CHAPTER TWO

Evolution of Vertebrate Cartilage Development

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Contents

Abstract

Major advances in the molecular genetics, paleobiology, and the evolutionary developmental biology of vertebrate skeletogenesis have improved our understanding of the early evolution and development of the vertebrate skeleton.

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These studies have involved genetic analysis of model organisms, human genetics, comparative developmental studies of basal vertebrates and nonvertebrate chordates, and both cladistic and histological analyses of fossil vertebrates. Integration of these studies has led to renaissance in the area of skeletal development and evolution. Among the major findings that have emerged is the discovery of an unexpectedly deep origin of the gene network that regulates chondrogenesis. In this chapter, we discuss recent progress in each these areas and identify a number of questions that need to be addressed in order to fill key gaps in our knowledge of early skeletal evolution.

1. Introduction

The vertebrate skeleton consists of two predominant tissue types: cartilage and bone. Although generally considered a vertebrate character, cartilage is found across a broad range of animal taxa, indicating a long and complex evolutionary history (Hall, 2005). Cartilage differs from bone in several ways; cartilage has a lower metabolic rate, is mostly avascular, and contains different cellular and extracellular components that give it unique structural properties. Classically, true cartilage was defined by three criteria (1) it contains chondrocytes suspended in rigid matrix, (2) the matrix has a high content of collagen, and (3) the matrix is rich in acidic polysaccharides (Person and Mathews, 1967). The proposal that the cartilage of some vertebrates, such as lampreys and hagfishes, is noncollagenous led to a revision of this definition to substitute "fibrous proteins" for "collagen" (Cole and Hall, 2004a); however, recent work has shown that these jawless fishes also have collagen-based cartilage (Ohtani *et al.*, 2008; Zhang and Cohn, 2006; Zhang *et al.*, 2006). Such studies of cartilage in nontetrapod lineages have revealed that a deeply conserved genetic system underlies a diverse array of cartilage types. These discoveries have enhanced our understanding of the early evolution of cartilage and raised new questions about the homologies of animal connective tissues. Here, we review these advances in the context of skeletal developmental genetics and the evolutionary history of vertebrates, and discuss how changes to developmental and genomic programs may have contributed to the origin of the vertebrate skeleton.

2. Skeletal Cell Lineage Determination and the Skeletogenic Gene Network

Vertebrate cartilage and bone are composed of three major cell lineages, chondrocytes, osteoblasts, and osteoclasts. The former two cell types are derived from common mesenchymal progenitor cells, whereas

osteoclasts are of hematopoietic origin. After condensation, mesenchymal cells start to differentiate into chondrocytes. These chondrocytes may remain as cartilage throughout life, or the cartilage template may undergo hypertrophy and eventually be replaced by bone, a process termed endochondral ossification. Alternatively, the mesenchymal cells may differentiate directly into bone, through a process termed intramembranous ossification, as seen in the membrane bones of the skull, such as the calvaria. In both intramembranous and endochondral ossification, osteoblasts first aggregate as mesenchymal condensations (Karsenty and Wagner, 2002; Yang and Karsenty, 2002; Zelzer and Olsen, 2003). The cell fate decisions made by aggregating mesenchymal cells are regulated by a skeletogenic gene network (Fig. 2.1), and understanding the hierarchy, regulation, and function of these factors is critical to our discussion of the evolution of skeletogenic mechanisms. Below, we review the major components of this network and describe their functions and interactions during embryonic development of the skeleton.

2.1. Sox9

As cells in mesenchymal condensations begin to differentiate into chondrocytes, the earliest marker of chondrogenesis is *Sox9*, a member of the vertebrate SoxE family that contains a high-mobility-group (HMG)-box

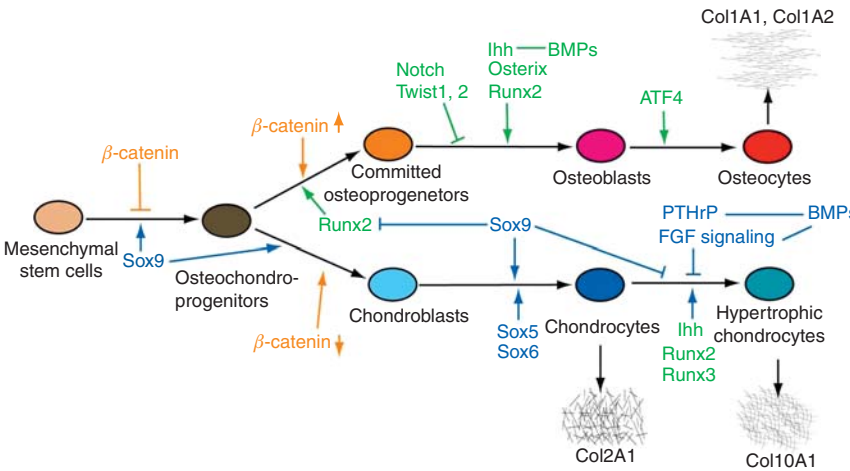

Figure 2.1 Schematic representation of gene network that directs mesenchymal cells along chondrogenic (bottom) and osteogenic (top) differentiation pathways. Arrows indicate positive regulation, lines indicate interaction, and bars indicate negative regulation. Data represented in this schematic are taken from multiple sources cited in the text. The scheme depicts hierarchical arrangement of genes in the network and does not necessarily indicate direct transcriptional regulation at each step.

DNA-binding domain (Fig. 2.1) (Healy *et al.*, 1996; Wright *et al.*, 1995). *Sox9* directly regulates expression of two genes that code for major matrix proteins, type II collagen (Col2α1) and aggrecan, and is required for expression of genes that encode minor matrix proteins, including type IX and XI collagen (Lefebvre and de Crombrugghe, 1998; Lefebvre *et al.*, 1997; Liu *et al.*, 2000; Ng *et al.*, 1997; Zhang *et al.*, 2003b; Zhou *et al.*, 1998). Haploinsufficiency of *Sox9* in humans underlies campomelic dysplasia, a congenital malformation of the skeleton characterized by shortening and bowing of the limbs, and similar anomalies occur in mice with loss-of-function mutation in *Sox9* (Foster *et al.*, 1994; Wagner *et al.*, 1994). Reciprocal experiments involving ectopic expression of *Sox9* in chick embryos can induce dermomyotomal or neural crest-derived cells to form cartilage (Healy *et al.*, 1999; Eames *et al.*, 2004). Sox9 function is enhanced by Sox5 and Sox6, which can bind to Sox9 and act as cofactors in the activation of *Col2α1* (Ikeda *et al.*, 2004; Lefebvre and de Crombrugghe, 1998; Lefebvre *et al.*, 1998, 2001; Smits and Lefebvre, 2003; Stolt *et al.*, 2006). The Sox5/6/9 trio also has been shown to bind S100A1 and S100B, two novel targets that mediate the trio's ability to inhibit chondrocyte differentiation (Saito *et al.*, 2007). Sox9 can form complexes with the CREB-binding protein CBP/P300, and the association of these proteins may be required for chondrocyte-specific expression of *Col2α1* (Tsuda *et al.*, 2003). Interestingly, the chondrogenic activity of TGFβ/Bmp signaling (described below) may be mediated, at least in part, by the ability of Smad3 to promote binding of Sox9 with the CBP/P300 coactivator (Furumatsu *et al.*, 2005). These interactions may account for the ability of Sox9 to activate *Col2α1* in some cell lineages (e.g., limb bud, sclerotome, and cranial neural crest) but not others (e.g., genital ridge).

2.2. Runx2

The vertebrate *Runx2* gene [also known as *PEBP2A (polyoma enhancer-binding protein 2A)*, *Osf2 (osteoblast-specific factor 2)*, *AML3 (acute myelogenous leukemia 3)*, and *Cbfa1 (core-binding factor alpha 1)*] is an ortholog of the fly *runt* gene and a master regulator of osteoblast differentiation (Fig. 2.1) (van Wijnen *et al.*, 2004). In addition to its role in osteoblast differentiation (Ducy *et al.*, 1997, 1999; Komori *et al.*, 1997; Otto *et al.*, 1997), *Runx2* is required for chondrocyte hypertrophy (Fig. 2.1). In *Runx2*-null mice, the entire skeleton remains cartilaginous due to the maturational arrest of osteoblasts, and there is a failure of chondrocyte hypertrophy (Inada *et al.*, 1999; Kim *et al.*, 1999; Takeda *et al.*, 2001). Reciprocally, ectopic expression of *Runx2* in chick head mesenchyme can drive excess bone formation and ectopic chondrocyte hypertrophy (Eames *et al.*, 2004). Haploinsufficiency of *Runx2* in humans causes cleidocranial dysplasia, a rare skeletal malformation characterized by short stature, distinctive facial features and

narrow, sloping shoulders associated with defective or absent clavicles (Mundlos and Olsen, 1997a,b; Mundlos *et al.*, 1996). *Runx1* and *Runx3*, two genes closely related to *Runx2*, also are expressed in chondrocytes and participate in the progression of chondrocytes to the hypertrophic stage (Karsenty, 2008; Levanon *et al.*, 2001; Lian *et al.*, 2003; Smith *et al.*, 2005; Stricker *et al.*, 2002; Wang *et al.*, 2005).

2.3. Interaction of Sox9 and Runx2

Several lines of evidence have shown that in many cases, condensed mesenchymal cells have chondrogenic and osteogenic potential, since they express both *Sox9* and *Runx2* (Bi *et al.*, 1999; Ducy *et al.*, 1997; Eames and Helms, 2004; Otto *et al.*, 1997; Yamashiro *et al.*, 2004). Moreover, cultured embryonic cells may form both bone and cartilage (Fang and Hall, 1997; Toma *et al.*, 1997; Wong and Tuan, 1995). Inactivation of *Sox9* in the cranial neural crest-derived mesenchymal cells blocks cartilage differentiation, but this also leads to ectopic expression of osteoblast-specific genes such as *Runx2*, *Osterix*, and *Col1α1*(Mori-Akiyama *et al.*, 2003). Conversely, it was reported that in *Osterix* mutants, ectopic chondrocytes formed at the expense of the bone collar in long bones and in some membrane bones (Nakashima *et al.*, 2002). These data support the idea that the common skeletal mesenchymal progenitors have three possible differentiation fates in the skeleton, chondrogenesis, intramembranous ossification or endochondral ossification (it is noteworthy, however, that these mesenchymal cells also can take on other, nonskeletal cell fates, such as adipose tissue) (Karsenty, 2003; Karsenty and Wagner, 2002). In mesenchymal osteochondrogenic progenitors, removal of *Sox9* will abolish cartilage and endochondral bone formation, indicating that *Sox9* is required for skeletal differentiation (Akiyama *et al.*, 2005). Experiments in chick embryos demonstrated that higher levels of *Sox9* will commit cells to chondrogenesis, whereas higher levels of *Runx2* will push them toward osteogenesis (Fig. 2.1) (Eames *et al.*, 2004). *Sox9* has been shown to be dominant to *Runx2* (Zhou *et al.*, 2006), which suggests that if these transcription factors are expressed at similar levels, then skeletal progenitor cells may differentiate preferentially into cartilage.

2.4. Parathyroid hormone-related protein and Indian hedgehog

During long bone growth, chondrocyte proliferation and differentiation is tightly regulated by a negative feedback loop between Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP) (Fig. 2.1) (Karp *et al.*, 2000; Lanske *et al.*, 1996; St-Jacques *et al.*, 1999; Vortkamp *et al.*, 1996). PTHrP is a peptide hormone that is secreted by the most distal perichondrium, and its G protein-coupled receptor, *PPR*, localizes to the

proliferative prehypertrophic zone. PTHrP acts to maintain proliferation and to inhibit differentiation (St-Jacques *et al.*, 1999). In humans, activating mutations of *PPR* cause Jansen's metaphyseal chondrodysplasia, which involves delayed skeletal differentiation and abnormal growth plates (Schipani *et al.*, 1995). Loss-of-function mutations in *PTHrP* in mice result in dwarfism due to accelerated hypertrophy (Karaplis *et al.*, 1994; Lanske *et al.*, 1996). *Ihh* is expressed along with *PPR* in the prehypertrophic zone and controls expression of *PTHrP* (Vortkamp *et al.*, 1996). Deletion of *Ihh* results in reduced chondrocyte proliferation and failure of perichondral osteoblast formation, ultimately leading to dwarfism. In the *Ihh*-null mutants, *PTHrP* expression is lost (Razzaque *et al.*, 2005; St-Jacques *et al.*, 1999), and *Ihh* overexpression results in upregulation of *PTHrP*, promoting proliferation and delaying hypertrophy. PTHrP feeds back to negatively regulate *Ihh* expression. This Ihh–PTHrP feedback loop maintains the balance between proliferation and differentiation (Kronenberg, 2006). Very recent work has shown that Ihh can promote chondrocyte hypertrophy independently of PTHrP (Mak *et al.*, 2008). Bapx1 (Nk3.2) is a downstream target of Ihh–PTHrP loop and, at least in part, mediates chondrocyte hypertrophy (Provot *et al.*, 2006). Interestingly, Runx2 and Runx3 can induce *Ihh* expression (St-Jacques *et al.*, 1999; Yoshida *et al.*, 2004) and Ihh can feed back to inhibit *Runx2* expression through the PKA pathway (Iwamoto *et al.*, 2003; Li *et al.*, 2004).

2.5. Wnt signaling

The canonical Wnt pathway is a key regulator for mesenchymal cell lineage determination (Fig. 2.1). *Wnt* genes are vertebrate orthologs of the *Drosophila wingless* gene, and there are 19 known *Wnt* genes in humans (Logan and Nusse, 2004; Miller, 2002). This group of secreted molecules is highly conserved in metazoan animals ranging from cnidarians to humans, and they have critical functions both in normal development and tumorigenesis (Kusserow *et al.*, 2005; Lee *et al.*, 2006; Logan and Nusse, 2004; Prud'homme *et al.*, 2002). Wnt proteins that bind to Frizzed receptors transduce the input into the cell together with the coreceptor, LDL receptor-related protein 5/6 (LRP5/6). There are at least three intracellular pathways for Wnt signaling; the canonical pathway mediated by β-catenin, the Ca–PKC pathway, and the planar cell polarity pathway (Miller, 2002). Interestingly, Sox9 also interacts with β-catenin. Sox9 can inhibit β-catenin-dependent promoter activation through the interaction between HMG-box and Armadillo repeats. Sox9 also promotes degradation of β-catenin by ubiquitation or the proteasome pathway (Akiyama *et al.*, 2004).

Canonical Wnt signaling has been implicated in skeletal development (Bodine *et al.*, 2004; Boyden *et al.*, 2002; Gong *et al.*, 2001; Hartmann and Tabin, 2001; Kato *et al.*, 2002; Little *et al.*, 2002; Rawadi *et al.*, 2003).

Several lines of evidence have revealed that the canonical Wnt pathway regulates skeletogenic cell fate determination through a cell-autonomous mechanism to induce osteoblast differentiation and to repress chondrocyte differentiation (Fig. 2.1) (Day *et al.*, 2005; Glass *et al.*, 2005; Hill *et al.*, 2005; Hu *et al.*, 2005; Rodda and McMahon, 2006). When β-catenin is conditionally removed from skeletogenic mesenchyme using the *Prx1-Cre* allele, osteoblast differentiation arrests, and neither cortical nor membrane bone forms (although this can be rescued by Ihh and Bmp2). Similar phenotypes were found when β-catenin was deleted from the skeletal primordium using *Dermo1-Cre* and *Col2a1-Cre* mouse lines, in which ectopic chondrocytes formed at the expense of osteoblasts (Day *et al.*, 2005; Hu *et al.*, 2005). Moreover, micromass cell culture experiments showed that β-catenin levels can control the expression of *Sox9* and *Runx2 in vitro* (Day *et al.*, 2005). Collectively, β-catenin controls early osteochondroprogenitor differentiation into chondrocytic or osteoblastic lineages. High levels of β-catenin lead to osteogenic differentiation and low levels lead to chondrogenic differentiation (Day *et al.*, 2005; Hill *et al.*, 2005). The process is summarized in Fig. 2.1. These studies suggest that variation in skeletal composition, both developmentally and evolutionarily, may be accomplished by tinkering with the temporal and spatial expression of canonical Wnt signals.

2.6. Fibroblast growth factor signaling

Fibroblast growth factors (Fgfs) and their receptors are also critical regulators of chondrocyte proliferation and differentiation (Fig. 2.1). In humans and mice, there are 22 Fgf genes and 4 Fgf receptors (Fgfr), many of which are involved in skeletal development, including those that signal through Fgfr1, Fgfr2, and Fgfr3 (Ornitz and Marie, 2002). Fgf9 has been shown to regulate differentiation of hypertrophic chondrocytes and to direct vascularization of the limb skeleton (Hung *et al.*, 2007). *Fgf18* is expressed in the perichondrium, and it signals to the chondrocytes through Fgfr3. *Fgfr1* is found in prehypertrophic and hypertrophic zone, and *Fgfr2* and *Fgfr3* are expressed, respectively, in perichondral cells and in the proliferating zone. Each of the three receptors has a unique function. Human genetic studies first revealed the importance of Fgf signaling in skeletal development, when *Fgfr3* mutations were shown to underlie achondroplasia, hypochondroplasia, and thanatophoric dysplasia (Olsen *et al.*, 2000). In *Fgfr3*-null mice, the proliferative rate is accelerated, which causes the chondrocyte column length to be increased (Colvin *et al.*, 1996; Deng *et al.*, 1996). Moreover, activating mutations in mouse *Fgfr3* cause reduced proliferation and increased apoptosis of chondrocytes (Sahni *et al.*, 1999). These studies suggested that Fgfr3 is a negative regulator of proliferation in the growth plate, and this process is mediated through STAT1–P21 pathway (Sahni *et al.*, 1999). As with Fgfr3, conditional removal of Fgfr1 in chondrocytes results in

expansion of the hypertrophic chondrocyte zone, indicating that Fgfr1 is also a negative regulator of proliferation (Jacob *et al.*, 2006).

2.7. Bone morphogenetic protein signaling

Bone morphogenetic proteins (BMPs) and their receptors play multiple roles in chondrocyte differentiation and proliferation, and have been reviewed extensively elsewhere (Li and Cao, 2006; Pogue and Lyons, 2006). *Bmp7* is found mainly in the proliferating chondrocytes, whereas *Bmp2–Bmp5* are expressed primarily in the perichondrium (Lyons *et al.*, 1995; Minina *et al.*, 2001), although hypertrophic chondrocytes also express *Bmp2* and *Bmp6* (Solloway *et al.*, 1998). These distinctive expression patterns suggest that each of these Bmps has a unique function. The relationship of Bmp and Indian hedgehog is somewhat unclear. Although *in vitro* experiments in chick and mouse and *in vivo* studies in chick showed that Bmp receptor IA is an upstream regulator of *Ihh*, other *in vivo* and *in vitro* studies in mouse failed to detect changes in *Ihh* following activation of Bmp receptors or treatment with Bmp protein (Kobayashi *et al.*, 2005; Seki and Hata, 2004; Zhang *et al.*, 2003a). Different experimental approaches also have led to curious findings regarding the function of BmpR1A and BmpR1B. Studies in the chick limb suggested that BmpR1A and BmpR1B may have very different functions (Zou *et al.*, 1997), although more recent studies in mice found them to be interchangeable (Kobayashi *et al.*, 2005). Kobayashi *et al.* (2005) used multiple experimental strategies to overexpress *BmpR1A* in chondrocytes and found that BmpR1A has different roles at different stages of cartilage development. According to their findings, constitutive activation of *BmpR1A* stimulates chondrocyte hypertrophy and also promotes differentiation of prechondrogenic mesenchyme into chondrocytes.

3. Structure of Vertebrate Cartilage Matrix

3.1. Collagens

Most of the connective tissues of vertebrates are formed from extracellular fibers, matrix, and ground substance. For example, up to 90% of the dry weight of cartilage is extracellular matrix (Hardingham and Fosang, 1992). In jawed vertebrates, cartilage extracellular matrix typically is composed of mucopolysaccharides (in the form of proteoglycans) deposited within a meshwork of collagen fibers (Bruckner and van der Rest, 1994). Collagens are the main components of animal extracellular matrix (Exposito *et al.*, 2002), and the expansion of this gene family within the vertebrate clade coincided with evolution of a broad range of vertebrate skeletal tissues. For example, 29 different collagen genes have been identified in humans thus far

(Soderhall *et al.*, 2007), and the resultant proteins can be divided into two major groups, fibrillar and nonfibrillar collagens. The fibrillar collagen proteins, in which multiple collagen fibrils are assembled into collagen fibers, are further divisible into three clades, designated A, B, and C (Aouacheria *et al.*, 2004). Clade A collagens are the major fibril-forming collagens, including types I, II, III, and V (Aouacheria *et al.*, 2004). Clade A fibril procollagens consist of an N-propeptide, an N-telopeptide, a triple helix, a C-telopeptide, and a C-propetide (from N- to C-terminus). The triple helix domain consists of a Gly–X–Y triplet repeat, with X and Y usually being proline and hydroxyproline. The propeptide is removed during the maturation of collagen through posttranslational processing by N- and C-proteinase (Exposito *et al.*, 2002; Kadler *et al.*, 1996). Type II collagen is encoded by *Col2α1*, and nearly 40 years ago this was shown to be the major matrix protein found in cartilage (Miller and Matukas, 1969). Each type II collagen fibril is made of three identical chains that provide tensile strength and a scaffolding network for proteoglycans (van der Rest and Garrone, 1991). Cartilage also contains minor collagens type IX and XI, which belong to the clade B fibrillar collagen family and participate in the process of fibril formation (Eyre *et al.*, 2004; Kadler *et al.*, 1996; Li *et al.*, 1995). Different types of cartilage are characterized by different combinations and quantities of collagen proteins. In addition, the profile of collagen expression can be dynamic during skeletal development. During long bone development, for example, the major matrix protein found in proliferative cartilage is type II collagen, whereas type X collagen is most abundant during the hypertrophic stage and type I collagen dominates bony matrix (Olsen *et al.*, 2000).

3.2. Proteoglycans

Proteoglycans are the second-most abundant proteins (after the fibrillar collagens) in cartilage matrix. Glycosaminoglycan side chains of proteoglycans become heavily sulfated, which increases their retention of water, giving cartilage its characteristic resistance to compression. Chondroitin sulfate was shown to be the predominant glycosaminoglycan in cartilage, and one of its substrates, aggrecan, was found to be the most abundant cartilage proteoglycan (Doege *et al.*, 1991). Deposition of aggrecan has been considered a hallmark of chondrogenesis (although it is also present in aorta, intervertebral disks, and tendons) (Schwartz *et al.*, 1999). Aggrecan not only contributes to the physical properties of cartilage, but also it protects cartilage collagen from degradation by stabilizing collagen protein (Pratta *et al.*, 2003). In addition to the large aggregating proteoglycan aggrecan, there are many small leucine-rich proteoglycans in cartilage, including biglycan, decorin, fibromodulin, lumican, and epiphycan, which have a variety of functions in cartilage development and maintenance (Iozzo, 1998; Knudson and Knudson, 2001). Chondrocytes also express cell surface

proteoglycans, such as syndecans and glypican, which can bind growth factors during cell–cell and cell–matrix interactions (Iozzo, 1998; Song *et al.*, 2007).

4. Evolutionary History of the Vertebrate Skeleton

For extant deuterostomes, mapping the key characters of skeletogenesis onto a phylogeny provides a window into the distribution and pattern of skeletal evolution (Fig. 2.2), but what does the fossil record reveal about the evolution of cartilage and bone within vertebrates? Obviously, most preserved specimens will reflect the existence of mineralized tissues, since they are most easily fossilized, but some samples reveal unmineralized cartilage as well. Although studies of invertebrates indicate that cartilage had an earlier origin than did bone in metazoans, it is less clear which of these tissues appeared first in vertebrate skeletal evolution. Conodonts lacked a dermal skeleton and early descriptions of bone in conodonts have been disputed, although their dental elements were rich in dentine and enamel (Donoghue *et al.*, 2006). The 530-million-year-old fossil *Haikouella* is one of the earliest examples of unmineralized vertebrate cartilage, and comparison with modern lamprey cartilage shows striking morphological similarity (Mallatt and Chen, 2003). Jawless fishes dominate the vertebrate fossil record through the upper Paleozoic, and most possessed a heavily armored dermoskeleton, a character that has been lost in lampreys and hagfishes (Sansom *et al.*, 2005). Histological and microscopic studies of dermoskeletons have identified a variety of tissue types, including bone, dentine, and enamel, although neither cartilage nor perichondral bone have been observed (Donoghue and Sansom, 2002; Donoghue *et al.*, 2006; Patterson *et al.*, 1977). Most crown-group vertebrates show few similarities between the mineralized tissues of the teeth and those of the skeleton. Interestingly, the dermal skeletons of early vertebrates were composed of both ''dental'' and ''skeletal'' tissue types, and the presence of dentine and enamel in dermal armor has led some investigators to suggest that the evolutionary origin of teeth may be traced to the dermal skeleton (Smith and Johanson, 2003). The earliest examples of mineralized endoskeletons are found in galeaspids and pteraspidomorphs (Donoghue and Sansom, 2002; Donoghue *et al.*, 2006; Janvier, 1996; Stensio, 1927). Galeaspids had dermal armor of unmineralized cartilage and acellular bone. In heterostracans, the dermal skeleton contained dentine, acellular bone, and enameloid tissues. Cellular bone is found in the dermal skeletons of osteostracans, which was combined with dentine in their head shields. The dermoskeleton of thelodonts consisted of scales that were made up of dentine and also may have contained acellular bone (Donoghue and Smith, 2001; Donoghue

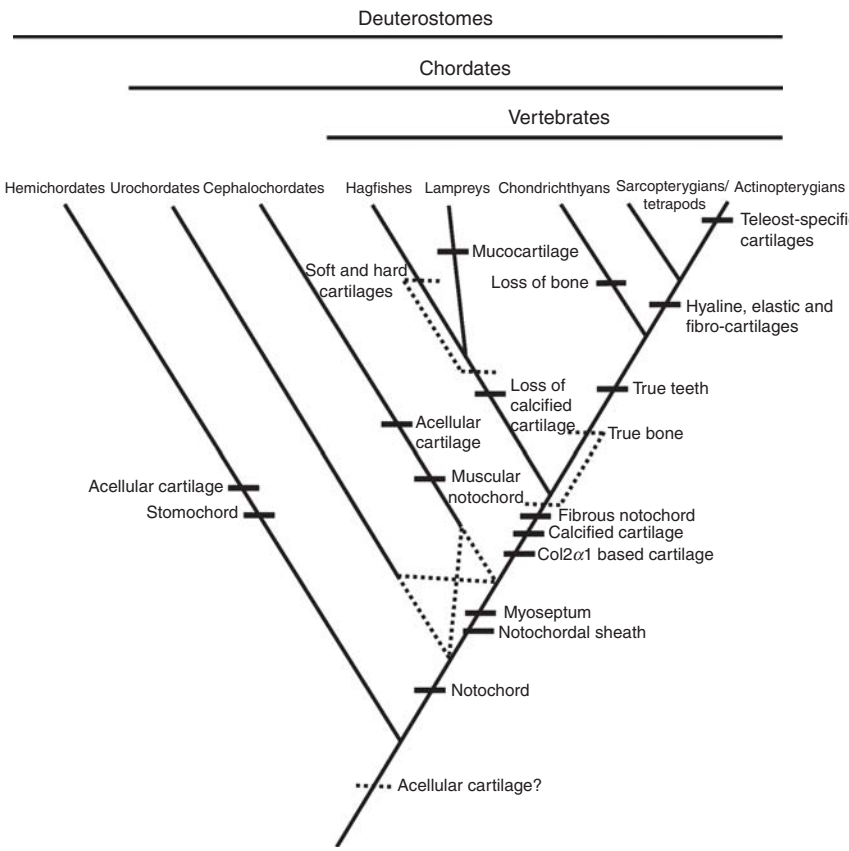

Figure 2.2 Phylogenetic distribution of key skeletogenic characters in deuterostomes. Dotted lines at the base of the cephalochordate and urochordate branches indicate ambiguous positions and these may be transposed. Dotted horizontal bar at base of tree indicates a possible early origin of acellular cartilage in stem deuterostomes (see Rychel and Swalla, 2007). Alternatively, acellular cartilage may have arisen independently in hemichordates and cephalochordates. Dotted horizontal bar in cyclostome (hagfish + lamprey) clade indicates uncertainty regarding the origin of classically defined "hard" and "soft" cartilage (see Cole, 1905; Parker, 1883; Zhang and Cohn, 2006 for further details).

et al., 2006). The almost exclusively cartilaginous skeletons of extant cyclostomes and sharks have been misinterpreted as evidence that cartilage predated bone in vertebrate evolution; however, this is a derived condition that followed an evolutionary loss of bone (Carroll, 1988; Daniel, 1934; Donoghue and Sansom, 2002; Goodrich, 1930; Hall, 1975; Janvier, 1996; Maisey, 1988; Moss, 1977; Orvig, 1951; Romer, 1985; Smith and Hall, 1990). The fossil record of sharks shows abundant evidence of exoskeletal

bone (Coates and Sequeira, 2001; Hall, 1975; Maisey, 1988; Moss, 1977; Zangerl, 1966) and limited examples of endoskeletal bone (Coates *et al.*, 1998). Indeed, true bone has persisted in some extant chondrichthyans, in subchondral linings, neural arches, and dermal denticles (Bordat, 1987; Eames *et al.*, 2007; Kemp and Westrin, 1979; Moss, 1970, 1977; Peignoux-Deville *et al.*, 1982; Reif, 1980; Sire and Huysseune, 2003). Thus far, despite the rich diversity of skeletal tissues in the fossil record, the question of whether the earliest vertebrate skeletons were cartilaginous, bony, or both remains unclear.

5. Diversification of Cartilaginous Tissues

A major challenge has been the classification of different cartilage types at the molecular and biochemical levels, and understanding the interrelationships among this diverse family of tissues. Depending on relative amounts of cells and extracellular matrix, there are generally four kinds of cartilage in vertebrates and invertebrates: matrix-rich cartilage, cell-rich cartilage, vesicular cartilage, and acellular cartilage, although skeletal tissues with an intermediate or mosaic composition have been identified in some vertebrates, such as the cartilage-like chondroid tissues, which possess characters of both bone and cartilage (Cole and Hall, 2004a). Whether these four cartilage types evolved independently or diversified from a single type of ancestral connective tissue is unknown (Fig. 2.2). The similarities in matrix composition, histological properties, gene expression profiles, and cell biology of notochord cells and chondrocytes have led some to propose that vertebrate cartilage may have evolved from the notochord of early chordates (Stemple, 2004; Zhang and Cohn, 2006). Alternatively, vertebrate cartilage may have its origins in the secretion of acellular matrix by ectodermal cells. Acellular cartilage, which lacks chondrocytes, has been found in hemichordates, cephalochordates, and vertebrates (e.g., rays) (Cole and Hall, 2004b; Meulemans and Bronner-Fraser, 2007; Rychel and Swalla, 2007; Rychel *et al.*, 2006; Wright *et al.*, 2001). Rychel *et al.* proposed that ectodermally derived acellular cartilage is an ancestral mode of pharyngeal cartilage development in deuterostomes (Fig. 2.2) (Rychel and Swalla, 2007; Rychel *et al.*, 2006). The conservation of cartilage matrix genes in invertebrates could be interpreted as evidence for an unexpectedly deep origin of cartilage, or may simply reflect the limited number of tools in the genetic toolkit for making cartilaginous tissues. According to the latter idea, the molecular program for chondrogenesis has a single origin, but the tissue itself may have evolved many times. Resolving this question will require comparative studies of the molecular mechanisms of chondrogenesis across

metazoa. In the next two sections, we review the diversity of cartilaginous tissues in vertebrates and invertebrates.

5.1. Cartilage variation within vertebrates

5.1.1. Tetrapods

Cartilage exists in a variety of forms in vertebrates (Fig. 2.2). In tetrapods, cartilage is broadly divisible into three major subtypes: hyaline cartilage, elastic cartilage, and fibrocartilage (Hall, 2005). *Hyaline cartilage* is the primary component of the endoskeleton and serves as the scaffold for bone that develops by endochondral ossification. Sometimes termed ''true cartilage,'' hyaline cartilage derives its structural integrity mainly from glycosaminoglycans and type II collagen fibrils. *Elastic cartilage*, such as that found in the mammalian ear pinnae and epiglottis, is also rich in glycosaminoglycans and collagen proteins, but additionally contains thick bundles of elastic fibrils and elastin-rich extracellular matrix (Naumann *et al.*, 2002). This combination of matrix proteins gives elastic cartilage the toughness of hyaline cartilage but with increased elasticity. *Fibrocartilage* is found at the attachment points of tendons and ligaments, in intervertebral disks, and at the pubic symphysis. Fibrocartilage matrix contains large amounts of type I collagen, which makes it both tensile and tough (Benjamin and Evans, 1990; Benjamin and Ralphs, 2004; Eyre and Wu, 1983). Even in tetrapods, some cartilage can demonstrate intermediate tissue properties that do not adhere to this tidy classification scheme. For example, secondary cartilage, which forms from osteoblast precursors at stressed joint regions, is similar to hyaline cartilage, but expresses high amounts of type I collagen (Fang and Hall, 1997; Fukada *et al.*, 1999; Fukuoka *et al.*, 2007; Ishii *et al.*, 1998).

5.1.2. Teleosts

Teleost fishes exhibit an even richer diversity of cartilage types (Fig. 2.2). According to one classification scheme, there are five ''cell-rich'' cartilages and three ''matrix-rich'' cartilages (Benjamin, 1989, 1990). The ''cell-rich'' cartilages, which are defined by cells or lacunae making up >50% of a cartilage tissue's volume, include (1) hyaline-cell cartilage, (2) cell-rich hyaline cartilage, (3) fibrocell cartilage, (4) elastic/cell-rich cartilage, and (5) Schaffer's Zellknorpel. *Hyaline-cell cartilage*, which is found in the lips, rostral folds, and other cranial cartilages, is characterized by compact chromophobic chondrocytes and hyaline cytoplasm with little matrix (Benjamin, 1989). Hyaline-cell cartilage is divisible into three subtypes; fibro/hyaline has greater quantities of collagen than elastin, elastic/hyaline contains more elastin in the matrix, and lipo/hyaline contains adipocytes as well as chondrocytes. *Cell-rich hyaline cartilage* is more cellular than hyaline-cell cartilage, with lacunae occupying more than half of the total volume. Parts of neurocranium and Meckel's cartilage belong in this category

(Benjamin, 1990). *Fibrocell cartilage* is a highly cellular (nonhyaline) fibrocartilage that is rich in collagen, lacks a perichondrium, and is commonly found on articular surfaces. *Elastic/cell-rich cartilage*, which is usually found in the barbels and maxillary oral valves, is dense with elastin, the cells are not hyaline, and these elements are surrounded by a thick fibrous perichondrium (Benjamin, 1990). The fifth type of "cell-rich" cartilage is known as Schaffer's Zellknorpel and occurs in teleost gill filament rays and the basal plate. Zellknorpel chondrocytes are more chromophilic than those of hyaline-cell cartilage and are shrunken within large lacunae (Benjamin, 1990).

The "matrix-rich cartilages" of teleosts are defined by cells or lacunae making up <50% total volume. In teleosts, like tetrapods, the "matrix-rich cartilages" are divisible into three subtypes (1) matrix-rich hyaline cartilage, (2) fibrocartilage, and (3) elastic cartilage. Each of these cell-rich and matrix-rich cartilages can be found in the cranial and postcranial skeletons, with the exception of the cranially restricted Zellknorpel (Benjamin *et al.*, 1992). Scleral cartilage is particularly interesting, as it has been described as a composite structure, in which a central zone of cell-rich hyaline cartilage is surrounded by a cortex of matrix-rich hyaline cartilage (Benjamin and Ralphs, 1991; Franz-Odendaal *et al.*, 2007). This classification system is based on histological/structural characters, and little is known about their molecular composition or development. The observation that teleosts have a broader variety of cartilage tissue types than do tetrapods may relate to the larger number of matrix (and other skeletogenic) genes that resulted from the teleost genome duplication event. Accordingly, the increased number of gene expression combinations that are possible in teleosts may underlie the diversity of cartilage types. Alternatively, similar patterns of gene expression in chondrogenic tissues may yield different structural and histological patterns due to differences in the local environment during chondrogenesis. Molecular characterization of the different cartilaginous tissues of teleosts is needed to uncover the developmental basis of their diversity.

5.1.3. Chondrichthyans

Chondrichthyan skeletons are almost entirely cartilaginous; however, their cartilage undergoes extensive mineralization (Dean and Summers, 2006; Eames *et al.*, 2007; Hall, 2005). The majority of the shark skeleton appears to be true hyaline cartilage, staining strongly for sulfated proteoglycans and type II collagen (Eames *et al.*, 2007). The cartilaginous nature of chondrichthyan skeletons is likely a derived condition that followed an evolutionary loss of bone (Fig. 2.2). Catsharks, for example, retain true bone in their neural arches, and the fossil record of sharks shows evidence of both exoskeletal and endochondral bones (Coates *et al.*, 1998; Kemp and Westrin, 1979; Moss, 1970, 1977; Peignoux-Deville *et al.*, 1982). Biochemical studies showed that shark and skate cartilage may contain type I collagen in addition to type II collagen (Mizuta *et al.*, 2003; Moss, 1977;

Rama and Chandrakasan, 1984); however, antibodies to type I collagen did not react to shark cartilage immunohistochemically (Eames *et al.*, 2007). It has been suggested that biochemical identification of type I collagen in shark cartilage may have resulted from contamination from shark bone (Eames *et al.*, 2007). Cartilage development within chondrichthyans has not received the level of scrutiny provided to teleost skeletal tissues, and a comprehensive and comparative analysis of gene expression, regulation and function is needed.

As an aside, the widely held belief that sharks do not develop tumors is false (neoplasias in sharks have been known for over 150 years) and there is no scientific basis to support the notion that consumption of crude shark cartilage affects tumor development in cancer patients (reviewed in Ostrander *et al.*, 2004). Some general features of cartilage (not restricted to sharks) that may contribute to the rarity of tumor invasion into cartilaginous tissues are that it is hypoxic, has poor vascularity, produces collagenase inhibitors, and may contain antiangiogenic factors.

5.1.4. Cyclostomes

Cartilaginous skeletons are also present in both extant groups of jawless (agnathan) vertebrates, lampreys and hagfishes. Lamprey and hagfish have mucocartilage (Fig. 2.2) and were described as lacking collagen (Wright *et al.*, 2001). Instead, their matrix was reported to contain the elastin-like molecules *lamprin* and *myxinin*, respectively (Wright *et al.*, 2001). Recent molecular developmental studies have overturned the idea that agnathans lack collagenous cartilage by demonstrating that both lampreys and hagfishes do indeed have type II collagen-based cartilage (Ohtani *et al.*, 2008; Zhang and Cohn, 2006; Zhang *et al.*, 2006). Lamprey cartilages are found mainly in the cranial region. The postcranial skeleton is limited to paired axial cartilage nodules (termed arcualia) and caudal fin rays (Morrison *et al.*, 2000). In the head of larval lamprey, the proteoglycan-rich mucocartilage occurs as a transient, avascular cartilage that is surrounded by perichondrium (Hall, 2005). During metamorphosis, mucocartilage is transformed into the pistal and tongue cartilages (Hall, 2005). In the nineteenth century, two kinds of cartilages, ''soft'' and ''hard,'' were identified in lampreys (Parker, 1883). The hard cartilage is similar structurally to mammalian hyaline cartilage. In hagfishes, cartilage is also present in the cranium and median fin rays, although they lack the paired arcualia found along the trunks of lampreys. Like lampreys, hagfish were reported to contain soft and hard cartilages (Cole, 1905). Cole (1905) described hagfish ''soft'' cartilage as containing large hypertrophic chondrocytes that stain with hematoxylin (blue) and are surrounded by a thin extracellular matrix, whereas ''hard'' cartilage contains smaller chondrocytes that are surrounded by an abundance of extracellular matrix. Biochemical analysis also supported the two types of hagfish cartilage, designated type I and

type II cartilage, with only type I containing myxinin and type II being more similar to adult lamprey cartilage (Wright *et al.*, 1984). Neither lamprey nor hagfish cartilage is mineralized, but lamprey cartilage can be calcified *in vitro* (Langille and Hall, 1988). Interestingly, calcified cartilage was reported in the fossil lamprey *Euphanerops*, suggesting that mineralized cartilage in this group persisted at least to the Devonian (Janvier and Arsenault, 2002). These recent analyses of extant and extinct agnathans suggest that cartilage containing high amounts of type II collagen and sulfated proteoglycans was present in the common ancestor of jawed and jawless vertebrates (Fig. 2.2).

5.2. Invertebrate cartilage

Cartilaginous tissues are not restricted to the vertebrates; examples of cellular and/or acellular cartilage exist in such diverse taxa as cephalochordates, hemichordates, annelids, mollusks, brachiopods, arthropods, and cnidaria (see Cole and Hall, 2004a for a detailed review). Some of these tissues bear striking similarities to vertebrate cartilage at the structural, morphological, and histological levels. In general, there are three kinds of cartilages found in invertebrates: central cell-rich cartilage, vesicular cartilage with large vesicles or vacuoles, and acellular cartilage. Within deuterostomes, fibrillar collagens are expressed in the developing acellular cartilage of hemichordates and cephalochordates, in the cellular cartilage and the notochord of cephalochordates, and in the notochordal sheath of urochordates (Rychel *et al.*, 2006; Wada *et al.*, 2006; Zhang *et al.*, 2006). Vesicular cartilage has been identified in polychaete worms, horseshoe crabs, and mollusks (Cole and Hall, 2004b). To some degree, the vertebrate notochord can be considered a vesicular cell-rich cartilage, since notochordal cells are vacuolated and surrounded by cartilage-like extracellular matrix containing type II collage, type I collagen, type X collagen, aggrecan, and polysaccharides (Domowicz *et al.*, 1995; Eikenberry *et al.*, 1984; Linsenmayer *et al.*, 1986; Welsch *et al.*, 1991). How similar or different are the developmental processes and molecular mechanisms involved in chondrogenesis in invertebrates and vertebrates? The paucity of molecular and even embryological data on invertebrate cartilage development makes it difficult to answer this question. The structural similarities are striking, and given the conservatism of developmental evolution and the limited number of "toolkit genes," it would be surprising if entirely different mechanisms were utilized to build this tissue type in different lineages. Nonetheless, the possibility of convergence using different mechanisms remains, and comparative analyses of chondrogenesis will be required to resolve this evolutionary mystery.

6. Elaborating the Chondrogenetic Toolkit: Gene/Genome Duplication and the Origin of Collagenous Cartilage

Given the dominant role that fibrillar collagens play in constructing the matrices of a diverse array of vertebrate connective tissues, it seems likely that expansion of this gene family would have been a critical step toward the evolutionary diversification of skeletal tissues. Molecular phylogenetic analyses of deuterostome collagens indicate that a gene ancestral to the vertebrate clade A collagens had arisen in chordates before the origin of vertebrates, but the duplication and divergence of clade A collagens (*Col1α1*, *Col1α2*, *Col2α1*, *Col3α1*, and *Col5α2*) and clade B collagens (*Col5α1*, *Col5α3*, *Col11α1*, and *Col11α2*) occurred within the vertebrate lineage (Boot-Handford and Tuckwell, 2003; Zhang and Cohn, 2006, 2008). A number of findings point to deep conservation of chondrogenic mechanisms, such as the evidence that horseshoe crab cartilage contains chondroitin-6-sulfate (Sugahara *et al.*, 1996) and that squid and cuttlefish cartilages may contain collagen, although these appear to be different than type II collagen (Bairati and Gioria, 2004; Bairati *et al.*, 1999; Kimura and Karasawa, 1985; Kimura and Matsuura, 1974). Fibrillar collagens also have been identified in cartilage-like tissues of protostomes, including sponge, sea urchin, abalone, and hydra. Similarities have been described between the sea urchin α1 and vertebrate α2(I) chains, and between hydra Hcol1 and vertebrate collagen type I/II (Deutzmann *et al.*, 2000; Exposito *et al.*, 1992). Indeed, some invertebrate cartilage-like tissues crossreact with antibodies against vertebrate types II, V, and X collagen, and proteoglycans (Bairati *et al.*, 1999; Cole and Hall, 2004a,b; Sivakumar and Chandrakasan, 1998), although published phylogenies of the collagen family suggest it unlikely that these vertebrate antibodies are detecting strict orthologs of Col2, Col5, or Col10 in invertebrates (Boot-Handford and Tuckwell, 2003; Rychel *et al.*, 2006; Wada *et al.*, 2006; Zhang *et al.*, 2006, 2007). Nonetheless, the fundamental structure of fibrillar collagens was established early in metazoan evolution (Boot-Handford and Tuckwell, 2003). The phylogenetic distribution of cartilage and cartilage-like tissues suggests that this tissue type evolved independently and multiple times in metazoans (Cole and Hall, 2004a), and while the evidence for convergent evolution precludes structural homologies of invertebrate and vertebrate cartilages, it does not rule out the possibility of homologous developmental mechanisms. The striking structural and molecular similarities between invertebrate and vertebrate cartilages, such as utilization of fibrillar collagens and chondroitin-6-sulfate, suggests that a common suite of developmental tools was used repeatedly by metazoans to generate cartilaginous tissues, much like the deeply conserved

eye development program involving Pax6 has been redeployed time and again to build eyes. The area of invertebrate cartilage biology is ripe for comparative studies using modern molecular developmental approaches.

REFERENCES

Akiyama, H., Lyons, J. P., Mori-Akiyama, Y., Yang, X., Zhang, R., Zhang, Z., Deng, J. M., Taketo, M. M., Nakamura, T., Behringer, R. R., McCrea, P. D., and de Crombrugghe, B. (2004). Interactions between Sox9 and beta-catenin control chondrocyte differentiation. *Genes Dev.* **18,** 1072–1087.

Akiyama, H., Kim, J. E., Nakashima, K., Balmes, G., Iwai, N., Deng, J. M., Zhang, Z., Martin, J. F., Behringer, R. R., Nakamura, T., and de Crombrugghe, B. (2005). Osteochondroprogenitor cells are derived from Sox9 expressing precursors. *Proc. Natl. Acad. Sci. USA* **102,** 14665–14670.

Aouacheria, A., Cluzel, C., Lethias, C., Gouy, M., Garrone, R., and Exposito, J. Y. (2004). Invertebrate data predict an early emergence of vertebrate fibrillar collagen clades and an anti-incest model. *J. Biol. Chem.* **279,** 47711–47719.

Bairati, A., and Gioria, M. (2004). Collagen fibrils of an invertebrate (Sepia officinalis) are heterotypic: Immunocytochemical demonstration. *J. Struct. Biol.* **147,** 159–165.

Bairati, A., Comazzi, M., Gioria, M., Hartmann, D. J., Leone, F., and Rigo, C. (1999). Immunohistochemical study of collagens of the extracellular matrix in cartilage of Sepia officinalis. *Eur. J. Histochem.* **43,** 211–225.

Benjamin, M. (1989). Hyaline-cell cartilage (chondroid) in the heads of teleosts. *Anat. Embryol. (Berl.)* **179,** 285–303.

Benjamin, M. (1990). The cranial cartilages of teleosts and their classification. *J. Anat.* **169,** 153–172.

Benjamin, M., and Evans, E. J. (1990). Fibrocartilage. *J. Anat.* **171,** 1–15.

Benjamin, M., and Ralphs, J. R. (1991). Extracellular matrix of connective tissues in the heads of teleosts. *J. Anat.* **179,** 137–148.

Benjamin, M., and Ralphs, J. R. (2004). Biology of fibrocartilage cells. *Int. Rev. Cytol.* **233,** 1–45.

Benjamin, M., Ralphs, J. R., and Eberewariye, O. S. (1992). Cartilage and related tissues in the trunk and fins of teleosts. *J. Anat.* **181**(Pt. 1), 113–118.

Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R., and de Crombrugghe, B. (1999). Sox9 is required for cartilage formation. *Nat. Genet.* **22,** 85–89.

Bodine, P. V., Zhao, W., Kharode, Y. P., Bex, F. J., Lambert, A. J., Goad, M. B., Gaur, T., Stein, G. S., Lian, J. B., and Komm, B. S. (2004). The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. *Mol. Endocrinol.* **18,** 1222–1237.

Boot-Handford, R. P., and Tuckwell, D. S. (2003). Fibrillar collagen: The key to vertebrate evolution? A tale of molecular incest. *Bioessays* **25,** 142–151.

Bordat, C. (1987). Ultrastructural study of the vertebrae of the selachian *Scyliorhinus canicula*. *Can. J. Zool.* **65,** 1435–1444.

Boyden, L. M., Mao, J., Belsky, J., Mitzner, L., Farhi, A., Mitnick, M. A., Wu, D., Insogna, K., and Lifton, R. P. (2002). High bone density due to a mutation in LDL-receptor-related protein 5. *N. Engl. J. Med.* **346,** 1513–1521.

Bruckner, P., and van der Rest, M. (1994). Structure and function of cartilage collagens. *Microsc. Res. Tech.* **28,** 378–384.

Carroll, R. L. (1988). "Vertebrate Paleontology and Evolution." Freeman, New York.

Coates, M. I., and Sequeira, S. E. K. (2001). A new stethacanthid chondrichthyan from the Lower Carboniferous of Bearsden, Scotland. *J. Vertebr. Paleontol.* **21,** 438–459.

Coates, M. I., Sequeira, S. E. K., Sansom, I. J., and Smith, M. M. (1998). Spines and tissues of ancient sharks. *Nature* **396,** 729–730.

Cole, F. J. (1905). A monograph on the general morphology of the myxinoid fishes based on a study of Myxine. 1. The anatomy of the skeleton. *Trans. R. Soc. Edinburgh* **41,** 749–791.

Cole, A. G., and Hall, B. K. (2004a). Cartilage is a metazoan tissue; integrating data from nonvertebrate sources. *Acta Zool. (Stockholm)* **85,** 69–80.

Cole, A. G., and Hall, B. K. (2004b). The nature and significance of invertebrate cartilages revisited: Distribution and histology of cartilage and cartilage-like tissues within the Metazoa. *Zoology (Jena)* **107,** 261–273.

Colvin, J. S., Bohne, B. A., Harding, G. W., McEwen, D. G., and Ornitz, D. M. (1996). Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. *Nat. Genet.* **12,** 390–397.

Daniel, J. F. (1934). ''The Elasmobranch Fishes.'' University of California Press, Berkeley.

Day, T. F., Guo, X., Garrett-Beal, L., and Yang, Y. (2005). Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Dev. Cell* **8,** 739–750.

Dean, M. N., and Summers, A. P. (2006). Mineralized cartilage in the skeleton of chondrichthyan fishes. *Zoology (Jena)* **109,** 164–168.

Deng, C., Wynshaw-Boris, A., Zhou, F., Kuo, A., and Leder, P. (1996). Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell* **84,** 911–921.

Deutzmann, R., Fowler, S., Zhang, X., Boone, K., Dexter, S., Boot-Handford, R. P., Rachel, R., and Sarras, M. P., Jr. (2000). Molecular, biochemical and functional analysis of a novel and developmentally important fibrillar collagen (Hcol-I) in hydra. *Development* **127,** 4669–4680.

Doege, K. J., Sasaki, M., Kimura, T., and Yamada, Y. (1991). Complete coding sequence and deduced primary structure of the human cartilage large aggregating proteoglycan, aggrecan. Human-specific repeats, and additional alternatively spliced forms. *J. Biol. Chem.* **266,** 894–902.

Domowicz, M., Li, H., Hennig, A., Henry, J., Vertel, B. M., and Schwartz, N. B. (1995). The biochemically and immunologically distinct CSPG of notochord is a product of the aggrecan gene. *Dev. Biol.* **171,** 655–664.

Donoghue, P. C., and Sansom, I. J. (2002). Origin and early evolution of vertebrate skeletonization. *Microsc. Res. Tech.* **59,** 352–372.

Donoghue, P. C. J., and Smith, M. P. (2001). The anatomy of *Turinia pagei* (Powrie) and the phylogenetic status of the Thelodonti. *Tran. R. Soc. Edinburgh (Earth Sci.)* **92,** 15–37.

Donoghue, P. C., Sansom, I. J., and Downs, J. P. (2006). Early evolution of vertebrate skeletal tissues and cellular interactions, and the canalization of skeletal development. *J. Exp. Zool. B Mol. Dev. Evol.* **306,** 278–294.

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L., and Karsenty, G. (1997). Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. *Cell* **89,** 747–754.

Ducy, P., Starbuck, M., Priemel, M., Shen, J., Pinero, G., Geoffroy, V., Amling, M., and Karsenty, G. (1999). A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. *Genes Dev.* **13,** 1025–1036.

Eames, B. F., and Helms, J. A. (2004). Conserved molecular program regulating cranial and appendicular skeletogenesis. *Dev. Dyn.* **231,** 4–13.

Eames, B. F., Sharpe, P. T., and Helms, J. A. (2004). Hierarchy revealed in the specification of three skeletal fates by Sox9 and Runx2. *Dev. Biol.* **274,** 188–200.

Eames, B. F., Allen, N., Young, J., Kaplan, A., Helms, J. A., and Schneider, R. A. (2007). Skeletogenesis in the swell shark *Cephaloscyllium ventriosum*. *J. Anat.* **210,** 542–554.

Eikenberry, E. F., Childs, B., Sheren, S. B., Parry, D. A., Craig, A. S., and Brodsky, B. (1984). Crystalline fibril structure of type II collagen in lamprey notochord sheath. *J. Mol. Biol.* **176,** 261–277.

Exposito, J. Y., D'Alessio, M., Solursh, M., and Ramirez, F. (1992). Sea urchin collagen evolutionarily homologous to vertebrate pro-alpha 2(I) collagen. *J. Biol. Chem.* **267,** 15559–15562.

Exposito, J. Y., Cluzel, C., Garrone, R., and Lethias, C. (2002). Evolution of collagens. *Anat. Rec.* **268,** 302–316.

Eyre, D. R., and Wu, J. J. (1983). Collagen of fibrocartilage: A distinctive molecular phenotype in bovine meniscus. *FEBS Lett.* **158,** 265–270.

Eyre, D. R., Pietka, T., Weis, M. A., and Wu, J. J. (2004). Covalent cross-linking of the NC1 domain of collagen type IX to collagen type II in cartilage. *J. Biol. Chem.* **279,** 2568–2574.

Fang, J., and Hall, B. K. (1997). Chondrogenic cell differentiation from membrane bone periostea. *Anat. Embryol. (Berl.)* **196,** 349–362.

Foster, J. W., Dominguez-Steglich, M. A., Guioli, S., Kowk, G., Weller, P. A., Stevanovic, M., Weissenbach, J., Mansour, S., Young, I. D., Goodfellow, P. N., Brook, J. D., and Schafer, A. J. (1994). Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature* **372,** 525–530.

Franz-Odendaal, T. A., Ryan, K., and Hall, B. K. (2007). Developmental and morphological variation in the teleost craniofacial skeleton reveals an unusual mode of ossification. *J. Exp. Zool. B Mol. Dev. Evol.* **308,** 709–721.

Fukada, K., Shibata, S., Suzuki, S., Ohya, K., and Kuroda, T. (1999). *In situ* hybridisation study of type I, II, X collagens and aggrecan mRNas in the developing condylar cartilage of fetal mouse mandible. *J. Anat.* **195**(Pt. 3), 321–329.

Fukuoka, H., Shibata, S., Suda, N., Yamashita, Y., and Komori, T. (2007). Bone morphogenetic protein rescues the lack of secondary cartilage in Runx2-deficient mice. *J. Anat.* **211,** 8–15.

Furumatsu, T., Tsuda, M., Taniguchi, N., Tajima, Y., and Asahara, H. (2005). Smad3 induces chondrogenesis through the activation of SOX9 via CREB-binding protein/p300 recruitment. *J. Biol. Chem.* **280,** 8343–8350.

Glass, D. A., II, Bialek, P., Ahn, J. D., Starbuck, M., Patel, M. S., Clevers, H., Taketo, M. M., Long, F., McMahon, A. P., Lang, R. A., and Karsenty, G. (2005). Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Dev. Cell* **8,** 751–764.

Gong, Y., Slee, R. B., Fukai, N., Rawadi, G., Roman-Roman, S., Reginato, A. M., Wang, H., Cundy, T., Glorieux, F. H., Lev, D., Zacharin, M., Oexle, K., *et al.* (2001). LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell* **107,** 513–523.

Goodrich, E. S. (1930). ''Studies on the Structure and Development of the Vertebrates.'' Macmillan, London.

Hall, B. K. (1975). Evolutionary consequences of skeletal differentiation. *Am. Zool.* **15,** 329–350.

Hall, B. K. (2005). ''Bone and Cartilage: Development and Evolutionary Skeletal Biology.'' Elsevier Academic Press, San Diego.

Hardingham, T. E., and Fosang, A. J. (1992). Proteoglycans: Many forms and many functions. *FASEB J.* **6,** 861–870.

Hartmann, C., and Tabin, C. J. (2001). Wnt-14 plays a pivotal role in inducing synovial joint formation in the developing appendicular skeleton. *Cell* **104,** 341–351.

Healy, C., Uwanogho, D., and Sharpe, P. T. (1996). Expression of the chicken Sox9 gene marks the onset of cartilage differentiation. *Ann. N. Y. Acad. Sci.* **785,** 261–262.

Healy, C., Uwanogho, D., and Sharpe, P. T. (1999). Regulation and role of Sox9 in cartilage formation. *Dev. Dyn.* **215,** 69–78.

Hill, T. P., Spater, D., Taketo, M. M., Birchmeier, W., and Hartmann, C. (2005). Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes. *Dev. Cell* **8,** 727–738.

Hu, H., Hilton, M. J., Tu, X., Yu, K., Ornitz, D. M., and Long, F. (2005). Sequential roles of Hedgehog and Wnt signaling in osteoblast development. *Development* **132,** 49–60.

Hung, I. H., Yu, K., Lavine, K. J., and Ornitz, D. M. (2007). FGF9 regulates early hypertrophic chondrocyte differentiation and skeletal vascularization in the developing stylopod. *Dev. Biol.* **307,** 300–313.

Ikeda, T., Kamekura, S., Mabuchi, A., Kou, I., Seki, S., Takato, T., Nakamura, K., Kawaguchi, H., Ikegawa, S., and Chung, U. I. (2004). The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. *Arthritis Rheum.* **50,** 3561–3573.

Inada, M., Yasui, T., Nomura, S., Miyake, S., Deguchi, K., Himeno, M., Sato, M., Yamagiwa, H., Kimura, T., Yasui, N., Ochi, T., Endo, N., *et al.* (1999). Maturational disturbance of chondrocytes in Cbfa1-deficient mice. *Dev. Dyn.* **214,** 279–290.

Iozzo, R. V. (1998). Matrix proteoglycans: From molecular design to cellular function. *Annu. Rev. Biochem.* **67,** 609–652.

Ishii, M., Suda, N., Tengan, T., Suzuki, S., and Kuroda, T. (1998). Immunohistochemical findings type I and type II collagen in prenatal mouse mandibular condylar cartilage compared with the tibial anlage. *Arch. Oral Biol.* **43,** 545–550.

Iwamoto, M., Kitagaki, J., Tamamura, Y., Gentili, C., Koyama, E., Enomoto, H., Komori, T., Pacifici, M., and Enomoto-Iwamoto, M. (2003). Runx2 expression and action in chondrocytes are regulated by retinoid signaling and parathyroid hormone-related peptide (PTHrP). *Osteoarthr. Cartil.* **11,** 6–15.

Jacob, A. L., Smith, C., Partanen, J., and Ornitz, D. M. (2006). Fibroblast growth factor receptor 1 signaling in the osteo-chondrogenic cell lineage regulates sequential steps of osteoblast maturation. *Dev. Biol.* **296,** 315–328.

Janvier, P. (1996). ''Early Vertebrates.'' Oxford University Press, Oxford.

Janvier, P., and Arsenault, M. (2002). Palaeobiology: Calcification of early vertebrate cartilage. *Nature* **417,** 609.

Kadler, K. E., Holmes, D. F., Trotter, J. A., and Chapman, J. A. (1996). Collagen fibril formation. *Biochem. J.* **316**(Pt. 1), 1–11.

Karaplis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L., Kronenberg, H. M., and Mulligan, R. C. (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev.* **8,** 277–289.

Karp, S. J., Schipani, E., St-Jacques, B., Hunzelman, J., Kronenberg, H., and McMahon, A. P. (2000). Indian hedgehog coordinates endochondral bone growth and morphogenesis via parathyroid hormone related-protein-dependent and -independent pathways. *Development* **127,** 543–548.

Karsenty, G. (2003). The complexities of skeletal biology. *Nature* **423,** 316–318.

Karsenty, G. (2008). Transcriptional control of skeletogenesis. *Annu. Rev. Genomics Hum. Genet.* **9,** 183–196.

Karsenty, G., and Wagner, E. F. (2002). Reaching a genetic and molecular understanding of skeletal development. *Dev. Cell* **2,** 389–406.

Kato, M., Patel, M. S., Levasseur, R., Lobov, I., Chang, B. H., Glass, D. A., II, Hartmann, C., Li, L., Hwang, T. H., Brayton, C. F., Lang, R. A., Karsenty, G., *et al.* (2002). Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. *J. Cell Biol.* **157,** 303–314.

Kemp, N. E., and Westrin, S. K. (1979). Ultrastructure of calcified cartilage in the endoskeletal tesserae of sharks. *J. Morphol.* **160,** 75–109.

Kim, I. S., Otto, F., Zabel, B., and Mundlos, S. (1999). Regulation of chondrocyte differentiation by Cbfa1. *Mech. Dev.* **80,** 159–170.

Kimura, S., and Karasawa, K. (1985). Squid cartilage collagen: Isolation of type I collagen rich in carbohydrate. *Comp. Biochem. Physiol. B* **81,** 361–365.

Kimura, S., and Matsuura, F. (1974). The chain compositions of several invertebrate collagens. *J. Biochem. (Tokyo)* **75,** 1231–1240.

Knudson, C. B., and Knudson, W. (2001). Cartilage proteoglycans. *Semin. Cell Dev. Biol.* **12,** 69–78.

Kobayashi, T., Lyons, K. M., McMahon, A. P., and Kronenberg, H. M. (2005). BMP signaling stimulates cellular differentiation at multiple steps during cartilage development. *Proc. Natl. Acad. Sci. USA* **102,** 18023–18027.

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R. T., Gao, Y. H., Inada, M., Sato, M., Okamoto, R., *et al.* (1997). Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* **89,** 755–764.

Kronenberg, H. M. (2006). PTHrP and skeletal development. *Ann. N. Y. Acad. Sci.* **1068,** 1–13.

Kusserow, A., Pang, K., Sturm, C., Hrouda, M., Lentfer, J., Schmidt, H. A., Technau, U., von Haeseler, A., Hobmayer, B., Martindale, M. Q., and Holstein, T. W. (2005). Unexpected complexity of the Wnt gene family in a sea anemone. *Nature* **433,** 156–160.

Langille, R. M., and Hall, B. K. (1988). The organ culture and grafting of lamprey cartilage and teeth. *In Vitro Cell Dev. Biol.* **24,** 1–8.

Lanske, B., Karaplis, A. C., Lee, K., Luz, A., Vortkamp, A., Pirro, A., Karperien, M., Defize, L. H., Ho, C., Mulligan, R. C., Abou-Samra, A. B., Juppner, H., *et al.* (1996). PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* **273,** 663–666.

Lee, P. N., Pang, K., Matus, D. Q., and Martindale, M. Q. (2006). A WNT of things to come: Evolution of Wnt signaling and polarity in cnidarians. *Semin. Cell Dev. Biol.* **17,** 157–167.

Lefebvre, V., Behringer, R. R., and de Crombrugghe, B. (2001). L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. *Osteoarthr. Cartil.* **9** (Suppl. A), S69–S75.

Lefebvre, V., and de Crombrugghe, B. (1998). Toward understanding SOX9 function in chondrocyte differentiation. *Matrix Biol.* **16,** 529–540.

Lefebvre, V., Huang, W., Harley, V. R., Goodfellow, P. N., and de Crombrugghe, B. (1997). SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. *Mol. Cell Biol.* **17,** 2336–2346.

Lefebvre, V., Li, P., and de Crombrugghe, B. (1998). A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. *EMBO J.* **17,** 5718–5733.

Levanon, D., Brenner, O., Negreanu, V., Bettoun, D., Woolf, E., Eilam, R., Lotem, J., Gat, U., Otto, F., Speck, N., and Groner, Y. (2001). Spatial and temporal expression pattern of Runx3 (Aml2) and Runx1 (Aml1) indicates non-redundant functions during mouse embryogenesis. *Mech. Dev.* **109,** 413–417.

Li, X., and Cao, X. (2006). BMP signaling and skeletogenesis. *Ann. N. Y. Acad. Sci.* **1068,** 26–40.

Li, Y., Lacerda, D. A., Warman, M. L., Beier, D. R., Yoshioka, H., Ninomiya, Y., Oxford, J. T., Morris, N. P., Andrikopoulos, K., Ramirez, F., *et al.* (1995). A fibrillar collagen gene, Col11a1, is essential for skeletal morphogenesis. *Cell* **80,** 423–430.

Li, T. F., Dong, Y., Ionescu, A. M., Rosier, R. N., Zuscik, M. J., Schwarz, E. M., O'Keefe, R. J., and Drissi, H. (2004). Parathyroid hormone-related peptide (PTHrP) inhibits Runx2 expression through the PKA signaling pathway. *Exp. Cell Res.* **299,** 128–136.

Lian, J. B., Balint, E., Javed, A., Drissi, H., Vitti, R., Quinlan, E. J., Zhang, L., Van Wijnen, A. J., Stein, J. L., Speck, N., and Stein, G. S. (2003). Runx1/AML1 hematopoietic transcription factor contributes to skeletal development *in vivo*. *J. Cell. Physiol.* **196,** 301–311.

Linsenmayer, T. F., Gibney, E., and Schmid, T. M. (1986). Segmental appearance of type X collagen in the developing avian notochord. *Dev. Biol.* **113,** 467–473.

Little, R. D., Carulli, J. P., Del Mastro, R. G., Dupuis, J., Osborne, M., Folz, C., Manning, S. P., Swain, P. M., Zhao, S. C., Eustace, B., Lappe, M. M., Spitzer, L., *et al.* (2002). A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. *Am. J. Hum. Genet.* **70,** 11–19.

Liu, Y., Li, H., Tanaka, K., Tsumaki, N., and Yamada, Y. (2000). Identification of an enhancer sequence within the first intron required for cartilage-specific transcription of the alpha2(XI) collagen gene. *J. Biol. Chem.* **275,** 12712–12718.

Logan, C. Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20,** 781–810.

Lyons, K. M., Hogan, B. L., and Robertson, E. J. (1995). Colocalization of BMP 7 and BMP 2 RNAs suggests that these factors cooperatively mediate tissue interactions during murine development. *Mech. Dev.* **50,** 71–83.

Maisey, J. G. (1988). Phylogeny of early vertebrate skeletal induction and ossification patterns. *In* "Evolutionary Biology" (M. K. Hecht, B. Wallace, and G. T. Prance, Eds.), pp. 1–36. Plenum Publishing Corporation, New York.

Mak, K. K., Kronenberg, H. M., Chuang, P. T., Mackem, S., and Yang, Y. (2008). Indian hedgehog signals independently of PTHrP to promote chondrocyte hypertrophy. *Development* **135,** 1947–1956.

Mallatt, J., and Chen, J. Y. (2003). Fossil sister group of craniates: Predicted and found. *J. Morphol.* **258,** 1–31.

Meulemans, D., and Bronner-Fraser, M. (2007). Insights from amphioxus into the evolution of vertebrate cartilage. *PLoS ONE* **2,** e787.

Miller, J. R. (2002). The Wnts. *Genome Biol.* **3,** REVIEWS3001.

Miller, E. J., and Matukas, V. J. (1969). Chick cartilage collagen: A new type of alpha 1 chain not present in bone or skin of the species. *Proc. Natl. Acad. Sci. USA* **64,** 1264–1268.

Minina, E., Wenzel, H. M., Kreschel, C., Karp, S., Gaffield, W., McMahon, A. P., and Vortkamp, A. (2001). BMP and Ihh/PTHrP signaling interact to coordinate chondrocyte proliferation and differentiation. *Development* **128,** 4523–4534.

Mizuta, S., Hwang, J.-H., and Yoshinaka, R. (2003). Molecular species of collagen in pectoral fin cartilage of skate (Raja Kenojei). *Food Chem.* **80,** 1–7.

Mori-Akiyama, Y., Akiyama, H., Rowitch, D. H., and de Crombrugghe, B. (2003). Sox9 is required for determination of the chondrogenic cell lineage in the cranial neural crest. *Proc. Natl. Acad. Sci. USA* **100,** 9360–9365.

Morrison, S. L., Campbell, C. K., and Wright, G. M. (2000). Chondrogenesis of the branchial skeleton in embryonic sea lamprey, *Petromyzon marinus*. *Anat. Rec.* **260,** 252–267.

Moss, M. L. (1970). Enamel and bone in shark teeth: With a note on fibrous enamel in fishes. *Acta Anat. (Basel)* **77,** 161–187.

Moss, M. L. (1977). Skeletal tissues in sharks. *Am. Zool.* 335–342.

Mundlos, S., and Olsen, B. R. (1997a). Heritable diseases of the skeleton. Part I. Molecular insights into skeletal development-transcription factors and signaling pathways. *FASEB J.* **11,** 125–132.

Mundlos, S., and Olsen, B. R. (1997b). Heritable diseases of the skeleton. Part II. Molecular insights into skeletal development-matrix components and their homeostasis. *FASEB J.* **11,** 227–233.

Mundlos, S., Huang, L. F., Selby, P., and Olsen, B. R. (1996). Cleidocranial dysplasia in mice. *Ann. N. Y. Acad. Sci.* **785,** 301–302.

Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J. M., Behringer, R. R., and de Crombrugghe, B. (2002). The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* **108,** 17–29.

Naumann, A., Dennis, J. E., Awadallah, A., Carrino, D. A., Mansour, J. M., Kastenbauer, E., and Caplan, A. I. (2002). Immunochemical and mechanical characterization of cartilage subtypes in rabbit. *J. Histochem. Cytochem.* **50,** 1049–1058.

Ng, L. J., Wheatley, S., Muscat, G. E., Conway-Campbell, J., Bowles, J., Wright, E., Bell, D. M., Tam, P. P., Cheah, K. S., and Koopman, P. (1997). SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. *Dev. Biol.* **183,** 108–121.

Ohtani, K., Yao, T., Kobayashi, M., Kusakabe, R., Kuratani, S., and Wada, H. (2008). Expression of Sox and fibrillar collagen genes in lamprey larval chondrogenesis with implications for the evolution of vertebrate cartilage. *J. Exp. Zool. B Mol. Dev. Evol.* **310,** 596–607.

Olsen, B. R., Reginato, A. M., and Wang, W. (2000). Bone development. *Annu. Rev. Cell Dev. Biol.* **16,** 191–220.

Ornitz, D. M., and Marie, P. J. (2002). FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. *Genes Dev.* **16,** 1446–1465.

Orvig, T. (1951). Histologic studies of Placoderms and fossil Elasmobranchs. I. The endoskeleton, with remarks on the hard tissues of lower vertebrates in general. *Ark. Zool.* **2,** 321–456.

Ostrander, G. K., Cheng, K. C., Wolf, J. C., and Wolfe, M. J. (2004). Shark cartilage, cancer and the growing threat of pseudoscience. *Cancer Res.* **64,** 8485–8491.

Otto, F., Thornell, A. P., Crompton, T., Denzel, A., Gilmour, K. C., Rosewell, I. R., Stamp, G. W., Beddington, R. S., Mundlos, S., Olsen, B. R., Selby, P. B., and Owen, M. J. (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* **89,** 765–771.

Parker, W. (1883). On the skeleton of the marsipobranch fishes. Part II. Petromyzon. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **174,** 411–457.

Patterson, C. M., Kruger, B. J., and Daley, T. J. (1977). Lipid and protein histochemistry of enamel—Effects of fluoride. *Calcif. Tissue Res.* **24,** 119–123.

Peignoux-Deville, J., Lallier, F., and Vidal, B. (1982). Evidence for the presence of osseous tissue in dogfish vertebrae. *Cell Tissue Res.* **222,** 605–614.

Person, P., and Mathews, M. B. (1967). Endoskeletal cartilage in a marine polychaete, *Eudistylia polymorpha*. *Biol. Bull.* **132,** 244–252.

Pogue, R., and Lyons, K. (2006). BMP signaling in the cartilage growth plate. *Curr. Top. Dev. Biol.* **76,** 1–48.

Pratta, M. A., Yao, W., Decicco, C., Tortorella, M. D., Liu, R. Q., Copeland, R. A., Magolda, R., Newton, R. C., Trzaskos, J. M., and Arner, E. C. (2003). Aggrecan protects cartilage collagen from proteolytic cleavage. *J. Biol. Chem.* **278,** 45539–45545.

Provot, S., Kempf, H., Murtaugh, L. C., Chung, U. I., Kim, D. W., Chyung, J., Kronenberg, H. M., and Lassar, A. B. (2006). Nkx3.2/Bapx1 acts as a negative regulator of chondrocyte maturation. *Development* **133,** 651–662.

Prud'homme, B., Lartillot, N., Balavoine, G., Adoutte, A., and Vervoort, M. (2002). Phylogenetic analysis of the Wnt gene family. Insights from lophotrochozoan members. *Curr. Biol.* **12,** 1395.

Rama, S., and Chandrakasan, G. (1984). Distribution of different molecular species of collagen in the vertebral cartilage of shark (*Carcharias acutus*). *Connect Tissue Res.* **12,** 111–118.

Rawadi, G., Vayssiere, B., Dunn, F., Baron, R., and Roman-Roman, S. (2003). BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. *J. Bone Miner. Res.* **18,** 1842–1853.

Razzaque, M. S., Soegiarto, D. W., Chang, D., Long, F., and Lanske, B. (2005). Conditional deletion of Indian hedgehog from collagen type 2alpha1-expressing cells results in abnormal endochondral bone formation. *J. Pathol.* **207,** 453–461.

Reif, W. E. (1980). Development of dentition and dermal skeleton in embryonic *Scyliorhinus canicula*. *J. Morphol.* **166,** 275–288.

Rodda, S. J., and McMahon, A. P. (2006). Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development* **133,** 3231–3244.

Romer, A. S. (1985). The vertebrate body. *In* "Saunders Series in Organismic Biology." Saunders College Publishing, Philadelphia.

Rychel, A. L., and Swalla, B. J. (2007). Development and evolution of chordate cartilage. *J. Exp. Zool. B Mol. Dev. Evol.* **308,** 325–335.

Rychel, A. L., Smith, S. E., Shimamoto, H. T., and Swalla, B. J. (2006). Evolution and development of the chordates: Collagen and pharyngeal cartilage. *Mol. Biol. Evol.* **23,** 541–549.

Sahni, M., Ambrosetti, D. C., Mansukhani, A., Gertner, R., Levy, D., and Basilico, C. (1999). FGF signaling inhibits chondrocyte proliferation and regulates bone development through the STAT-1 pathway. *Genes Dev.* **13,** 1361–1366.

Saito, T., Ikeda, T., Nakamura, K., Chung, U. I., and Kawaguchi, H. (2007). S100A1 and S100B, transcriptional targets of SOX trio, inhibit terminal differentiation of chondrocytes. *EMBO Rep.* **8,** 504–509.

Sansom, I. J., Donoghue, P. C., and Albanesi, G. (2005). Histology and affinity of the earliest armoured vertebrate. *Biol. Lett.* **1,** 446–449.

Schipani, E., Kruse, K., and Juppner, H. (1995). A constitutively active mutant PTH–PTHrP receptor in Jansen-type metaphyseal chondrodysplasia. *Science* **268,** 98–100.

Schwartz, N. B., Pirok, E. W., III, Mensch, J. R., Jr., and Domowicz, M. S. (1999). Domain organization, genomic structure, evolution, and regulation of expression of the aggrecan gene family. *Prog. Nucleic Acid Res. Mol. Biol.* **62,** 177–225.

Seki, K., and Hata, A. (2004). Indian hedgehog gene is a target of the bone morphogenetic protein signaling pathway. *J. Biol. Chem.* **279,** 18544–18549.

Sire, J. Y., and Huysseune, A. (2003). Formation of dermal skeletal and dental tissues in fish: A comparative and evolutionary approach. *Biol. Rev. Camb. Philos. Soc.* **78,** 219–249.

Sivakumar, P., and Chandrakasan, G. (1998). Occurrence of a novel collagen with three distinct chains in the cranial cartilage of the squid Sepia officinalis: Comparison with shark cartilage collagen. *Biochim. Biophys. Acta* **1381,** 161–169.

Smith, M. M., and Hall, B. K. (1990). Development and evolutionary origins of vertebrate skeletogenic and odontogenic tissues. *Biol. Rev. Camb. Philos. Soc.* **65,** 277–373.

Smith, M. M., and Johanson, Z. (2003). Separate evolutionary origins of teeth from evidence in fossil jawed vertebrates. *Science* **299,** 1235–1236.

Smith, N., Dong, Y., Lian, J. B., Pratap, J., Kingsley, P. D., van Wijnen, A. J., Stein, J. L., Schwarz, E. M., O'Keefe, R. J., Stein, G. S., and Drissi, M. H. (2005). Overlapping expression of Runx1(Cbfa2) and Runx2(Cbfa1) transcription factors supports cooperative induction of skeletal development. *J. Cell. Physiol.* **203,** 133–143.

Smits, P., and Lefebvre, V. (2003). Sox5 and Sox6 are required for notochord extracellular matrix sheath formation, notochord cell survival and development of the nucleus pulposus of intervertebral discs. *Development* **130,** 1135–1148.

Soderhall, C., Marenholz, I., Kerscher, T., Ruschendorf, F., Esparza-Gordillo, J., Worm, M., Gruber, C., Mayr, G., Albrecht, M., Rohde, K., Schulz, H., Wahn, U., *et al.* (2007). Variants in a novel epidermal collagen gene (COL29A1) are associated with atopic dermatitis. *PLoS Biol.* **5,** e242.

Solloway, M. J., Dudley, A. T., Bikoff, E. K., Lyons, K. M., Hogan, B. L., and Robertson, E. J. (1998). Mice lacking Bmp6 function. *Dev. Genet.* **22,** 321–339.

Song, S. J., Cool, S. M., and Nurcombe, V. (2007). Regulated expression of syndecan-4 in rat calvaria osteoblasts induced by fibroblast growth factor-2. *J. Cell. Biochem.* **100,** 402–411.

St-Jacques, B., Hammerschmidt, M., and McMahon, A. P. (1999). Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev.* **13,** 2072–2086.

Stemple, D. L. (2004). The notochord. *Curr. Biol.* **14,** R873–R874.

Stensio, E. A. (1927). The Devonian and Downtonian vertebrates of Spitsbergen. Part I. Family Cephalaspidae. *Skr. Svalbard Ishavet* **12,** 1–391.

Stolt, C. C., Schlierf, A., Lommes, P., Hillgartner, S., Werner, T., Kosian, T., Sock, E., Kessaris, N., Richardson, W. D., Lefebvre, V., and Wegner, M. (2006). SoxD proteins influence multiple stages of oligodendrocyte development and modulate SoxE protein function. *Dev. Cell* **11,** 697–709.

Stricker, S., Fundele, R., Vortkamp, A., and Mundlos, S. (2002). Role of Runx genes in chondrocyte differentiation. *Dev. Biol.* **245,** 95–108.

Sugahara, K., Tanaka, Y., Yamada, S., Seno, N., Kitagawa, H., Haslam, S. M., Morris, H. R., and Dell, A. (1996). Novel sulfated oligosaccharides containing 3-O-sulfated glucuronic acid from king crab cartilage chondroitin sulfate K. Unexpected degradation by chondroitinase ABC. *J. Biol. Chem.* **271,** 26745–26754.

Takeda, S., Bonnamy, J. P., Owen, M. J., Ducy, P., and Karsenty, G. (2001). Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. *Genes Dev.* **15,** 467–481.

Toma, C. D., Schaffer, J. L., Meazzini, M. C., Zurakowski, D., Nah, H. D., and Gerstenfeld, L. C. (1997). Developmental restriction of embryonic calvarial cell populations as characterized by their *in vitro* potential for chondrogenic differentiation. *J. Bone Miner. Res.* **12,** 2024–2039.

Tsuda, M., Takahashi, S., Takahashi, Y., and Asahara, H. (2003). Transcriptional co-activators CREB-binding protein and p300 regulate chondrocyte-specific gene expression via association with Sox9. *J. Biol. Chem.* **278,** 27224–27229.

van der Rest, M., and Garrone, R. (1991). Collagen family of proteins. *FASEB J.* **5,** 2814–2823.

van Wijnen, A. J., Stein, G. S., Gergen, J. P., Groner, Y., Hiebert, S. W., Ito, Y., Liu, P., Neil, J. C., Ohki, M., and Speck, N. (2004). Nomenclature for Runt-related (RUNX) proteins. *Oncogene* **23,** 4209–4210.

Vortkamp, A., Lee, K., Lanske, B., Segre, G. V., Kronenberg, H. M., and Tabin, C. J. (1996). Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* **273,** 613–622.

Wada, H., Okuyama, M., Satoh, N., and Zhang, S. (2006). Molecular evolution of fibrillar collagen in chordates, with implications for the evolution of vertebrate skeletons and chordate phylogeny. *Evol. Dev.* **8,** 370–377.

Wagner, T., Wirth, J., Meyer, J., Zabel, B., Held, M., Zimmer, J., Pasantes, J., Bricarelli, F. D., Keutel, J., Hustert, E., Wolf, U., and Tommerup, N. (1994).

Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell* **79,** 1111–1120.

Wang, Y., Belflower, R. M., Dong, Y. F., Schwarz, E. M., O'Keefe, R. J., and Drissi, H. (2005). Runx1/AML1/Cbfa2 mediates onset of mesenchymal cell differentiation toward chondrogenesis. *J. Bone Miner. Res.* **20,** 1624–1636.

Welsch, U., Erlinger, R., and Potter, I. C. (1991). Proteoglycans in the notochord sheath of lampreys. *Acta Histochem.* **91,** 59–65.

Wong, M., and Tuan, R. S. (1995). Interactive cellular modulation of chondrogenic differentiation *in vitro* by subpopulations of chick embryonic calvarial cells. *Dev. Biol.* **167,** 130–147.

Wright, G. M., Keeley, F. W., Youson, J. H., and Babineau, D. L. (1984). Cartilage in the Atlantic hagfish, *Myxine glutinosa*. *Am. J. Anat.* **169,** 407–424.

Wright, E., Hargrave, M. R., Christiansen, J., Cooper, L., Kun, J., Evans, T., Gangadharan, U., Greenfield, A., and Koopman, P. (1995). The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos. *Nat. Genet.* **9,** 15–20.

Wright, G. M., Keeley, F. W., and Robson, P. (2001). The unusual cartilaginous tissues of jawless craniates, cephalochordates and invertebrates. *Cell Tissue Res.* **304,** 165–174.

Yamashiro, T., Wang, X. P., Li, Z., Oya, S., Aberg, T., Fukunaga, T., Kamioka, H., Speck, N. A., Takano-Yamamoto, T., and Thesleff, I. (2004). Possible roles of Runx1 and Sox9 in incipient intramembranous ossification. *J. Bone Miner. Res.* **19,** 1671–1677.

Yang, X., and Karsenty, G. (2002). Transcription factors in bone: Developmental and pathological aspects. *Trends Mol. Med.* **8,** 340–345.

Yoshida, C. A., Yamamoto, H., Fujita, T., Furuichi, T., Ito, K., Inoue, K., Yamana, K., Zanma, A., Takada, K., Ito, Y., and Komori, T. (2004). Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. *Genes Dev.* **18,** 952–963.

Zangerl, R. (1966). A new shark in the family Edestidae, *Ornithoprion hertwigi* from the Pennsylvania Mecca and Logan Quarry Shales of Indiana. *Fieldiana Geol.* **16,** 1–43.

Zelzer, E., and Olsen, B. R. (2003). The genetic basis for skeletal diseases. *Nature* **423,** 343–348.

Zhang, G., and Cohn, M. J. (2006). Hagfish and lancelet fibrillar collagens reveal that type II collagen-based cartilage evolved in stem vertebrates. *Proc. Natl. Acad. Sci. USA* **103,** 16829–16833.

Zhang, G., and Cohn, M. J. (2008). Genome duplication and the origin of the vertebrate skeleton. *Curr. Opin. Genet. Dev.* **18,** 387–393.

Zhang, D., Schwarz, E. M., Rosier, R. N., Zuscik, M. J., Puzas, J. E., and O'Keefe, R. J. (2003a). ALK2 functions as a BMP type I receptor and induces Indian hedgehog in chondrocytes during skeletal development. *J. Bone Miner. Res.* **18,** 1593–1604.

Zhang, P., Jimenez, S. A., and Stokes, D. G. (2003b). Regulation of human COL9A1 gene expression. Activation of the proximal promoter region by SOX9. *J. Biol. Chem.* **278,** 117–123.

Zhang, G., Miyamoto, M. M., and Cohn, M. J. (2006). Lamprey type II collagen and Sox9 reveal an ancient origin of the vertebrate collagenous skeleton. *Proc. Natl. Acad. Sci. USA* **103,** 3180–3185.

Zhang, X., Boot-Handford, R. P., Huxley-Jones, J., Forse, L. N., Mould, A. P., Robertson, D. L., Lili, M., Athiyal, M., and Sarras, M. P., Jr. (2007). The collagens of hydra provide insight into the evolution of metazoan extracellular matrices. *J. Biol. Chem.* **282,** 6792–6802.

Zhou, G., Lefebvre, V., Zhang, Z., Eberspaecher, H., and de Crombrugghe, B. (1998). Three high mobility group-like sequences within a 48-base pair enhancer of the Col2a1 gene are required for cartilage-specific expression *in vivo*. *J. Biol. Chem.* **273,** 14989–14997.

Zhou, G., Zheng, Q., Engin, F., Munivez, E., Chen, Y., Sebald, E., Krakow, D., and Lee, B. (2006). Dominance of SOX9 function over RUNX2 during skeletogenesis. *Proc. Natl. Acad. Sci. USA* **103,** 19004–19009.

Zou, H., Wieser, R., Massague, J., and Niswander, L. (1997). Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev.* **11,** 2191–2203.