Hatori et al. 1995; Rajpurohit et al. 1999). The hypertrophic cartilage is replaced by bone cells both on the outside and inside, and blood vessels invade the cartilage model (Figure 1E and 1F). On the *outside*, the osteoblasts begin forming bone matrix, constructing a bone collar around the calcified and partially degraded cartilage matrix (Bruder and Caplan 1989; Hatori et al. 1995; St-Jacques et al. 1999). The osteoblasts become responsive to Wnt signals that upregulate Osterix, a transcription factor that instructs the osteoblasts to become mature bone cells, or osteocytes (Nakashima et al. 2002; Hu et al. 2005).



After W. A. Horton. 1990. *Growth Genet Horm* 6: 1-3.

Figure 1 Schematic diagram of endochondral ossification. (A) Mesenchymal cells commit to becoming cartilage cells (chondrocytes). (B) Committed mesenchyme condenses into compact nodules. (C) Nodules differentiate into chondrocytes and proliferate to form the cartilage model of bone. (D) Chondrocytes undergo hypertrophy and apoptosis while they change and mineralize their extracellular matrix. (E) Apoptosis of chondrocytes allows blood vessels to enter. (F) Blood vessels bring in osteoblasts, which bind to the degenerating cartilaginous matrix and deposit bone matrix. (G) Bone formation and

growth consist of ordered arrays of proliferating, hypertrophic, and mineralizing chondrocytes. Secondary ossification centers also form as blood vessels enter near the tips of the bone. (After Horton 1990.)



A (ossification stain) from M. C. Naski and D. M. Ornitz. 1998. *Frontiers in Bioscience* 3, d781-794;
 B after F. Long. 2012. *Nat Rev Mol Cell Biol* 13: 27-38

Figure 2 Endochondral ossification. (A) Long bone undergoing endochondral ossification. The cartilage is stained with alcian blue, and the bone is stained with alizarin red. Below is a diagram of the transition zone wherein the chondrocytes (cartilage cells) divide, enlarge, die, and are replaced by osteocytes (mature bone cells). (B) Paracrine and transcription factors active in the transition from cartilage to bone. The mesenchymal sclerotome cell can become a chondrocyte (characterized by the Sox9 transcription factor) or an osteocyte (characterized by the Osterix transcription factor), depending on the types of paracrine factors it experiences. The paracrine factor Indian hedgehog (Ihh), secreted by the growing chondrocytes, appears to repress Twist, an inhibitor of Runx2. Runx2 is critical for directing cell fate into the bone pathway; it activates Osterix, which in turn activates bone-specific proteins. (After Long 2012.)

New bone material is added peripherally from the *internal surface* of the periosteum, a fibrous sheath covering the developing bone. The periosteum contains connective tissue, capillaries, and bone progenitor cells (Long et al. 2012). At the same time, there is a hollowing out of the internal region of the bone to form the bone marrow cavity. As cartilage cells die, they alter the extracellular

matrix, releasing VEGF, which stimulates the formation of blood vessels around the dying cartilage. If the blood vessels are inhibited from forming, bone development is significantly delayed (Karsenty and Wagner 2002; Yin et al. 2002). The blood vessels bring in both osteoblasts and osteoclasts, multinucleated cells that eat the debris of the apoptotic chondrocytes and thus create the hollow bone marrow cavity (Kahn and Simmons 1975; Manolagas and Jilka 1995). Osteoclasts are not derived from the somite; rather, they are derived from a blood cell lineage (in the lateral plate mesoderm) and come from the same precursors as macrophage blood cells (Ash et al. 1980; Blair et al. 1986).

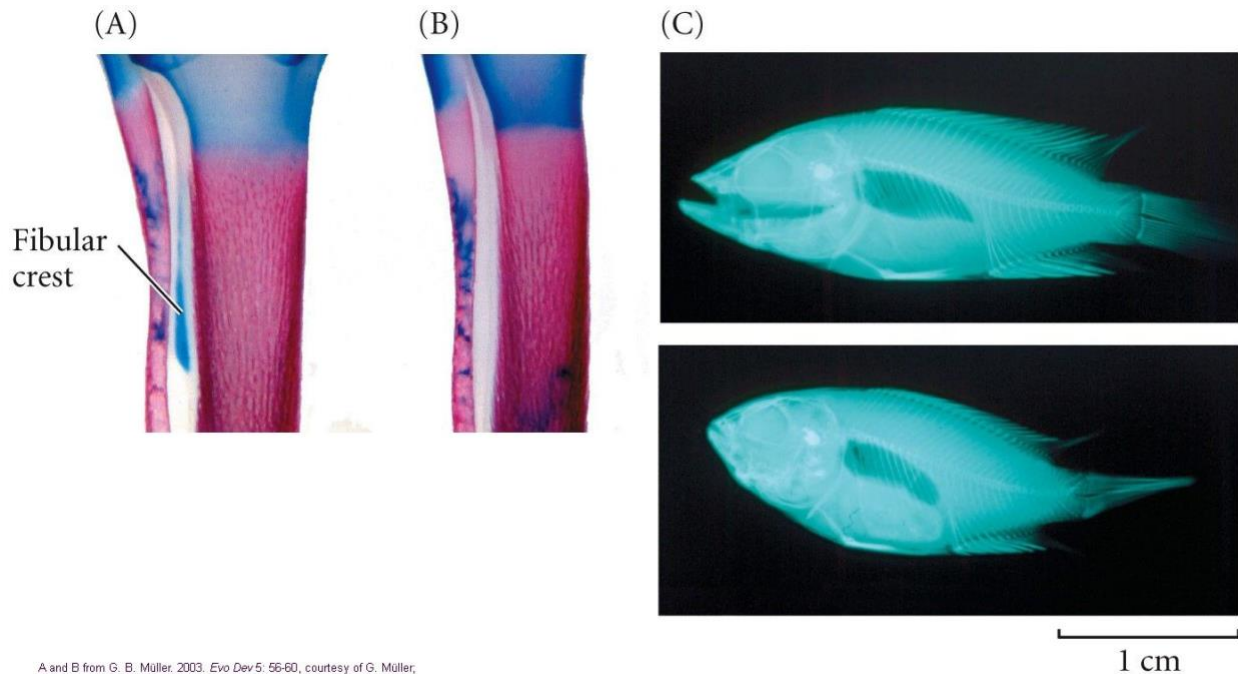
Mechanotransduction and vertebrate bone development

The ability of cells to sense their environment and convert mechanical forces into molecular signals is called mechanotransduction, and the importance of mechanotransduction to development is just beginning to be recognized. We saw this importance in the discussion of how extracellular mechanical signals changed the differentiation of stem cells, and mechanical forces appear to be significant in the formation of bones, muscles, and tendons, and perhaps also for their repair and regeneration in the adult. However, very little is known about how mechanical stress is sensed, quantified, and transmitted as a change in cytoplasmic chemicals.

Vertebrate skeletal bone development shows some dependence on mechanotransduction. Tension and stress forces activate the gene for Indian hedgehog (Ihh), a paracrine factor that activates the bone morphogenetic proteins (BMPs; Wu et al. 2001). In the chick, several bones do not form if embryonic movement in the egg is suppressed. One of these bones is the fibular crest, which connects the tibia directly to the fibula (Figure 3A and 3B). This direct connection is believed to be important in the evolution of birds, and the fibular crest is a universal feature of the bird hindlimb (Müller and Steicher 1989; Müller 2003).

The jaws of cichlid fish differ enormously depending on the food they eat (Figure 3C; Meyer 1987). Similarly, normal primate jaw development may be predicated on how much tension is produced by grinding food: mechanical tension appears to stimulate *Indian hedgehog* expression in mammalian mandibular cartilage (Tang et al. 2004). If an infant monkey is given soft food, its lower jaw is smaller than normal. Corruccini and Beecher (1982, 1984) and Varrela (1992) have shown that people in cultures where infants are fed hard food have jaws that “fit” better, and these researchers speculate that soft baby food may explain why so many children in Western societies need braces. Indeed, the notion that mechanical tension can change jaw size and shape is the basis of the functional hypothesis of modern orthodontics (Moss 1962, 1997).

In mammals, muscle force within the embryo is critical for the normal shaping of bone and the development of load-bearing capacity (Sharir et al. 2011). After birth, the patella (kneecap) is formed by pressure on the skeleton, and it is thought that the aberrant skeletal development seen in persons with cerebral palsy is caused by the absence of pressure on these bones (Shefelbine and Carter 2004; Ward et al. 2006).



A and B from G. B. Müller. 2003. *Evo Dev* 5: 56-60, courtesy of G. Müller;
 C from A. Meyer 1987. *Evolution* 41: 1357-1369, courtesy of A. Meyer.

Figure 3 Phenotypes can be produced by stress force on muscular and skeletal tissues. (A,B) The avian fibular crest (syndesmosis tibiofibularis) connects the tibia directly to the fibula. The fibular crest is formed when the movement of the active embryo inside the egg puts physical stress on the tibia. (A) Fibular crest forming in the connective tissue of a 13-day chick embryo. (B) Absence of fibular crest in the connective tissue of a 13-day embryo whose movement was inhibited. The blue dye stains cartilage; the red dye stains the bone elements. (C) The jaws of cichlid fish are shaped by the hardness of the food they eat. Different diets give different jaw structures. (A,B from Müller 2003, courtesy of G. Müller; C from Meyer 1987, courtesy of A. Meyer.)

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