HHS Public Access

Author manuscript

Bone. Author manuscript; available in PMC 2016 November 01.

Published in final edited form as:

Bone. 2015 November ; 80: 14–18. doi:10.1016/j.bone.2015.04.035.

Bone development

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Abstract

The development of the vertebrate skeleton reflects its evolutionary history. Cartilage formation came before biomineralization and a head skeleton evolved before the formation of axial and appendicular skeletal structures. This review describes the processes that result in endochondral and intramembranous ossification, the important roles of growth and transcription factors, and the consequences of mutations in some of the genes involved. Following a summary of the origin of cartilage, muscle, and tendon cell lineages in the axial skeleton, we discuss the role of muscle forces in the formation of skeletal architecture and assembly of musculoskeletal functional units. Finally, ontogenetic patterning of bones in response to mechanical loading is reviewed.

Keywords

bone development; endochondral ossification; intramembranous ossification; transcription factors; somite differentiation; skeletal patterning; tendon-bone attachment

Introduction

The development of the vertebrate skeleton reflects its evolutionary history in that different parts utilize different cellular sources and differentiation mechanisms for their formation (Karsenty, 2003; Olsen et al., 2000). The development of cranial bones can be traced back to the evolution of Craniates in the early Cambrian period, when chordates evolved a head with a brain, eyes and other sensory organs protected by a cartilaginous non-mineralized skull (Zhang, 2009). In the late Cambrian, Euconodont fossils show mineralization of tooth-like structures and a cartilage head skeleton, but no axial skeleton. Cartilage formation appears therefore to predate biomineralization, and a head skeleton evolved before axial and appendicular skeletal elements. The axial skeleton evolved first as a structure of unmineralized cartilage around the notochord that later (after lampreys diverged from other vertebrates) became mineralized and replaced by bone. Evolution of gnathostomes with jaws, a shoulder girdle and two paired sets of appendages represented a breakthrough in the mid-Ordovician period.

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Endochondral and intramembranous ossification

Given the stepwise progress of skeletal evolution over millions of years it is not surprising that different cell lineages contribute to different parts of the mammalian skeleton. Neural crest cells from the dorsal margins of the closing neural tube migrate into the anterior region of the skull, giving rise to dentin of teeth and the connective tissue and some of the bones and cartilages of the face and anterior skull. Prechordal mesodermal cells produce cartilages and bones in the posterior part of the skull. Paraxial mesoderm (somites) is the cellular source of the axial skeleton, whereas lateral plate mesodermal cells form the appendicular skeleton (Figure 1). The initiation of skeletogenesis starts with migration of mesenchymal cells derived from these embryonic lineages to the sites of the future bones. Here they form condensations of high cellular density that outline the shape and size of the future bones, *i.e.* the anatomic identity of the skeletal element is predetermined (Hall and Miyake, 1992). Within the condensations, the mesenchymal cells either differentiate into chondrocytes and form cartilage models (anlagen) of the future bones (endochondral bone formation) or differentiate into osteoblasts to directly form bone (intramembranous bone formation). Endochondral bone formation occurs in the skull base and the posterior part of the skull, the axial skeleton and the appendicular skeleton. Intramembranous bone formation takes place in the membranous neuro- and viscerocranium and in part of the clavicle.

The replacement of cartilage with mineralized bone in endochondral bones (endochondral ossification) is a complex process, triggered by differentiation of proliferating chondrocytes in the center of cartilage anlagen to a non-proliferative hypertrophic state. This is followed by invasion of osteoblast progenitors, osteoclasts, blood vessel endothelial cells and hematopoietic cells from the perichondrium into the hypertrophic cartilage. The hypertrophic cartilage is resorbed, the incoming osteoblast progenitors differentiate into trabecular bone-forming osteoblasts, and hematopoietic and endothelial cells establish bone marrow in what becomes the primary ossification center (Maes et al., 2010). Osteoblast progenitors in the perichondrium differentiate into osteoblasts that deposit cortical bone around the cartilage anlage. As the fetus grows, the primary ossification center expands and secondary ossification centers form in one or both ends of the developing bone. This results in the development of epiphyseal growth plate cartilage, responsible for the longitudinal growth of bones (Karsenty and Wagner, 2002; Kronenberg, 2003). Within growth plates, chondrocytes are organized into structural and functional zones, each with distinct gene expression patterns (Mundlos, 1994). Small and relatively inactive cells are located in the reserve zone close to the secondary ossification center, whereas proliferating chondrocytes are present in the adjacent proliferative zone. These cells undergo clonal expansion and align themselves into columns parallel to the direction of longitudinal growth. As the cells enter the hypertrophic zone, they stop proliferating and may undergo apoptosis. Recent data suggest that hypertrophic cells are not all undergoing apoptosis, but may also become osteoblasts (Yang et al., 2014). With age, the growth plates get thinner and are eventually replaced by bone at various times after puberty in humans.

Local and systemic factors regulate proliferation, differentiation and apoptosis of growth plate chondrocytes. A local factor, Indian hedgehog (Ihh), is produced by prehypertrophic chondrocytes, promotes hypertrophy and stimulates proliferation of chondrocytes in the

proliferative zone (Mak et al., 2008). It also stimulates osteoblast differentiation and synthesis of parathyroid hormone-related peptide (PTHrP) in the perichondrium. In turn, PTHrP inhibits the transition of proliferative chondrocytes to prehypertrophic, Indian hedgehog-expressing cells. This establishes a negative feed-back loop that controls the size and activity of the proliferative zone in the growth plate and couples cortical bone formation to the longitudinal growth of the bone. Synthesized in the perichondrium, fibroblast growth factor 18 (Fgf18) acts as a negative regulator of chondrocyte proliferation by activating fibroblast growth factor receptor 3 (Fgfr3) signaling in proliferating and prehypertrophic chondrocytes (Haque et al., 2007). Proliferation is also controlled by growth hormone and insulin-like growth factor 1 (IGF-1) (Baker et al., 1993; Ohlsson et al., 1992). Deficiencies or activating gene mutations in these factors result in phenotypes with reduced growth and dwarfism without effects on bone shape or structure.

Regulation of skeletal patterning and differentiation of chondrocytes and osteoblasts

During evolution many genes and associated signaling pathways have been recruited as regulators of the phylogenetic patterning of the vertebrate skeleton (Karsenty, 2003; Kronenberg, 2003; Olsen et al., 2000). Use of genetic approaches in mice and studies of the genetic basis of hereditary skeletal diseases in humans have been important for their identification (Mundlos and Olsen, 1997a, b; Zelzer and Olsen, 2003). A number of skeletal dysplasias are associated with defects in production or functionality of cartilage and bone matrix; others cause defects in differentiation or function of chondrocytes, osteoblasts and osteoclasts. This results in compromised skeletal growth or too much or too little cartilage and bone. Conditions known as dysostoses affect particular skeletal elements and not skeletal tissues in general. They include mutations in patterning genes that affect the size and shape of specific bones. The phenotypic consequences of such defects can develop at different stages, such as during mesenchymal condensations, differentiation into proliferative chondrocytes or maturation into hypertrophic chondrocytes. Studies of such disorders have identified transcription factors that are crucial for patterning and formation of cartilage and bone, including members of the homeobox (*Hox*) and paired-box (*Pax*) families (for review see (Helms and Schneider, 2003; Krumlauf, 1994; Mariani and Martin, 2003; Mundlos and Olsen, 1997a)). Mutations in *HOXD13* result in shortening of phalanges and/or metacarpals (brachydactyly; OMIM 113200 and 113300 and brachydactyly-syndactyly; OMIM 610713 syndromes) or syndactyly with or without polydactyly (syndactyly; OMIM 186300 and synpolydactyly; OMIM 186000) (Kan et al., 2003; Muragaki et al., 1996). Mutations in *HOXA13* are associated with unusually short great toes and abnormal thumbs (hand-foot-genital syndrome; OMIM 140000) (Mortlock and Innis, 1997). Mutations in *PAX3* may result in a musculoskeletal phenotype (craniofacial-deafness-hand syndrome; OMIM 122880) in which all fingers show ulnar deviation as a result of a muscle defect, combined with sensorineural hearing loss, underdeveloped or absent nasal bones, hypertelorism and a small upper jaw, and permanently bent third, fourth and fifth fingers in some patients. *PAX3* mutations can also cause abnormalities in the upper limbs, associated with hearing loss and changes in pigmentation (Type III Waardenburg syndrome; OMIM 148820) (Tassabehji et al., 1992; Zlotogora et al., 1995).

Members of the transforming growth factor beta (TGF-β) superfamily, such as growth and differentiation factors (GDFs) and bone morphogenetic proteins (BMPs), regulate bone shape (Kingsley, 1994). Characterization of several mutant mouse phenotypes, including the autosomal recessive short ear (se; BMP5) and brachypodism (bp; GDF5) mutants, revealed the importance of BMP family members in formation of mesenchymal condensations (King et al., 1994; Storm et al., 1994). BMP signaling induces the expression of the transcription factor SRY (sex determining region Y)-box 9 (SOX9), which is required for commitment of undifferentiated mesenchymal cells in the condensations to chondrocytes (Akiyama et al., 2002; Kawakami et al., 2006; Mori-Akiyama et al., 2003). Mutations resulting in loss of *SOX9* activity cause lack of cartilage in campomelic dysplasia (CMPD1; OMIM 114290) (Wagner et al., 1994). Interestingly, in addition to its essential role in inducing chondrocyte differentiation, SOX9 is also indirectly a regulator of maturation of the appendicular vasculature plexus in developing limbs via its ability to induce vascular endothelial growth factor A (VEGF) expression in the condensing mesenchymal cells (Eshkar-Oren et al., 2009).

Endochondral bone formation is regulated by many morphogens and growth factors, such as Wnts, Hedgehogs, Notch, VEGF, FGFs, IGF-1, TGF-β, and PTHrP (Kronenberg, 2003). These factors and their associated signaling pathways interact with each other and coordinate many cellular processes, including chondrocyte and osteoblast differentiation and proliferation. Many of the pathways regulate the expression of Runx2 and its downstream target Osterix, transcription factors that are important for the progression of proliferating chondrocytes to hypertrophy and essential for differentiation of mesenchymal stem cells to osteoblasts (Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997). Their expression patterns are high in the late condensation stage of chondrogenesis, in prehypertrophic and hypertrophic chondrocytes, in perichondrial cells and osteoblasts. Heterozygous loss-of-function mutations in *RUNX2* cause Cleidocranial dysplasia (OMIM 119600), characterized by insufficient ossification of the calvarial bones and the clavicles (Mundlos et al., 1997). A recessive form of Osteogenesis imperfecta (type XII; OMIM 613849) has been associated with a frame-shift mutation in *SP7*, the gene encoding Osterix (Lapunzina et al., 2010).

Origin of cartilage, muscle and tendon cell lineages in the axial skeleton

The tetrapod axial skeleton with the associated skeletal muscles and dorsal dermis are derived from the somites, the specialized blocks of paraxial mesodermal cells that form on each side of the neural tube and notocord in the developing embryo (for review see (Brent and Tabin, 2002)). In response to various patterning signals from surrounding tissues somites differentiate and the multipotent somitic cells are organized into epithelial dorsal dermomyotomes and mesenchymal ventral sclerotomes (Brent et al., 2003). Upon further maturation, the dermomyotome develops into myotome and dermatome and the sclerotome is divided into a ventral sclerotome and dorsal syndetome (Figure 2). Sclerotomal cells wrap around the notochord to form the vertebral bodies and intervertebral discs and ligaments of the vertebral column. The more lateral sclerotomal cells form the neural arches and ribs. The dorsal dermis and skeletal muscle originate from the dermomyotome and tendons associated with the axial skeleton are formed from cells of the syndetome. Multiple signals are crucial for somite development. The formation and maintenance of the sclerotome depends on

secretion of two morphogens, Sonic hedgehog (Shh) and Noggin, by cells in the notochord and neural tube floor plate, competing with Wnts and BMPs that are expressed by more lateral and dorsal cells and act to suppress sclerotome formation while stimulating formation of the dermomyotome (Figure 2). Sclerotome cells express the transcription factors Pax1, Pax9, FoxC1 and FoxC2 in response to Shh and Noggin signaling. These factors are important for proliferation and survival of sclerotome cells, and FoxC2 is involved in the formation of vertebral dorsal neural arches and spine processes (Kume et al., 2001). The two Pax genes are required for induction of a key transcription factor (NK3.2) that initiates skeletogenesis by regulating Sox9 and Runx2 expression (Kokubu et al., 2003; Peters et al., 1999).

Shh signaling from the notochord, combined with Wnt signaling from the dorsal neural tube and BMP4 signaling from the adjacent lateral plate mesoderm, restricts expression of myogenic regulatory factors, MyoD and Myf5, to the myotome region of the dermomyotome. These factors and Mrf4 drive the development of epaxial and hypaxial muscles of the spine (Brent and Tabin, 2002). MyoD and Myf5 also induce expression of Fgf4 and Fgf5 in the center of the myotome and secretion of the two growth factors results in specification of tendon progenitor cells and their expression of the bHLH transcription factor Scleraxis (Scx) in the syndetome (Brent et al., 2005; Brent et al., 2003). The myotome also expresses Fgf8, which induces proliferation of the ventrolateral rib-forming region of the sclerotome (Brent and Tabin, 2004) (Figure 2).

Muscle force and musculoskeletal assembly

The development and functionality of bones, cartilage and joints, require precise coordination with other components such as muscles, tendons, and ligaments (Shwartz et al., 2013). Regulatory interactions are therefore needed during the assembly into functional units. Such interactions include muscle-induced mechanical forces that regulate morphogenesis and maintenance of skeletal tissues, tendons and the development of tendon-to-bone attachment sites. For example, the size of the proliferative zone and the number of proliferating chondrocytes in growth plates are greatly affected in paralyzed chick and mouse embryos (for review see (Shwartz et al., 2013)). Furthermore, mechanical stress affects the elongation of chondrocyte columns (Shwartz et al., 2012) and production of extracellular matrix (ECM) (Mikic et al., 2004). Both Indian hedgehog (Ihh) and PTHrP have been found to be mechanosensitive molecules (Ng et al., 2006; Wu et al., 2001; Wu and Chen, 2000), suggesting that complex mechanisms involving both biological and mechanical signals are integrated during growth plate formation and longitudinal growth of long bones. Muscle contraction is also required for normal joint development, and fusion of multiple joints was observed in limbs of paralyzed embryos (Lelkes, 1958). Muscle activity is not needed for the specification of joint progenitor cells (Kahn et al., 2009; Mikic et al., 2000; Mitrovic, 1982), but cavitation of the joint does not occur and the flattened joint cells dedifferentiate to chondrocytes, resulting in joint fusion, in the absence of muscle contraction (Kahn et al., 2009). Mechanical load regulates tendon development and the maintenance of tendon-specific extracellular matrix by inducing Scleraxis (Scx) expression through TGF-β-mediated signaling pathways (Maeda et al., 2011). Muscle force also regulates the circumferential shapes of bones and local control of cortical bone thickness,

thereby optimizing their capacity to bear load during development (Carter et al., 1996; Main, 2007; Sharir et al., 2011).

Development of tendon-bone attachment sites

Each bone has a unique size and shape with numerous protrusions that are optimal for tendon attachments and coordination with muscle activities. Different types of protrusions include rough elevations along the bone surface forming bone ridges, providing stable anchoring points for insertion of muscles to bones via tendons (Blitz et al., 2009). Bone ridges, also referred to as bone eminences, are important structures for absorption and dissipation of mechanical loads exerted on the bones via tendons by muscle contractions (Benjamin et al., 2002; Biewener et al., 1996). Bone eminences form after the development of the primary cartilage model of the future bone. Cells occupying the area of the forming eminence are not derived from the chondrocytes of the cartilage anlage but rather an external pool of progenitor cells close to the cartilage. Specification of the progenitor cells requires TGF-β signaling and blocking Sox9 expression in Scx-positive cells results in loss of bone ridges. The tendon-bone attachment unit therefore develops from progenitor cells expressing both Scx and Sox9 (Blitz et al., 2013).

Subsequent differentiation of these cells into eminence-forming cells is controlled by mechanisms that require both Scx and BMP. Loss of either *Scx* or *Bmp4* expression in limbs prevents the development of eminences (Blitz et al., 2009). However, the embryonic origin of the cell lineage forming the bone eminences is not clear. The tendon-bone attachment unit starts to mineralize about a week after birth in mice via mechanisms similar to growth plate mineralization (Shwartz et al., 2012). Several signaling pathways play important roles in this process, including Ihh and PTHrP, as changes in their expression levels in Scx-expressing cells affect the enthesis. These factors and others that are critical to growth plate function, e.g. SOX9 and collagen type X, are expressed at the developing enthesis (Bland and Ashhurst, 1997, 2001; Fujioka et al., 1997; Galatz et al., 2007). Muscle contraction also regulates the tendon-bone attachment formation (for review see (Zelzer et al., 2014)). Further studies are needed to identify the molecules involved in the different aspects of the formation and growth of bone eminences and tendon-bone attachment units. In fact, information on the development of all different aspects of the musculoskeletal system and the various developmental mechanisms of tendon attachment is crucial in order to develop therapeutic strategies for repair and regeneration in cases of compromised body movement.

Bone ridges that are large and irregular in size are referred to as tuberosities. They develop through endochondral ossification in a coordinated manner with the development of muscles and tendons. Bone formation in a tuberosity involves formation of a tuberosity-specific growth plate that depends on both tendon and muscle interactions with the developing bone. Initiation of the deltoid tuberosity formation in mice occurs at E13.5 through a Scx-BMP4-dependent mechanism in tendon cells while its subsequent growth is under the control of muscle contractions (Blitz et al., 2009). In the initiation process, Scx in tendon cells directly regulates the Bmp4 promoter and secretion of Bmp4 in turn activates Bmp signaling in tuberosity-forming chondrocytes.

Ontogenetic patterning of the skeleton in response to mechanical loading

The postnatal adaptation of the skeleton to a rapidly changing mechanical environment results in modeling and remodeling responses that change cortical and trabecular bone patterns and the size and structure of tendon insertion sites. An interesting example of the extent to which such postnatal modeling can change the skeleton is the ontogenetic change in skull morphology in the spotted hyena (Tanner et al., 2010). Hyenas compete for food in an extreme environment where ability to crack and consume large amounts of bones in a short period of time favors survival. As an adaptation to such an environment, the animals develop a strong dentition, large jaw muscles, increased cortical thickness of the dental bone, and a large sagittal bone crest on the calvarium. Remarkably, the full development of these structural features, clearly driven by muscle forces on the bones involved, is not completed until long after weaning and puberty. Less impressive but equally important is the age-dependent changes in modeling and remodeling in different parts of the human skeleton. The osteocyte network within bone is likely playing a major role in sensing the changes in strain generated by alterations in muscle mass, bone length and muscle activity. The extent to which cortical and trabecular bone models and remodels in response to these changes in strain depends on ontogenetic age; young individuals show strong responses to changes in loading, whereas old adults exhibit little or no response (Pearson and Lieberman, 2004). Many factors contribute to this difference, but age-dependent loss of mesenchymal stem cells and osteoprogenitors are likely playing a role. Also important may be age-dependent decreases in responsiveness to hormones and growth factors. Finally, osteoprogenitor cells respond to the stiffness of their environment by organizing their cytoplasmic (actin) and nuclear (lamin A) cytoskeletal networks into an osteoblast-inducing mode; in senescent cells, reduced levels of lamin A and mediators of its effect on osteoblast differentiation may contribute as well (Pekovic and Hutchison, 2008; Swift et al., 2013).

Concluding remarks

Research during the last 25 years led to remarkable progress in our understanding of cellular processes and molecular mechanisms underlying development and postnatal growth of the vertebrate skeleton. Several specific advances prompted the development of novel treatments for both rare and common bone disorders. However, relatively little is still known about how the musculoskeletal system operates as a set of functional units, from early development to an old age. Questions related to how the system works in health and disease will require studies of integrated cell and extracellular matrix processes at the cross roads between skeletal, muscle, tendon, vascular and hematopoietic tissue biology.

Acknowledgments

Work in the authors' laboratory was supported by R01AR36819 (to BRO) from the National Institutes of Health.

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Highlights

- Development of the vertebrate skeleton reflects its evolutionary history.
- Processes of endochondral and intramembranous ossification are described.
- Consequences of mutations affecting skeletal development are discussed.
- Cell lineages axial skeletal development are described.
- Skeletal architecture and patterning require muscle forces and mechanical loading.

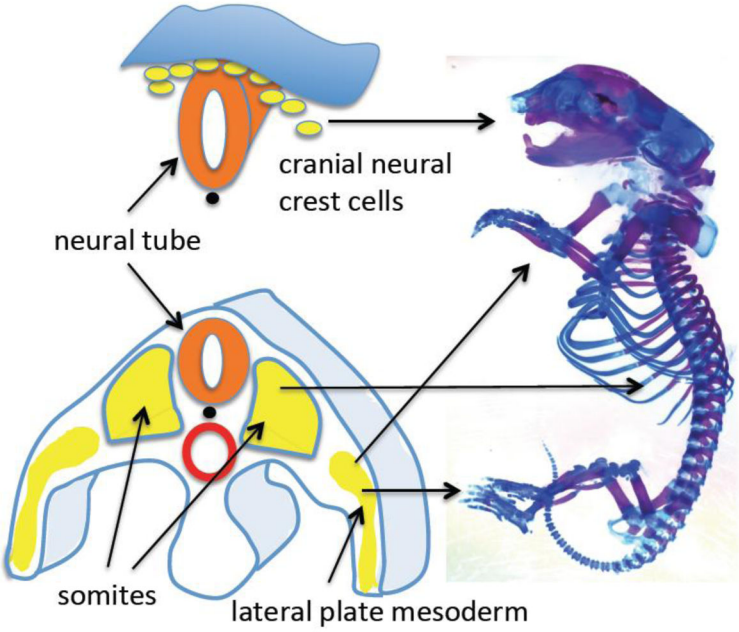

Figure 1.

Diagrams illustrating the contributions of cranial neural crest cells, somite-derived cells and lateral plate mesodermal cells to the craniofacial, axial and appendicular parts of the mouse skeleton. The notochord under the neural tube (top part) and between the neural tube and the aorta (red ring; bottom part) is indicated by a black dot.

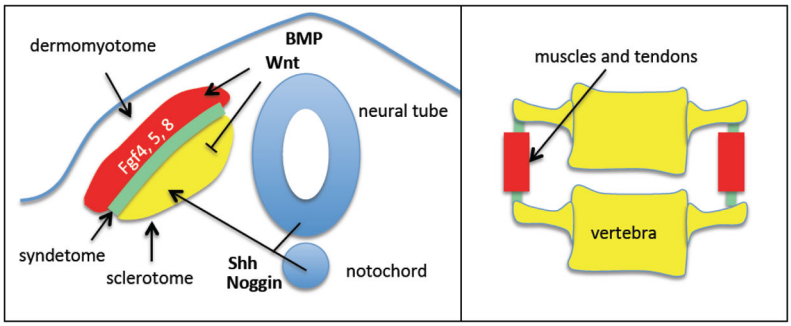

Figure 2.

Diagrams illustrating how BMP, Wnt, Shh, Noggin and Fgf4, 5, and 8 control the differentiation of somites into dermomyotome, syndetome and sclerotome compartments (at left). Sclerotomal cells give rise to vertebral bodies, while syndetomal and sclerotomal cells give rise to intervertebral tendons and muscles (at right).