EVOLUTION OF VERTEBRATE CARTILAGE DEVELOPMENT

By

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The phylogenetic relationships of the vertebrates were established largely based on anatomical characters, particularly those of the skeleton. Skeletal development has been well studied in higher vertebrates (e.g., mouse and chicken), however, little is known about the developmental mechanisms responsible for evolutionary origin of the vertebrate skeleton. Here I investigate cartilage development in two jawless vertebrates, hagfishes and lampreys, and in a sister group to the vertebrates, lancelets (amphioxus). I show that both lampreys and hagfishes have type II collagen based cartilage, suggesting that this type of cartilage was present in common ancestor of all crown-group vertebrates. My analysis of lancelets revealed the presence of an ancestral clade A fibrillar collagen (*ColA*) gene that is expressed in the notochord. The results suggest that, during the chordate-vertebrate transition, an ancestral Clade A fibrillar collagen gene underwent duplication and diversification, and this process may underlie the evolutionary origin of vertebrate skeletal tissues.

By comparing the axial skeletal developmental genetic program in lamprey and catshark, I found that the network of sclerotomal genes is conserved in mediovental part of somites. These results suggested that lamprey possesses a sclerotome. The conservation of this sclerotomal gene network, the later chondrogenetic program, and the structure of lamprey arcualia and

gnathostome vertebrae suggest that the arcualia develop from sclerotome. Phylogenetic analysis showed all of the axial skeletal genes examined here arose from gene duplication events. I propose that the sclerotome, like other vertebrate novelties may have resulted from the same gene duplication events. These duplications allowed differentiation of developmental control genes, which facilitated both subfunctionalization and acquisition of new expression domains.

CHAPTER 1
GENERAL INTRODUCTION

Vertebrate Cartilage Structures

Cartilage as a skeletal tissue type is found mainly in the endoskeleton system. Cartilage is diversely distributed across animal taxa, suggesting it may potentially have a long evolutionary history (Hall, 2005). Although cartilage has some unique characters compared with bone, such as its low metabolic rate and avascular structure, cartilage is defined by its components. Person and Mathews' definition includes three criteria of true cartilage; chondrocytes suspended in rigid matrix; high content of collagen and acidic polysaccharides (Person and Mathews, 1967). More recently, this definition was modified by replacing collagen with fibrous protein, in order to accommodate the non-collagenous proteins found in lamprey and hagfish (Cole and Hall, 2004a).

Tetrapods

Mammalian cartilage has been studied extensively. In terapods, there are three major kinds of cartilages according the physical characters and matrix components. Hyaline cartilage is the most widespread type in mammals. Hyaline cartilage is named by its semi-transparent and bluish-white color appearances. It forms the scaffold for the endochondral bones in early embryos. During later development, most cartilages are replaced by bone during development, although some may remain as cartilage throughout the whole lifespan. In the adults, hyaline cartilages are found mostly in epiphyses, which cap the long bones at their proximal and distal ends. Other less common types of cartilage are fibrocartilage and elastic cartilage, which contain numerous thick bundles of collagen fibers and elastic fibers, respectively (Hall, 2005). Similar with hyaline cartilage, elastic cartilage is stiff but is also more elastic than hyaline cartilage since its matrix contains more elastin protein fibrils. Elastic cartilages usually are distributed in the

pinna of the ear, larynx and epiglottis (Naumann et al., 2002). The third type of cartilage is fibrocartilage, which contains type I collagen in its matrix. The type I collagen makes the fibrocartilage more tensile and tough. Fibrocartilage is usually found in the intervertebral disks and in the joints with tendons or ligaments attached 1(Benjamin and Evans, 1990; Benjamin and Ralphs, 2004; Eyre and Wu, 1983). I wish to note that this cartilage classification system does not necessarily include all the cartilages in the vertebrate lineage.

Teleosts

Whereas in mammals three major cartilage types are found, teleosts have many more diverse types of cartilages. Most of the histological work on teleost cartilage types comes from one laboratory, and Benjamin and co-workers divided teleost cartilages into at least eight types, most of which are absent in mammals (1989, 1990). Hyaline-cell cartilage is composed of compact chromophobic chondrocytes and hyaline cytoplasm with little matrix. It was found in the lips, rostral folds and other cranial cartilages (Benjamin, 1989). Hyaline-cell cartilage can be further classified into the following sub-types: fibrohyaline-cell cartilage with greater quantities of collagen; elastic hyaline-cell cartilage with more elastin matrix; and lipohyaline-cell cartilage which contains adipose cells as well as chondrocytes (Benjamin, 1990). Zellknorpel chondrocytes are more chromophilic than those of hyaline-cell cartilage and are shrunken within the large lacunae. Zellknorpel is usually found in the gill filaments, basal plate, etc (Benjamin, 1990). Elastic/cell-rich cartilage is a kind of cartilage with highly cellular elastic fibers, and the cells are not hyaline. This type of cartilage can be distinguished from Zellknorpel and hyaline-cell cartilage by elastic staining. Elastic/cell-rich cartilage is usually found at the barbels and maxillary oral valves, and is surrounded by a thick fibrous perichondrium (Benjamin, 1990). Cell-rich hyaline cartilage is a hyaline-like cartilage with more cells and lacunae that occupy more than half of the total volume. Parts of neurocranium and Meckel's cartilage belong in this

category (Benjamin, 1990). Matrix-rich hyaline cartilage is the typical mammalian hyaline cartilage type. It is common in gill arches and part of neurocranium (Benjamin, 1990). Scleral cartilage is a special cartilage, which is located in the sclera. Although a COL2A1 antibody can label most of the fish matrix-rich cartilages, cell-rich cartilages is not stained (Benjamin, 1991).

Chondrichthyans

The skeletons of chondrichthyans are entirely cartilaginous, although it was reported that shark may retain some bone, and primitive chondrichthyans possessed both exoskeleton endochondral bones (Coates et al., 1998; Kemp and Westrin, 1979; Moss, 1970; Moss, 1977; Peignoux-Deville et al., 1982). One obvious character of shark cartilage is that it can be calcified, and biochemical studies showed that shark and skate cartilage contains type I collagen in addition to type II collagen (Mizuta et al., 2003; Moss, 1977; Rama and Chandrakasan, 1984).

Agnathans

Cartilage structures are also present in the only two extant jawless fishes, lamprey and hagfish. In the head of larval lamprey, the mucocartilage occurs as a kind of temporal cartilage without blood vessel invasion and is surrounded by perichondrium (Hall, 2005). During metamorphosis, the mucocartilage is transformed into pistal and tongue cartilages (Hall, 2005). In the19th century, two kinds of cartilages were identified, soft and hard in adult lampreys. The hard cartilage is kind of like mammalian hyaline cartilage (Parker, 1883). The lamprey cartilages are mainly found in the cranial region except the axial cartilage nodes named arcualia and caudal fin rays (Morrison et al., 2000). Similar with lamprey, hagfish cartilage is also mainly present in the cranium and median fin rays. Cole described two types of cartilages in hagfishes; “soft” cartilage contains 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 (Cole, 1905).

Biochemical analysis also supported the two types of hagfish cartilages, which were named Type I and Type II cartilage, with Type II being more similar to adult lamprey cartilage (Wright et al., 1984). Although they have similar morphological structures, adult lamprey and hagfish cartilages were reported to lack collagen. Instead, lamprey and hagfish cartilage was argued to be composed of elastin-like molecules which were named lamprin and myxinin, respectively (Wright et al., 2001). Like chondrichthyans, lamprey cartilage can be calcified in vitro (Langille and Hall, 1988). Calcified cartilage also was reported in the fossil agnathan *Euphanerops* (Janvier and Arsenault, 2002).

Invertebrates

True cartilage is usually assumed to be found only in the vertebrate lineage, although cartilage-like tissues (chondoid or chondroid) were also identified in invertebrates (Wright, 2001 and Cole, 2004). Morphologically, some invertebrate cartilages are indistinguishable from vertebrate cartilage. Thus far, cartilage tissues were identified in Cnidaria (*Metridium*); Molluska (*Sepia*), Arthropoda (*Limulus*), Brachiopoda (*Terebratulina*), Polychaeta (*Potamilla*), Hemichordata (Saccoglossus), Urochordata (Styella), and Cephalochordata (*Branchiostoma*) (Cole and Hall, 2004a; Cole and Hall, 2004b; Wright et al., 2001). In general, there are three kinds of cartilages found in invertebrates; central cell-rich cartilage; vesicular cartilage with large vesicles or vacuoles; and acelluar cartilage. The cartilage-like tissues cross reacted with vertebrate type II and X collagen and proteoglycan antibodies (Cole and Hall, 2004a; Cole and Hall, 2004b). It was also reported that squid cartilage collagens were type V like instead of type II, although the details of invertebrate collagen types were not known (Sivakumar 1998 and Bairati 1999). Acelluar cartilage may have evolved on several independent occasions, since it occurs in different lineages in metazoa (Junqueira et al., 1983). Acelluar cartilage was also found in lancelets, however lancelet cartilage was thought to be non-collagenous due to its

incomplete digestion by cyanogen bromide, a chemical that digests collagen protein (Wright et al., 2001). In general, cartilages show extensive variation across different taxa, and I summarize the major chordate cartilage structures in the Figure 1-1.

Compositions of Vertebrate Cartilage Matrix

Most of the supportive tissues of vertebrates are formed from extracellular fibers and matrix, both of which are produced by the cells of various connective tissues. Up to 90% of the dry weight of cartilage is extracellular matrix, indicating that matrix proteins are particularly rich in cartilage (Hardingham and Fosang, 1992). In jawed vertebrates, cartilage extracellular matrix is composed of mucopolysaccharides (proteoglycans) deposited on collagen fibers, mainly COL2A1 and the less abundant "minor" collagens IX and XI (Bruckner and van der Rest, 1994). A diagram of cartilage matrix composition is illustrated in Figure 1-2, which is adapted from Knudson and Knudson (Knudson and Knudson, 2001).

Collagens

Collagens are the main components of animal extracellular matrix (Expositio JY, 2002). Thus far, 27 t collagen genes have been identified (Pace et al., 2003). The collagens are divisible into two major groups, fibril and non-fibril collagens. The fibril collagens are further divisile into clade A, B and C (Aouacheria, A. et al. 2004). Clade A fibrillar collagens are the major fibril-forming collagen, including type, I, II, III and V (Aouacheria, A. et al. 2004). Clade A fibril procollagens are made of N-propeptide, N-telopeptide, triple helix, C-telopeptide, C-propetide from N to C terminus. Triple helix is the collagenous domain consisting of Gly-Xaa-Yaa triplet repeat. X and Y usually are proline and hydroproline. The propeptide will be removed during the maturation of collagen through posttranscriptional process by N- and C-proteinase (Ladler, KE, 1996; Expositio JY, 2002). Type II collagen is encoded by Col2a1 gene and was found to the major matrix protein in cartilage at the end of 1960s (Miller 1969). Each

type II collagen fibril is made of three identical chains, conferring tensile strength and providing a scaffolding network for proteoglycans (van der Rest and Garrone, 1991). As minor collagens in cartilage, type IX and XI collagens belong to the clade B fibrillar collagen. They participate in the process of fibril formation (Eyre et al., 2004; Kadler et al., 1996; Li et al., 1995). The collagen types and quantities are different in different types of cartilage. The fibrocartilage contains certain amount of type I collagen (Benjamin and Evans, 1990). During the long bone development, there is also a collagen type transition. In the proliferative cartilage, the major matrix is type II collagen, the hypertrophic cartilage major collagen type is type X collagen, and type I collagen is the major matrix of bone (Olsen et al., 2000).

Proteoglycans

Proteoglycans are the second-most abundant proteins after the fibrillar collagens in cartilage matrix. Chondroitin sulfate has long been known to be the predominant glycosaminoglycan in cartilage, and aggrecan was found to be the most abundant proteoglycan in cartilage (Doege et al., 1991). Deposition of aggrecan is thought a hallmark of chondrogenesis, although it is also present in aorta, disks and tendons (Schwartz et al., 1999). Aggrecan not only contributes to the physical characters of cartilage, it also protects cartilage collagen from degradation (Pratta et al., 2003). In addition to aggrecan, there are many small leucine-rich proteoglycans in cartilage, including biglycan, decorin, fibromodulin, lumican, epiphycan and perlecan. These small leucine-rich proteoglycans have a variety of functions in cartilage development and maintenance (Iozzo, 1998; Knudson and Knudson, 2001). Chondrocytes also express cell surface proteoglycans: syndecans and glypican which may have the potential to interact with FGF and TGF-β signals in cell–cell and cell–matrix interactions (Iozzo, 1998; Song et al., 2007). In addition to the proteoglycans and collagen proteins, elastic cartilage contains

elastin proteins. Recently, more minor proteins were identified in human articular cartilage by proteomic analyses, especially the posttranscriptional modification proteins. (Belluoccio et al., 2006; Lammi et al., 2006).

Evolutionary History of Cartilage

As I described above, the cartilage structures are widely distributed in metazoan taxa and show great diversity. The dominance of cartilage in vertebrate skeletons has led to the idea that cartilage is vertebrate-specific character. The existence of invertebrate cartilage suggested this specific tissue appeared before vertebrates and has a more ancient evolutionary history (Cole and Hall, 2004a). It has long been debated whether bone or cartilage occurred first, although the earliest vertebrate skeletons are believed to be unmineralized cartilaginous splanchnic noncranial endoskeletons (Donoghue and Sansom, 2002; Donoghue et al., 2006). Obviously, cartilage has a longer history than bone if we take into consideration of invertebrate cartilage, whereas bone and dental skeletons are limited only to the vertebrate lineage.

In higher vertebrates, there are some intermediate skeletal types (Hall, 2005). Chondroid tissues are cartilage-like and possess both characters of bone and cartilage (Cole and Hall, 2004a). Squid cartilage is very similar to mammalian hyaline cartilage morphologically, but there are some desmosomes and other direct cell-cell connections in squid cartilage cells, while such cell-cell connections are not found among vertebrate chondrocytes (although they are in osteocytes) (Bairati et al., 1999). These combined characters suggest an ancestral state of vertebrate skeleton, and raise the possibility that the vertebrate skeleton may have evolved from invertebrate cartilage.

In their comprehensive examination of invertebrate cartilage distributions, Cole and Hall proposed that all the metazoan animals may potentially have the ability to make cartilage-like tissues, and some taxa may have lost this ability during evolution (Cole and Hall, 2004a).

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. Vesicular cartilage was proposed first by Benjamin as a special cell-rich cartilage with large vesicles or vacuoles with chondrocytes (Benjamin, 1990; Cole and Hall, 2004b). Polychaetes, horseshoe crabs and mollusks have been reported to possess vesicular cartilage. To some degree, the vertebrate notochord is a kind of vesicular cell-rich cartilage, since notochordal cells are vacuolated and surrounded by cartilage spectrum of extracellular matrix incuding 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). Interestingly, the notochord is usually thought to be an epithelial structure instead of cartilaginous.

It has been proposed that vertebrate cartilage may has evolved from the notochord based on the evidence that matrix similarity and notochord predate the vertebrate cartilage (Stemple, 2005). Acellular cartilage is another interesting type of cartilage with no chondrocytes. It is has been found in hemichordates, cephalochordates and vertebrates (stingray) (Cole and Hall, 2004b; Rychel et al., 2006). Epithelial cells may secrete the matrix since there are no chondrocytes. Rychel has proposed that ectodermal acellular cartilage is an ancestral model in making pharyngeal cartilage in deuterostomes (Rychel et al., 2006; Rychel and Swalla, 2007). Cell-rich, matrix-rich or acellular cartilage may have evolved independently or from a single type of connective tissue. The sequence of occurrence of these four cartilage types in a phylogenetic context is important for understanding the origin and evolution of cartilage. The detailed distribution of cartilage in metazoa is illustrated in Figure 1-3. It seems that vertebrate cartilage may have two possible origins; notochord and/or ectoderm.

Cartilage is a kind of connective tissue. Connective tissues are widely distributed in metazoan animals. At the most basic level, there are two groups of extracellular molecules, ground substances (glycosaminoglycans, proteoglycans) and fibrous proteins (collagen fibrils) in all kinds of connective tissues. In histological sections, the vertebrate connective tissues can be distinguished by relative quantities of ground and fibril molecules and orientation of fibrils in addition cell types (Cole and Hall, 2004a). Like the hyaline cartilage matrix is dominated by type II collage fibril, while the fibrocartilage contains more type I collagen. The elastic cartilage contains elastin fibrils in addition to type II collagen fibril. The hypertrophic cartilage mainly contains type X collagen. Bone and tendon contain mainly type I collagen, but the former is also composed of osteonectin, osteopotin and other unique proteins. Although none of the collagen types are expressed exclusively in certain connective tissues, they are still widely used as molecular indicators. Due to the uniqueness of extracellular matrix types, the evolutionary history of each connective tissue will be well reflected by the natural history matrix molecules.

The current view on the evolution of vertebrate cartilage matrix contrasts with the idea of an invertebrate origin. The gnathostomes, from sharks to mammals, express the same major matrix, COL2A1, in their chondrocytes (Wright et al., 2001). The agnathans, hagfishes and lampreys have been reported to possess the non-collagenous cartilage. Their cartilage matrix was said to be composed by elastin-like molecules, named lamprin and myxinin respectively. The lancelets were thought to be non-collagenous due to the insolubility of their cartilage in cyanogen bromide. These differences in matrix structure suggested that the ancestor of vertebrates is non-collagenous and that agnathans and gnathostomes evolved their cartilages independently (Cole and Hall, 2004a; Wright et al., 2001). The similar non-collagenous biochemical matrixes of their skeleton provide some unifying characters, since putative

homologies between the endoskeleton elements remain dubious and made it difficult to ascertain the gross structure of vertebrate skeletons (Donoghue and Sansom, 2002; Donoghue et al., 2006). The controversy of agnathan and gnathostome cartilage matrix composition raised the possibility that vertebrate cartilage has a dual origin, either non-collagen-based or collagen-based. Although it is difficult to sort out the vertebrate cartilage matrix origins, there is still some evidence that squid and cuttlefish cartilage contain some collagen, although they are different than type II collagen (Bairati et al., 1999; Bairati and Gioria, 2004; Kimura and Karasawa, 1985; Kimura and Matsuura, 1974). In addition, horseshoe crab cartilage was shown to contain chondroitin sulphate (Sugahara et al., 1996). The cartilage matrix genes in invertebrates suggested that the chondrogenetic genetic program might be deeply conserved. So, further investigation of agnathan cartilage matrix will shed light on the origin of vertebrate cartilage.

Vertebrate Skeletal Development

Embryonic Origins and Mesenchymal Condensation

Vertebrate skeletons mainly came from different three embryonic lineages. The cranial neural crest cells contribute most of the head skeleton, which is also derived form cranial mesoderm and the first five somites (Couly et al., 1993); axial skeletons are made from paraxial mesoderm; appendicular skeletons are formed by lateral plate mesoderm. However, not all the vertebrate skeletons are composed by these three lineages. Especially in teleosts, and early fossil vertebrates, even in some tetrapod, like armadillo, the dermal skeletons are derived from dermomyotome or ectoderm (Vickaryous and Hall, 2006). No matter what the cell lineages, mesenchymal cells undergo condensation as a characteristic early step towards skeletogenesis.

Skeletogenesis occurs in four steps; cells migrate to the right sites, epithelial-mesenchymal tissue interactions occur, mesenchymal condensations form and cells differentiate into chondroblasts and osteoblasts (Hall and Miyake, 2000). Cells committed to skeletal fate

have no obvious morphological characters prior to condensation, when the cells begin to aggregate together, reduce their sizes and appear rounded with higher density once condensation begins (Wezeman, 1998). By regulating proliferative cell rate through growth factor signalling, extracellular matrix is necessary for the condensation (Streuli et al., 1993). It was reported Type I collagen is expressed in these mesenchymal cells, but is downregulated once condensation begins. At this stage, type II collagen became the major fibrillar protein and type I collagen persists only in the perichondrium (Dessau et al., 1980; Wezeman, 1998). Mesenchymal condensation is also characterized by cell surface adhesion molecules, like *N-cadherin*, N-CAM, *Hyaladherin, Versican, Tenascin*, *Sydecan, Heparin Sulphate* and *Chondroitin Sulphate Proteoglycans* (Hall and Miyake, 2000). The sequence of matrix molecule expression is well summarized by Shum at al (Shum et al., 2003). These adhesive molecules have important functions in addition to mediating the condensation process. For example, *N-Cadherin* has been shown to recruit cells into condensations and disruption of *N-cadherin* led to condensation inhibition (DeLise and Tuan, 2002; Haas and Tuan, 1999). *Syndecan-3* and *Tenascin-C* were shown to set boundaries and are involved in periosteum development (Koyama et al., 1995). Most of these adhesive markers are downregulated at the commencement of overt differentiation (Wezeman, 1998).

Skeletal Cell Lineage Determination

Vertebrate cartilage and bone are primarily composed of three cell lineages, chondrocytes, osteoblasts and osteoclasts. The former two cell types are derived from the common mesenchymal progenitor cell condensation, whereas the osteoclasts are of blood-borne hematopoietic origin. After condensation, mesenchymal cells start to differentate into chondrocytes. These chondrocytes may remain as cartilage throughout life, or the cartilage templates may undergo hypertrophy and eventually be replaced by bone. This process is called

endochondral ossification. Alternatively, the mesenchymal cells may form bone directly in a process named intramembranous ossification, which is usually seen in cranial skeletons. In both ossification types -- intramembranous and endochondral -- osteoblasts aggregate as mesenchymal condensations. (Karsenty and Wagner, 2002; Yang and Karsenty, 2002; Zelzer and Olsen, 2003).

Several lines of evidence have suggested that the condensed mesenchymal cells have both chondrogenic and osteogenic potential, since they express both the chondrogenesis and osteogenesis master genes, *Sox9* and *Runx2* (Bi et al., 1999; Ducy et al., 1997; Otto et al., 1997; Yamashiro et al., 2004). Moreover, cultured embryonic calvarial 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 block the cartilage differentiation, but this also leads to ectopic expression of osteoblast specific genes such as *Runx2*, *Osterix*, and *Col1a1*(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 intramembrane bones (Nakashima et al., 2002). These data support the idea that the common mesenchymal progenitors have three possible differentiation fates, chondrogenesis, intramembranous ossification or endochondral ossification.

The mesenchymal cells also can take other cell fates like adipose tissues (Karsenty, 2003; Karsenty and Wagner, 2002). Only recently have the molecular mechanisms underlying these different pathways become clear. The canonical WNT pathway was shown to be a key regulator for this mesenchymal cell lineage determination. *Wnt* genes are the vertebrate orthologs of fruit fly's *wingless* gene, and there are at least 19 *Wnt* genes in humans (Logan and Nusse, 2004; Miller, 2002). This group of secreted molecules is highly conserved in metazoan animals from

cnidarians to humans, and play major roles in normal development and tumorigenesis (Kusserow et al., 2005; Lee et al., 2006; Logan and Nusse, 2004; Prud'homme et al., 2002). WNT proteins bind to their receptor, Frizzed, and transduce the input into the cell with the co-receptor, named LDL Receptor related Protein 5/6 (LRP5/6). There are at least three intracellular pathways, the canonical pathway, which is mediated by ß-Catenin, Ca-PKC pathway and planar cell polarity pathway (Miller, 2002). Canonical Wnt signals previously were shown to be involved in the 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). Recently several critical lines of evidence revealed that the WNT canonical pathway regulates cell fate determination through a cell-autonomous mechanism to induce osteoblast differentiation and repress chondrocyte differentiation (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 in early mesenchyme (skeletal progenitors) using Prx-Cre mouse line, osteoblast differentiation was arrested and no cortical and intramembrane bones were formed, however this could be rescued by IHH and BMP2. In the mutants, periosteal cells turned into chondrocytes instead of osteoblasts, and some ectopic cartilages were found in the membranous bone region (Hill et al., 2005). Similar phenotypes were found when ß-Catenin was deleted from skeletal primordium (mesenchymal condensation) using *Dermo1*-Cre and *Col2a1*-Cre mouse lines (Day et al., 2005). The ectopic chondrocytes formed at the expense of osteoblasts (Day et al., 2005; Hu et al., 2005). Moreover, micromass cell culture experiments showed that ß-Catenin levels can control the *Sox9* and *Runx2* expression in vitro (Day et al., 2005). Collectively, ß-Catenin controls early osteo-chondroprogenitor differentiation into chondrocytic or osteoblastic lineages. High level of ß-Catenin lead to

osteogenic differentiation and low level lead to chondrogenic differentiation (Day et al., 2005; Hill et al., 2005). The process is summarized in the Figure 1-4. This mechanism is not only important developmentally to designate skeletal types, but also is important for skeletal evolution. It seems that vertebrate skeletal variation can be achieved through tinkering with the temporal and spatial expression of canonical WNT signals.

Both *Sox9* and *Runx2* are expressed in mesenchymal osteo-chondrogenic progenitors; removal of *Sox9* will abolish bone and cartilage formation. This suggested that *Sox9* is required for their overt skeletal differentiation (Akiyama et al., 2005). Very recently, *Sox9* was found to be dominant to *Runx2* (Zhou et al., 2006), this finding leads us to assume that skeletal progenitor cells will automatically differentiate into cartilage if there are no other transcriptional factors to upregulate *Runx2*. In chicken embryos, it was shown that high levels of *Sox9* and *Runx2* commit the cells to chondrogenesis and osteogenesis, respectively (Eames et al., 2004). The cross talk between *Sox9* and *ß-Catenin* was proven recently. *Sox9* can inhibit ß-Catenin-dependent promoter activation through the interaction between HMG-box and Armadillo repeats. *Sox9* also promotes degradation of ß-Catenin by ubiquitation or the proteasome pathway (Akiyama et al., 2004). Naturally, we expect more evidence to ensure about the interactions between ß-Catenin and RUNX2. Although *Wnt* and *Sox9* were clearly shown to play major roles in the cell fate determination process, it does not exclude other possibilities that may be found in the future.

Chondrogenesis

Embryonic cartilage has at least two potential fates. A part of cartilage remains permanent cartilage, like the articular cartilage. Most cartilages eventually are replaced by bone through endochondral ossification process. Although there are three types of cartilage, as mentioned at the beginning of this dissertation, only hyaline cartilage was well characterized, especially in limb development. Chondrocytes are not simply a single cell population, but as suggested by

Karsenty and Wagner, there are three subpopulation cells; non-hypertrophic chondrocytes, hypertrophic chondrocytes and articular chondrocytes, and each cell type has a unique morphology, gene expression profile and appears sequentially at certain developmental stages (Karsenty and Wagner, 2002).

Non-hypertrophic chondrocyte differentiation and proliferation

After overt differentiation, the cells in mesenchymal condensation then develop into chondrocytes, which is marked by Type II collagen and aggrecan as major matrix proteins and type IX and XI collagens as minor matrix proteins. The master transcription factor that regulates this process is Sox9, one of the vertebrate SoxE family members, which contains a high-mobility-group (HMG)-box DNA binding domain. *Sox9* was the earliest marker of chondrogenesis as it starts to express during the mesenchymal condensation (Healy et al., 1996; Wright et al., 1995). *Sox9* is required for cartilage-specific extracellular matrix component such as COL2A1, COL9, COL11 and AGGRECAN, and was proved to be the direct regulator of Col2a1 and aggrecan in mice and its major roles was chondrocyte differentiation (Lefebvre and de Crombrugghe, 1998; Lefebvre et al., 1997; Liu et al., 2000; Ng et al., 1997; Zhang et al., 2003; Zhou et al., 1998). Haploinsufficiency of *Sox9* in human and mice will cause campomelic dysplasia (CD) (Foster et al., 1994; Wagner et al., 1994). *Sox9* homozygous knockout mice died prior to birth and *Sox9* heterozygous mutants exhibited severe cartilage hypoplasia. The cartilage matrix maker genes were downregulated in the mutants too. Furthermore, the *Sox9* null embryonic stem cells could not form cartilage in teratomas (Bi et al., 1999; Bi et al., 2001). Conversely, ectopically expressed *Sox9* in chick using RCAS retroviral vector induced cartilage formation in dermomyotome (Healy et al., 1999). Detailed analysis revealed that *Sox9* did not work by itself, and that its normal function during overt chondrocyte differentiation requires two important cofactors, *Sox5* and *Sox6*, both members of the *SoxD* group (Lefebvre et al., 1998).

These two *SoxD* genes modulate the function of *Sox9* (Stolt et al., 2006). Collectively, the Sox trio (*Sox9, Sox5* and *Sox6*) is a very important regulator for chondrocyte differentiation (Ikeda et al., 2004; Lefebvre et al., 2001; Lefebvre and de Crombrugghe, 1998; Lefebvre et al., 1998; Smits and Lefebvre, 2003; Stolt et al., 2006). The targets of *Sox* trio was unclear until recently, when two targets, *Sox100A1* and *Sox100B* were discovered (Saito et al., 2007). In addition to *Sox5* and *Sox6*, there are some other *Sox9* cofactors, like *CBP/P300, TRAP230* and *PGC-1a*, which are important for the functions of *Sox9* (Kawakami et al., 2005; Liu et al., 2007; Rau et al., 2006; Zhou et al., 2002). Given the importance of *Sox9*, its temporal and spatial regulation is very important. Unfortunately, few genes have been identified that regulate *Sox9*, although there are some genome-wide analyses targeted this direction (Bagheri-Fam et al., 2006; Bagheri-Fam et al., 2001). BMP, TGF-ß and ß-Catenin are implicated in this process (Akiyama et al., 2004; Bagheri-Fam et al., 2006; Chimal-Monroy et al., 2003; Yoon et al., 2005; Zou et al., 1997).

Chondrocyte proliferation and hypertrophy

Cartilage anlagens have two potential pathways for differentiation; they can remain as permanent cartilage, in which case the cells never express IHH and BMP6, or they can undergo endochondral ossification (Eames and Helms, 2004). During the endochondral ossification, the specified chondrocytes in the center of skeletal anlagen proliferate and develop successively into prehypertrophic, hypertrophic and terminal hypertrophic chondrocytes. The chondrocytes were surrounded by perichondrium, which contains flattened fibroblastic cells. The perichondrium is also influenced by hypertrophy of the underlying chondrocytes, and the fibroblastic cells will differentiate into osteoblasts, which are involved in bone collar formation (Olsen et al., 2000; Provot and Schipani, 2005). The prehypertrophic and hypertrophic cell subpopulations are marked by Ihh and BMP6, or type X collagen and VEGF, respectively. Interestingly, the terminal hypertrophic chondrocytes do express bone markers, like *Bsp, Osteocalcin, Osteonectin,*

Osteopotin, and *AlkalinePhosphatase* (Eames and Helms, 2004; Goldring et al., 2006; Karsenty and Wagner, 2002).

Runx2 is a *Runx* domain containing gene that is also named *PEBP2A* (polyoma enhancer binding protein), *Osf2* (osteoblast specific factor 2), *AML3* (acute myelogenous leukemia 3) and *Bcfa1* (core-binding factor) (van Wijnen et al., 2004). It was shown to control chondrocyte maturation in addition to osteoblast differentiation (Ducy et al., 1999; Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997). Cleidocranial dysplasia (CCD) is a rare skeletal dysplasia characterized by short stature, distinctive facial features and narrow, sloping shoulders caused by defective or absent collarbones (clavicles). Haploinsufficiency of *Runx2* causes CCD in human and mice (Mundlos et al., 1996; Mundlos and Olsen, 1997a; Mundlos and Olsen, 1997b), In *Runx2* null mice, the entire skeleton is cartilaginous due to the maturational arrest of osteoblasts, and there is a complete loss of chondrocyte hypertrophy in most of these skeletons (Inada et al., 1999; Kim et al., 1999; Takeda et al., 2001). More interestingly, either *Runx2* was conditionally over-expressed or knocked out from chondrocytes using transgenic mice, the chondrocytes maturation rates were changed (Ueta et al., 2001). There are three closely related *Run*x genes in mouse, *Runx1*, *Runx2* and *Runx3*, and all the three genes are expressed in chondrocytes, suggesting that *Runx1* and *Runx3* are also involved in the hypertrophy and maturation (Levanon et al., 2001; Lian et al., 2003; Smith et al., 2005; Stricker et al., 2002; Wang et al., 2005). In fact, this was proved by *Runx1* and *Runx2* double knockout experiments, in which the chondrocyte hypertrophy was inhibited more completely (Yoshida et al., 2004). Collectively, these observations suggested that *Runx2* is an essential transcription factor for chondrocyte maturation, and that *Runx1* and *Runx3* are also important and may have distinct roles. In contrast to the function of *Runx2* in chondrocytes, *Runx2* in perichondrium was shown to inhibit

chondrocyte proliferation and hypertrophy during later stages of chondrogenesis (Hinoi et al., 2006). These results suggest that *Runx2* has complex functions during chondrogenesis. It will be very interesting to know the targets, cofactors and regulators of *Runx2*. One good example is histone deacetylase 4 (*HDAC4*), which regulate chondrocyte hypertrophy through its interaction with *Runx2* as a corepressor (Vega et al., 2004). *HDAC4* normally is expressed in the prehypertrophic zone. In the null mutant mice, ectopic and premature chondrocyte hypertrophy was observed. Over expression of *HDAC4* led the opposite phenotypes (Vega et al., 2004).

The chondrocyte proliferation and differentiation is tightly regulated by IHH and PTHrP negative loop (Karp et al., 2000; Lanske et al., 1996; St-Jacques et al., 1999; Vortkamp et al., 1996). The PTHrP is a peptide hormone that is secreted by the most distal perichondrium. Its G-protein coupled receptors are located at the prehypertrophic zone, which the cells proliferate. Normally, PTHrP prevents the chondrocytes from exiting the proliferative cell cycle, then inhibits hypertrophy (St-Jacques et al., 1999). Ectopic expression of PTHrP or its receptor severely inhibits cartilage maturation (Schipani et al., 1997; Weir et al., 1996). Moreover, mutations of PTHrP and its receptor cause Jansen's chondrodysplasia, in which the patients have reduced skeletal growth and abnormal metaphases (Schipani et al., 1995). The mutant mice had phenotypes similar to the human disease, and exhibit long bone dwarfism (Karaplis et al., 1994; Lanske et al., 1996). Ihh is a member of vertebrate hedgehog family genes, and localizes at the prehypertrophic zone, along with the PTHrP receptor. Mice without Ihh in their skeletons exhibited severe dwarfism due to proliferation reduction and osteoblast absence. This suggested that *Ihh* plays a major role in chondrocyte maturation (Vortkamp A, et al 1996). In the IHH mutants, PTHrP expression was lost (Razzaque et al., 2005; St-Jacques et al., 1999). Conversely, *Ihh* over-expression data showed that IHH can induce expression of PTHrP in distal

chondrocytes, thereby promoting the proliferation and antagonizing hypertrophy. As a result of the sustained proliferation and delayed maturation, *Ihh* expression is reduced. Collectively, the IHH-PTHrP feedback loop is necessary for proliferation and differentiation. Since *Runx2* and the IHH-PTHrP loop are both involved in the regulation of chondrocyte hypertrophy, it is not a surprise that *Runx2* and *Runx3* was shown to induce *Ihh* expression (St-Jacques et al., 1999; Yoshida et al., 2004). It is interesting that *Ihh* was also reported to inhibit *Runx2* expression through the PKA signaling pathway (Iwamoto et al., 2003; Li et al., 2004). Very recently, it was reported that *Bapx1* (*Nk3.2*) is a downstream target of IHH-PTHrP loop and, at least in part, mediates chondrocyte hypertrophy (Provot et al., 2006).

Fgfs and their receptors are also critical regulators of chondrocyte proliferation and differentiation. In humans and mice there are 22 *Fgf* genes and 4 FGF receptors (*Fgfr*) , many of which are involved in the skeletal genesis, 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, *Fgfr2* and *Fgfr3* are expressed in perichondrial cells and the proliferating zone, respectively. Each of the three receptors has a unique function. *Fgfr3* mutation will cause achondroplasia, hypochondroplasia and thanatophoric dysplasia in humans (Olsen et al., 2000). The chondrocyte column length was increased in *Fgfr3* null mice and this is caused by an increase in the proliferative rate (Colvin et al., 1996; Deng et al., 1996). This 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). Moreover, if FGFR3 signaling is increased, there will be more apoptosis and a reduction of chondrocyte proliferation

(Sahni et al., 1999). As with *Fgfr3*, conditional removal of *Fgfr1* using *Col2*-Cre and *Col1*-Cre in mice results in the hypertrophic chondrocyte zone being expanded (Jacob et al., 2006). This suggested Fgfr1 is also a negative regulator of proliferation. In contrast to *Fgfr1* and *Fgfr3*, *Fgfr2* and *Fgfr4* were positive regulators of skeletal development (Eswarakumar et al., 2002; Weinstein et al., 1998; Yu et al., 2003).

Another group of important chondrocyte regulators are the BMPs and their receptors, which play multiple roles in chondrocyte differentiation and proliferation (Zou et al., 1997). *Bmp7* is mainly found in the proliferating chondrocytes, whereas *BMP2, 3, 4* and *5* are primarily expressed in the perichondrium (Lyons et al., 1995; Minina et al., 2001). *Bmp2* and *Bmp6* are expressed in hypertrophic chondrocytes (Solloway et al., 1998). These distinctive expression patterns suggest that each of these Bmps has a unique function. Manipulating the BMP antagonist Noggin showed that BMP2 has anti-proliferation roles in the growth plate. In Noggin over-expressing experiment, the BMP signal was attenuated and chondrocyte proliferation was reduced (De Luca et al., 2001; Minina et al., 2001). Conversely, skeletons overgrew in the Noggin null mice (Brunet et al., 1998). Bmp also was proven to be able to increase Collagen X promoter activity (Volk et al., 1998), thus Bmps may also be involved in the chondrocyte differentiation. Bmp receptor IA was shown to work downstream of IHH and regulate chondrocyte differentiation, while Bmp receptor IB was related to cell death (Zou et al., 1997). Recently, there is further evidence that Bmps promote chondrocyte differentiation in vivo. Kobayashi *et. al.* investigated the roles of Bmp receptor IA during chondrocyte development by overexpressing it using *Col2a1*-Promoter driven and UAS-Gal binary systems. In the over-expression mice, long bones were shortened and, in the growth plate, the proliferating chondrocytes in the columnar layer were reduced (Kobayashi et al., 2005).

The BMP signal, FGF signal, IHH-PTHrP signals and the transcription factors Runx2, Runx3 and Sox trio do not function independently. In fact, they have to work as a network. There is increasing evidence that links the functions of these genes together. *Runx2* was upregulated and *Sox9* was downregulated in hypertrophic chondrocytes, suggesting that *Sox9* plays a negative role in hypertrophic maturation (Eames, BF 2003). Bmps can interact with IHH-PTHrP loop by promoting Ihh expression, and in turn, Ihh maintains Bmp expression level (Minina et al., 2001; Pathi et al., 1999). In general, Fgf pathways have opposite functions to Bmp pathways (Minina et al., 2002). Thus, the balance of BMP and FGF signals are important regulators of chondrocyte proliferation (Ornitz 2005).

Osteogenesis

As discussed above, mesenchymal cells are committed to the osteoblast when there are high levels of *β-Catenin* and *Runx2* expression. In tetrapods, these osteoblasts continue to mature and eventually become osteocytes regardless of ossification mode, be it intramembranous, perichondrial or endochondral. The osteoblasts do not always become osteocytes, especially in advanced teleosts (Franz-Odendaal et al., 2006). The last step of endochondral bone formation is blood vessel invasion, in which the osteoclasts are introduced. Here, I will mainly focus on endochondral bone formation since it was most extensively studied and is most relevant to the topic of this dissertation.

The roles of *ß -catenin* have been discussed as they relate to the skeletal cell lineage determination. Another master gene that controls the osteoblast is *Runx2*. *Runx2* is involved multiple roles in both chondrogenesis and osteogenesis. In the *Runx2* null mice, no intramembranous or endochondral ossification was observed, and the calvarial cells transformed into chondrocytes or adipocytes (Kobayashi et al., 2000; Komori et al., 1997; Otto et al., 1997).

Osterix (*Sp7*), the downstream gene of *Runx2*, is also important for osteoblast determination, since its null mutant phenotypes are similar to those of *Runx2* mutants (Nakashima et al., 2002). In ß-catenin conditional knockout mice, *Osterix* is absence although the *Runx2* was intact, suggesting *Osterix* is a target gene of ß-catenin. Collectively these three genes collaborate to determine the osteoblast lineage and inhibit chondrogenesis and adipogenesis.

IHH is required for perichondrial mesenchyme to differentiate into osteoblasts in the perichondrium. Instead, the perichondrial mesenchyme cells adopt chondrogenic fates in the absence of IHH (Long et al., 2004). In the *Ihh* mutants, *Runx2* was present in chondrocytes, but not in the perichondrium, indicating that *Ihh* works upstream of *Runx2* and is required for *Runx2* expression in the perichondrium. Similar to *Runx2*, ß-catenin was absent in the perichondrium in *Ihh* mutants (Hu et al., 2005). Taken together, spatial and temporal signaling by *Ihh* and *Wnt/ß-catenin* promote the osteogenesis and inhibit chondrogenesis through their interaction with *Runx2*, *Osterix* and *Sox9*.

Osteoblastic lineage determination

Unlike *Ihh*, both *Runx2* and *ß-Catenin* signaling are required for osteoblast differentiation after the osteogenic fate is determined. As discussed above, *Runx2* promotes osteoblast formation from the mesenchymal skeletal progenitors. Consistently, many *in vitro* experiments revealed that *Runx2* upregulates the bone matrix gene promoters, like Col1A1, Col1A2, osteopontin, bone sialoprotein, osteocalcin, fibronectin, MMP13 and OPG (Komori, 2002). This was further supported by the finding that conditional deletion of *Runx2* from mature osteoblasts resulted in down-regulation of *Col1a1*, *Col1a2*, *osteopontin*, *osteocalcin* and *Bsp* (Ducy et al., 1999). It is interesting that the same gene was shown to have some negative regulatory functions at later stages. When *Runx2* was over-expressed under the *Col1a1* promoter, multiple fractures and osteopenia were observed in mice, and most of the osteoblasts were immature (Geoffroy et

al., 2002; Liu et al., 2001). Taken together, *Runx2* is important for osteoblast differentiation; it promotes differentiation at early stages and inhibits maturation at late stages.

Another important osteoblast differentiation regulator is ATF2, a basic leucine-zipper transcription factor, which belongs to CREB family. It is required for the late osteoblast development, since in the *Atf2* deficient mice only the late markers of osteoblast differentiation, Bsp and osteocalcin, were down regulated and *Runx2*, *Osterix* and *Col1a1* were not affected (Yang and Karsenty, 2004). These data also suggested that *Atf2* works independently of *Runx2*. *Atf2* is capable to interact with *Ap*1 (*Fos* and *Jun*), other *Creb* gene and *Bcl-2* to control cell cycle and proliferation (Luvalle et al., 2003). It is not a surprise that the mutation of Ribosomal serine/threonine kinase 2 (*RSK2*), a growth factor regulated kinase cause Coffin-Lowry Syndrome, an X-linked mental retardation with skeletal abnormalities because *Atf2* is the direct target of RSK2 (Yang and Karsenty, 2004). RSK2 also is required for the phosphorylation of *Ap1*, which is also important for bone formation. Ap1 transcription factor complex is composed of *Fos* (*c-Fos, FosB, Fra-1, Fra-2*), *Jun* (*c-Jun, JunB, JunD*) and ATF family (Jochum et al., 2001). Conditional knockout *Fra-1* leads to bone matrix reduction and overexpression of *Fra-1* or *Delta-FosB* in mice results in the opposite phenotype, osteopetrosis due to increased osteoblast number and bone formation (Eferl et al., 2004; Karreth et al., 2004; Kenner et al., 2004; Kveiborg et al., 2004). Very recently, a new regulator of *Atf2* and *Runx2*, *Satb2* was reported (Dobreva et al., 2006). *Satb2* not only regulates osteoblast differentiation through its interaction with *Runx2* and *Atf2*, but also patterns the craniofacial skeleton by repressing *Hoxa2* (Dobreva et al., 2006). Other genes were also reported to regulate osteoblast differentiation through interaction with *Runx2*, like *Msx1*, *Msx2*, *Dlx5* and *Dlx6* etc (Ichida et al., 2004; Ishii et al., 2005; Lee et al., 2005; Robledo et al., 2002; Satokata et al., 2000). However, most of these

genes' functions remain unclear, with the exception of *Twist1* and *Twist2*. *Twist* genes are transcription factors containing the basic helix-loop-helix (*b-HLH*). Both *Twist1* and *Twist2* are co-expressed with *Runx2* in the osteoprogenitors in the head and trunk, respectively, during early developmental stages. And osteoblasts start to differentiate after Twist expression is downregulated (Bialek et al., 2004). In the *Twist1* or *Twist 2* null mice, osteoblasts differentiated prematurely, and overexpression of *Twist1* resulted in delayed osteoblast differentiation. Further analysis showed twist genes directly interact with *Runx2* through their twist box domain (Bialek et al., 2004). Not all the osteoblast regulators need the interaction with *Runx2* and *Atf2*. Recently, *Calcineurin/Nfat* was identified to be able to regulate osteoblast proliferation and osteoclastogenesis, probably through the interaction with Wnt pathway (Winslow et al., 2006).

Blood vessel invasion and osteoclast development

The final step of endochondral osteogenesis is replacement of hypertrophic cartilage by bone (Erlebacher et al., 1995; Olsen et al., 2000). At least four major events feature in this process: blood vessel invasion (angiogenesis), osteoclast differentiation, osteoblast development into osteocytes and bone marrow formation. Hypertrophic chondrocytes secrete agiogenic factors like vascular endothelial growth factor (VEGF) and specific metalloproteinase (MMP) 9 and 13 to facilitate the vascular invasion into the skeleton (Gerber and Ferrara, 2000; Inada et al., 1999; Stickens et al., 2004). Then hypertrophic chondrocytes die through apoptosis, the perichondrial osteoblasts and blood-borne osteoclasts are introduced through blood vessels, and finally the osteoblasts further develop into osteocytes, which are buried by bone specific matrices such as type I collagen. *Runx2* is a potential regulator of *Vegf* and *Mmp13*, therefore, *Runx2* is also important for bone angiogenesis (Inada et al., 1999) (Otto et al., 1997; Zelzer et al., 2001; Zelzer et al., 2004). Since these major events were already reviewed in detail, and my review mainly focuses on the cartilage development, I will not discuss these processes further. For further

details, the reader is referred to the following references: angiogenesis (Gerber and Ferrara, 2000), osteoclasts (Boyle et al., 2003; Karsenty and Wagner, 2002; Kobayashi and Kronenberg, 2005; Shinohara and Takayanagi, 2007; Wagner and Karsenty, 2001) and osteocytes (Franz-Odendaal et al., 2006).

Complexity of Skeletal Development, Secondary Cartilage and Influences of Epigenetic Factors

Our current knowledge of skeletal developmental is mainly based on work in mouse and chicken. The development of many intermediate skeletal tissues, especially in fishes, remains mysterious. Even in mice, development of fibril cartilage and elastic cartilage is not well studied. For bony tissues, not all bones are formed through either endochondral and intramembranous ossification; the clavicle, for example, has a complex development that probably involves a mixed mechanism (Huang et al., 1997).

Secondary cartilages are named after their position, which is at the margin of intramembranous bones. Phylogenetically, secondary cartilages are found in birds, mammals and teleosts, suggesting that they are not homologous tissues (Hall, 2005). They are usually found in several human sutures, where epigenetic factors, such as oxygen saturation and mechanical loading, influence this kind of cartilage development (Hall, 1967; Hall, 1970). The developmental process of secondary cartilage is like the reverse process of endochondral bone formation: cartilages are formed from the periosteum. This was further confirmed by the finding that upregulation of *Sox9* and downregulation of *Runx2* lead to the formation of secondary cartilages. This induced the chondrocytes to rapidly exit the cell cycle and increase the expression of *Ihh* (Buxton et al., 2003). *Ihh* expression in prehypertrophic chondrocytes works like a bone formation center without a cartilage template. The mechanical forces stimulate the formation of secondary cartilages (Buxton et al., 2003). There are some reports that physical

strain is able to change gene expression profiles of skeletal cells. After application of mechanical force, osteoblasts increased *cyclooxygenase-2* and *c-fos* transcripts in an hour and *osteopontin* and *osteocalcin* level in one day (Pavalko et al., 1998; Walker et al., 2000).

Recently it was proved that mechanical force across the midpalatal suture promotes osteoblast maturation in the periosteum and induces osteoclast activity in vivo (Hou et al., 2007). In the same mice, secondary cartilage formation was inhibited and new cartilage formation was promoted (Hou et al., 2007). Another well-characterized epigenetic factor is hypoxia, which is mediated through the transcription factor hypoxia inducible factor 1 (*Hif1*). Hif1 is required for the chondrocyte survival through its interaction with *Vegf* (Schipani et al., 2001). Recently evidence was provided that the growth plate is hypoxic during fetal development and *Hif1* was turned on in the hypoxic environment to regulate chondrocyte differentiation, joint development, autophagy and apoptosis (Bohensky et al., 2007; Provot et al., 2007). Hypoxia and *Hif-1* are also involved in tumor progression, survival of tissues with low blood supply, and many other pathological events (Schipani, 2006).

Summary

In this dissertation, I investigate the evolutionary origin of cartilage development in vertebrates. Based on the extensive model system work described above, I take a comparative approach and ask when the key components of the skeletogenic gene network were recruited for chondrogenesis. In particular, I focus on the origin of collagen2-based cartilage, which has long been thought to be a unique character of the jawed vertebrates. My results challenge this view and uncover an unexpectedly ancient origin of a conserved skeletogenic program in vertebrates. I also examine the evolutionary origin of the axial skeleton, by determining when the embryonic precursor of vertebrae, the sclerotome, first appeared in vertebrate evolution. The results presented below show that the collagenous skeleton evolved at an earlier node than previously

expected, and rather than being a unique feature of gnathostomes, is a character that unites the extant jawed and jawless vertebrates. Moreover, I provide molecular evidence for the presence of a sclerotome in lampreys, which supports the century-old hypothesis that lamprey arcualia and gnathostome vertebrae may have a common developmental origin. I discuss the implications of these results for the question of homology of these structures, and propose a new model for the role of gene duplication in the evolutionary origin of the skeleton.

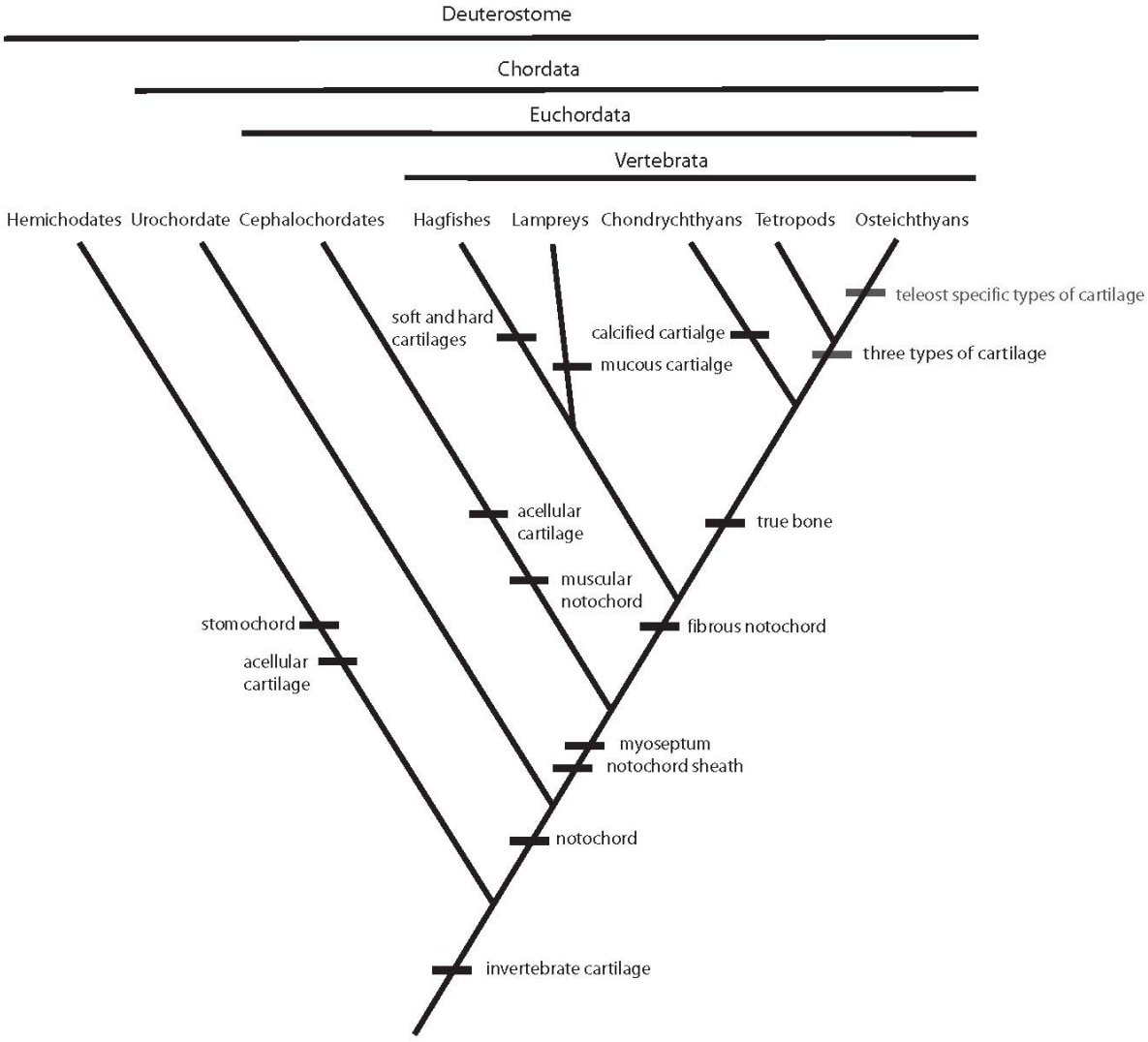


Figure 1-1. Distribution of vertebrate cartilage structures. The phylogenetic relationships are based on Bourlat and Neidert (Bourlat et al., 2006; Mallatt and Winchell, 2007; Neidert et al., 2001). The bars indicate the emergence of the cartilage structures.

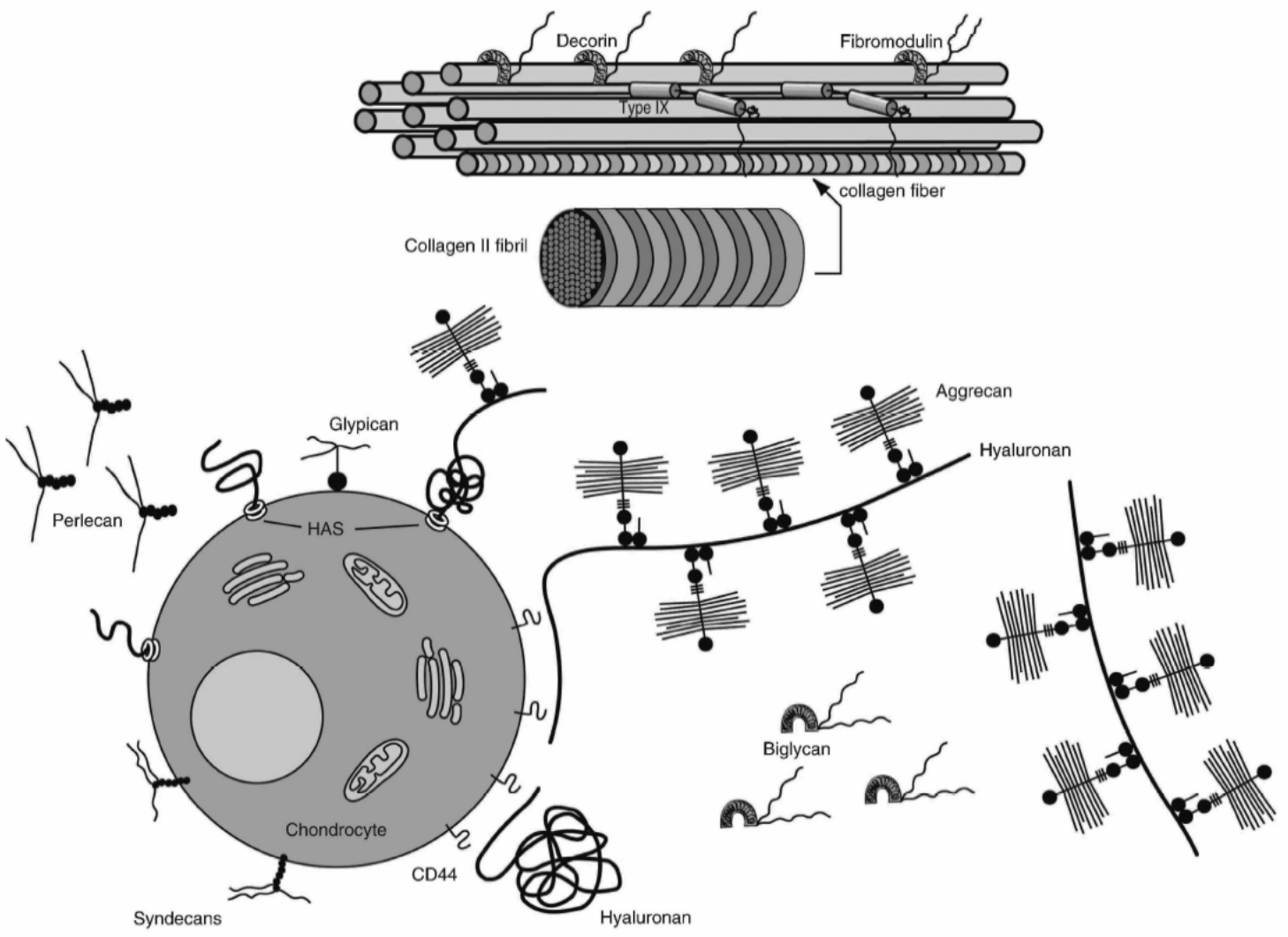


Figure 1-2. Overview of extracellular composition of cartilage. Depicted is a cartilage chondrocyte. Cartilage collagen fibrils (collagens types II, IX and XI) have been indicated. The proteoglycans and their positions are marked respectively. Modified from Knudson and Knudson (Knudson and Knudson, 2001).

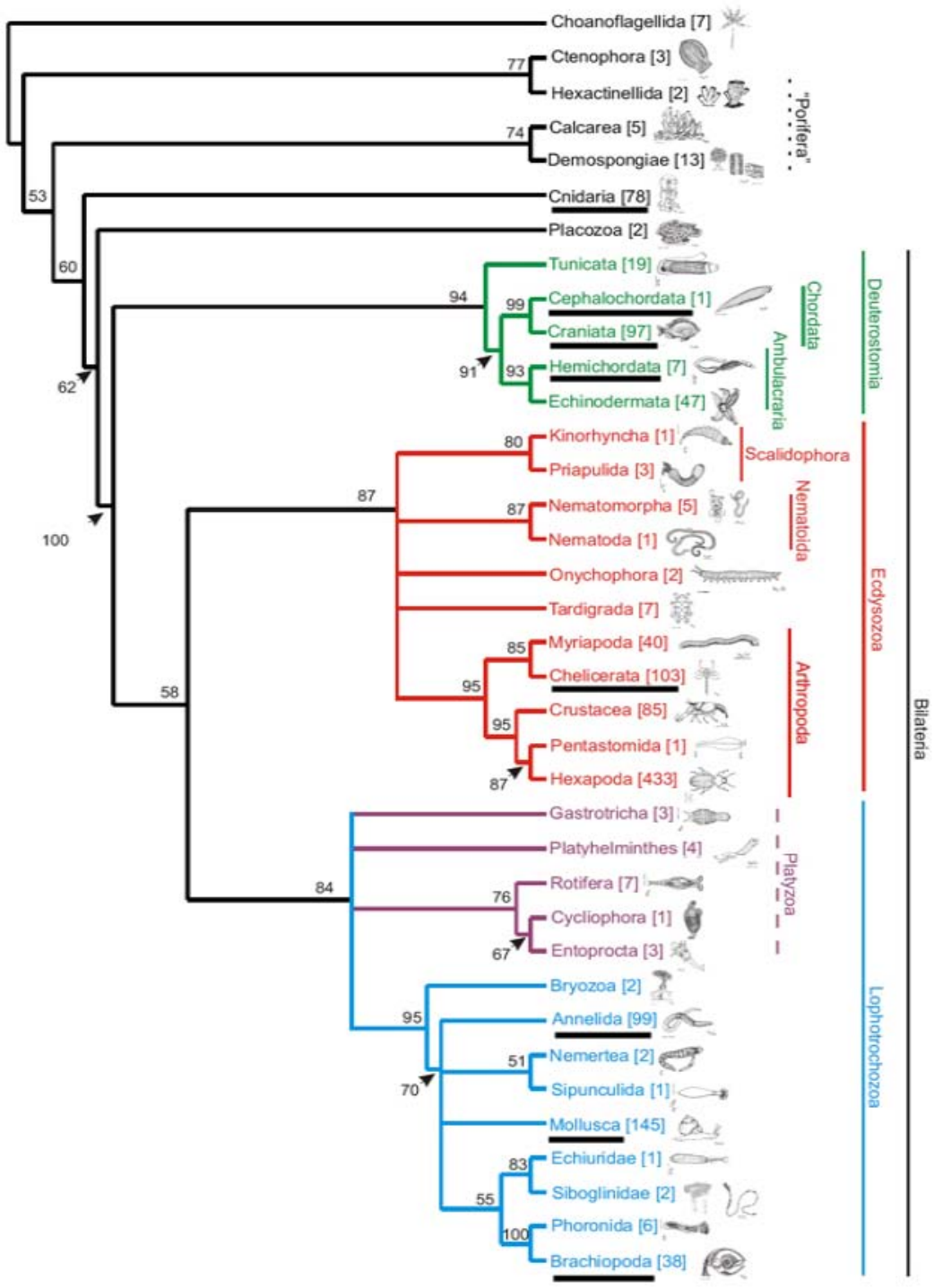


Figure 1-3. Distribution of cartilage tissue within metazoa. The phylogenetic relationships were based on Gerlach, D et. al. (Gerlach et al., 2007). The taxa with cartilage were highlighted with black line underneath the taxa name. The taxa with cartilage were based on the previous reports (Cole and Hall, 2004a; Wright et al., 2001)

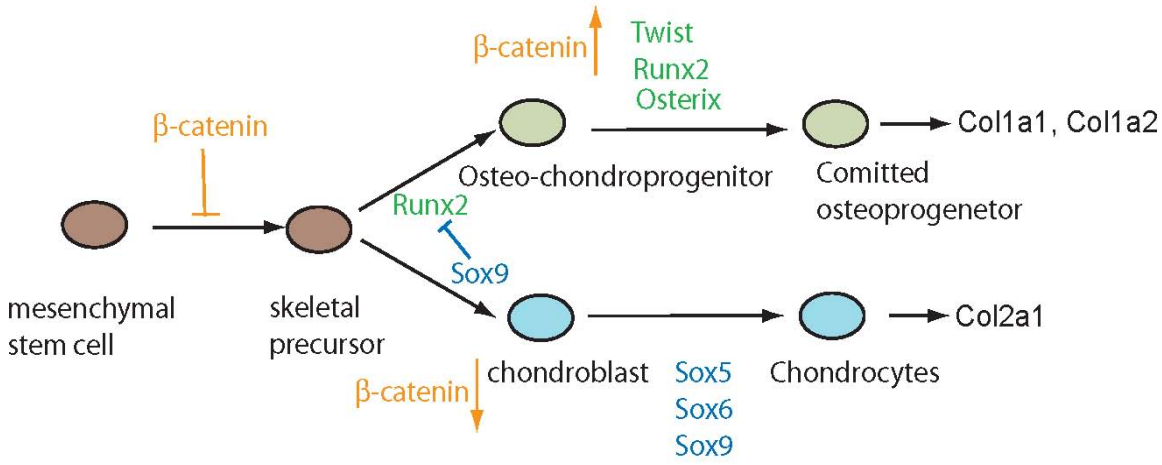


Figure 1-4. Vertebrate skeletal cell lineage determination. The early skeletal cells co-express *Sox9* and *Runx2*, and *Sox9* dominantely inhibits *Runx2*. Dependings on the expression level of *β-catenin*, the skeletal precursor cells go to the chondrogenic or osteogenic pathway. Adapted from Hartmann C. 2006.

CHAPTER 2
LAMPREY TYPE II COLLAGEN AND SOX9 REVEAL AN ANCIENT ORIGIN OF THE VERTEBRATE COLLAGENOUS SKEELTON

Introduction

The earliest known vertebrates are jawless fishes that date to the Lower Cambrian (~520 Ma) (Shu et al., 1999). The remarkable preservation of these skeletons has revealed the patterns of early vertebrate skeletal evolution, however little is known about the developmental processes and molecular mechanisms that gave rise to these patterns. In the cartilage of jawed vertebrates (gnathostomes), the major extracellular matrix molecule is type II collagen (COL2A1). By contrast, the extant jawless fishes (lampreys and hagfishes) have been reported to lack collagen-based cartilage (Janvier and Arsenault, 2002; Wright et al., 2001; Wright and Youson, 1983). In lampreys and hagfishes, some skeletal elements are composed of elastin-like molecules, such as lamprin and myxinin (Robson et al., 2000; Robson et al., 1993). This difference in skeletal structure has raised the hypothesis that the collagenous skeleton is a gnathostome synapmorphy, and the earliest vertebrates have been presumed to have non-collagen based cartilage (Wright et al., 2001).

Collagens are estimated to have appeared in the late Proterozoic (about 800 ma) and then diversified into two major groups, the fibril-forming and non fibril-forming collagens (Morvan-Dubois et al., 2003). The fibril-forming group, consisting of collagen types I, II, III, V and XI, share a high degree of sequence and structural similarity and provide stiffness to a variety of tissues, whereas the non-fibril-forming group is structurally and functionally heterogeneous (Exposito et al., 2002; Morvan-Dubois et al., 2003; van der Rest and Garrone, 1991). The former then split into two phylogenetic groups that are designated (A) for types I, II, III, and Vα2 versus (B) for types Vα1, Vα3, Vα4, Vα5, and XI (Morvan-Dubois et al., 2003) .

During chondrocyte differentiation in gnathostomes, expression of the *Col2a1* gene leads to secretion of Col2a1 protein into the extracellular matrix, where it mineralizes. Expression of *Col2a1* is regulated directly by *Sox9*, which binds to a chondrocyte-specific enhancer to activate *Col2a1* transcription (Bell et al., 1997; Lefebvre et al., 1997). The Sox family of transcription factors consists of at least ten subgroups (A-H) that are characterized by a specific 79-amino acid DNA-binding region, termed the high-mobility-group (HMG) box (Bowles et al., 2000). *Sox9*, a member of the SoxE subgroup, is required for COL2A1 expression and for chondrogenesis in jawed vertebrates (Bi et al., 1999; Yan et al., 2005). Mutation of *Sox9* in zebrafish disrupts the stacking of chondrocytes and the separation and shaping of individual cartilage elements, and in humans causes campomelic dysplasia (Wagner et al., 1994; Yan et al., 2002).

Despite the different compositions of lamprey and gnathostome cartilage matrix, the genetic pathway that regulates early development of the cranial skeleton is well conserved (Cohn, 2002; McCauley and Bronner-Fraser, 2003; Neidert et al., 2001; Shigetani et al., 2002). This raises the question of how the same cascade of gene expression can lead to activation of different cartilage matrix gene targets in these two lineages. Although studies of lamprey cartilage matrix have identified non-collagenous proteins in particular skeletal elements (McBurney et al., 1996a; McBurney et al., 1996b; Robson et al., 1993), it is not clear whether these elastin-related proteins exist in place of or in addition to collagen. Indeed, comparative anatomical studies from the 19th century identified true hyaline cartilage in lampreys and noted striking structural similarities to gnathostome cartilage (Parker, 1883), suggesting that there may be hitherto undiscovered molecular similarities in lamprey and gnathostome cartilage matrix. Here we revisit the evolutionary origin of collagenous cartilage from a molecular developmental perspective, and we report that development of the lamprey skeleton involves type II collagen.

Our experiments show that lampreys have two type II collagen genes, and that both are expressed in the developing skeleton. We also find COL2A1 protein in cranial and postcranial cartilages. We go on to show that lampreys have a true orthologue of *Sox9* that is co-expressed with both *Col2a1* genes during development of the lamprey skeleton. Thus, we conclude that lampreys have collagen-based cartilage, and that the genetic pathway for chondrogenesis is conserved all the way to cartilage matrix gene activation in lampreys and gnathostomes. The results indicate that a collagenous skeleton evolved prior to the divergence of the lamprey and gnathostome lineages and suggest that collagen-based cartilage may be a unifying character of crown vertebrates.

Materials and Methods

Gene Cloning and Sequence Analyses

Degenerate RT-PCR was performed to amplify fragments of lamprey *Col2a1* and *Sox9* orthologues from a *Petromyzon marinus* cDNA library. PCR products were cloned into pDrive vector (Qiagen) and sequenced in both directions. These sequences have been submitted to GenBank (Accession numbers: DQ136023-DQ136025). The inferred protein sequences for the new lamprey cDNAs were initially assigned to the *Col2a1* and *Sox9* families on the basis of BLAST searches and conserved domains (Bowles et al., 2000; Koopman et al., 2004; Valkkila et al., 2001). These initial assignments were followed by estimates of their amino acid identities and phylogenetic relationships. Multiple sequence alignments for available fibrillar A collagens and SoxE proteins, including the new lamprey sequences, were generated with CLUSTAL X and then refined according to their known tertiary structures (Bowles et al., 2000; Koopman et al., 2004; Valkkila et al., 2001). GenBank accession numbers for all sequences used in these phylogenetic analyses: human COL2a1, NP_001006952; mouse COL2A1, B41182; rat COL2A1, NP_037061; chicken COL2A1, NP_989757; frog COL2A1, B40333; salamander

COL2A1, BAA82043; Zebrafish Col2a1, XP_692723; Lamprey COL2A1a, DQ136024; Lamprey COL2A1b, DQ136025; human COL1A1, BAD92834; mouse COL1A1, CAI25880; zebrafish COL1A1, BC063249; Human COL1A2, AAH42586; mouse COL1A2, NP_031769; zebrafish Col1a2, NP_892013; human COL3A1, AAL13167; mouse COL3A1, NP_034060; human COL5A2, NP_000384; mouse COL5A2, NP_031763; sea uchin ColP2a, NP_999675; human Sox8, AAH31797; mouse SOX8, BAC28299; rat SOX8, XP_220283; chicken SOX8; NP_990062; frog SOX8, AAQ67212; zebrafish Sox8, AAX73357; pufferfish, SOX8, AAT42231; human SOX9, AAP35521; mouse SOX9, NP_035578; pig SOX9, AAB81431; chicken SOX9, NP_989612; frog SOX9, BAA95427; alligator SOX9, AAD17974; zebrafish Sox9a, AAM13696; zebrafish Sox9b, AAH67133; pufferfish SOX9, CAG00200; medaka SOX9, BAC06353; trout SOX9, AB006448; stickleback SOX9a, AAQ62978; stickleback SOX9b, AAQ62979; sturgeon SOX9, AAW78521; lamprey SOX9, DQ136023; human SOX10, NP_008872; mouse SOX10; XP_128139; rat SOX10; AAH62067; chicken SOX10, NP_990123; frog SOX10; AAO13216; zebrafish Sox10, NP_571950; pufferfish Sox10a, AAQ18509; pufferfish SOX10b, AAQ18510; lamprey SOXE1, AAW34332; sea squirt SOXE, CAD58841.

Phylogenetic analyses of these multiple protein alignments were conducted with maximum parsimony (MP), minimum evolution (ME), maximum likelihood (ML), and Bayesian phylogenetics (BP) methods (Felsenstein, 2004). The MP analyses included both the equal and unequal weighting of amino acid replacements, with the latter relying on the “ProtPars” cost matrix. The former relied on branch-and-bound searches, whereas the latter was based on heuristic ones with tree-bisection-and-reconnection branch swapping and 1,000 starting trees that were generated from different random sequence additions. The pairwise distances in ME were corrected for multiple replacements with the JTT rate matrix and the gamma (Γ) distribution for

site-to-site heterogeneity in rates. The ME analyses relied on heuristic searches with close-neighbor-interchanges starting from neighbor joining trees. The ML and BP analyses also relied on Γ, but in combination with the improved WAG rate matrix available in their computer programs (but not in that for ME; see below). In ML and BP, the α parameter for Γ was estimated, whereas it was fixed to its ML estimates in ME. The ML analyses relied on a fast heuristic procedure that simultaneously searched for both optimal branch lengths and topologies. The BP analyses were based on three independent runs of 2,000,000 generations apiece, with each run consisting of one cold and three heated chains (T=0.2) and with samples taken from the former every 100 generations. The reliability of groups was evaluated in MP, ME, and ML with 1,000 bootstrap replicates apiece and in BP with posterior probabilities that were calculated after discarding the first 1,000 samples of each run as burnin. The MP, ME, ML, and BP analyses were conducted with PAUP*4.0b10 (Swofford, 2002), MEGA3 (Kumar et al., 2004), PHYML2.4.4 (Guindon and Gascuel, 2003), and MRBAYES3.1.1 (Ronquist and Huelsenbeck, 2003) respectively.

***In Situ* Hybridization**

Whole mount *in situ* hybridization was performed as described for chick embryos (Nieto et al., 1996), with the following modifications: embryos were treated with proteinase K (10 mg/ml) for 15-30 min at room temperature, and 10% dimethylformamide was added to color reaction solution. For histological analysis, specimens were equilibrated in 15% sucrose then 30% sucrose in 20% gelatin, after which they were embedded in 20% gelatin for cryosectioning (10mm).

Immunohistochemistry

Lamprey specimens were fixed in 70% ethanol and processed for paraffin sectioning using standard methods. Sections were cut at 6 mm and antigen retrieval was performed by autoclaving slides in 0.01 M citrate buffer (pH6). Antibody staining was performed using the Vectastain ABC kit according to manufacturer's instructions. Primary antibodies against human COL2A1 (Santa Cruz Biotechnology Inc.) were used at concentrations of 1:500-1:1000.

Results

Lampreys have Two Col2a1 Orthologues

As a first step towards resolving whether collagen genes are involved in lamprey skeletal development, we searched for expressed orthologues of *Col2a1* using a degenerate PCR screen of a *P. marinus* embryonic cDNA library. We isolated two 1.74 kb clones with 75% nucleotide sequence identity to one another, and their deduced amino acid sequences were 80% identical to mouse Col2a1. Absence of gaps in the nucleotide alignments suggested that the two transcripts were not splice variants from a single gene. Molecular phylogenetic analyses with MP, ME, ML and BP methods consistently placed the lamprey sequences within the vertebrate Col2a1 family, and supported their position as a sister group to the gnathostome COL2A1 clade (Figure 2-1. and Figure 2-2.). These results indicate the presence of two *Col2a1* genes in lamprey, which we designate *Col2a1a* and *Col2a1b*. This represents the first demonstration of true type II collagen orthologues outside of jawed vertebrates, and reveals that an independent duplication of the ancestral *Col2a1* gene occurred within the lamprey lineage.

Col2a1a and *Col2a1b* are Expressed during Lamprey Chondrogenesis

If the ancestral *Col2a1* gene was involved in skeletogenesis in the common ancestor of lampreys and gnathostomes, then an evolutionary signature may be detectable during lamprey embryonic development. We therefore examined whether *Col2a1a* and *Col2a1b* are expressed

in tissues that give rise to the lamprey skeleton. Using whole mount *in situ* hybridization, we found that *Col2a1a* is expressed during somitogenesis in a segmental pattern along the lamprey trunk (Figure 2-3.A). Histological sections showed that *Col2a1a* transcripts in the somites were localized ventromedially to the sclerotome, which gives rise to the axial skeleton, and dorsolaterally to the dermatome, which gives rise to the dermis (Figure 2-3. B). In the midline, lamprey *Col2a1a* expression was observed in the floor plate of the neural tube and in the hypochord, but not in the notochord (Figure 2-3. B). This represents a subset of the zebrafish *Col2a1* expression pattern, which occurs along the midline in three domains, the floor plate, notochord, and hypochord (Yan et al., 1995). The second lamprey orthologue, *Col2a1b*, was expressed in a pattern similar to *Col2a1a* along the anteroposterior axis and in the midline; however *Col2a1b* transcripts also localized to the notochord and to endoderm immediately ventral to the hypochord (Figure 2-3. C and D).

During differentiation of the lamprey skeleton, both *Col2a1a* and *Col2a1b* were expressed in chondrogenic cells. The axial skeleton of lampreys consists of a series of paired cartilaginous vertebrae or arcualia, which are serially repeated on either side of the notochord and have long been thought to be homologous to the neural arches of gnathostome vertebrae (Gadow, 1933). *Col2a1a* expression persisted in sclerotomal cells (Figure 2-3. E) and could be seen in the pre-vertebral condensations by stage 30 (Figure 2-3. F). *Col2a1b* expression appeared in the sclerotome by stage 26, but expression in the midline was downregulated anteriorly (Figure 2-3. G-I). In the developing head skeleton, *Col2a1a* expression was detected throughout the cartilaginous branchial basket and posterior to the oral cavity (Fig. 2J). Transcripts were most abundant in the chondrocyte stacks that make up each gill bar (Figure 2-3. K). By contrast, *Col2a1b* expression could not be detected in the pharyngeal arches (Figure 2-3. L). During

development of the median fin skeleton, *Col2a1a* and *Col2a1b* were expressed respectively in the posterior and anterior parts of the finfold (data not shown). Thus, both lampreys and jawed vertebrates exhibit widespread expression of *Col2a1* genes during skeletal development.

Adult Lamprey Cartilage Contains COL2A1 Protein

The discovery that two *Col2a1* genes are transcribed during lamprey skeletal development raised the possibility that lampreys may possess a collagenous skeleton. As a direct test of whether lamprey differentiated cartilage contains type II collagen protein, we performed immunohistochemical analysis of an adult using an antibody specific to COL2A1. We detected COL2A1 protein in the extracellular matrix of pharyngeal cartilages, notochord, notochordal sheath and arcualia (Figure 2-4.), confirming that COL2A1 protein is abundant in the lamprey skeleton. These results demonstrate that a collagenous skeleton evolved prior to the divergence of the lamprey and gnathostome lineages, and suggest that COL2A1 was involved in skeletal development at least as early as the common ancestor of crown vertebrates.

Upstream of *Col2a1*: a Lamprey Orthologue of *Sox9*

In gnathostomes, transcription of *Col2a1* is regulated directly by SOX9, and these two genes are co-expressed during chondrogenesis (Ng et al., 1997; Yan et al., 1995; Yan et al., 2002; Yan et al., 2005). Given the critical role of SOX9 in the development of cartilages derived from both neural crest and mesoderm, we next asked whether lampreys have a *Sox9* orthologue. Using degenerate RT-PCR, we isolated a 1.3KB clone whose inferred amino acid sequence most closely matched those of the gnathostome SOX9 proteins. For example, this inferred protein sequence was 96% identical to human SOX9 within the HMG-box (Figure 2-5.). Furthermore, this new amino acid sequence included the SOX9-specific signature motif (Bowles et al., 2000; Koopman et al., 2004) that occurs immediately 3' to the HMG-domain. Molecular phylogenetic analyses consistently joined this new lamprey protein to the base of the SOX9 clade, with BP

providing a posterior probability of 98% for this assignment (Figure. 2-6.). In concert with its overlapping gene expression pattern with that of SOX9 in jawed vertebrates (see below), these results collectively provide clear evidence for the designation of this clone as lamprey *Sox9*.

Sox9 Expression Co-Localizes with *Col2a1a* and *Cola1b* in the Developing Skeleton

We next investigated whether lamprey *Sox9* is expressed in a pattern consistent with a role in regulation of *Col2a1*. Lamprey *Sox9* expression along the primary body axis resembled the lamprey *Col2a1b* pattern at stage 23 (compare Figure 2-7.A with Figure 2-3. C). Transcripts were localized to the ventral neural tube, notochord and hypochord along the midline, as well as the sclerotome and dorsal endoderm at stage 24 (Figure 2-7. C). Neural expression of *Sox9* extended from the spinal cord to the forebrain (Figure 2-7. B). At stage 23, the pharyngeal arches were negative for *Sox9*, however by stage 24, expression was detected in streams of neural crest cells extending from the hindbrain towards the arches (Figure 2-7. B). At stage 26, *Sox9* was expressed throughout the developing branchial basket, and in the otic and optic placodes (Figure 2-7. D). Like *Col2a1b*, *Sox9* expression in the notochord later retracted from anterior to posterior, but transcripts remained in sclerotome and neural tube (Figure 2-7. E-G). Thus, lamprey *Sox9* expression co-localizes with *Col2a1* transcripts during chondrogenesis, and closely follows the pattern described for gnathostomes (Ng et al., 1997; Yan et al., 2005).

Discussion

The data presented here demonstrate that lampreys have two orthologues of *Col2a1* that are expressed during development of the cartilagenous skeleton. Our discovery of type II collagen protein throughout the adult lamprey skeleton challenges the view that collagen-based cartilage is a gnathostome character and indicates that a collagenous skleleton evolved prior to the divergence of lampreys and gnathostomes. Expression of lamprey SOX9 in chondrogenic cells suggests that a common suite of genes is targeted during skeletal differentiation in jawed

and jawless vertebrates. We conclude that a collagenous skeleton is a shared, derived feature of all vertebrates and not of jawed vertebrates only.

Molecular Evolution of Lamprey Collagens

Our molecular phylogenetic analyses of fibril A collagens leads us to conclude that *Col1* to *Col5* duplicated before the speciation of lampreys and jawed vertebrates. Thus, one prediction of our phylogenetic results is that future studies will recover lamprey orthologues of *Col1*, *Col3*, and/or *Col5*, which should join at the bases of their respective gnathostome clades in the fibril A collagen tree (Fig. 1). An independent duplication of the *Col2a1* gene then occurred in the lamprey lineage. The expression patterns of *Col2a1a* and *Col2a1b* suggest that subfunctionalization followed this duplication of the ancestral *Col2*a1 gene, with the ancestral expression pattern being partitioned between *Col2a1a* and *Col2a1b*. Force and colleagues proposed a mechanism by which duplicated genes are preserved during evolution by subfunctionalization, in which both members of the pair undergo reduction of their activity and expression patterns such that together they equal that of their single ancestral gene (Force et al., 1999; Lynch et al., 2001). Our finding that the expression domains of *Col2a1a* and *Col2a1b* in lampreys correspond to that of the single *Col2a*1 gene in jawed vertebrates supports their duplication-degeneration-complementation (DDC) model. It is also noteworthy that collagen genes are physically linked to the *Hox* clusters in gnathostomes (Bailey et al., 1997; Morvan-Dubois et al., 2003). In lampreys, there are at least three *Hox* clusters (Force et al., 2002; Fried et al., 2003; Irvine et al., 2002). Our identification of two *Col2a1* genes supports the suggestion that one of their three *Hox* clusters may have arisen by an independent duplication in the lamprey lineage (Force et al., 2002; Fried et al., 2003; Irvine et al., 2002). If the fibril A collagens are linked with Hox clusters in all chordates, then the presence of a single Hox cluster in amphioxus

makes it tempting to speculate that, barring tandem duplications, cephalochordates may possess a single fibril A collagen gene.

Evolution of Collagen-Based Cartilage

Localization of type II collagen mRNA and protein in the lamprey skeleton reveals that a collagenous skeleton is not restricted to the gnathostome lineage, but instead is a character shared by the crown vertebrates. This may provide a molecular explanation for Parker's observation in 1883 that lampreys have hard hyaline cartilage (Parker, 1883). We suggest that the additional cartilage matrix molecules (e.g., lamprin and myxinin) of agnathans may represent derived character states that that were added onto the more ancient collagenous skeleton. Expression of elastin-related molecules in a subset of lamprey cranial cartilages that also express COL2A1 may underlie the different structural and mechanical properties within the lamprey skeleton. Expression of *Col2a1a* in the lamprey dermatome was unexpected, as vertebrate dermis is generally characterized by type I collagen (van der Rest and Garrone, 1991). While this may be a derived feature of lampreys, it could also reflect their descent from agnathans with dermal armor (Forey and Janvier, 1993).

Five independent molecular phylogenetic analyses joined lamprey SOX9 to the gnathostome Sox9 clade. Thus, the duplication of the ancestral SOXE gene that gave rise to *Sox9* occurred before the divergence of the lamprey and gnathostome lineages. *Sox9* is expressed in strikingly similar patterns in lamprey and gnathostome embryos. The co-expression of *Sox9* with *Col2a*1 during skeletogenesis in both lineages raises the possibility that the regulatory relationship between these two genes had already been established in their common ancestor. In gnathostomes, Sox9 is a target of parathyroid hormone-related protein (PTHrP), which regulates chondrocyte differentiation through a negative feedback loop with Indian hedgehog (Huang et al., 2001; Vortkamp et al., 1996). Interestingly, PTHrP expression has

recently been detected in lamprey cartilage (Trivett et al., 2005). Our discovery of conserved expression of *Sox9* and *Col2a1*, taken together with the extensive conservation of upstream regulatory genes such as *AP2, Dlx, Msx, Id* and *PTHrP* (Cohn, 2002; McCauley and Bronner-Fraser, 2003; Neidert et al., 2001; Shigetani et al., 2002; Trivett et al., 2005), suggests that the genetic program for chondrogenesis -- from the initial induction of chondrogenic mesenchyme to the synthesis of collagen matrix -- was assembled surprisingly early in vertebrate evolution. Comparative analyses of fibrillar collagen and SOXE genes in hagfishes, amphioxus, ascidians and hemichordates will further refine the evolutionary history of the collagenous skeleton.

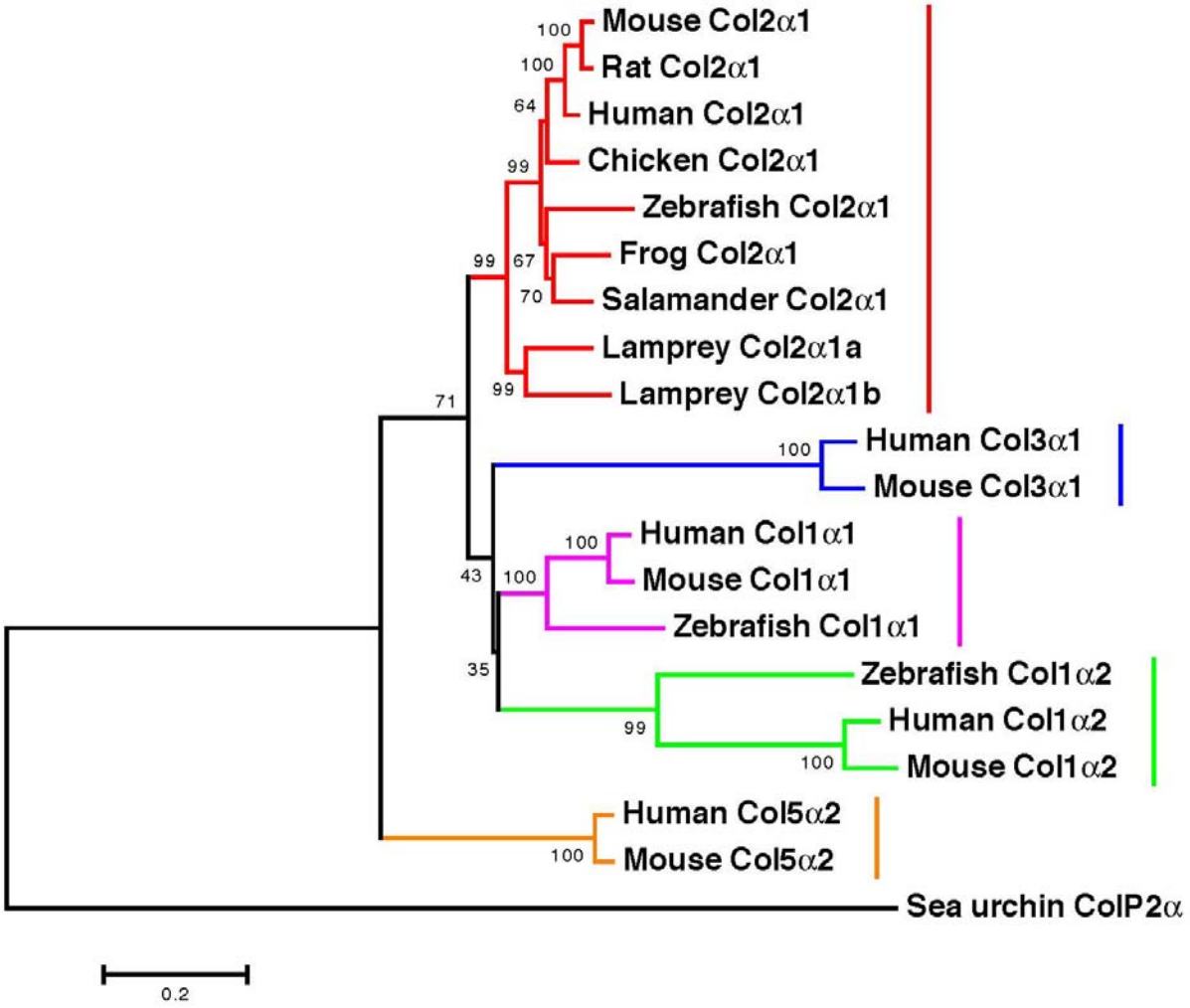


Figure 2-1. Minimum evolution phylogeny for fibril A collagen proteins as obtained with JTT+ Γ distances (α = 0.906). Numbers indicate bootstrap scores for each node, based on 1,000 replicates, whereas branch lengths are proportional to expected replacements per site. This tree is rooted by sea urchin COLP2. Equally and unequally weighted MP, ML, and BP also place the two lamprey sequences together at the base of the COL2A1 clade, with bootstrap scores or a posterior probability of 93%, 77%, 71%, and 87%, respectively. In contrast to this consistent support for a Col2A1 grouping, this ME tree is unique among the different phylogenies in its placement of the root along the Col5 clade. This discrepancy over the root is illustrated with the BP phylogeny and discussed further in Figure. 2-2.

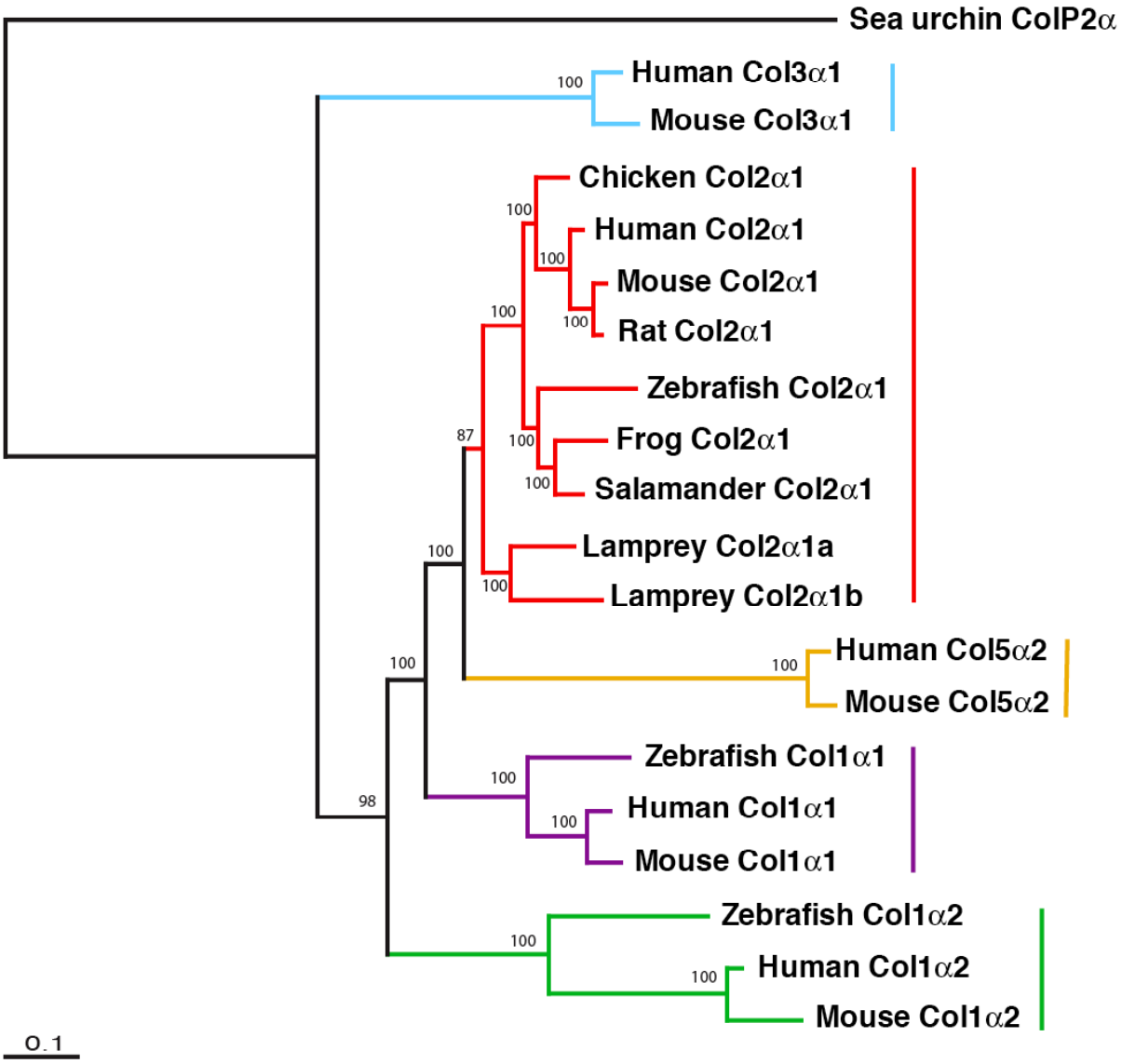


Figure 2-2. Extended majority-rule consensus tree for the BP analysis of the fibril A collagen proteins. Numbers indicate posterior probabilities that are given for each group with >50% credibility and for those clades that are combinable with this first set. Branch lengths are proportional to the means of the posterior probability densities for their expected replacements per site. This tree is rooted by sea urchin COLP2. This root along the COL3 clade illustrates an alternative placement for MP, ML, and BP relative to the COL5 rooting for ME (Figure. 2-1.). In contrast to this new position, the latter is consistent with available phylogenetic and linkage data for the physically linked Hox gene clusters (Bailey et al., 1997; Morvan-Dubois et al., 2003). Nevertheless, this discrepancy over the root does not diminish the fact that all phylogenetic methods converge onto the same placement of the two lamprey collagens at the base of the COL2A1 clade. Thus, no ambiguity exists over the identification of the two new lamprey sequences as orthologues of COL2A1.

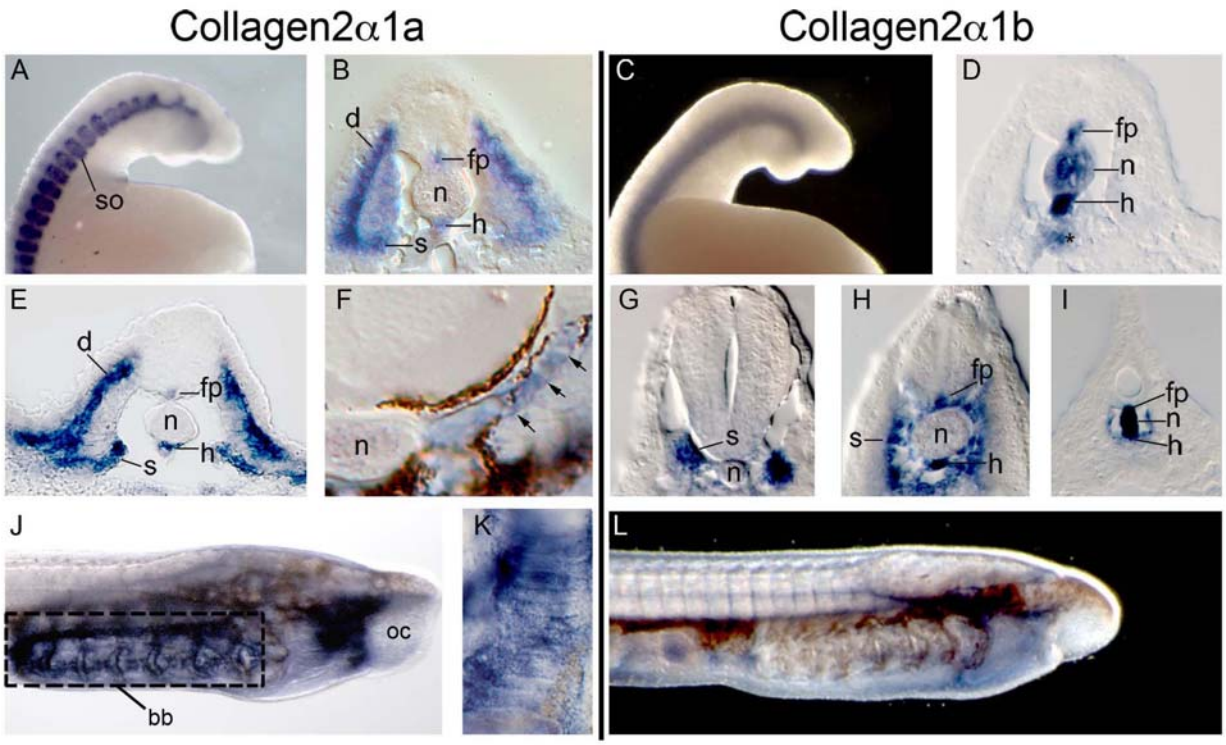


Figure 2-3. *Col2a1a* and *Col2a1b* expression during lamprey development. Whole mount *in situ* hybridization of lamprey embryos at stages 23 (A-D), 25 (E), 26 (G-I) and 30 (F, J-L). Anterior is to right in (A, C, J and L); All sections are transverse with dorsal to the top. A, B, *Col2a1a* expression is evident in the somites, within the dermatome and sclerotome. Expression in the midline is restricted to floor plate and hypochord (B). C, D, *Col2a1b* is expressed in the floor plate, notochord, hypochord and dorsal endoderm (asterisk). E. *Col2a1a* in the dermatome, sclerotome, floor plate and hypochord. F. *Col2a1a* expression in a prevertebral condensation (arrows). G-I, Sections through the hindbrain (G), mid-trunk (H) and tail (I) show an anterior to posterior retraction of the *Col2a1b* domain in the notochord, hypochord and floor plate. J, *Col2a1a* is expressed throughout the branchial skeleton (boxed) and posterior to the oral cavity. K, *Col2a1a* expression in a stack of chondrocytes in a branchial bar. (L). *Col2a1b* is not expressed in the branchial skeleton. Abbreviations: bb, branchial basket; d, dermatome; fp, floor plate; h, hypochord; n, notochord; oc, oral cavity; s, sclerotome; so, somite.

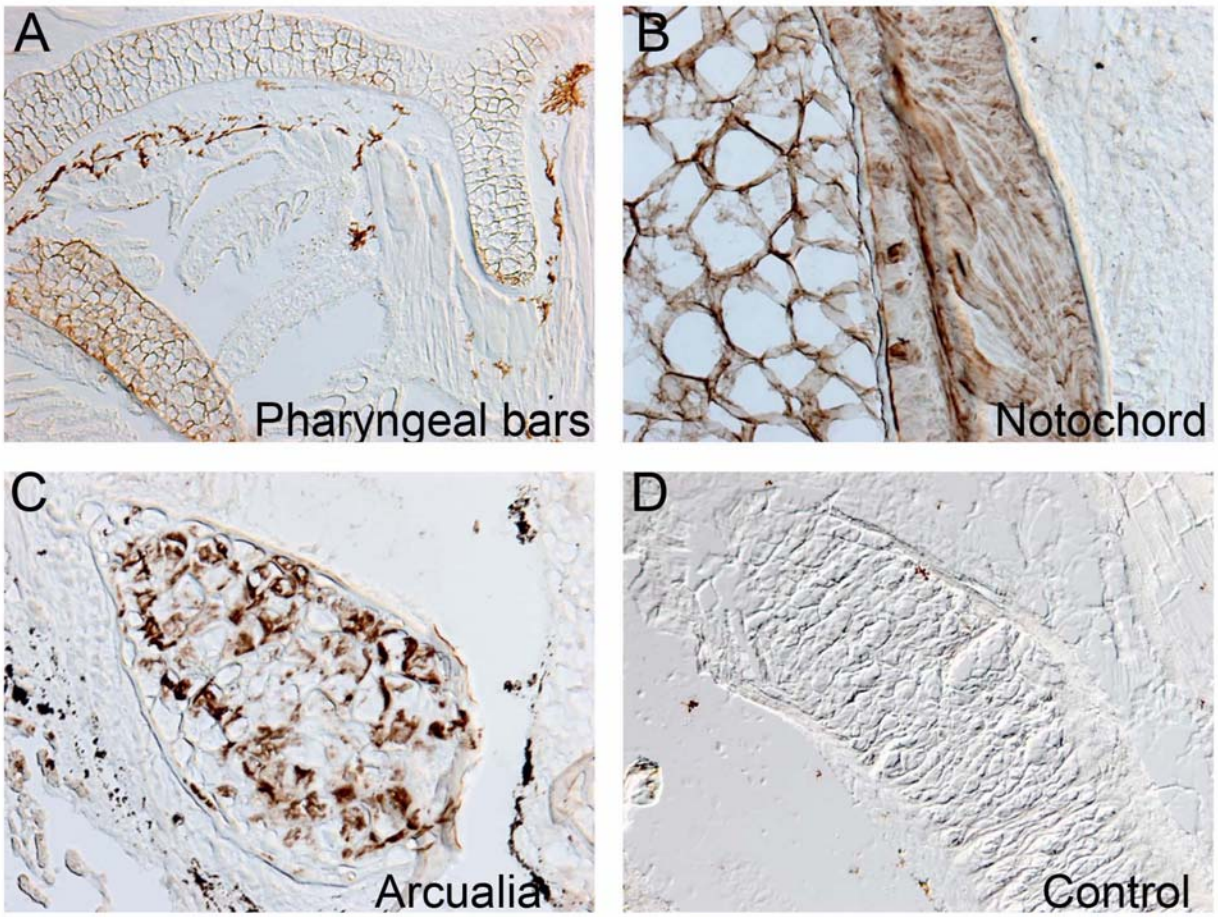
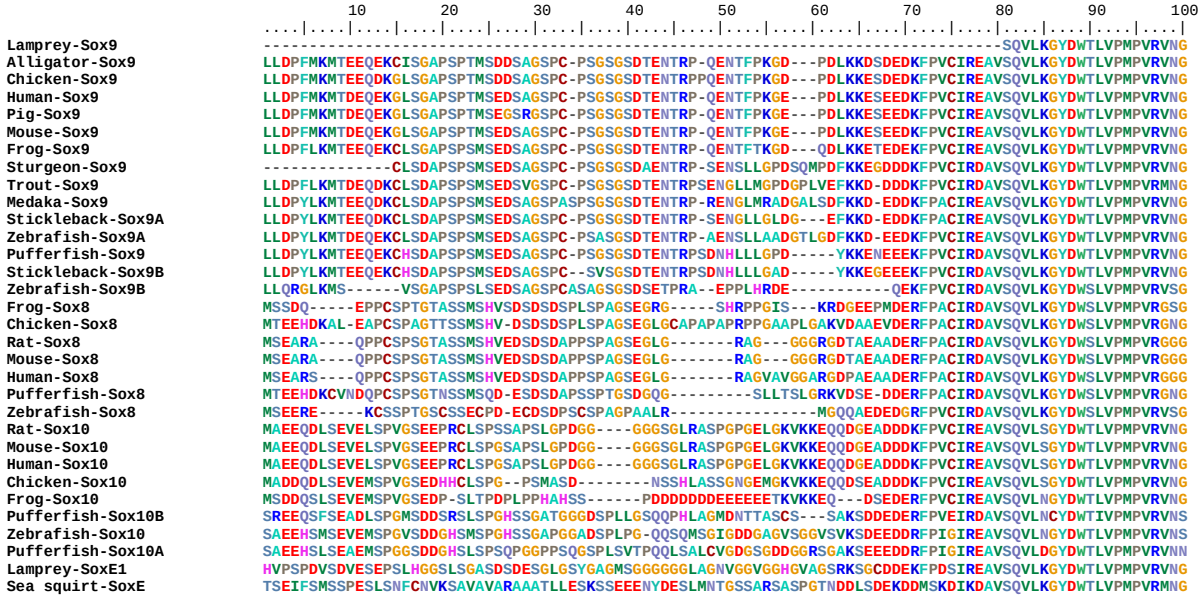


Figure 2-4. COL2A1 protein is abundant in adult lamprey cartilage. Immunohistochemical staining of lamprey cartilage with COL2A1 antibody. A. Sagittal section through pharyngeal cartilage bars. B. Transverse section through notochord. Note staining of notochord cells and notochordal sheath. C. Transverse section through arcualia on ventrolateral side of notochord. D. Control section through pharyngeal cartilage, after omission of primary antibody.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Alligator-Sox9
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Medaka-Sox9
Stickleback-Sox9A
Zebrafish-Sox9A
Pufferfish-Sox9
Stickleback-Sox9B
Zebrafish-Sox9B
Frog-Sox8
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Mouse-Sox10
Human-Sox10
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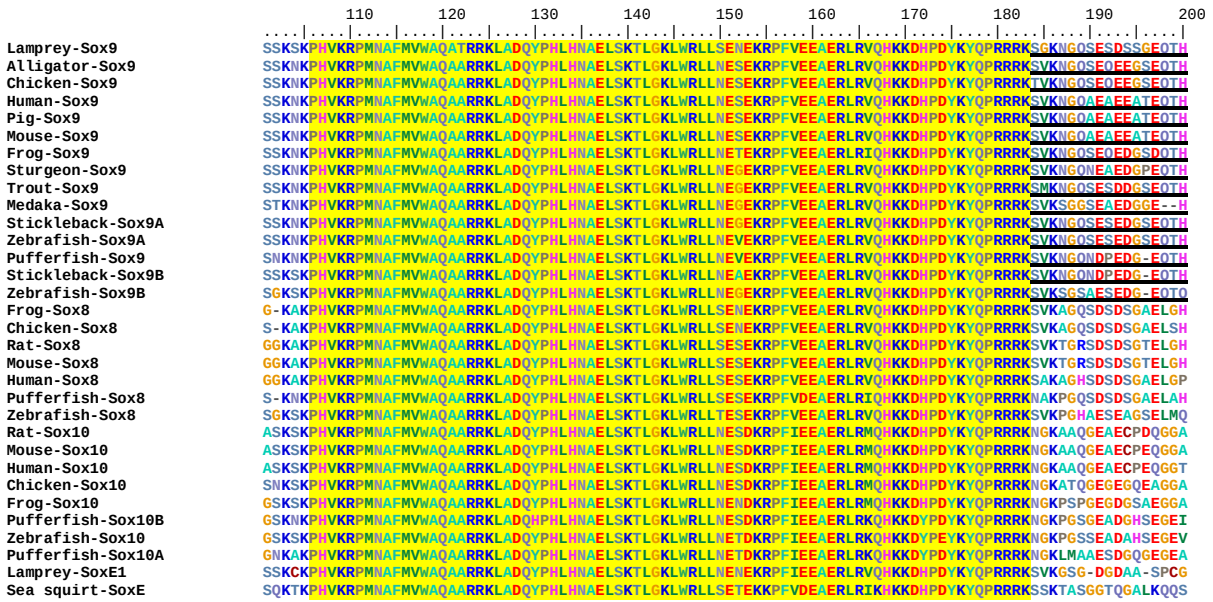
Sea squirt-SoxE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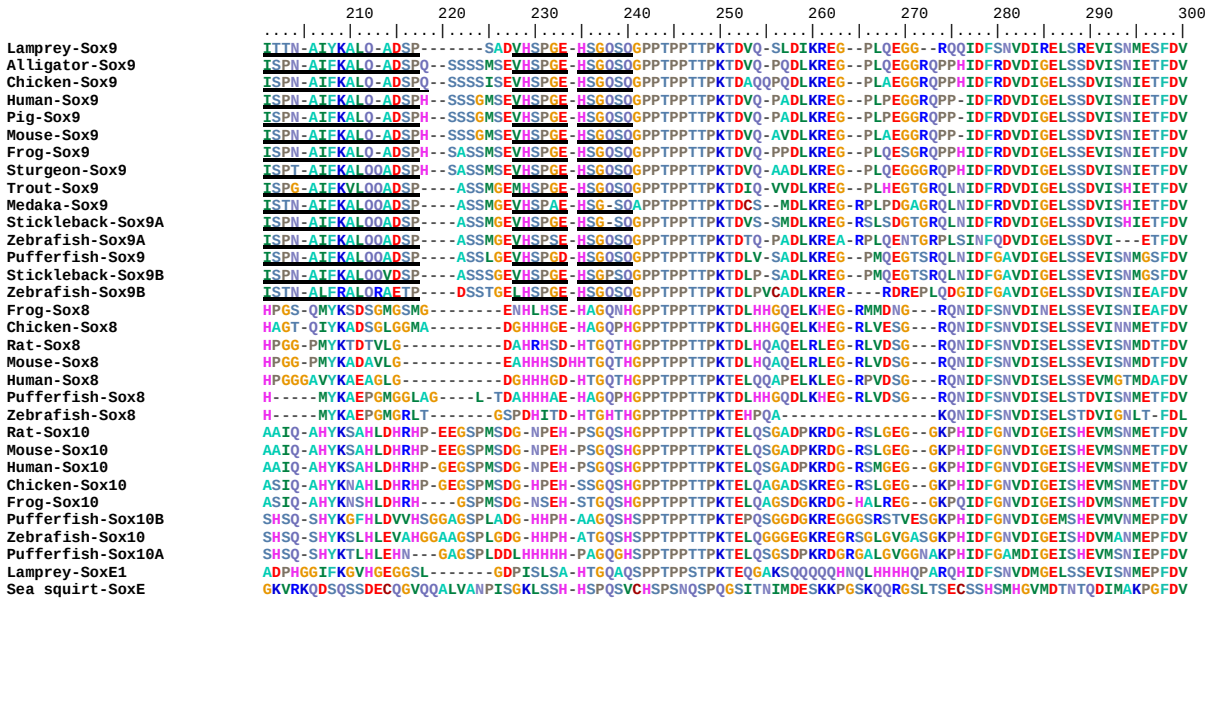
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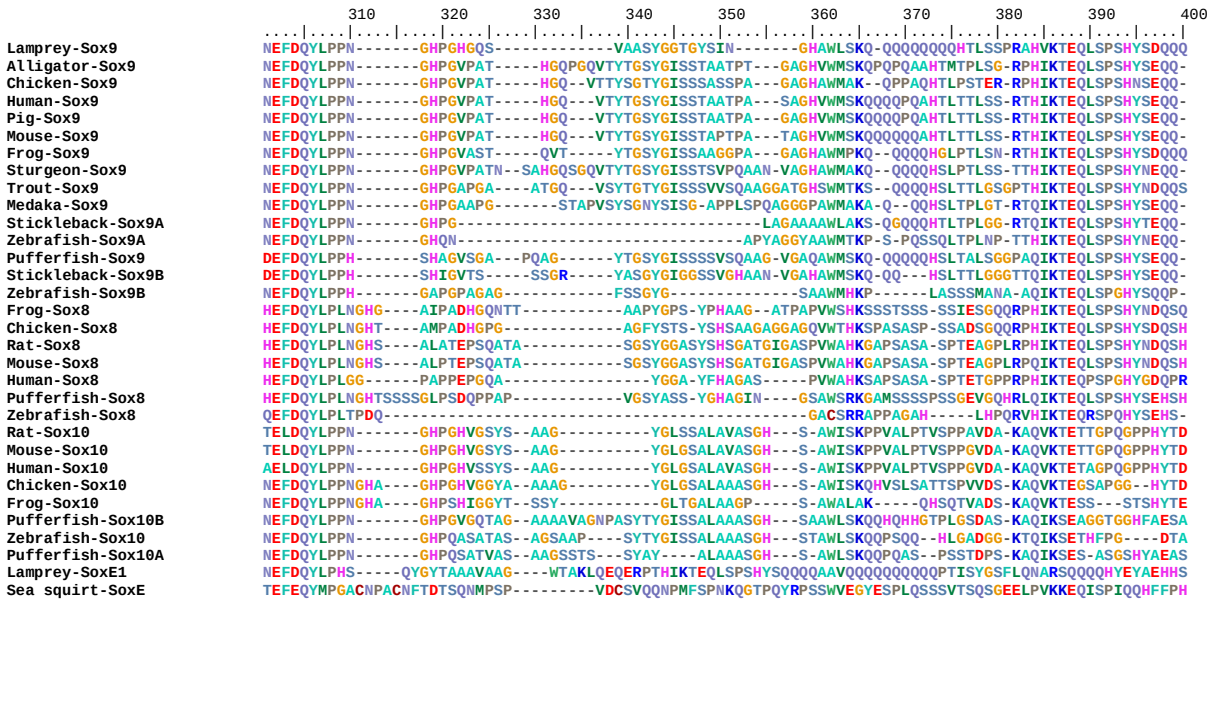
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Mouse-Sox9
Frog-Sox9
Sturgeon-Sox9
Trout-Sox9
Medaka-Sox9
Stickleback-Sox9A
Zebrafish-Sox9A
Pufferfish-Sox9
Stickleback-Sox9B
Zebrafish-Sox9B
Frog-Sox8
Chicken-Sox8
Rat-Sox8
Mouse-Sox8
Human-Sox8
Pufferfish-Sox8
Zebrafish-Sox8
Rat-Sox10
Mouse-Sox10
Human-Sox10
Chicken-Sox10
Frog-Sox10
Pufferfish-Sox10B
Zebrafish-Sox10
Pufferfish-Sox10A
Lamprey-SoxE1
Sea squirt-SoxE

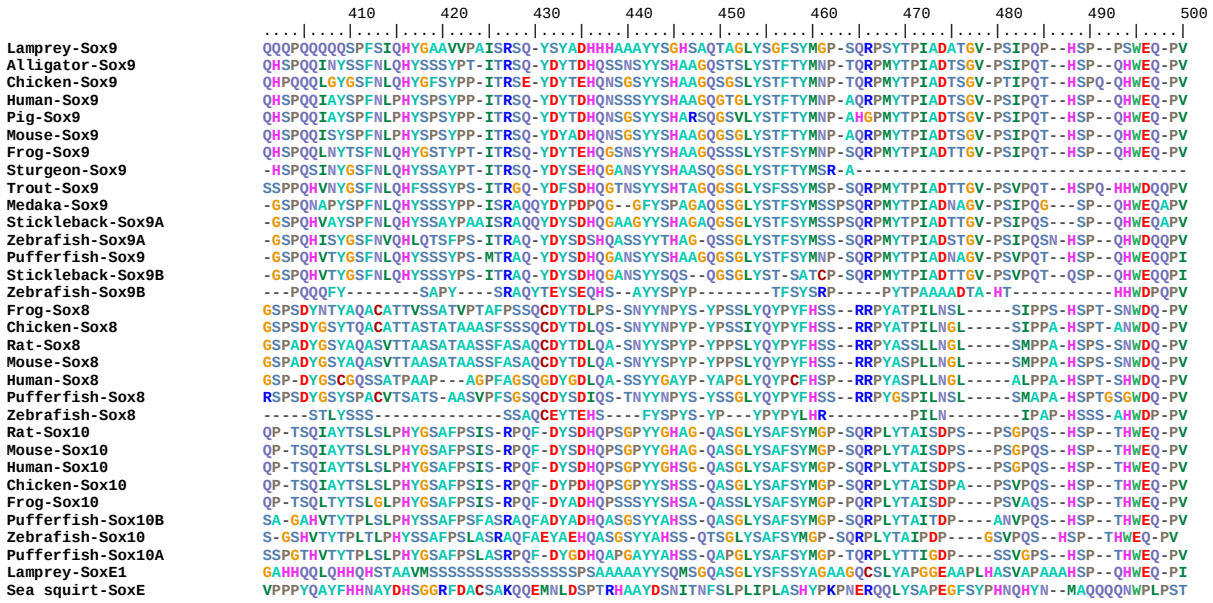

Figure 2-5. Alignment of inferred amino acid sequence for lamprey SOX9 with chordate SOXE proteins. The inferred amino acid sequence of the new lamprey Sox clone most closely matched those of the gnathostome SOX9 family. For example, this new lamprey sequence was 96% identical to human SOX9 within the HMG-box domain (highlighted here in yellow). Outside of this domain, the available SOXE proteins often showed considerable length variation, thereby making these regions difficult to align. Such problematic regions were removed prior to phylogenetic analyses (Swofford et al., 1996), resulting in the multiple sequence alignment shown here with 495 aligned positions. In ME, gapped positions were subjected to complete deletion (i.e., the removal of their entire column in the multiple sequence alignment) to ensure uniform comparisons among all pairwise distances (Nei and Kumar, 2004). However, this approach also reduced the number of aligned positions for ME to 219 sites, thereby resulting in even less information for the placement of the new lamprey sequence at the base of the SOX9 clade (as indicated by its low bootstrap score in Fig. 4). In the end, we based our current assignment of the new lamprey sequence on three different lines of evidence (Bowles et al., 2000; Koopman et al., 2004): (a) on the presence of a SOX9-signature motif (which is located immediately 3' to the HMG- box and is underlined in the figure); (b) on the consistent phylogenetic placement of the lamprey protein to the base of the SOX9 clade (Figure. 2-6.); and (c) on its overlapping gene expression pattern with that of SOX9 in jawed vertebrates (see text).

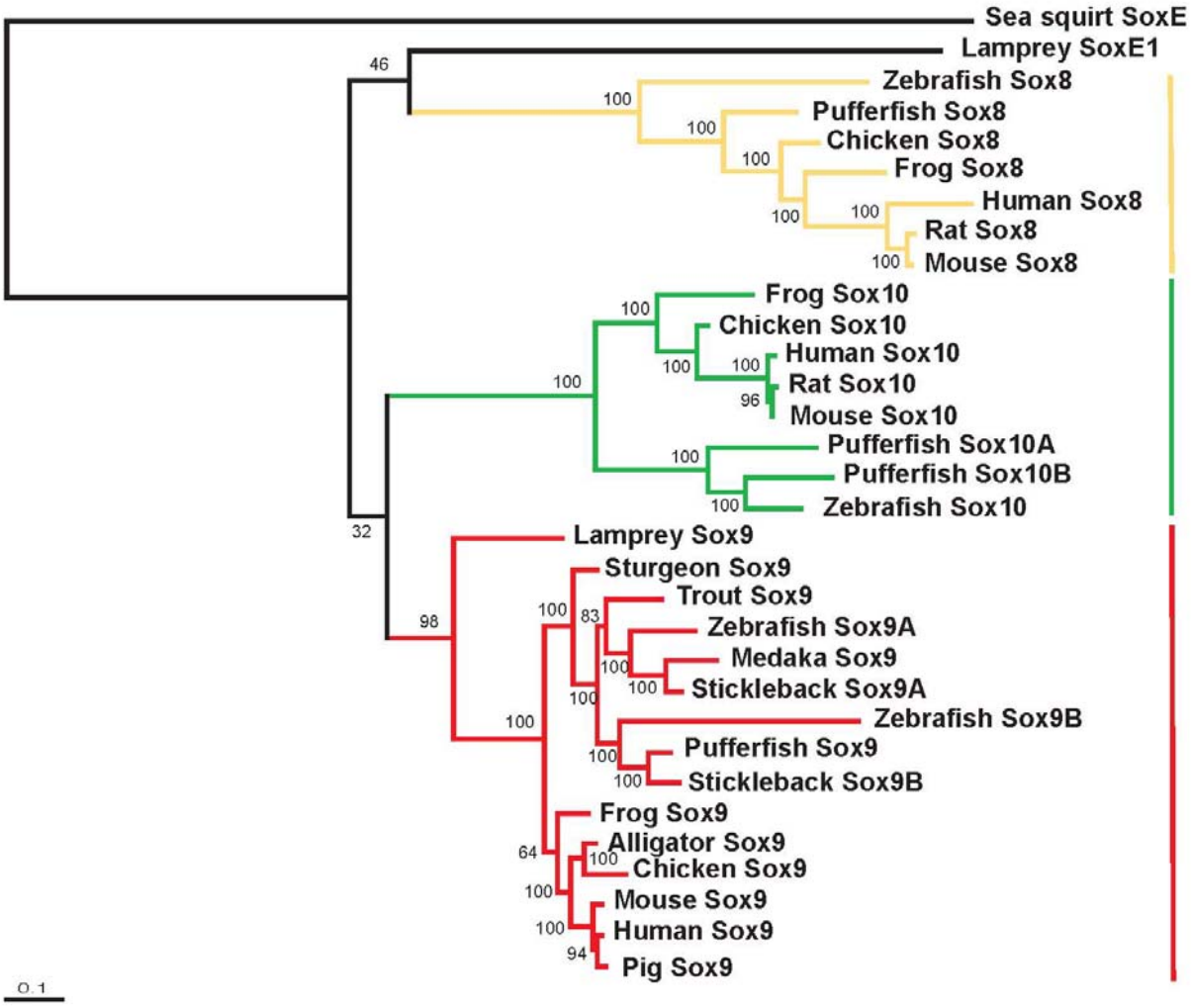

Figure 2-6. . Extended majority-rule consensus tree for the BP analysis of the chordate SOXE proteins. Numbers indicate posterior probabilities for groups with >50% credibility and for those clades that are combinable with this first set. Branch lengths are proportional to the means of the posterior probability densities for their expected replacements per site. This tree is rooted by sea squirt SOXE. Equally and unequally weighted MP, ME, and ML also place the new lamprey sequence at the base of the SOX9 clade, with bootstrap scores of 86%, 74%, 28%, and 75%, respectively. The surprisingly low bootstrap score for ME is related to our complete deletion of gapped positions in the pairwise distance calculations (Figure 2-5.). Despite this low score, the optimal ME phylogeny still supports as best a SOX9 assignment for the new lamprey protein and is therefore consistent with the other phylogenetic results.

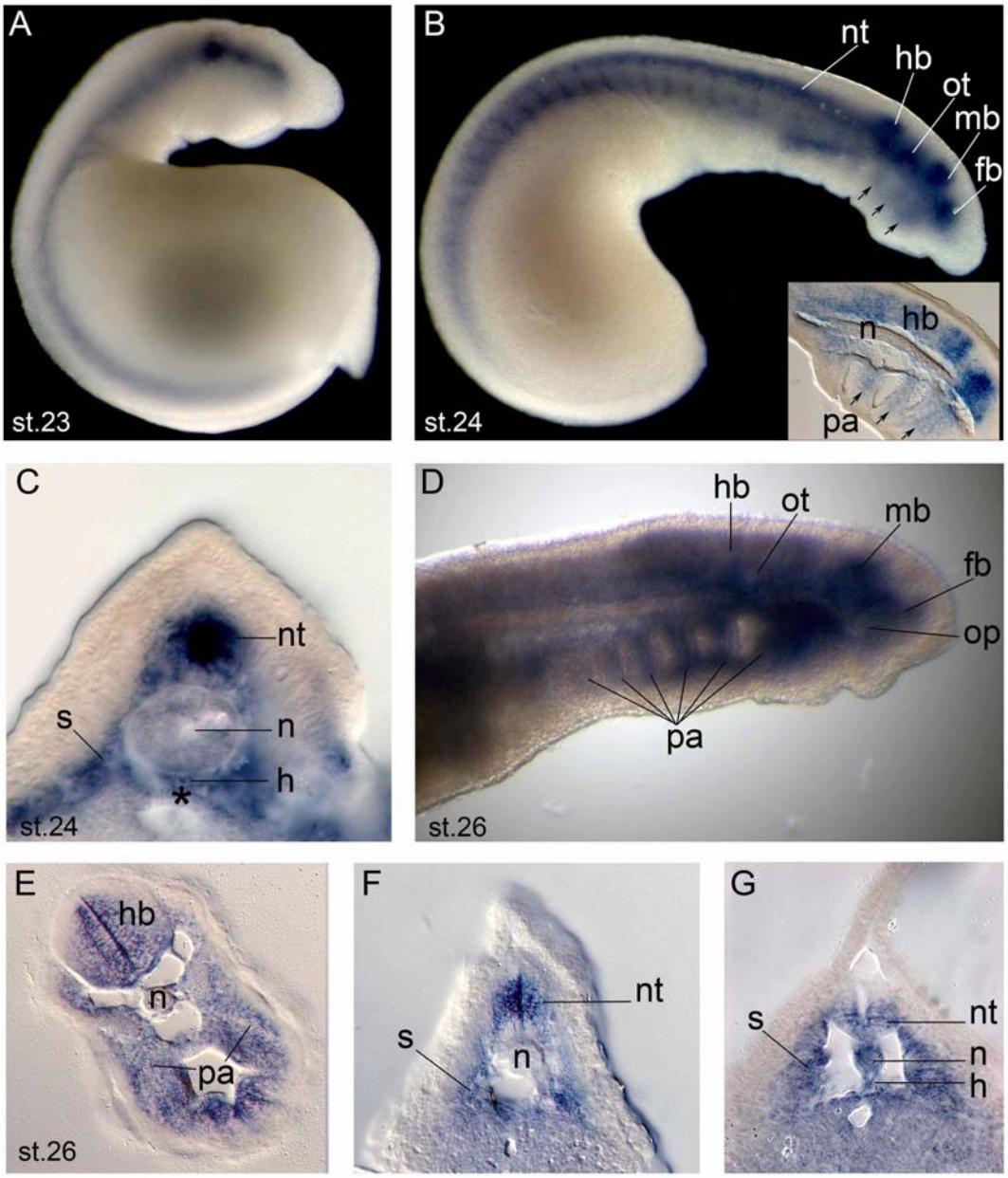

Figure 2-7. *Sox9* expression during lamprey development. Expression of lamprey *Sox9* in embryos at stages 23 (A) and 24 (B). Inset in (b) shows sagittal section through hindbrain and pharyngeal arches. Arrows indicate *Sox9* expression in streams of neural crest cells invading pharyngeal arches. C. Transverse section through trunk of embryo shown in (B). Note *Sox9* expression in notochord, hypochord, neural tube, sclerotome and dorsal endoderm (asterisk). D. *Sox9* expression in stage 26 lamprey head. E-G. Sections through the hindbrain (E), mid-trunk (F) and tail (G) at stage 26 show an anterior to posterior retraction of the *Sox9* domain in the notochord. Note persistant expression in neural tube, pharyngeal arch mesenchyme, sclerotome and hypochord. Abbreviations: fb, forebrain; hb, hindbrain; h, hypochord; mb, midbrain; n, notochord; nt, neural tube; op, optic placode; ot, otic placode; pa, pharyngeal arches; s, sclerotome.

CHAPTER 3
HAGFISH AND LANCELET FIBRILLAR COLLAGENS REVEAL THAT TYPE II COLLAGEN-BASED CARTILAGE EVOLVED IN STEM VERTEBRATES

Introduction

The phylogenetic relationships of the vertebrates were set up largely based on anatomical characters, particularly those of the skeleton. The skeletons of jawed vertebrates (gnathostomes) are composed of cartilage and bone, which contain high levels of COL2A1 and COL1A1 protein, respectively. By contrast, the cartilaginous skeletons of lampreys and hagfishes, the only extant jawless fishes (agnathans), have been reported to be non-collagenous and to contain instead the elastin-like proteins lamprin and myxinin (Wright et al., 2001). This difference in the histological matrices of vertebrate skeletons has led to the idea that type II collagen became the major structural component of gnathostome cartilage after the divergence of these two lineages (Wright et al., 2001). However, this view was challenged by our recent report that lampreys have two *Col2a1* orthologues and that both genes are expressed during chondrogenesis. Adult lamprey cartilage was also shown to be rich in COL2A1 protein (Chapter 2). Furthermore, we identified a lamprey orthologue of *Sox9*, a direct transcriptional regulator of *Col2a1* in gnathostomes, and showed that it is co-expressed with *Col2a1* (Chapter 2). This revealed that the genetic pathway for vertebrate chondrogenesis predated the divergence of lampreys and gnathostomes, although precisely how early this character arose is unknown.

Here we investigate the evolutionary origin of COL2A1-based cartilage by expanding our analysis to the most inclusive clade of vertebrates, which includes the hagfishes, and to a sister group to the vertebrates, the lancelets (Donoghue et al., 2000; Zeng and Swalla, 2005). We report that hagfishes also have COL2A1-based cartilage, suggesting that this type of cartilage was present in common ancestor of all crown vertebrates. Our analysis of lancelets revealed the presence of an ancestral clade A fibrillar collagen (COLA) gene that is expressed in the

notochord. Thus, during the chordate-vertebrate transition, an ancestral Clade A fibrillar collagen gene underwent duplication and diversification, and this process may underlie the evolutionary origin of vertebrate skeletal tissues.

Materials and Methods

Animals

Adult lancelets (*Branchiostoma floridae*) were collected in Tampa Bay, FL. and spawning was induced in the lab, according to published methods (Stokes and Holland, 1995). Atlantic hagfishes (*Myxine glutinosa*) were purchased from Wards Natural Science (Rochester).

Gene Cloning

RNA was extracted using TRIzol Reagent (Invitrogen). cDNA was made by reverse transcription reactions using SuperScript II first-strand cDNA synthesis kit (Invitrogen). Fibrillar collagen genes were amplified with degenerate primers designed using CODEHOP program (http://bioinformatics.weizmann.ac.il/ blocks/ codehop.html) (Rose et al., 2003). PCR reactions were carried out using BD Advantage 2 PCR enzyme system (Clontech) from cDNA. In 50-µl reactions containing 1µl forward (5'-GGCCCTCCCGGCCTGcarggnatgcc-3') and 1µl reverse (5'- GGGGCCGATGTCCACGccraaytcytg-3') primers (20pmol/µl), 5µl 10× buffer, 1µl cDNA template, 1µl dNTP at 10mM (each), 1µl Taq polymerase mixture and 40µl of double distilled water. Reactions were amplified as follows: 94℃-1 min followed by 35 cycles of 94℃-45 sec, 65℃-45 sec, 68℃-3min, and a final 10-min elongation at 68℃. PCR products were purified using QIAGEX II gel extraction kit (Qiagen) then cloned into pCRII-TOPO (Invitrogen) for sequencing. Hagfish COL2A1 and lancelet AmphiCOLA sequences have been submitted to GenBank, under accession numbers DQ647926 and DQ647925, respectively.

Sequence Analysis and Molecular Phylogenetics

Inferred protein sequences for hagfish and amphioxus cDNAs were initially assigned to the clade A fibrillar collagen families on the basis of BLAST searches and conserved domains. These preliminary assignments were followed by estimates of their amino acid identities and

phylogenetic relationships. Multiple sequence alignments for available fibrillar A, B and C collagen proteins, including the new hagfish and amphioxus sequences, were generated with CLUSTAL X. Phylogenetic analyses of these multiple protein alignments were conducted with Bayesian phylogenetics (BP), maximum likelihood (ML) and minimum evolution (ME) methods, as described previously (Chapter 2).

The following amino acid sequences were retrieved from GenBank for inclusion in our phylogenetic analyses: Mouse Col2a1: B41182; Rat COL2A1: NP_037061; Dog COL2A1: NP_001006952; Chick COL2A1: NP_989757; Horse COL2A1: T45467; Salamander COL2A1: BAA82043; Frog COL2A1: B40333; Zebrafish Col2a1:XP_692723; Lamprey COL2A1a: DQ136024; lamprey COL2A1b: DQ136025; Human COL1A1: BAD92834; Mouse COL1A1: CAI25880; Dog COL1A1: NP_001003090; Cow COL1A1: AAI05185; Salamander COL1A1: BAA36973; Frog COL1A1: BAA29028; Halibut COL1A1: BAD77968; Zebrafish Col1a1: AAH63249; Rainbow trout Col1a1: BAB55661; Human COL1A2: AAH42586; Mouse COL1A2: NP_034060; Dog COL1A2: NP_001003187; Chick COL1A2: XP_418665; Frog COL1A2:AAH49287; Salmon COL1A2: BAB79229; Halibut COL1A2: BAD77969; Fugu COL1A2: CAG11117; Zebrafish Col1a2: NP_892013; Human COL3A1: AAL13167; Mouse COL3A1: NP_031763; Dog COL3A1: XP_851009; Cow COL3A1: XP_588040; Frog COL3A1: AAH60753; Human COL5A2: NP_000384; Mouse COL5A2: NP_031763; Rat COL5A2: XP_343565; Dog COL5A2: XP_535998; Cow COL5A2: XP_581318; Pig COL5A2: BAD91584; Chick COL5A2: XP_421846; Sea urchin: NP_999675. acorn worm DQ233249; Human COL5A1: NP_000084; Mouse COL5A1: NP_056549; Chick COL5A1: NP_990121; Human COL5A3: NP_056534; Mouse COL5A3: P25940; Human COL11A1: NP_001845; Mouse COL11A1: NP_031755; Chick COL11A1: XP_422303; Human COL11A2: CAA20240;

Mouse COL11A2: NP_034056; Human COL24A1: NP_690850; Mouse COL24A1: NP_082046; Chick COL24A1: XP_422363; Human COL27A1: NP_116277; Mouse COL27A1: NP_079961; Chick COL27A1: XP_415514. The tunicate fibrillar collagen genes (clade A fibrillar collagen: ci0100150759; clade B fibrillar collagen: ci0100154301) were retrieved from the JGI Ciona intestinalis genome website (http://genome.jgi-psf.org/ciona4/ciona4. home.html).

Histology, Immunohistochemistry and *In Situ* Hybridization

Formalin-fixed hagfish specimens were washed in 70% ethanol, embedded in paraffin and sectioned (6μm). Sections were either used for immunohistochemistry or were stained with hematoxylin, eosin and fast green (Mallory's trichrome) using standard staining methods. Immunohistochemical staining for COL2A1 was carried out using an antibody against human COL2A1 protein, as described previously (Chapter 2) Whole-mount *in situ* hybridization of amphioxus embryos was performed following published methods (Shimeld, 1997).

Results

Identification of *Col2a1* in Hagfishes

We searched for an expressed *Col2a1* orthologue in hagfish using degenerate RT-PCR, and recovered a 2214bp cDNA fragment with a sequence that corresponds to the region between the major triple helix and the C-propeptide domains of gnathostome COL2A1. The deduced amino acid sequence of the hagfish clone was 76% identical to mouse COL2A1, and comparison with the two lamprey COL2A1 orthologues showed 80% identity to lamprey COL2A1a and 77% identity to lamprey COL2A1b. We next conducted molecular phylogenetic analyses using Bayesian phylogenetics (BP), minimum evolution (ME) and maximum likelihood (ML) methods. All three analyses placed the hagfish sequence within the vertebrate Col2a1 clade, supporting the designation of this gene as hagfish *Col2a1* (Figure 3-1, 3-2 and 3-3). Interestingly, each tree further positioned hagfish COL2A1 as a sister to lamprey COL2A1a,

with lamprey COL2A1b falling out as the sister branch (Figure 3-1, 3-2 and 3-3). The topology of these trees suggests that *Col2a1* was present in the common ancestor of agnathans and gnathostomes, and that an additional duplication gave rise to *Col2a1a* and *Col2a1b* in the cyclostome (lampreys + hagfishes) lineage.

COL2A1 Localizes to Hagfish Soft Cartilage

We next asked whether *Col2a1* is expressed in hagfish cartilage. Little is known about hagfish embryonic development because of difficulties in obtaining fertilized embryos (Ota and Kuratani, 2006; Powell, 2005; Wicht and Northcutt, 1995). *Eptatretus* embryos were last collected in 1930 (Wicht and Northcutt, 1995) and only three embryos of *Myxine* have ever been found (Ota and Kuratani, 2006; Powell, 2005). The unavailability of embryos therefore precludes analysis of hagfish *Col2a1* expression during development, however COL2A1 protein is known to be detectable in adult cartilage of gnathostomes and lampreys (Hamerman, 1989). We therefore investigated whether COL2A1 is present in hagfish cartilage using a human antibody against the highly specific N-terminal region of COL2A1 (Cotrufo et al., 2005; Guo et al., 2004; Yang et al., 2003). Immunohistochemical analysis revealed the presence of COL2A1 in the extracellular matrix of several cartilage elements in the hagfish head and tail (Figure 3-4). COL2A1 protein was also detected in the notochord (data not shown), which is known to contain collagen fibers (Welch et al., 1998). Although a number of cartilage elements in hagfish were rich in COL2A1 protein (Figure 3-4 A' and B'), others showed a mosaic distribution (Figure 3-4 C') or lacked COL2A1 altogether (Figure 3-4 D'). Hagfishes have been described as having two types of cartilage; "soft" cartilage contains 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 (Cole, 1905a; Robson et al., 2000). Cartilage elements that were positive for COL2A1 had the cellular

characteristics of "soft" cartilage (Figure 3-4 A, A', B and B'), whereas cartilages that lacked COL2A1 exhibited the features of "hard" cartilage (Figure 3-4 D and D'). Elements that showed a mosaic distribution of COL2A1 also showed a mosaicism of "hard" and "soft" features, and COL2A1 was restricted to the "soft" regions of these structures (Figure 3-4 C and C'). Hagfish "soft" cartilage has been proposed to be structurally similar to lamprey cartilage (Robson et al., 2000), and our finding that each is composed of COL2A1 protein supports this idea. The presence of COL2A1 in the cartilages of hagfishes, lampreys (Chapter 2) and gnathostomes (Yan et al., 1995) strongly suggests that their last common ancestor had a COL2A1-based endoskeleton.

Duplication and Divergence of Clade A Fibrillar Collagen Genes Occurred in Stem Vertebrates

COL2A1 belongs to the clade A fibrillar collagen family, which includes collagen types I, II, III and Va2 (Aouacheria et al., 2004). In order to investigate the relationship between evolution of the clade A collagens and the origin of the vertebrate skeleton, we extended our analysis to lancelets, a sister group to the vertebrates (Zeng and Swalla, 2005). Comparative studies of a multitude of genes in lancelets and vertebrates show that the lancelet lineage diverged prior to the duplication events that occurred in the vertebrate genome (Panopoulou et al., 2003). We screened for lancelet fibrillar collagen cDNAs using degenerate RT-PCR, and isolated a 2196bp clone with a deducted amino acid sequence that is 54% identical to mouse COL2A1 and 53% identical to mouse COL1A1 and COL3A1. To determine the relationship of the lancelet clone to the vertebrate fibrillar collagen proteins, we carried out molecular phylogenetic analyses using C-propeptide of clade A, B and C fibrillar collagens. BP, ML and ME methods each supported its designation as a clade A fibrillar collagen (*AmphiColA*; Figure 3-5). We then used the C-propeptide and triple helix domains to further refine the position of

AmphiColA within the chordate clade A family, and all three methods placed it as the sister clade to the vertebrate (including cyclostome) clade A collagens (Figure 3-1). These results suggest that duplication of the ancestral *ColA*, the precursor of the clade A fibrillar collagen multi-gene family, occurred in the vertebrate lineage after the divergence of lancelets.

AmphiColA is Expressed in the Lancelet Notochord and Neural Tube

To gain insight into the expression pattern of the ancestral *ColA* gene, we investigated the developmental expression of *AmphiColA* by RNA *in situ* hybridization on lancelet embryos. During neurulation, *AmphiColA* was expressed in the notochord (Figure 3-6 A). At 30 hr post-fertilization, *AmphiColA* remained in the notochord, and a new domain of expression was detected in the neural tube (Figure 3-6 B). By 36 hr, *AmphiColA* expression was being downregulated in the middle third of the neural tube and notochord, but remained strong anteriorly and posteriorly (Figure 3-6 C). In 5-day old larvae, *AmphiColA* was detected in the tail bud and in the anterior region of the neural tube, up to the base of the cerebral vesicle (Figure 3-6 D). Expression of *AmphiColA* in the lancelet notochord and neural tube is strikingly similar that of clade A fibrillar collagens in gnathostomes and lampreys. We did not detect *AmphiColA* in the embryonic and larval pharynx by *in situ* hybridization, although Rychel *et al*. showed that the gill bars of adult cephalochordates can be stained with a chicken COL2A1 antibody (Rychel et al., 2006). Our phylogenetic analyses indicate that AmphiColA is not a strict orthologue of COL2A1, but rather is a sister to the entire vertebrate COLA family, and whether the COL2A1 antibody can recognize AmphiColA protein is unclear. Nonetheless, our results cannot exclude the possibility that *AmphiColA* is activated in the pharynx during metamorphosis, when the gill region undergoes extensive remodeling.

Discussion

COL2A1-Based Cartilage is a Shared Character of Crown-group Vertebrates

Type II collagen based cartilage has long been considered a unifying character of gnathostomes that separates them from lampreys and hagfishes (Wright et al., 2001; Wright and Youson, 1983). Recently, we reported that lampreys possess two *Col2a1* orthologues, and that adult lamprey cartilage is composed of COL2A1 protein (Chapter 2). Here we have gone on to show that hagfishes, the sister group to lampreys, also possess a *Col2a1* orthologue, and we demonstrate that COL2A1 protein is localized to their soft cartilage. Taken together, these results suggest that the common ancestor of all crown-group (the living jawed and jawless) vertebrates had COL2A1-based cartilage. Presence of an undifferentiated clade A fibrillar collagen in lancelets and tunicates (Wada et al., 2006) suggests that the expansion of the COLA gene family occurred in stem vertebrates after the divergence of lancelets and tunicates. Thus, COL2A1-based cartilage is a synapomorphy of all crown-group vertebrates.

Our finding that hagfish "hard" cartilage lacks COL2A1 indicates that hagfishes also possess some non- COL2A1 based cartilage. This finding highlights a relationship between the profile of collagen expression and the structure of skeletal tissues in hagfishes. In gnathostomes, cartilage matrix is composed predominantly of COL2A1 whereas bone matrix is mostly COL1A1. During endochondral ossification, which involves the transition from cartilage to bone, COL2A1 is replaced by COL1A1 in the skeletal matrix. Our observation that a single skeletal element in the hagfish can have a mosaic structure (both "soft" and "hard" cartilage) that corresponds to mosaic distribution of COL2A1 raises the question of whether one type of cartilage may develop from the other, perhaps by altering the proportion of COL2A1 relative to other types of collagen or non-collagen matrix proteins. Alternatively, "soft" and "hard" cartilage elements may arise from distinct chondrocyte lineages (COL2A1-positive and

negative), with mosaic cartilages having a mixed lineage. Further characterization of hagfish hard cartilage will clarify whether it is composed of other types of collagen or whether this subset of the skeleton is entirely non-collagenous.

Did vertebrate Chondrocytes Evolve from the Notochord?

It has been suggested that the notochord may represent a primitive form of cartilage, based on their many shared structural, cellular and molecular properties, and that vertebrate chondrocytes may have evolved from notochordal cells (Cole and Hall, 2004a; Stemple, 2005). In gnathostomes, the notochord and/or notochordal sheath expresses most of the vertebrate clade A fibrillar collagen genes (Dubois et al., 2002; Ghanem, 1996; Yan et al., 1995; Zhao et al., 1997). Our finding that *AmphiColA* is expressed in the notochord and notochordal sheath of *Branchiostoma floridae*, taken together with the recently reported data on *Ciona intestinalis CiFCol1* and *Branchiostoma belcheri BbFCol1* (both clade A fibrillar collagens), supports the idea that an ancestral *ColA* gene was expressed in the notochord of stem-group chordates (Robson et al., 2000; Satou et al., 2001; Wada et al., 2006). We suggest that duplication and divergence of the clade A collagen genes in stem-group vertebrates may have facilitated the evolutionary origin of chondrocytes from notochordal cells. This hypothesis deals specifically with the origin of vertebrate chondrocytes, and it is important to note that cartilage is also found in several invertebrates, including cephalopods, snails, and horseshoe crabs (Cole and Hall, 2004a; Wright et al., 2001). Future work on the molecular basis of invertebrate chondrogenesis should reveal whether fibrillar collagens also were utilized in these independent evolutionary events.

Clade A fibrillar Collagen Duplication Facilitated Evolution of the Vertebrate Skeleton

The Identification of an undifferentiated, ancestral *ColA* gene in lancelets indicates that the duplication event that gave rise to *Col2a1* occurred after the divergence of lancelets and

vertebrates. We have focused our analysis on hagfishes and lancelets, however it has recently been suggested that tunicates are the closest sister group to vertebrates (Delsuc et al., 2006). As noted above, tunicates also have an undifferentiated *ColA* gene (Wada et al., 2006), indicating that the duplication also post-dates their divergence from the lineage leading to vertebrates. Taken together, the data show that the origin of *Col2a1* from the ancestral *ColA* gene must have occurred in stem vertebrates. We propose that this duplication event may have been critical for the origin of the vertebrate skeleton (Figure 3-7).

Our data fit with the hypothesis that at least one round of genome duplication occurred between the origin of chordates and the origin of vertebrates (Boot-Handford and Tuckwell, 2003; Panopoulou and Poustka, 2005). The expansion of the clade A fibrillar collagen gene family, particularly the origin of *Col2a1* and *Col1a1*, may account for the unique skeletal matrices of vertebrate cartilage and bone. Although cartilage has evolved multiple times in metazoa, it is unclear from the fossil record whether cartilage or bone evolved first in the vertebrates (Donoghue et al., 2006a). Our results raise the possibility that *Col1a1* and *Col2a1* arose from the same duplication event, and thus, the major matrix components of bone and cartilage may have evolved at the same time. Finally, it is noteworthy that clade A fibrillar collagens are physically linked with *Hox* gene clusters in vertebrates and in echinoderms (Cameron et al., 2006). Duplication of the clade A fibrillar collagen genes therefore may have coincided with the *Hox* cluster duplications (Bailey et al., 1997; Morvan-Dubois et al., 2003). Coordinated expansion of the *ColA* and *Hox* gene families, respectively, may have facilitated the diversification of vertebrate connective tissue types and provided a mechanism for differentially patterning them.

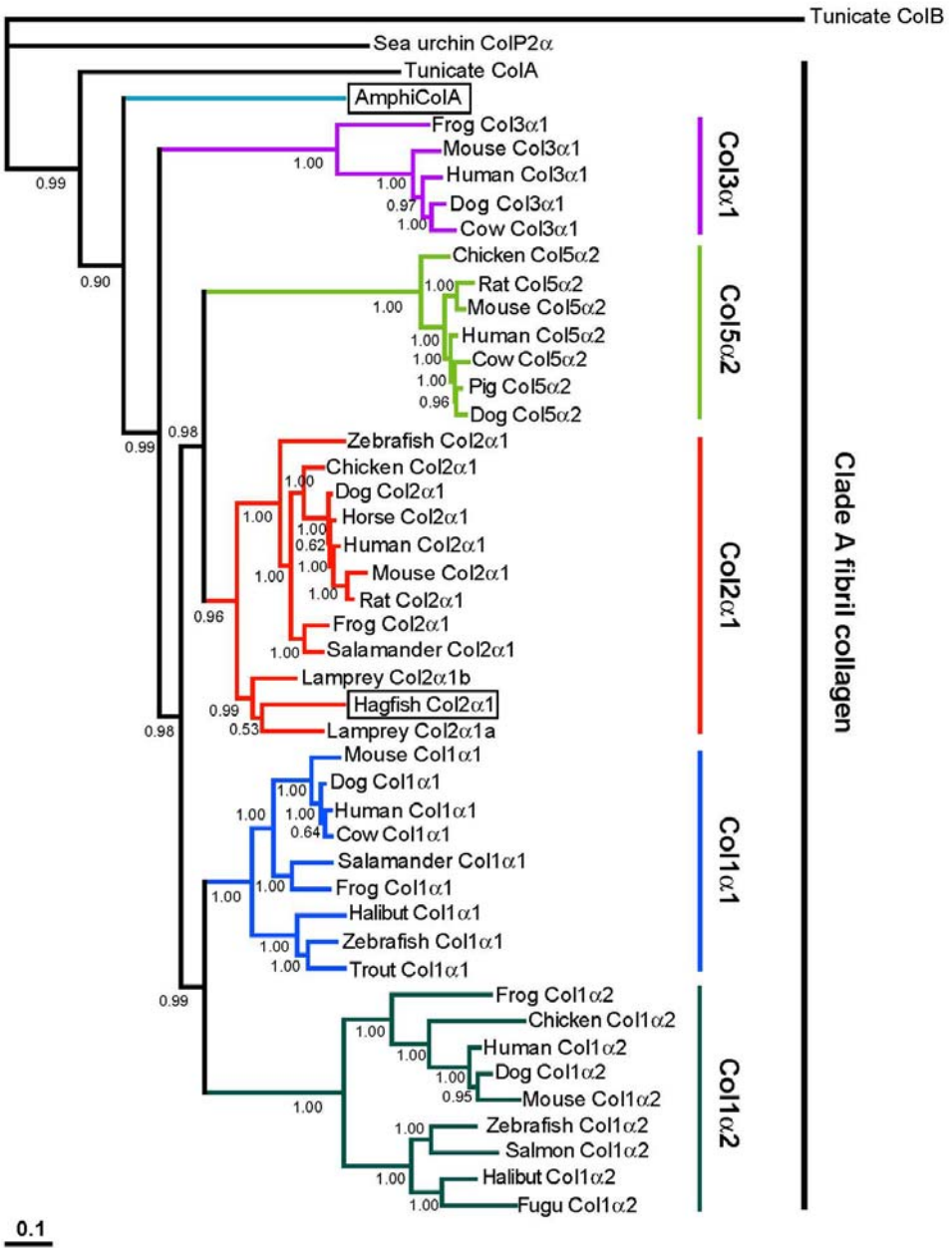

Figure 3-1. Extended majority-rule consensus tree for the Bayesian phylogenetic analysis of clade A fibrillar collagen proteins. Numbers at each node indicate posterior probability values based on 1,000,000 replicates. Branch lengths are proportional to means of the posterior probability (pp) densities for their expected replacements per site. The tree is rooted by tunicate (*Ciona intestinalis)* clade B fibrillar collagen (COLB) and sea urchin fibrillar collagen (COLP2a). Hagfish COL2A1 (boxed) is grouped with lamprey COL2A1a and COL2A1b with a pp of 0.99. This cyclostome COL2A1 clade joins to the base of the gnathostome COL2A1 clade with a pp of 0.96. Lancelet clade A fibrillar collagen (AmphiColA, boxed) is joined to the vertebrate clade A collagen family with a pp of 0.99. ML and ME methods confirm these positions for hagfish COL2A1 and AmphiColA (see also Figure 3-2. and 3-3).

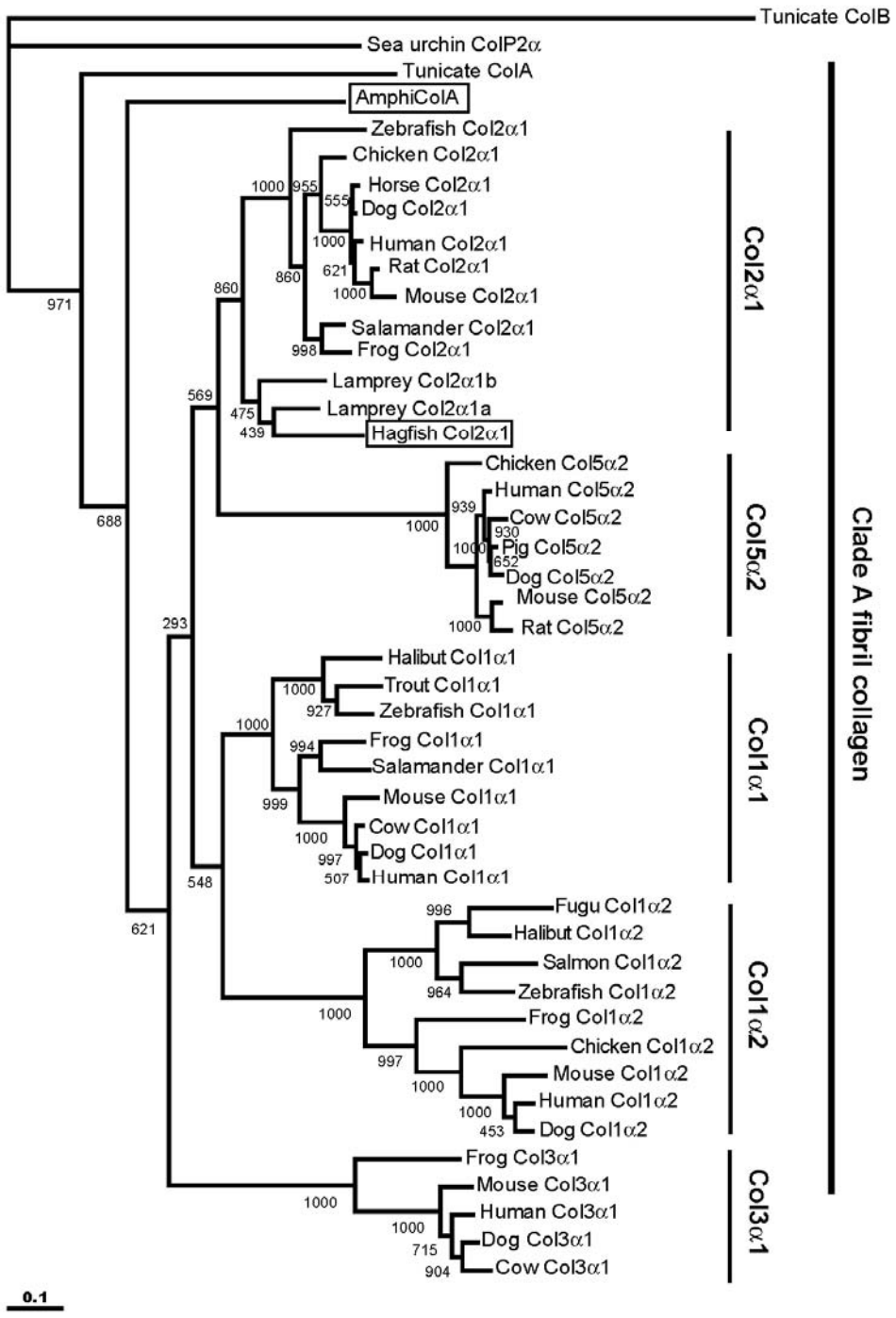

Figure 3-2. Maximum likelihood phylogeny of fibrillar collagen A proteins, as obtained with WAG plus gamma distances. Numbers indicate bootstrap values for each node, based on 1000 replicates. Branch lengths are proportional to expected replacements per site. The tree is rooted by tunicate (*Ciona intestinalis)* clade B fibrillar collagen (COLB). Consistent with BP analysis, the hagfish sequence is placed within the COL2A1 clade, as a sister of lamprey COL2A1a. The lancelet clade A fibrillar collagen (AmphiColA) joins to the root of the vertebrate clade A fibrillar collagens.

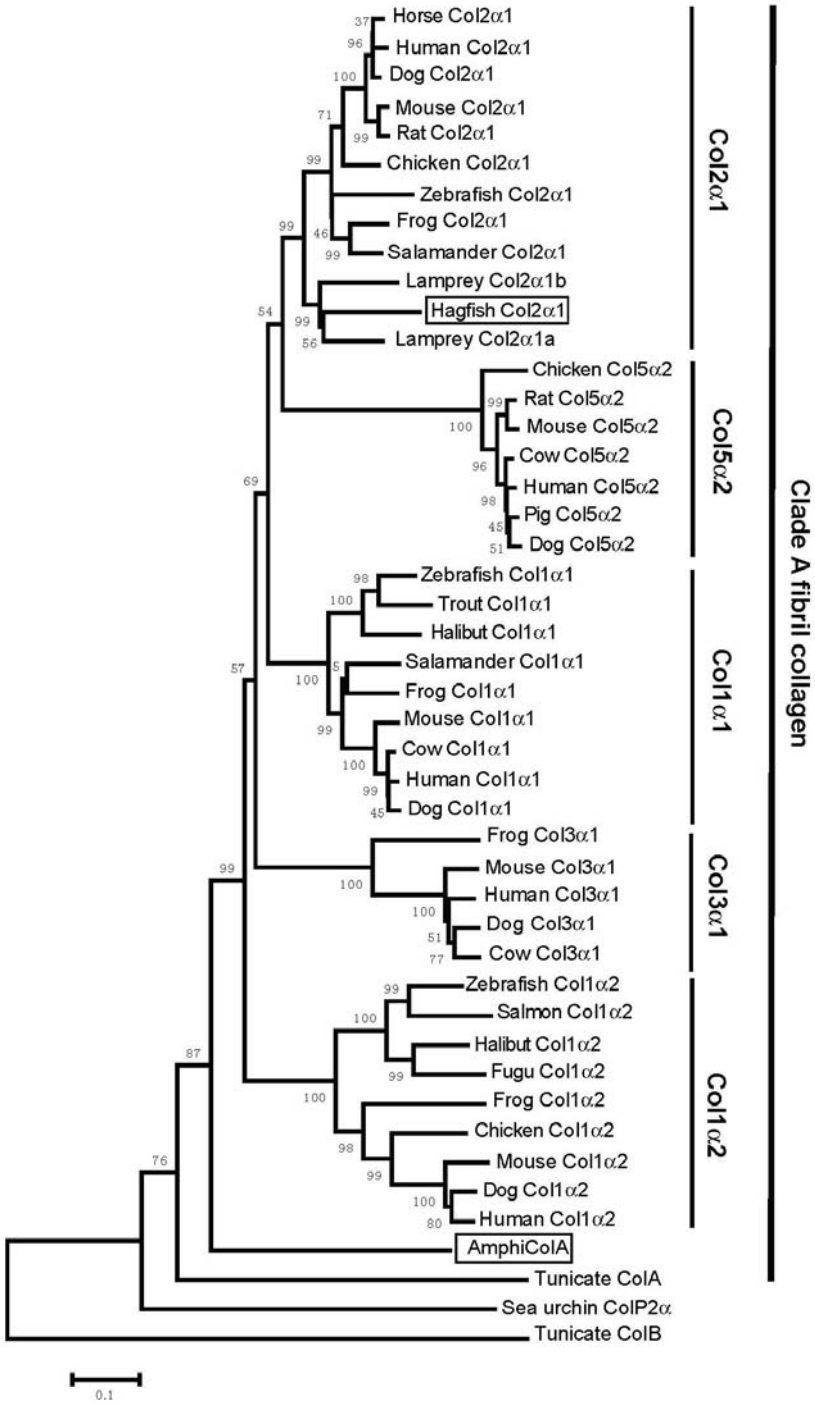

Figure 3-3. Minimum evolution phylogeny of fibrillar collagen A proteins as obtained with WAG plus gamma distances (a=0.906). Numbers indicate bootstrap values for each node, based on 10,000 replicates. Branch lengths are proportional to expected replacements per site. The tree is rooted by tunicate (*Ciona intestinalis)* clade B fibrillar collagen (COLB). The phylogenetic positions of hagfish COL2A1 and AmphiCOLA are consistent with the BP and ML analyses shown in Figure 3-1. and 3-2..

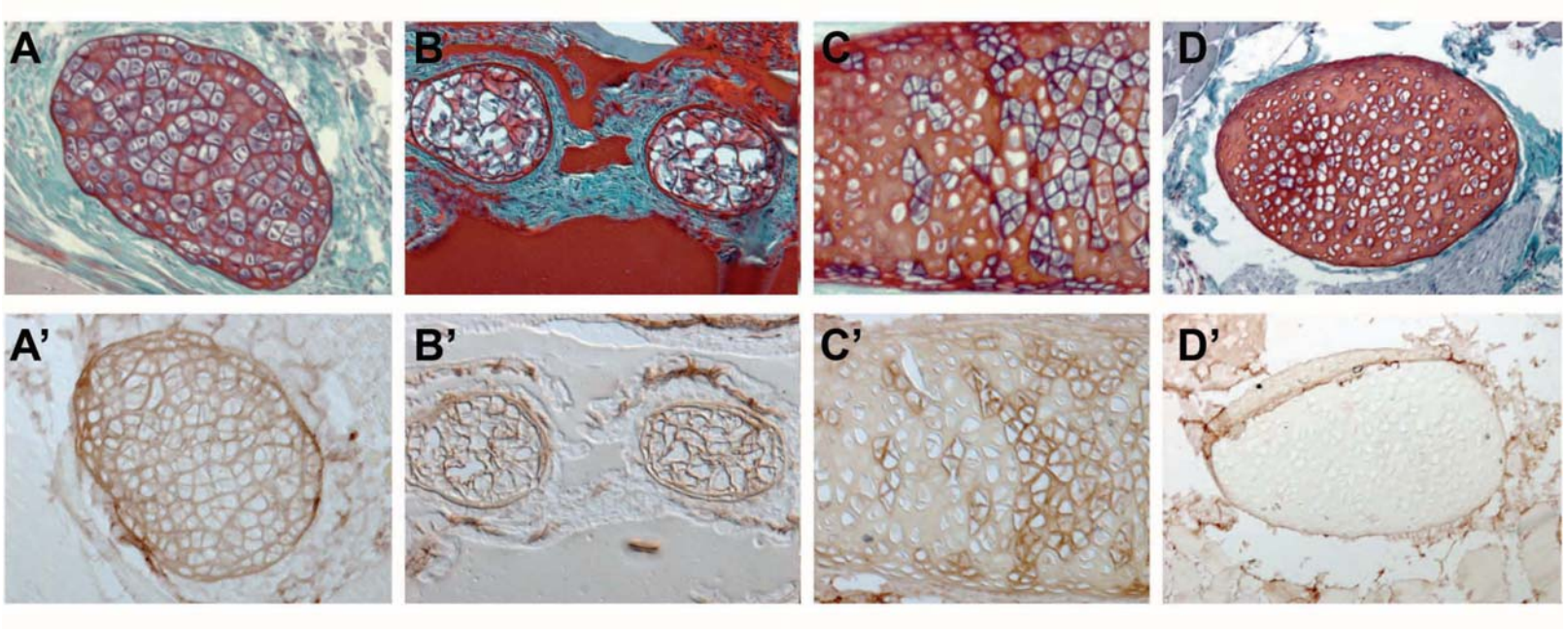

Figure 3-4. COL2A1 in cranial and tail fin cartilages of Atlantic hagfish. Transverse sections through adult hagfish stained with hematoxylin, eosin and fast green (A-D) or with COL2A1 antibody (A'-D'). (A, A') "Soft" cranial cartilage stains with hematoxylin, contains large hypertrophic chondrocytes surrounded by thin layer extracellular matrix (A), and is positive for COL2A1 (A'). (B, B') Section through caudal fin shows cartilaginous fin rays with "soft" cartilage characteristics (B) that are rich in COL2A1 (B'). (C, C') Cranial cartilage element exhibiting a mosaic distribution of "soft" (blue-stained chondrocytes) and "hard" (red-stained chondrocytes) cartilage (C). Note that COL2A1 protein is restricted to the "soft" cartilage region (C'). (D, D') "Hard" cranial cartilage stains with eosin but not hematoxylin, contains small chondrocytes surrounded by thick layer of extracellular matrix (D), and is negative for COL2A1 (D').

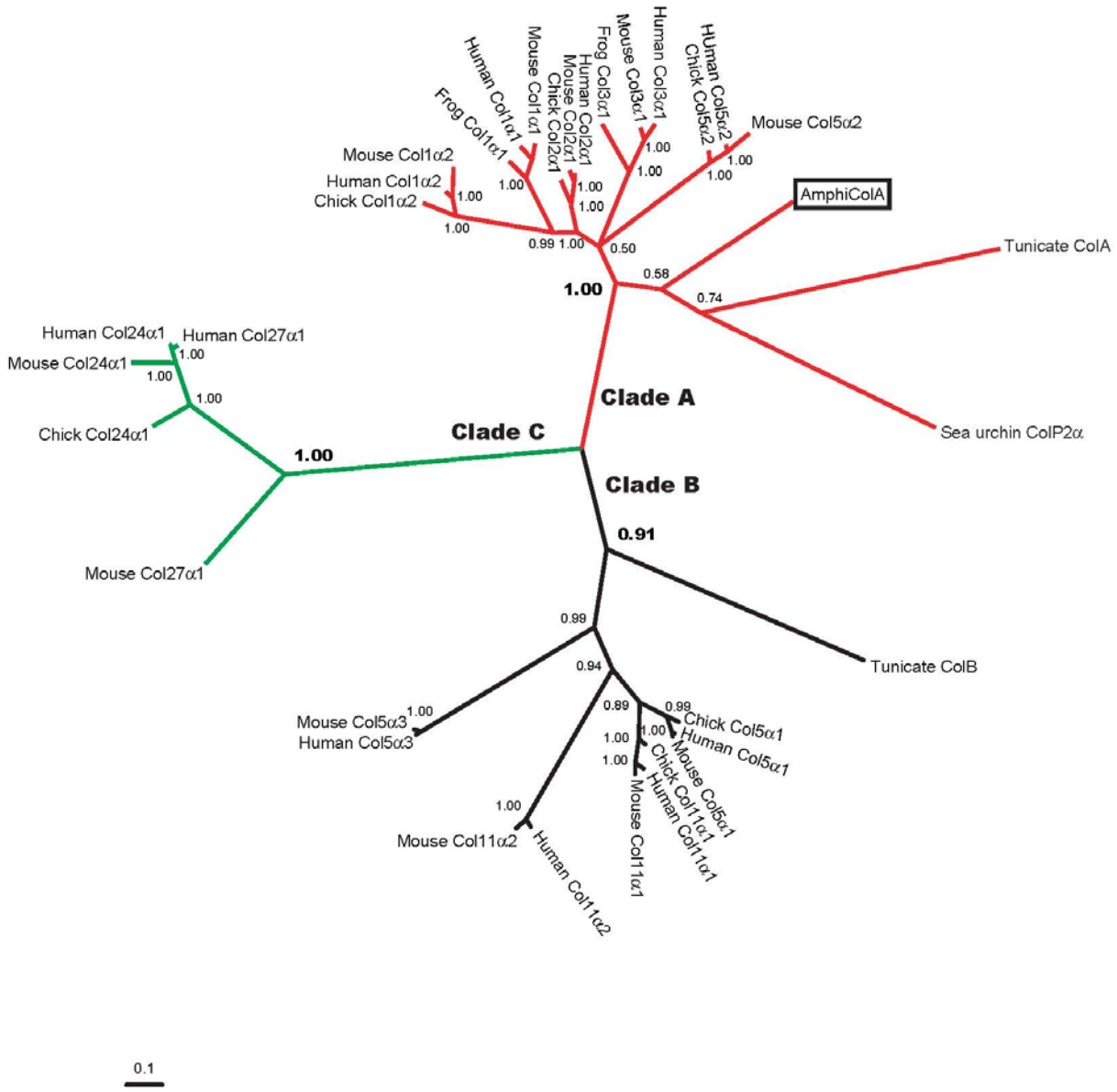

Figure 3-5. Unrooted extended majority-rule consensus tree for the Bayesian phylogenetic analysis of fibrillar A, B and C collagen proteins. Numbers at each node indicate posterior probability values based on 1,000,000 replicates. Branch lengths are proportional to means of the posterior probability (pp) densities for their expected replacements per site. AmphiCOLA joins the fibrillar collagen A clade, which is separated from the B and C clades with a pp of 1.00. ML and ME methods support this grouping of the clade A proteins, with bootstrap values of 97% and 85%, respectively.

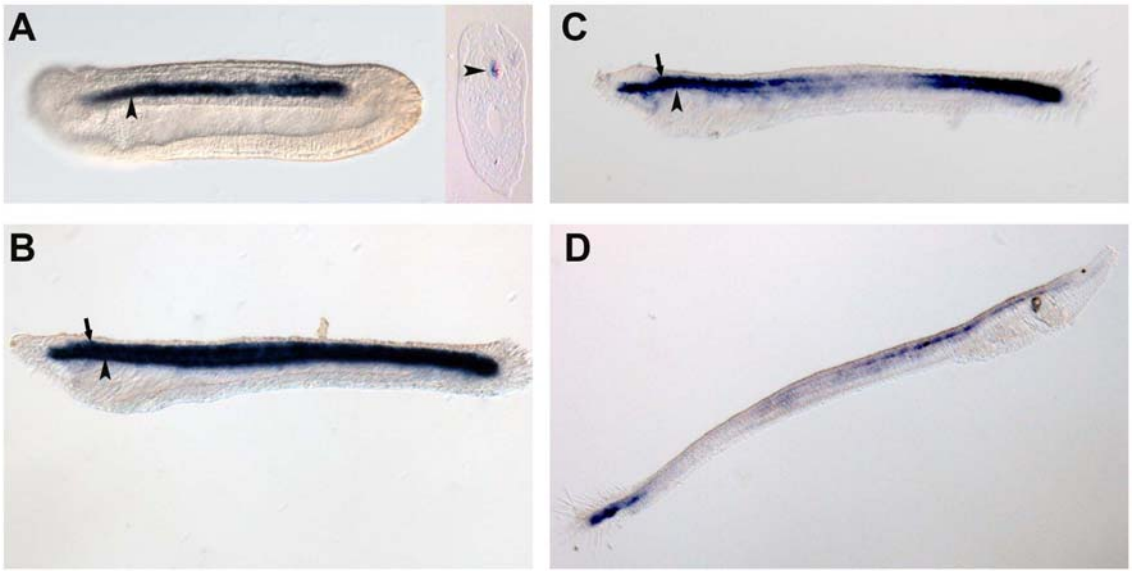

Figure 3-6. Expression of *AmphiColA* during lancelet development. Whole-mount *in situ* hybridizations of lancelet embryos with an *AmphiColA* antisense riboprobe. Anterior is to left in A-C and to the right in D. Stages shown are 18 hr (A), 30 hr (B), 36 hr (C) and 5 days (D) post-fertilization. Inset in A shows transverse section of 18 hr embryo. Arrowhead indicates notochord and arrows indicate neural tube.

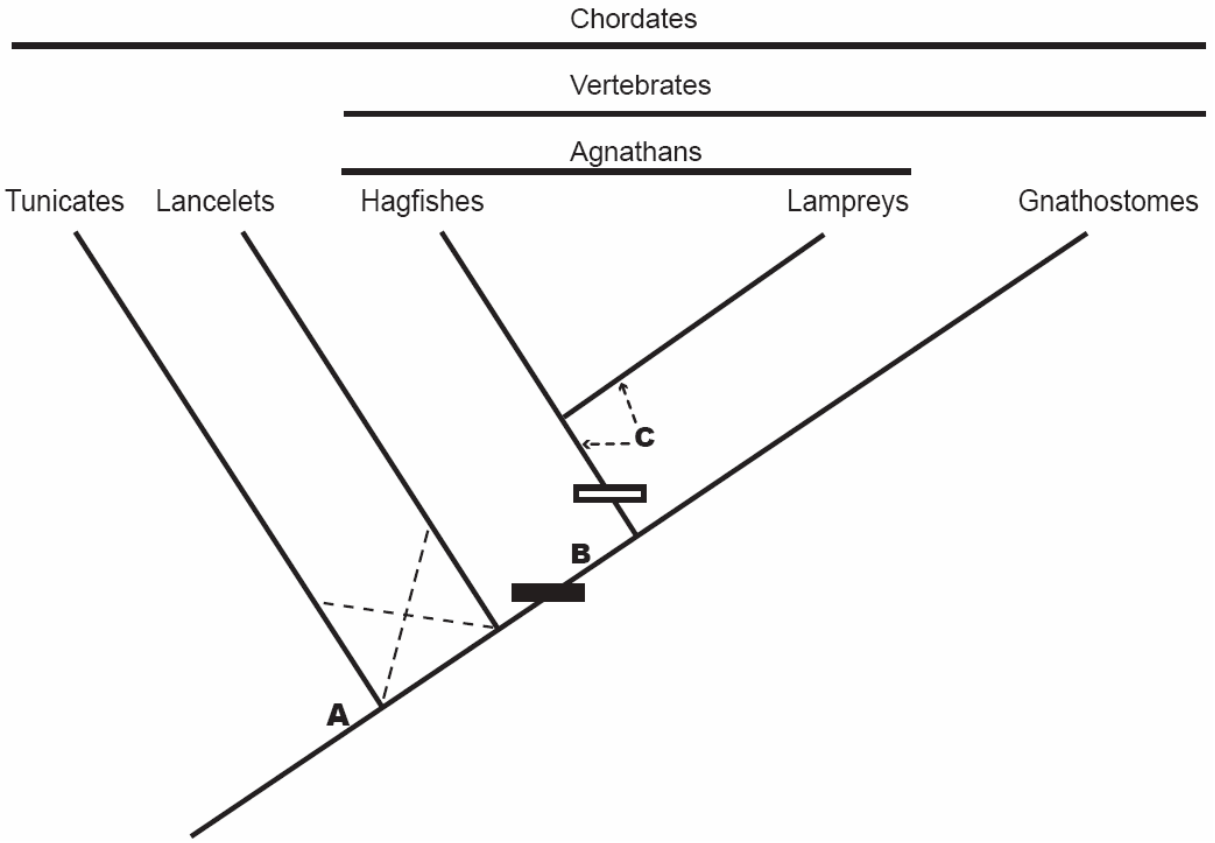

Figure 3-7. Origin of type II collagen-based cartilage. Phylogenetic relationships of tunicates, lancelets, hagfishes, lampreys and gnathostomes (Blair and Hedges, 2005; Donoghue et al., 2006b). Dashed lines indicate alternative branch positions (Delsuc et al., 2006). Letters indicate major steps in Col2a1-based cartilage evolution. Solid rectangle indicates origin of *Col2a1* gene by *ColA* gene duplication(s). Hollow rectangle indicates origin of *Col2a1a* and *Col2a1b* by *Col2a1* gene duplication A. Ancestral clade A collagen gene expression in notochord of stem chordates. B. Origin of COL2A1 based cartilage in stem vertebrates. C. Subfunctionalization of *Col2a1*, in which expression domain of ancestral gene is partitioned between the duplicate genes *(Col2a1a* and *Col2a1b)*; arrows indicate possible positions of this event.

CHAPTER 4
MOLECULAR IDENTIFICATION OF A SCLEROTOME IN LAMPREYS AND SHARKS: IMPLICATIONS FOR THE ORGIN OF THE VERTEBRAL COLUMN

Introduction

The vertebral column is one of the key characters that define vertebrates. A conserved feature of gnathostome vertebrae is the general design of a centrum, the central of the vetrebrae, a dorsal pair of neural arches surrounding the spinal cord, and a dorsal neural spine. Actinopterygians and chondrichthyans also possess a ventral pair of hemal arches and a hemal spine. In tetrapods, the vertebral column is differentiated into five regions: cervical, thoracic, lumbar, sacral and caudal. The structure and number of vertebrae shows a great diversity across taxa (Figure 4-1A). The phylogenetic transitions of axial structures are summarized in Figure 4-1A. This diversity is not only of interest for taxonomic purposes but also fascinating for understanding vertebrate morphological novelties from perspective of the phylogenetic change. The notochord serves as the primary functional axial supporting structure in all chordate embryos, although its fate is strikingly different in adults of different taxa. In chondrichthyans and bony fishes, the notochord persists, giving rise to the centra, whereas in amniotes does not contribute to vertebrae but instead forms a component of the intervertebral disk.

Embryonically, vertebrae develop from the sclerotome, the ventromedial part of somites. Most of our knowledge of vertebral developmental mechanisms derives from studies in amniotes, especially in chicken and mice (Christ et al., 2004; Christ et al., 2007; Scaal and Christ, 2004; Scaal and Wiegreffe, 2006). The paraxial mesoderm is segmented into somites, and somites are further divided into two parts, ventromedial sclerotome, which forms the vertebrae, and dorsolateral dermomyotome which give rises to the dermis and skeletal muscle of the trunk and limbs (Figure 4-1B). Ventromedial cells lose their epithelial characters aggregating as loose mesenchyme of the sclerotome. These sclerotomal cells migrate toward and

envelop the notochord, where they develop into the vertebral bodies and the connective tissue. The lateral sclerotomal cells contribute to the neural arches and ribs (Brand-Saberi and Christ, 2000). This specific subdivision of the somites depends on the relative positions to notochord and neural tube (Dockter and Ordahl, 2000; Pourquie et al., 1993).

Based on grafting experiments in chicken, it was reported that there are two midline signaling centers which are important in designating vertebral cell fates. The ventral signaling center consists of the floor plate of neural tube and notochord, which secrete the morphogen protein sonic hedgehog (Shh) and Noggin. The dorsal signaling center is located in the dorsal neural tube and surface ectoderm, which both secrete bone morphogen proteins (BMPs) and Wnts (Dietrich et al., 1997; Dockter and Ordahl, 2000; Monsoro-Burq and Le Douarin, 2000). *Shh* and *Noggin* signals promote sclerotome cell fate, whreas *BMPs* and *Wnts* have the opposite functions, thus the dorsalizing and ventralizing signals work antagonistically (Dietrich et al., 1997). The responses of the somite cells to the two signal centers are dose dependent, such as the cells close to notochord are induced to sclerotomal fate. The dorsal domain of Bmp also functions to induce differentiation of the spinous processes (Monsoro-Burq et al., 1994; Monsoro-Burq et al., 1996; Watanabe et al., 1998).

Sclerotome is defined both by its mesenchymal organization and expression of a network of sclerotomal genes (Christ et al., 2007). Two group I *Pax* genes, *Pax1* and *Pax9*, are expressed in similar, though not identical patterns. The *Pax1* first is detectable just before the de-epithelialization in the early developing ventral sclerotomes. This makes *Pax1* an identical sclerotome marker. *Pax9* expression begins a little bit later than *Pax1*. As the sclerotome differentiates, *Pax1* and *Pax9* are limited to the ventromedial and ventrolateral compartments of sclerotome. Both genes are downregulated once the sclerotomal cells undertake the

chondrogenenic process (Peters et al., 1995; Peters et al., 1999). Knockout mouse experiments have shown that *Pax1* is required for vertebral development, but *Pax9* is not necessary. Mice null for both *Pax1* and *Pax9* showed more severe vertebral defects than the individual knockouts (Peters et al., 1998; Peters et al., 1999). Similarly, another pair of homeobox transcription factors, *NK3.1* and *NK3.2*/*Bapx1* have indistinguishable expression patterns in the early sclerotome (Herbrand et al., 2002). *Nk3.2* is first detected at similar stages as *Pax1* in the mouse. *Bapx1* knockout mice show a similar phenotype to *Pax1* and *Pax9* double knockout mice (Lettice et al., 1999; Tribioli and Lufkin, 1999). Later, it was shown that *Bapx1* is the direct target of *Pax1/9* (Rodrigo et al., 2003). The expression group I *Pax* genes and *Nk3* genes in sclerotome have been shown to be *Shh*-dependent (Dietrich et al., 1993; Kos et al., 1998; Koseki et al., 1993; Murtaugh et al., 2001; Neubuser et al., 1995). *Bapx1* is a key transcription factor that links both the development of the sclerotome and its later chondrogenesis, and it plays major roles in regulating *Sox9* and *Runx2*, both themselves regulators of chondrogenesis (Lengner et al., 2005; Zeng et al., 2002). The transcription factor *Sox9* is expressed in all chondroprogenitors and has an essential role in chondrogenesis, as forced expression of *Sox9* can induce chondrogenic commitment in mouse. *Sox9* can bind the promoter of *Col2a1*, aggrecan and other cartilage matrix genes (Bi et al., 1999; Lefebvre et al., 1997).

In addition to the Group I *Pax* genes, *Nk3* genes and chondrogenic genes, there are also some other widely used molecular markers of these sclerotome, including the bHLH transcription factors *FoxC1* and *FoxC2*, and the T-box gene *Tbx18* (Brand-Saberi and Christ, 2000; Dockter, 2000; Lettice et al., 2001). The *FoxC1* and *FoxC2* compound null mice have been shown to lack somites, as the paraxial markers *Paraxis*, *Pax1*, and *Meox1* cannot be detected (Kume et al., 2001). Twist, *Scleraxis* and *Paraxis* are closed basic helix-loop-helix

(bHLH) transcription factors expressed in the developing somites. In chickens and mice, *Scleraxis* is expressed in a subset of the sclerotome, which was recently given the name syndetome because it gives rise to tendon cells (Brent et al., 2003). The detailed regulatory relationships between these sclerotome markers are summarized in Figure 4-1C.

Lampreys, one of only two extent jawless fishes, possess paired cartilaginous nodes known as arcualia on either side of the notochord and the have been proposed to be homologs of the primitive axial skeleton (Gadow and Abbott, 1895; Gadow, 1933). No vertebral centra have been reported in lampreys, although the primitive gnathostome vertebral column is usually thought to consist of a persistent notochord and cartilaginous centra (Goodrich, 1930; Romer, 1985). The embryonic origin of these arcualia remains unclear. It was reported that Japanese lamprey somitic mesoderm does not express the group I *Pax* gene *LjPax9,* and this result suggested that lampreys may lack a sclerotome (Ogasawara et al., 2000). We recently found the lamprey cartilage matrix genes (*Col2a1a* and *Col2a1b*) and the chondrogenic regulatory gene (*Sox9*) expressed in the lamprey paraxial mesoderm (Zhang et al., 2006). This conserved chondrogenic genetic pathway in somites raises the possibility that the arcualia may develop from a sclerotome in lamprey. In order to test our hypothesis, here we cloned several members of the sclerotome gene network from both lamprey and catshark. By comparing the expression patterns of these scletome markers in both species, we propose lampreys indeed possess a sclerotome, and that acquisition of sclerotome may have coincided with the origin of the primitive cartilaginous axial skeleton.

Materials and Methods

Fish Embryos

Lesser spotted catshark (*Scyliorhinus canicula*) eggs were collected from Menai Strait (North Wales). Embryos were isolated from the eggshells, dissected from the yolk sac in ice-

cold phosphate buffered saline solution (PBS) and staged according to Ballard et al (Ballard et al., 1993) Adult mountain brook lamprey (*Ichthymyzon greeleyi*) were colleted from Ohio River branches (Kentucky, USA). The eggs were artificially fertilized and raised in filtered river water. European brook lamprey (*Lampetra planeri*) eggs were colleted from New Forest, England. The lamprey embryos were staged according to Tahara et al. (Tahara, 1988)**.**

Gene Cloning and Phylogenetic Analysis

Degenerate RT-PCR was performed to amplify catshark gene fragments using cDNA from a mixed stage. RNA was extracted using TRIzol Reagent (Invitrogen). cDNA was made by reverse transcription reactions using SuperScript II first-strand cDNA synthesis kit (Invitrogen). Degenerate primers for the scleromte marker genes used in these chapter were designed using CODEHOP program (http://bioinformatics.weizmann.ac.il/ blocks/ codehop.html) (Rose et al., 2003). Lamprey genes were isolated by RT-PCR from a *Petromyzon marinus* cDNA library (a gift from J. Langeland) using Advantage GC-PCR Kit (Clontech). The amplified fragments were cloned into PGEM-T Easy Vector (Promega) or pDrive Cloning Vector (Qiagen). Some gene fragments were expanded to full-length sequences by rapid amplification of cDNA ends (RACE). For gene diagnosis, each gene was identified primarily by BLAST searches. The sequence alignments were generated with Clustal X. Phylogenetic analysis were done by using both minimum evolution, maximum likelihood and Bayesian analysis methods (Zhang et al., 2006). All three methods resulted in similar topologies and supported the robustness of our results. The sequences reported in this paper were deposited in the Genbank (accession number: EU196399-EU196410). The following amino acid sequences were retrieved from GenBank for inclusion in our phylogenetic analyses: human PAX1, NP_006183; mouse PAX1, NP_032806; rat PAX1, XP_001056369.1; cow PAX1, XP_617873; dog PAX1, XP_542866; horse PAX1, XP_001492632; fugu PAX1, CAG09888, frog PAX1, NP_001090451; Japanese lamprey

PAX1/9, BAB12396; lancelet PAX1/9, AAA81364; human PAX9, NP_006185; dog PAX9, XP_547776; mouse PAX9, EDL36706; rat PAX9, NP_001034628; fugu PAX9, AAG44703; frog PAX9, AAH84222; platypus PAX9, XP_001511262; zebrafish Pax9a, NP_571373; zebrafish Pax9b, AAC60035; human FOXC1, NP_001444; mouse FOXC1, NP_032618; frog FOXC1, NP_001007864; zebrafish FOXC1a, NP_571803; zebrafish Foxc1b, NP_571804; human FOXC2, NP_005242; cow FOXC2, XP_872296; mouse FOXC2, Q61850; horse FOXC2, NP_001077066; frog, FOXC2, NP_998857; lancelet FOXC, CAH69694; human NK3.1, NP_006158; cow NK3.1, XP_001250882; dog NK3.1, XP_543240; rat NK3.1, NP_001029316; frog NK3.1, AAH47968; see urchin NK3, XP_784735; cnidarian NK3, AAP88431; cow Nk3.2, XP_874137; dog Nk3.2, XP_545940; mouse NK3.2, AAI45875; fugu NK3.2, CAG08309; chicken Nk3.2, BAB40713; salamander NK3.2, AAC08704; zebrafish Nk3.2, NP_835233; human TWIST, CAA62850; mouse TWIST, AAA40514; rat TWIST, NP_445982; frog TWIST, NP_001079352; chicken TWIST, AAF00072; zebrafish Twist, NP_571059; lancelet TWIST, O96642; human SCLERAXIS, NP_001073983; mouse SCLERAXIS, NP_942588; chicken SCLERAXIS, NP_989584; human PARAXIS, CAC00470; fugu PARAXIS, CAG03890; chicken PARAXIS, AAC60208; zebrafish Paraxis, NP_571047; mouse MYOD, AAA39798; frog MYOD, CAE18108;chicken MYOD, AAA74374; zebrafish MyoD, NP_571337; lancelet MYOD, AAR12639.

***In Situ* Hybridization and Cryosections**

The catshark and lamprey whole mount in situ hybridization was performed as described for chicken method (Nieto et al., 1996) with the following modifications. Both catshark and lamprey embryos were treated with proteinase K (10ug/ml) for 15-30min at room temperature. Levamisol (2 mM) was added to both KTBT and NTMT washes. The pre-hybridization and hybridization was performed at 70°C. For the color reaction, 10% dimethyl formamide (DMF)

was added to maintain NBT and BCIP in solution. For histological analysis, specimens were equilibrated in 15% sucrose then 30% sucrose in 20% gelatin, after which they were embedded in 20% gelatin for cryosectioning (10um).

Results

Isolation and Analysis of Lamprey and Catshark Genes

In order to identify molecular compartments of the lamprey and shark somites, we set out to isolate orthologs of genes that both mark the sclerotome and regulate the skeletogenic pathway in jawed vertebrate embryos. Using degenerate RT-PCR on cDNA pools and cDNA libraries from the catshark *S. canicula* and the lampre*y P. marinus* to isolate cDNA fragments, followed by RACE to obtain full-length cDNAs, we isolated 11 clones from catshark and 10 clones from lamprey with putative orthology. Phylogenetic analyses using Minimum Evolution, Maximum Likelihood, and Baysian Phylogenetic analysis resolved the identities of catshark PAX1, BAPX11, FOXC1, FOXC2, TWIST, SCLERAXIS, PARAXIS, TBX18, GLI2, and lamprey PAX1/9, BAPX1, FOXC1/2, Twist, Parascleraxis, TBX15/18, GLI1/2/3 (Figure 4-2, 4-3 and 4-4). We then conducted whole mount and section in situ hybridizations to map the expression patterns of these genes during somite development in catshark and lamprey embryos. Below we describe the results of these experiments.

Expression of Sclerotomal Markers in the Somites of Lampreys and Catsharks

Shark *Pax1* and lamprey *Pax1/9*

Group I *Pax* genes (*Pax1* and *Pax9*) define the sclerotomal compartment of the somites in osteichthyans. We examined the expression of *Pax1* in catshark embryos at stage 26 and observed segmental domains of expression in the somites, along with strong expression in the pharyngeal pouches (Figure 4-5A). Analysis of *Pax1* expression in transverse sections confirmed the somitic expression and revealed that it was restricted to the ventral-medial regions

of the somites, in a domain that surrounded the notochord and extended dorsally around the neural tube, consistent with a sclerotomal pattern of expression (Figure 4-5B). Surprisingly, *Lampetra Pax1/9* was also expressed in the somites at stages 23 and 25 as well as in the pharyngeal pouches at stage 25 (Figure 4-5 C and E). Transverse sections show that the somitic domains are restricted to the ventral-medial margins at stage 23 (Figure 4-5 D), and at stage 25 the domains surround the notochord and extend to the ventral aspect of the neural tube (Figure 4-5 F). Expression was also observed in the ventral neural tube (Figure 4-5 F). Thus, in sharks and lampreys, *Pax1/9* expression marks the region of the somite that corresponds to the sclerotome of osteichthyans.

Analysis of a purported *Pax9* gene in the Japanese lamprey, *Lethteron japonicum*, revealed expression in the pharyngeal pouches, however Ogasawara and co-workers reported it to be absent from the sclerotome (Ogasawara et al., 2000). We re-examined the *L. japonica* sequence in our phylogenetic analyses and find that it is a sister gene to *Pax1* and *Pax9* clades and, as such, should be designated *Pax1/9*. To determine whether expression in the sclerotome is unique to *Lampetra planeri*, we examined *Pax1/9* expression in another lamprey genus, *Ichthyomyzon greelei*. We find that in both lamprey genera examined, *Pax1/9* is expressed in the ventromedial region of the somites (Figure 4-5 G, H). It is therefore unclear whether the previous report simply missed this expression domain, or whether *Lethenteron japonica* is a particularly derived genus, which has lost the sclerotomal expression of *Pax1/9*.

Bapx1

Bapx1 (*Nkx3.2*) was reported to be a direct downstream target gene of *Pax1/9* in the mouse (Rodrigo et al., 2003). We therefore asked whether *Bapx1* is expressed in a pattern similar to *Pax1/9* in catshark and lamprey. Whole mount in situ hybridization of stage 25 catshark embryos showed clear expression in the somites along the entire trunk and in the pharyngeal

pouches (Figure 4-6 A). Sections through the trunk indicated that expression in the somites was confined to the sclerotome, in domains that extended ventrally around the notochord and dorsally adjacent to the ventral neural tube (Figure 4-6 B). The catshark *Bapx1* is limited around the midpoints of pharyngeal arches (Figure 4-6 C). In lamprey embryos at stages 23 and 25, whole mount in situ hybridization revealed *Bapx1* expression in the regions of the pharynx and brain, and in two post-cranial domains in the regions of the neural tube and ventral somites (Figure 4-6 D, F). We also found lamprey *Bapx1* broadly expressed in the pharyngeal arches (Figure 4-6 H). Transverse sections through the lamprey trunk at stage 23 confirmed that *Bapx1* transcripts were localized to the ventral-medial aspect of the somites and to the ventral neural tube (Figure 4-6 E, G). As in the shark, lamprey *Bapx1* expression was detected in the pharyngeal pouches**.**

Shark *FoxC1, FoxC2* and lamprey *FoxC1/2*

At stage 26, the expression of *FoxC1* and *FoxC2* in catshark embryos was indistinguishable from the patterns reported for chickens and mice (Figure 4-7 A-D) (Sasaki and Hogan, 1993). Transcripts for both genes were also detected in the gut epithelium and in the pharyngeal arches. *FoxC1* and *FoxC2* expression persisted as the sclerotomal region surrounding the notochord. (Figure 4-7 B, D). The lamprey *FoxC1/2* was expressed in the paraxial mesoderm as in mice and chickens (Figure 4-7 E-H). The strongest expression in the trunk occurred in the ventral-medial aspects of the somites at stage 25 (Figure 4-7 H). Analysis of the single *FoxC1/2* gene that we isolated from lampreys showed that it is expressed in the ventral-medial regions of the somites at stages 23 and 25, similar to the sclerotomal expression observed in catsharks and tetrapods (Figure 4-7 F, H).

Twist

Twist is anther important somite marker in vertebrates and it has been shown to play important roles during axial skeletal development in medaka (Yasutake et al., 2004). In

catsharks, *Twist* was detected in head mesoderm, somites and fin folds (Figure 4-8 A). Somitic expression was confined to the sclerotome, which persisted as these cells surrounded the notochord (Figure 4-8 B). In lampreys, *Twist* was also found in the head region and somites (Figure 4-8 C, E), and transverse sections through the trunk at stage 23 showed that it was expressed in the lateral somite in the neural tube (Figure 4-8 D). Lamprey *Twist* is expressed in the neural tube and ventromedial region of the somites in the sections through both the stage 23 and stage 25 (Figure 4-8 C-F).

Shark *Scleraxis*, *Paraxis* and lamprey *Parascleraxis*

We previously reported median fin expression of catshark *Scleraxis* and lamprey *Parascleraxis*, the sister gene to the gnathostome *Scleraxis* and *Paraxis* genes (Freitas et al., 2006). We expanded that analysis to the entire embryo and found that catshark *Scleraxis* was expressed in the head, the somites and neural tube (Figure 4-9 A-D). *Scleraxis* expression in the somites was confined to the sclerotomal region (Figure 4-9 B, D), in a broader domain than reported for chick and mouse, where it marks the syndetome, which gives rise to tendon (Brent et al., 2003). Catshark *Paraxis* was found in newly developed somites (Figure 4-9 E), as reported in tetrapods. Both *Scleraxis* and *Paraxis* were expressed in the catshark neural tube (Figure 4-9 B, D and F), which was not reported in chicken and mice. In lampreys, we failed to isolate strict orthologs of the *Scleraxis* and *Paraxis* genes, but we previously identified an undifferentiated gene, which we named *Parascleraxis,* that is a sister to the *Scleraxis* and *Paraxis* clade (Freitas et al., 2006). Lamprey *Parascleraxis* was expressed throughout the somites and did not show compartmentally restricted expression (Figure 4-9 G-J). Lamprey *Parascleraxis,* like *Paraxis* and *Scleraxis* of the shark, also was detected in the neural tube (Figure 4-9 H and J).

Shark *TBX18* and lamprey *Tbx15/18*

Tbx18 is expressed in the anterior halves of developing somites in mouse and zebrafish (Begemann et al., 2002; Kraus et al., 2001), and we reported previously that catshark *Tbx18* and lamprey *Tbx15/18* (a sister to the *Tbx15/18* clade) are expressed in the median fins of both organisms (Freitas et al., 2006). Analysis of their expression patterns during somitogenesis shows that both genes are expressed in the ventral-medial region of the somites, immediately adjacent to the notochord in lamprey and catshark (Figure 4-10 B and D). In catsharks, we also noted *Tbx18* expression in the midbrain, hindbrain, heart, pectoral fin and around the eye and ear (Figure 4-10 A). Lamprey *Tbx15/18* was observed in the head and in the ventral neural tube (Figure 4-10 C and D). Anterior-posterior polarity of the somites was clearly seen in catshark although it was not apparent in lamprey.

Shark *Gli2* and lamprey *Gli1/2/3*

Gli1 and *Gli2* are expressed in the sclerotome and *Gli3* is expressed throughout the somite in mouse embryos (Buttitta et al., 2003). *Gli2* and *Gli3* mediate *Shh* induction of sclerotome, and are required for activation of *Pax1* and *Pax9* (Sasaki et al., 1999). Since expression of *Pax1/9* is conserved in lamprey and catshark (Figure 4-5), we investigated *Gli2* expression in both species. We found that both catshark *Gli2* and lamprey *Gli1/2/3* were expressed in the ventral-medial region of the somites, consistent with the sclerotomal pattern described for jawed vertebrates (Figure 4-11). Catshark *Gli2* and lamprey *Gli1/2/3* were also detected, excluded from floor plate in the neural tube, which is consistent with the pattern reported from tetropods. (Figure 4-11 B and D).

Discussion

The vertebral column is a key defining character for all vertebrates. In higher vertebrates, the vertebral columns are derived mainly from sclerotome. In lampreys, arcualia were purported

to be homologs of vertebral cartilages by comparative anatomists of the last century, who also suggested that lampreys might have a small sclerotome (Brand-Saberi and Christ, 2000; Maurer, 1906; Scott, 1882). However a recent study of the Japanese lamprey *LjPax9* gene showed that this sclerotome marker is absent in paraxial mesoderm (Ogasawara et al., 2000). This finding suggested that lampreys might lack a sclerotome. In addition, the major matrix components of lamprey cartilage were reported to be elastin-like molecules, named lamprin, instead of the Col2a1 protein found in jawed vertebrates (Wright et al., 2001). This chondrogenic difference suggested that lamprey cartilage development might involve a different genetic program. Thus, the embryonic origin of arcualia remained puzzling. Recently, we found the lamprey cartilage matrix contains *Col2a1* proteins and *Sox9*, which suggested that this chondrogenic genetic pathway is conserved in all vertebrates (lampreys and gnathostomes). More importantly, the *Col2a1* and *Sox9* genes in lamprey are also expressed in the positions of the arcualia (Zhang et al., 2006). Thus, the conserved chondrogenic pathway, the anatomical structure of these axial cartilages, and evidence from the early comparative embryology lead us to hypothesize that lamprey arcualia develop from sclerotome like the tetrapods.

Molecular Evidence for a Lamprey Sclerotome

Pax genes play important roles during animal development and all the *Pax* genes shared a conserved 128 amino acid paired domain. To date, in higher vertebrates, nine *Pax* genes have been classified into four subgroups based on these motifs (Dahl et al., 1997; Strachan and Read, 1994). Group I *Pax* genes include *Pax1* and *Pax9*, which possess an octapeptide in addition to the paired domain, but not a homeobox domain. *Pax1* and *Pax9* were all expressed in the pharyngeal slits in the living deuterostomes (Holland and Holland, 1996; Ogasawara et al., 2000; Ogasawara et al., 1999). In mouse, *Pax1* and *Pax9* are all expressed at the developing somites and play critical roles for the vertebral columns (Peters et al., 1999). So these two genes are

widely used as sclerotome markers (Balling et al., 1996). Previous studies on invertebrate deuterostomes and lampreys have suggested that axial expression of group I *Pax* genes are a novel event for the gnathostome lineage (Holland and Holland, 1996; Ogasawara et al., 2000; Ogasawara et al., 1999). Due to the conflict between the absence of *Pax1/9* in lamprey and presence of conserved chondrogenic program (Ogasawara et al., 2000) (Zhang et al., 2006), we investigated the *Pax1/9* gene in another two lamprey species, *Lampetra* and *Ichthymyzon*. In both *Lampetra* and *Ichthymyzon*, we detected their expression in the ventromedial somite, which was sustained as these cells surrounded the notochord. The *Pax1/9* pharyngeal expression was same as in *Lethenteron* (Ogasawara et al., 2000). Our catshark *Pax1* data showed pharyngeal and sclerotomal domains are typical of gnathostomes. The lamprey *Pax1/9* data suggests that this axial expression domain is a shared character of lampreys and gnathostomes and evolved coincident with the morphological sclerotome (Maurer, 1906). The difference between our results and those of previous publications may be caused by species differences, as proposed for lamprey *HoxL6* (Cohn, 2002; Takio et al., 2004), or simply the result the sensitivity of in situ hybridization methods, as suggested by Ogasawara et. al. (Ogasawara et al., 2000).

Since *Pax1/9* marks the sclerotome in amniotes, this suggests that lamprey possess a sclerotome. To confirm this, we investigated more sclerotome markers; consistently these makers were expressed in the ventromedial part of somites.

Bapx1

Bapx1 (*Nk3.2*) is one member of *NK3* genes that is homologous to the fruitfly *bagpipe* gene. There are two *Nk3* genes in human and mouse, *NK3.1* and *Nk3.2*. both genes are homeobox containing transcription factors and expressed in the sclerotome. Although the *Nk3.1* mutant does not show sclerotomal defects, the *Bapx1* mutant phenotypes were similar with *Pax1* and *Pax9* double mutants (Akazawa et al., 2000; Bhatia-Gaur et al., 1999; Lettice et al., 1999;

Schneider et al., 2000; Tribioli and Lufkin, 1999). *Bapx1* was shown to be a direct target of group I *Pax* genes in mouse (Rodrigo et al., 2003). Moreover, *Bapx1* was demonstrated to promote chondrogenesis in the somatic lineage (Murtaugh et al., 2001; Zeng et al., 2002). So the *Baxp1* is the key gene, which determines the sclerotomal cell fate to the chondrogenic lineage. *Bapx1* has been proposed to play important roles in the axial skeleton evolution after the split of jawless and jawed vertebrates (Lettice et al., 2001). Our lamprey *Nk3* expression in the somites suggests the co-option of *Nk3* from gut-associated mesenchyme to paraxial mesoderm happened before the split of jawed and jawless vertebrates. The *Bapx1* results also have implications for the origin of hinged jaws. The co-option of *Bapx1* expression in the first arch was proposed to be critical for the origin of a jaw since its expression resides at the mandibular joint of jawed vertebrates (Miller et al., 2003). Our finding that the lamprey *Nk3* gene is expressed in all the pharyngeal arches suggests there may have been a reduction of *NK3* gene expression from all gill arches of jawless vertebrates to first gill arch of gnathostomes. Modulation of the spatial extent of gene expression domains within the pharyngeal arches may have occurred with other genes during the agnathan-gnathostome transitions, as a similar reduction appears to have occurred with the pharyngeal *Hox* code (Cohn, 2002).

FoxC1/2

FoxC genes encode transcription factors with a winged helix/forkhead domain and are known to be required for proper sclerotome development (Iida et al., 1997; Winnier et al., 1999). Lamprey and shark *FoxC1/2* showed similar expression patterns to mouse, chicken and frog sclerotomes (Buchberger et al., 1998; El-Hodiri et al., 2001; Kume et al., 2001). Both genes were shown to be important in specifying the paraxial mesoderm (Wilm et al., 2004). The *FoxC2* null mutant axial skeletal defects are similar with *Bapx1* knockout mice, and the chondrogenic markers like *Sox9* and *Col2a1,* are severely reduced (Iida et al., 1997; Winnier et

al., 1999). Double mutants of *FoxC2* and *Pax1* showed more severe defects of vertebral columns than single mutants, and both genes are required for sclerotomal cell proliferation in a *Shh*-dependent manner (Furumoto et al., 1999). The conserved expression patter of *FoxC1/2* in catshark and lamprey suggests that they played a role in formation of the earliest sclerotomes.

Twist

Twist, a basic helix-loop-helix transcription factor, is expressed in the sclerotome and was shown to play critical roles in mesoderm differentiation in a variety of species (Castanon and Baylies, 2002; O'Rourke and Tam, 2002; Stickney et al., 2000) and during the development of vertebral columns, especially the neural arches in medaka (Yasutake et al., 2004). The expression of catshark *Twist* is similar with mouse, suggesting its conserved function in sclerotome development, pharyngeal arches and limb or fin formation. Lamprey *Twist* is not strictly homologous to the gnathostome twist gene; instead it is a paralogue to the higher vertebrate *Twist1* and *Twist2* genes. It was cloned very recently as *Twist* (Sauka-Spengler et al., 2007). Our finding is consistent with their data that lamprey *Twist* is expressed in the notochord and developing somites, especially in the sclerotome. Lamprey *Twist* is also found in the dermomyotome, suggesting it may play part of the roles of *Twist2* (*Dermo1*), or it may be related to the mesenchymal-epithelial transition. Taken together with lancelet twist expression in the differentiating notochordal and paraxial mesoderm, lamprey showed an intermedial state between lancelet and gnathostomes.

Paraxis* and *Scleraxis

Paraxis and *Scleraxis* are two closely-related bHLH transcription factors expressed in the early paraxial mesoderm and later in the sclerotome, but *Paraxis* declines after sclerotome formation whereas *Scleraxis* persists in a subset of the sclerotome (Burgess et al., 1995). In the absence of *Paraxis*, the axial skeleton and muscle formed but with abnormal patterns due to the

disruption of somite A/P polarity (Burgess et al., 1996; Johnson et al., 2001). *Scleraxis*-null mice are unable to form mesoderm and *Scleraxis*–null cells are excluded from the sclerotome in chimeric mouse analysis, suggesting *Scleraxis'* role in sclerotome development (Brown et al., 1999). Recently, *Scleraxis* was shown to be a early marker of tendons and ligaments, and the *Scleraxis* positive somite compartment of somitic tendon population was termed the syndetome (Brent et al., 2003; Schweitzer et al., 2001). Catshark *Paraxis* and *Scleraxis* are both expressed in the sclerotome. Interestingly both catshark *Paraxis* and *Scleraxis* are found in the neural tube. *Xenopus Paraxis* expression has been reported in the neural tube (Carpio et al., 2004). The lamprey *Paraxis* and *Scleraxis* orthologue, *Parascleraxis,* is also expressed in the sclerotome and neural tube. Taken together, *Scleraxis* and *Paraxis* sclerotome expression domains are conserved in all the living vertebrates, while the neural tube expression domain is lost in amniotes (chicken and mouse). The S*cleraxis* sclerotomal domain in catshark is similar to the *Pax1/9* expression domain, suggesting that differentiation of syndetome may be a more recent event in amniotes. Indeed, that syndetome forms a further molecular subdivision of the ventromedial somite within the *Pax1* expression domain. The roles of *Scleraxis* in shark myoseptum are very interesting since it has been proposed that tendon may have evolved from the myoseptum in gnathostomes (Gemballa et al., 2003; Summers and Koob, 2002).

Tbx15/18

Tbx18 or *Tbx15* are closely related T-box containing genes that are expressed in the pharyngeal arches, sclerotomes and limb/fin buds in mouse, chicken and zebrafish (Agulnik et al., 1998; Begemann et al., 2002; Haenig and Kispert, 2004; Kraus et al., 2001; Singh et al., 2005; Tanaka and Tickle, 2004). In chicken and mouse, *Tbx18* is important to maintain the somite boundary; in addition it marks the sclerotome (Bussen et al., 2004; Tanaka and Tickle, 2004). The *Tbx18* deficient mouse exhibits posterior lateral sclerotome derivative defects,

pedicles, and the proximal ribs are severely expanded (Bussen et al., 2004). The catshark *Tbx18* shows a typical gnathostome expression pattern and is found in the pharyngeal arches, sclerotome and fin buds. Lamprey *Tbx15/18* is found in the pharyngeal arches and perinotochordal region of the somites. Interestingly, the *Ciona Tbx15/18/22* was reported to be expressed in the paraxial mesoderm although *Ciona* do not have overt morphological segmentation (Takatori et al., 2004). In addition, lancelet *Tbx15/18/22* is also evident in the anterior part of the newly formed somites (Beaster-Jones et al., 2006). The current data suggests that *Tbx15/18/22* gene was originally deployed broadly in the mesoderm (as in *Ciona),* then became restricted to paraxial mesoderm (as in lancelets), and finally after the gene duplication events, *Tbx18* and *Tbx15* genes became confined to somite boundaries and used during sclerotome development.

Gli1/2/3

The *Ci* vertebrate homologues, *Gli1*, *Gli2* and *Gli3* are zinc finger transcription factors that mediate hedgehog signaling (Ingham and McMahon, 2001). In mouse, all three genes are expressed in the sclerotome, and *Gli2* and *Gli3* were demonstrated to be required for *Shh*-dependent sclerotome induction (Buttitta et al., 2003; Mo et al., 1997). In the *Gli2* and *Gli3* double mutants, axial skeletal defects are similar to *Pax1/Pax9* double mutants, whereas *Gli1* mutant mice exhibit no developmental defects (Buttitta et al., 2003). The catshark *Gli2* and the lamprey paralogous gene, *Gli1/2/3,* are all expressed at the neural tube and sclerotome, suggesting their ancient roles in mediating *Shh* signals for inducing sclerotome and perhaps motor neuron development. Recently, lancelet was reported to have a single *Gli* gene expressed in the neural tube and somites (Shimeld et al., 2007), suggesting that the vertebrate *Gli* gene duplication may have coincided with subdivision of the somites.

Embryonic Origin of Lamprey Arcualia

Vertebrate cartilage is characterized by the presence of chondrocytes and extracellular matrix molecules such as type II collagen fibrils and proteoglycans. The type II collagen protein is encoded by the *Col2a1* gene, and predominantly localize to cartilage as the major matrix protein. *Sox9* is a member of the Sox family of transcription factor that contains a high-mobility-group (HMG)-box DNA binding domain. Haploinsufficiency of *Sox9* in humans and mice causes the skeletal disease, campomelic dysplasia (CD) (Foster et al., 1994; Wagner et al., 1994). *Sox9* was proved to be the direct regulator of *Col2a1* and aggrecan in mice and its major roles during chondrocyte differentiation through direct regulating on the *Col2a1* promoter (Bi et al., 1999; Bi et al., 2001; Lefebvre and de Crombrugghe, 1998; Lefebvre et al., 1997). (Bi et al., 1999; Bowles et al., 2000; Lefebvre and de Crombrugghe, 1998). Recently we demonstrated that lamprey and hagfish have conserved this chondrogenic genetic program to build cartilage (Zhang and Cohn, 2006; Zhang et al., 2006).

Arcualia are paired cartilaginous nodules sitting on either side of the notochord. They are irregularly-shaped and variably distributed in the tail of ammocoetes and hagfish, and the trunk region of adult lampreys (Gadow and Abbott, 1895). If they were homologous to vertebrae, then they would be expected to have the same developmental origin. If lampreys lack a sclerotome, as suggested, this would cast doubt on the common origin of vertebrae and arcualia. Our eight molecular markers of the sclerotomal population strongly suggest that lamprey do possess sclerotome. This finding is consistent with the observations that arcualia develop from paraxial mesodermal cells (Gadow and Abbott, 1895). Hence, the genetic programs for sclerotome formation during chondrogenesis are conserved in the lampreys, strongly suggesting that arcualia derive embryonically from the sclerotome as axial endoskeletons do in gnathostomes. Recently, it has become possible to get live hagfish embryos (Ota et al., 2007), and thus it would be very

interesting to look at the sclerotomal molecular markers in hagfish embryos since some cartilaginous elements were reported in the hagfish tail region (Gadow and Abbott, 1895). This work could resolve questions over the phylogenetic positions of the two extent jawless fishes, since it is still controversial whether hagfish belong to vertebrata, or should be separated as craniata.

The Evolution of Sclerotome and Vertebral Columns

As pointed out by Scaal and Wiegreffe, modern embryologists have focused mostly on the amniote somites, contrasting to the late 19th and early 20th century embryologists who worked extensively on anamniotes. The basal vertebrates, like the cyclostomes, chondrychthians and chondroistei generally have been neglected (Scaal and Wiegreffe, 2006). Our experiments in lamprey and catshark demonstrated that sclerotome is a vertebrate character that unites gnathostomes and lampreys, suggesting its presence in their common ancestor. Analysis of hagfish somites may reveal whether the origin of sclerotome dates to the common ancestor of modern agnathans and gnathostomes, which is congruent with the large-scale gene duplication (Steinke et al., 2006). The size of sclerotome is highly variable in different taxa. In the amniotes, the sclerotome is comparatively bigger than the situation in fishes (Brand-Saberi and Christ, 2000). Clearly there is a trend of increasing the size of sclerotome from fish to tetrapods. The sclerotome size was determinated by the balance between the dorsal and ventral signals. The dorsal signals from the surface ectoderm and the dorsal neural tube promote the development of dermomyotome, but inhibit the sclerotome formation in amniotes. While the ventral signals promote sclerotome development and inhibit dermomyotome formation (Christ et al., 2004; Dockter, 2000). Currently, the dorsal signals are mediated by the WNT and BMP family members, while the ventral signals are mediated by *Shh* and *Noggin* secreted by notochord (Dockter, 2000; Lee et al., 2001; Lee et al., 2000; McMahon et al., 1998; Yusuf and Brand-

Saberi, 2006). The evolution of vertebrate sclerotome size may be a reflection the evolution of the interaction of these two signaling centers. Further functional studies on the *Wnt* and *Shh* gene interaction in fishes will shed light on this intriguing question.

Although composed mainly by arches and centra, vertebral columns are highly variable structures in different taxa. The major components of vertebrae appeared at different times, which are summarized in Figure 4-1A. The developmental mechanisms of centra and arches were shown to be different, which may reflect their modular evolutionary history. For example, *Pax1*, *Pax9*, and *Bapx1* null mice mainly showed centra defects (Lettice et al., 1999; Peters et al., 1999), while in *Twist*, *FoxC2* and *Zic1* knockout mice, the arch structures were mainly affected (Aruga et al., 1999; Furumoto et al., 1999; Yasutake et al., 2004). Vertebral centra are absent and only some arch structures are present in cyclostomes and chondrosteis (Gadow and Abbott, 1895). Hans Gadow classified the vertebral centra into two categories, according to the participation of notochordal sheath and sclerotomal cells, which he termed chorda-centra and arch-centra (Gadow and Abbott, 1895). He suggested that the former centra mainly develop from notochordal sheath, with some participation of sclerotomal cells, like the chondrychthians, whereas the latter centra are entirely made by sclerotomal cells and all the amniotes belong to this category (Gadow and Abbott, 1895). So, the diversity of vertebrae could be interpreted as the results of phylogenetic differences in the interactions between the notochord and sclerotome. Indeed, there is some functional evidence that support this idea. The notochord is required to induce *Pax1* expression (Brand-Saberi et al., 1993; Muller et al., 1996), and in *Pax1* mutant mice, the notochord is enlarged due to more notochordal cell proliferation, suggesting reciprocal interaction between sclerotome and notochord (Wallin et al., 1994). Further functional studies in different animal taxa are necessary to test this hypothesis.

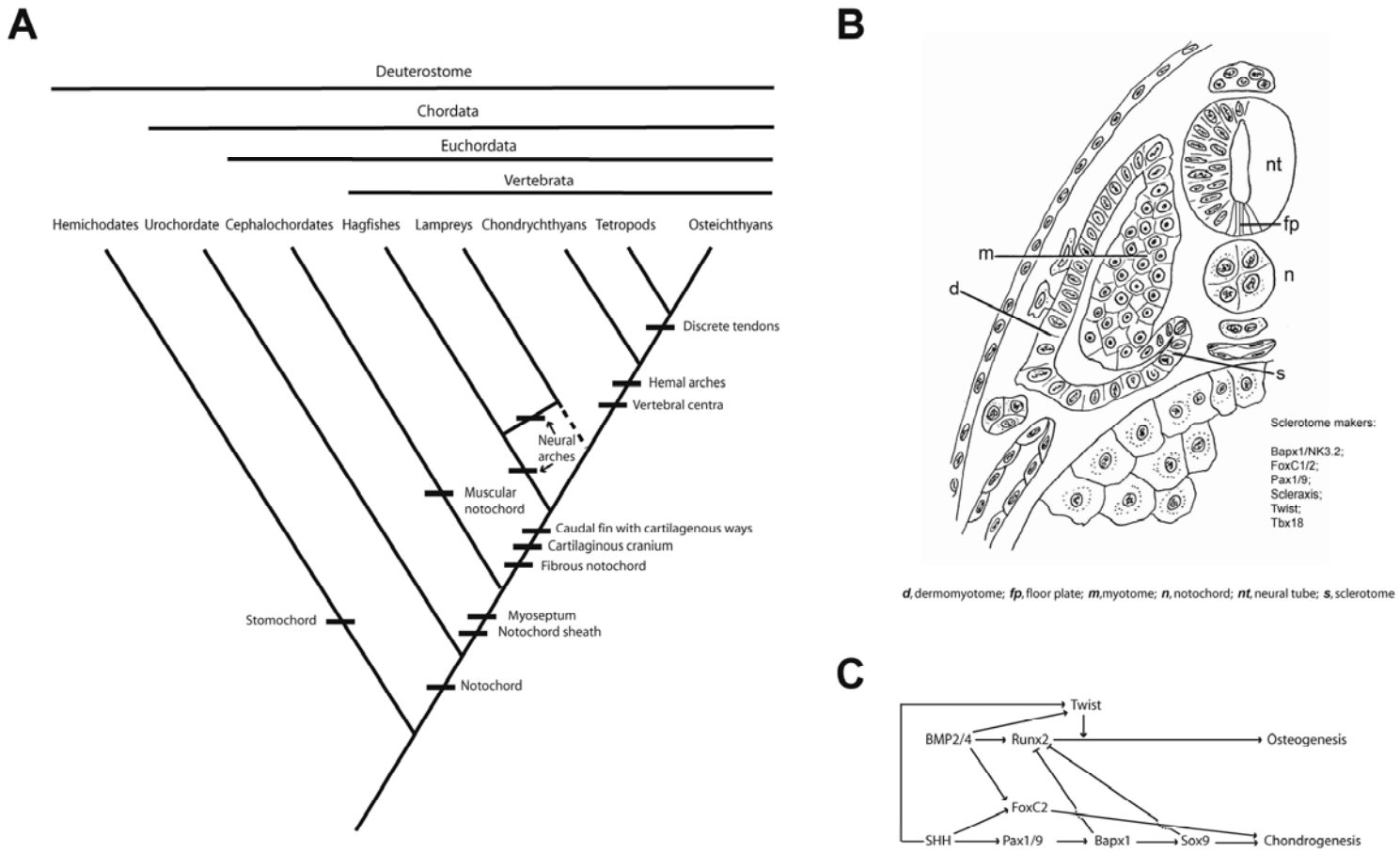

Figure 4-1. Evolution and development of axial morphological structures. (A) The evolution of axial structures in living deuterostomes. The phylogenetic relationships are based on Koob and Neidert (Koob and Long, 2000; Neidert et al., 2001). The bars on the tree indicate the emergence of the morphological structures. The dashed lines show the alternative possibility. The tree structures are based on Koob and Bourlat (Bourlat et al., 2006; Koob and Long, 2000). (B) Subdivisions of a somite, modified from Williams (Williams, 1959). Sclerotome markers are listed at the right bottom corner. (C) The genetic regulatory circuit of axial skeleton development. The relationships are based on several published studies (Bialek et al., 2004; Furumoto et al., 1999; Hornik et al., 2004; Kume et al., 2000; Lengner et al., 2005; Rodrigo et al., 2003; Zeng et al., 2002; Zhou et al., 2006).

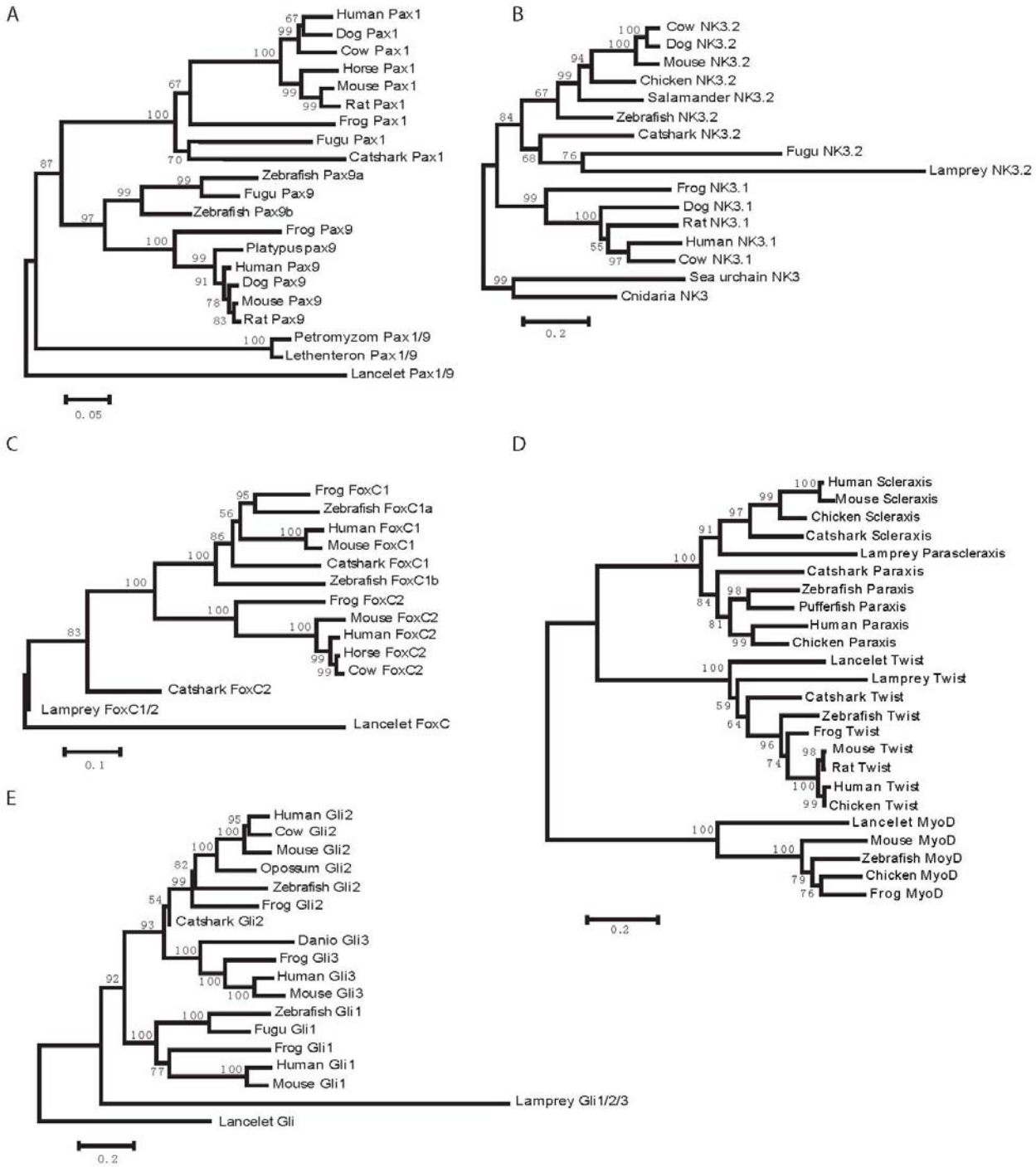

Figure 4-2. Minimum evolution phylogenetic analysis of the predicted protein sequences of catshark and lamprey clones. The numbers indicate bootstrap scores for each node, based on 10,000 replicates, whereas branch lengths are proportional to expected replacements per site. Each tree is rooted by a lancelet sequence. The phylogenetic relationships are consistent with maximum likelihood and Bayesian analyses that are provided as supplementary information. (A) Pax1 and Pax9. (B) Nk3.1 and Nk3.2. (C) FoxC1 and FoxC2. (D) Twist (E) Gli1, Gli2 and Gli3.

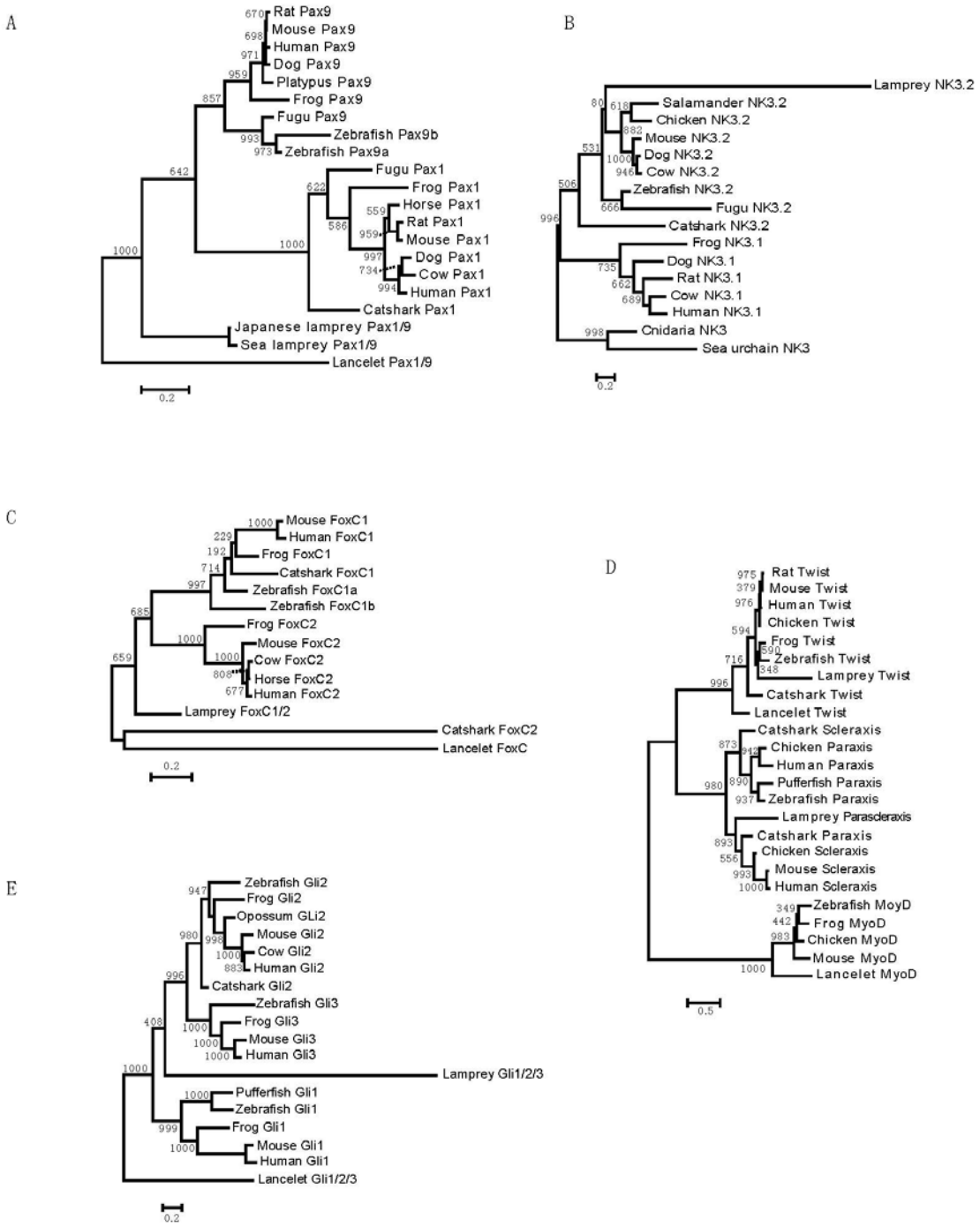

Figure 4-3. Maximum likelihood phylogenetic analysis of the predicted protein sequences of catshark and lamprey clones, as obtained with JTT plus gamma distances. The numbers indicate bootstrap scores for each node, based on 1,000 replicates, whereas branch lengths are proportional to expected replacements per site. Each tree is rooted by the lancelet sequence. The phylogenetic relationships are consistent with minimum evolution (Fig. 2) and Bayesian analysis (Fig. S2). (A) Pax1 and Pax9. (B) Nk3.1 and Nk3.2. (C) FoxC1 and FoxC2. (D) Twist (E) Gli1, Gli2 and Gli3.

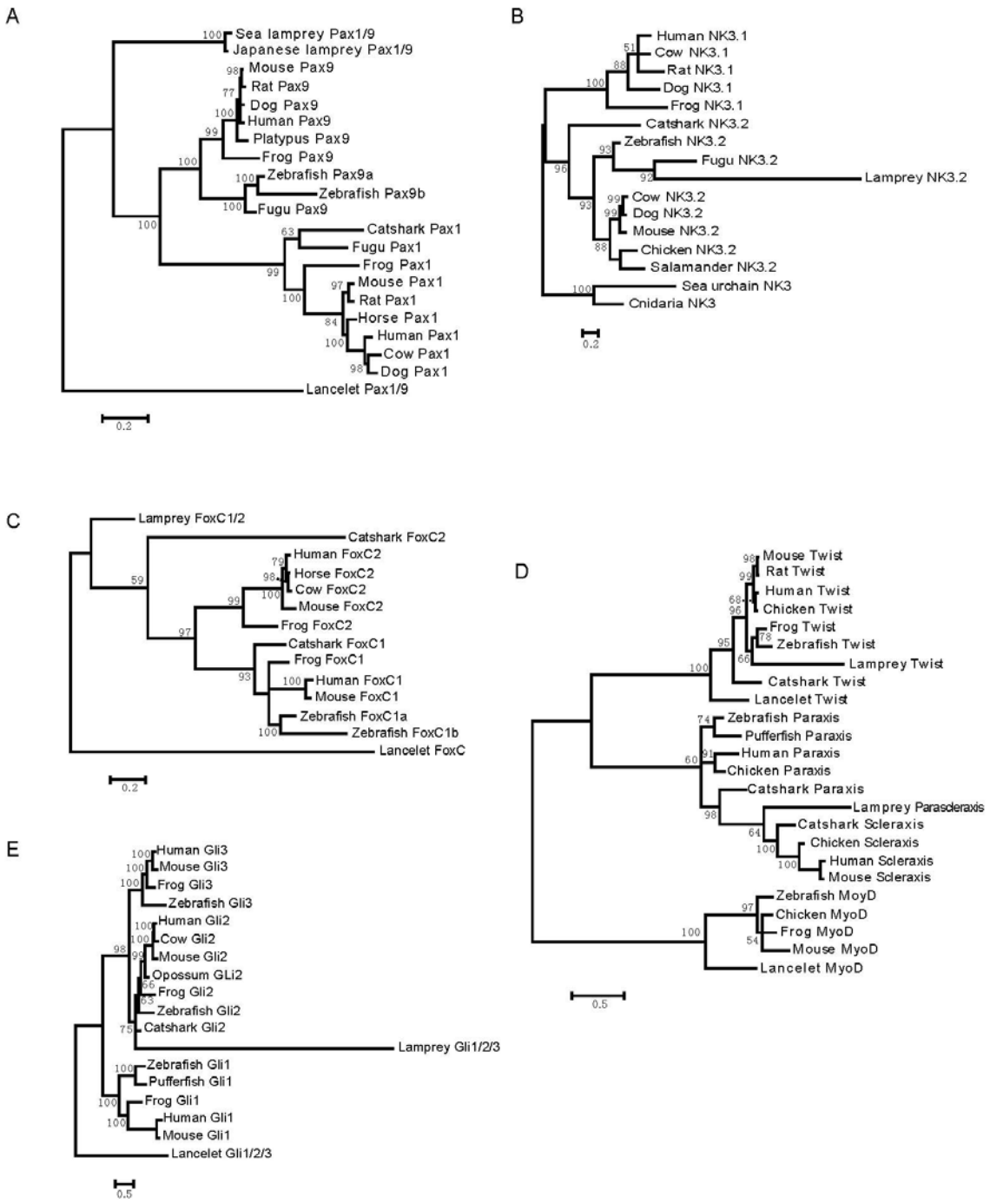

Figure 4-4. Bayesian phylogenetic analysis of the predicted protein sequences of catshark and lamprey clones, as obtained with WAG plus gamma distances. Numbers indicate posterior probabilities for groups with >50% credibility and for those clades that are combinable with this first set. Branch lengths are proportional to the means of the posterior probability densities for their expected replacements per site. Each tree is rooted by the lancelet sequence. The phylogenetic relationships are consistent with minimum evolution (Fig. 2) and maximum likelihood (Fig. S2). (A) Pax1 and Pax9. (B) Nk3.1 and Nk3.2. (C) FoxC1 and FoxC2. (D) Twist (E) Gli1, Gli2 and Gli3.

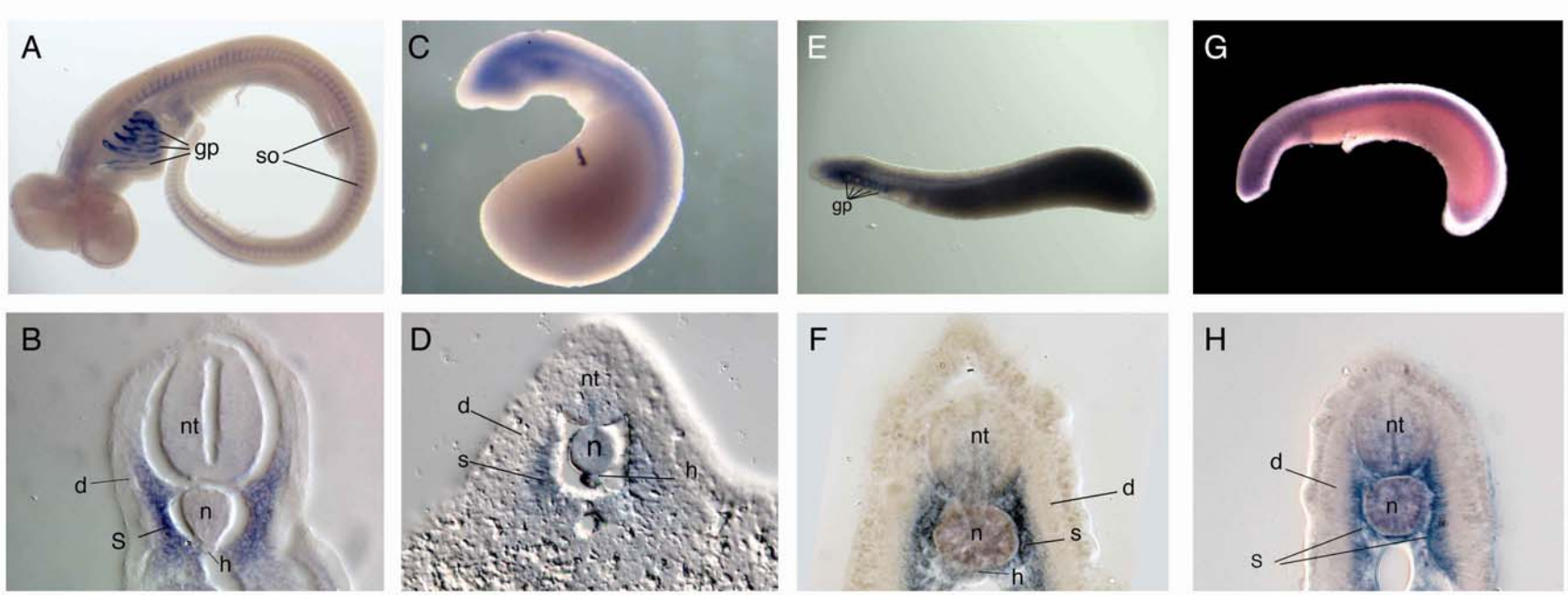

Figure 4-5. Group I *Pax* genes are expressed in the sclerotome of catshark (A, B) and lamprey (C-H). Catshark *Pax1* gene is expressed in the somites and the pharyngeal pouches at stage 26 (A). Transverse section shows *Pax1* is mainly located at the sclerotome (B). *Lampetra Pax1/9* expression patterns at stages 23 and 25, which is evident at the pharyngeal pouches and somites (C, E). In transverse sections, *Pax1/9* is seen at the ventromedial part of somite, sclerotome, and ventral neural tube (D, F). *Ichthymyzon Pax1/9* shows similar expression patterns with *Lampetra* (G, H). All the sections are with dorsal to the top. *d*, dermomyotome; *gp*, pharyngeal pouches; *h*, hypochord; *n*, notochord; *nt*, neural tube; *s*, sclerotome; *so*, somites.

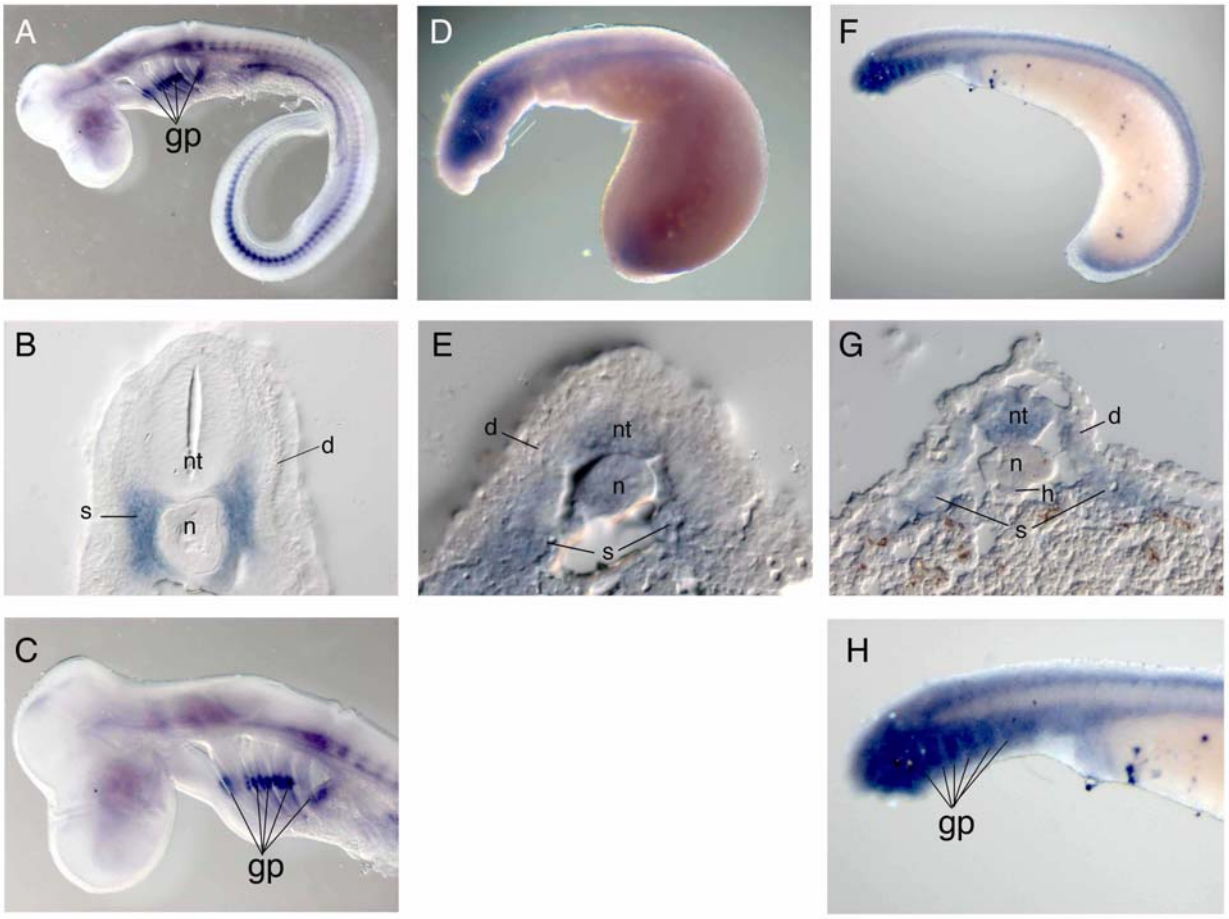

Figure 4-6. Expression of *Nk3.2* genes in catshark and lamprey. Catshark *Nk3.2* gene expression is evident in the somites and pharyngeal pouches at stage 26 (A), and a transverse section shows it is located at the sclerotome (B). It is not only evident at the mandible joint, but also in the midpoint of other pharyngeal arches in catshark (C). The lamprey *Nk3.2* gene is broadly expressed in the pharyngeal pouches and somites at stages 23 (D) and 25 (F, H). Transverse sections show lamprey *Nk3.2* is evident in the neural tube and sclerotome (E, G). All the sections are with dorsal to the top. *d*, dermomyotome; *gp*, pharyngeal pouches; *n*, notochord; *nt*, neural tube; *s*, sclerotome.

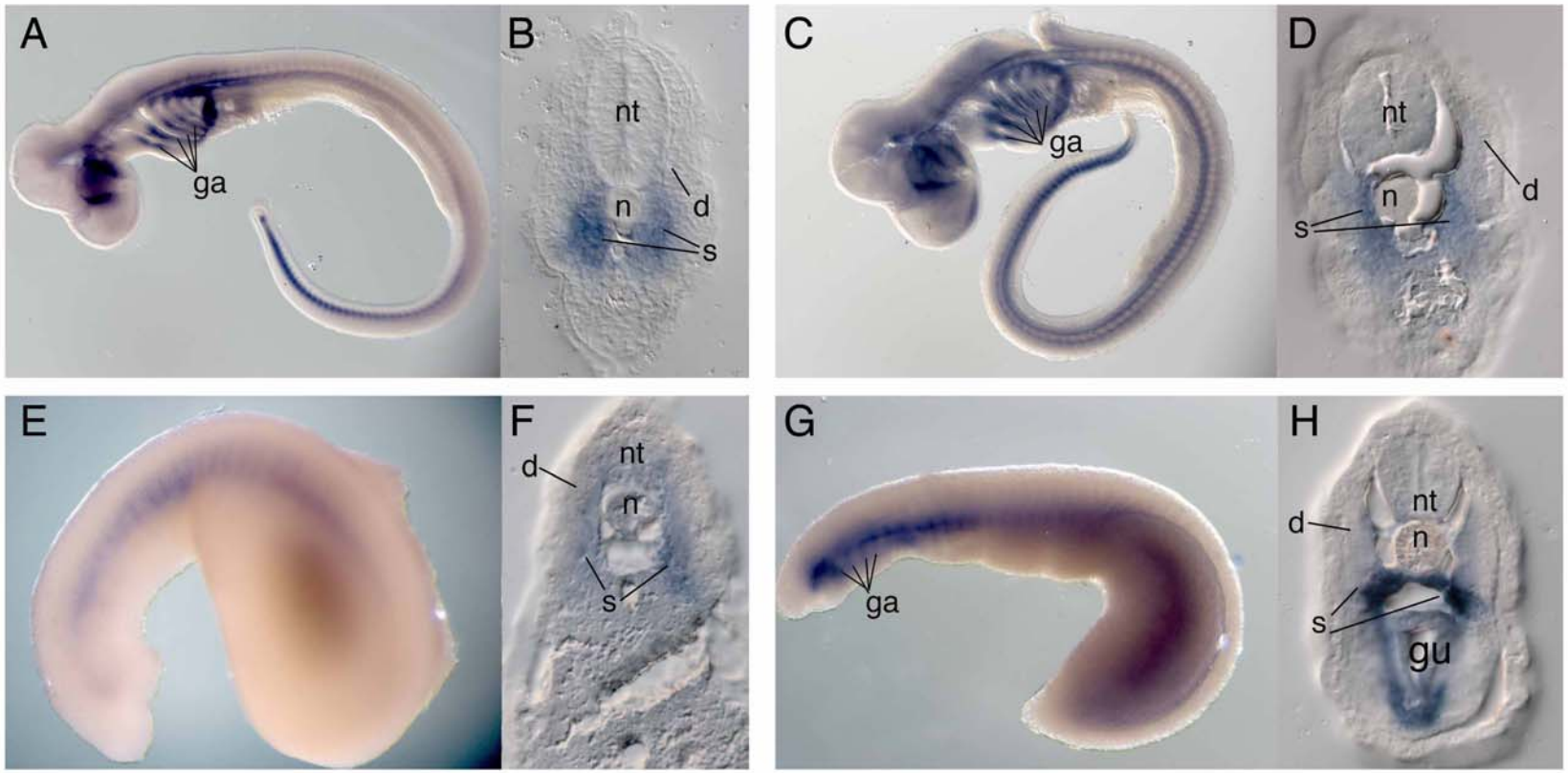

Figure 4-7. *FoxC1* and *FoxC2* expression patterns in catshark and lamprey. *FoxC1* in strongly expressed at the newly formed somites and pharyngeal arches at stage 26 (A). Transverse section shows catshark *FoxC1* is located at the sclerotome (B). Catshark *FoxC2* is evident in the somites and pharyngeal arches at stage 26 (C). Catshark *FoxC2* is located at the sclerotome in the somite (D). Lamprey *FoxC1/2* is evident at the somites at stages 23 (E) and 25 (G). It is also expressed at the pharyngeal arches at stage 25 (G). Transverse sections show lamprey *FoxC1/2* and catshark *FoxC2* expression in the sclerotome (F, H) and gut region (H). All the sections are with dorsal to the top. *d*, dermomyotome; *ga*, pharyngeal arches; *gu*, gut; *n*, notochord; *nt*, neural tube; *s*, sclerotome.

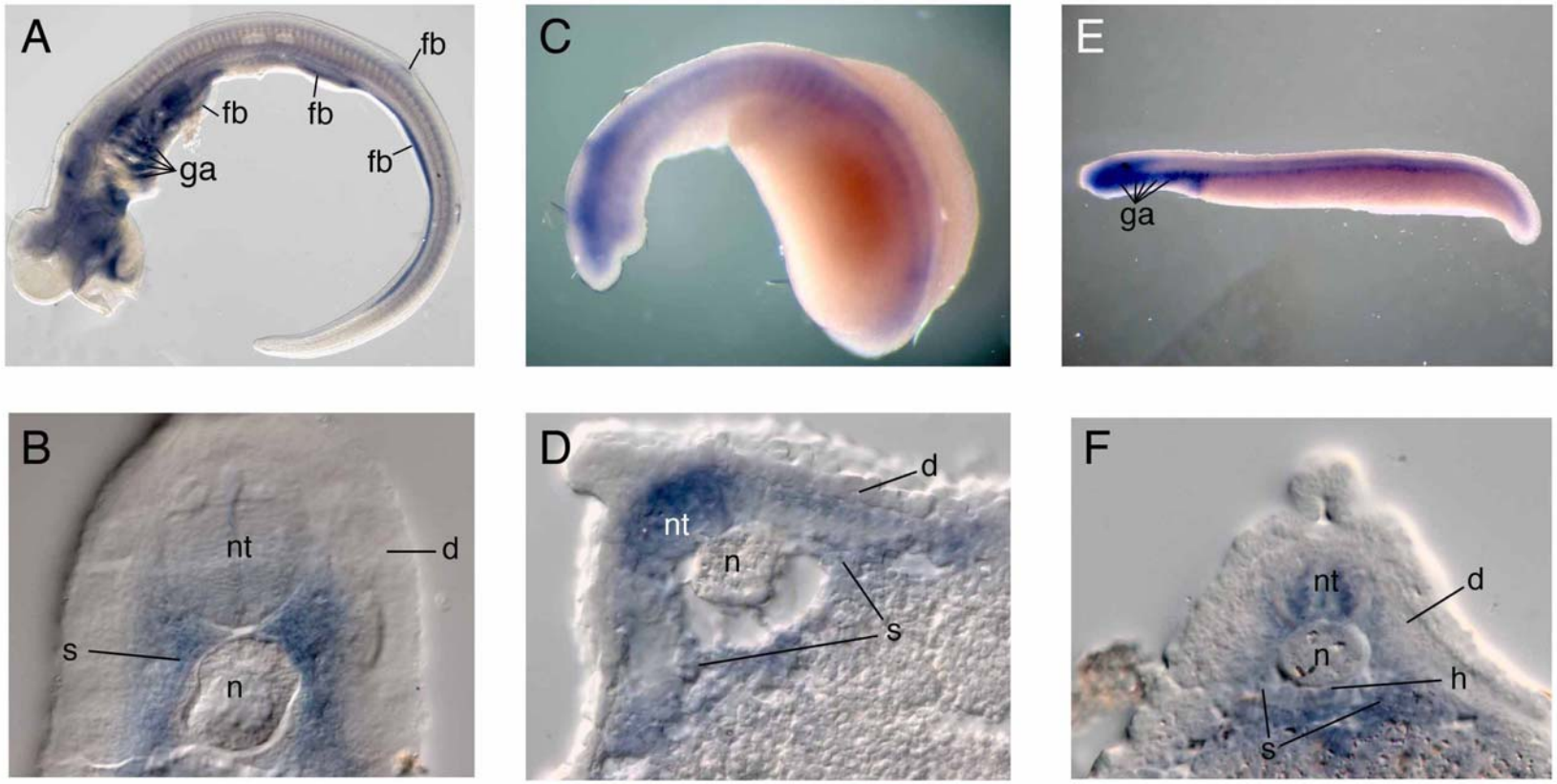

Figure 4-8. Expression of catshark and lamprey *Twist* genes. At stage 26, catshark *Twist* is expressed in the somites, fin fold and pharyngeal arches (A). Transverse section shows that *Twist* is limited to the sclerotome (B). The lamprey *Twist* is also expressed in the pharyngeal arches and somites at stage 23 (C) and stage 25 (E). Transverse section shows that lamprey *Twist* is evident at the neural tube, dermomyotome and sclerotome at stage 23 (D). While at stage 25, lamprey *Twist* is limited in the sclerotome and neural tube (F). All the sections are with dorsal to the top. *d*, dermomyotome; *fb*. fin bud; *ga*, pharyngeal arches;*h*, hypochord; *n*, notochord; *nt*, neural tube; *s*, sclerotome.

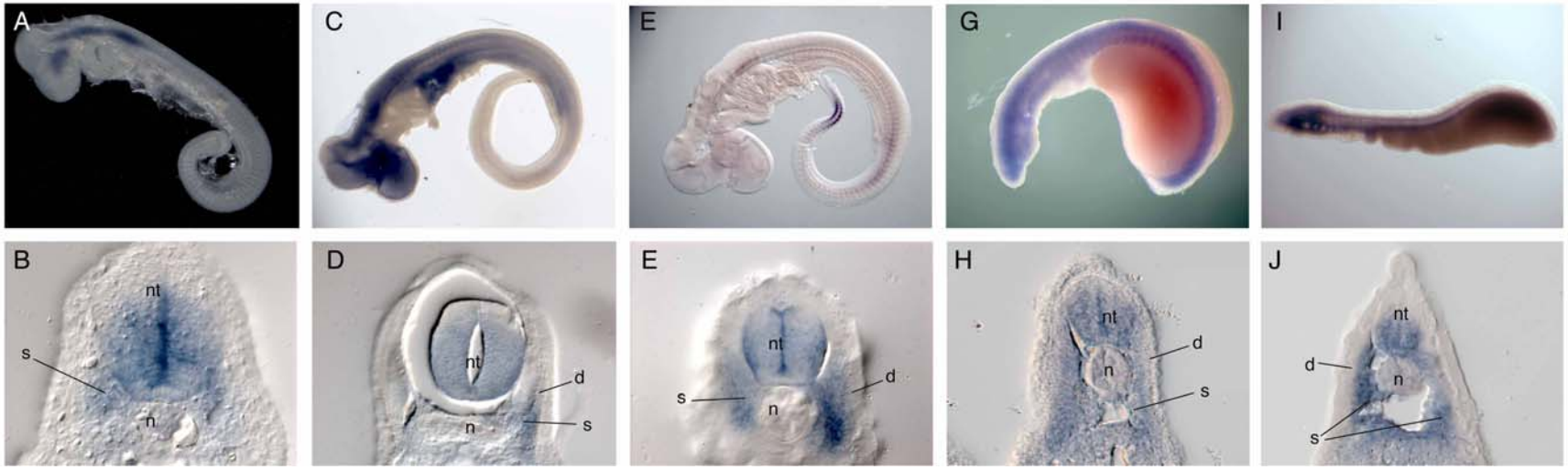

Figure 4-9. *Scleraxis* and *Parascleraxis* expression in catshark and lamprey. Catshark *Scleraxis* is expressed in the neural tube and head paraxial mesoderm at stage 21 (A, B). At stage 26, catshark *Scleraxis* is found in the craniofacial region, neural tube and sclerotome (C, D). Catshark *Paraxis* is expressed in the newly formed somites at stage 26 (C) and transverse section shows it is evident at the neural tube and sclerotome (D). Lamprey *Parascleraxis* is evident in the pharyngeal region and somites at stages 23 and 25 (D, E). Transverse sections show that lamprey *Parascleraxis* is expressed in the neural tube and sclerotome (I, J). All the sections are with dorsal to the top. *d*, dermomyotome; *n*, notochord; *nt*, neural tube; *s*, sclerotome.

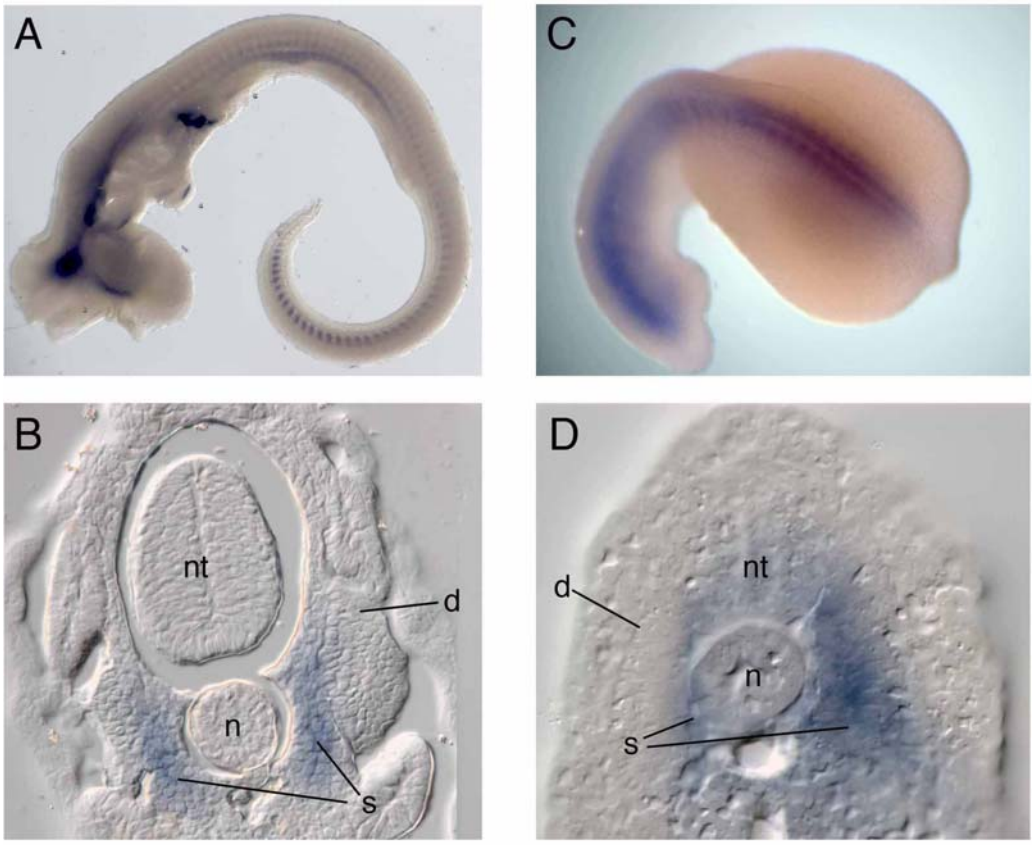

Figure 4-10. Expression of *Tbx18* in catshark and lamprey. The catshark *Tbx18* is evident in the somites at stage 26 (A). Transverse sections show its expression in the sclerotome (B). Lamprey *Tbx15/18* is expressed at the somite region (C, D). All the sections are with dorsal to the top. *d*, dermomyotome; *n*, notochord; *nt*, neural tube; *s*, sclerotome.

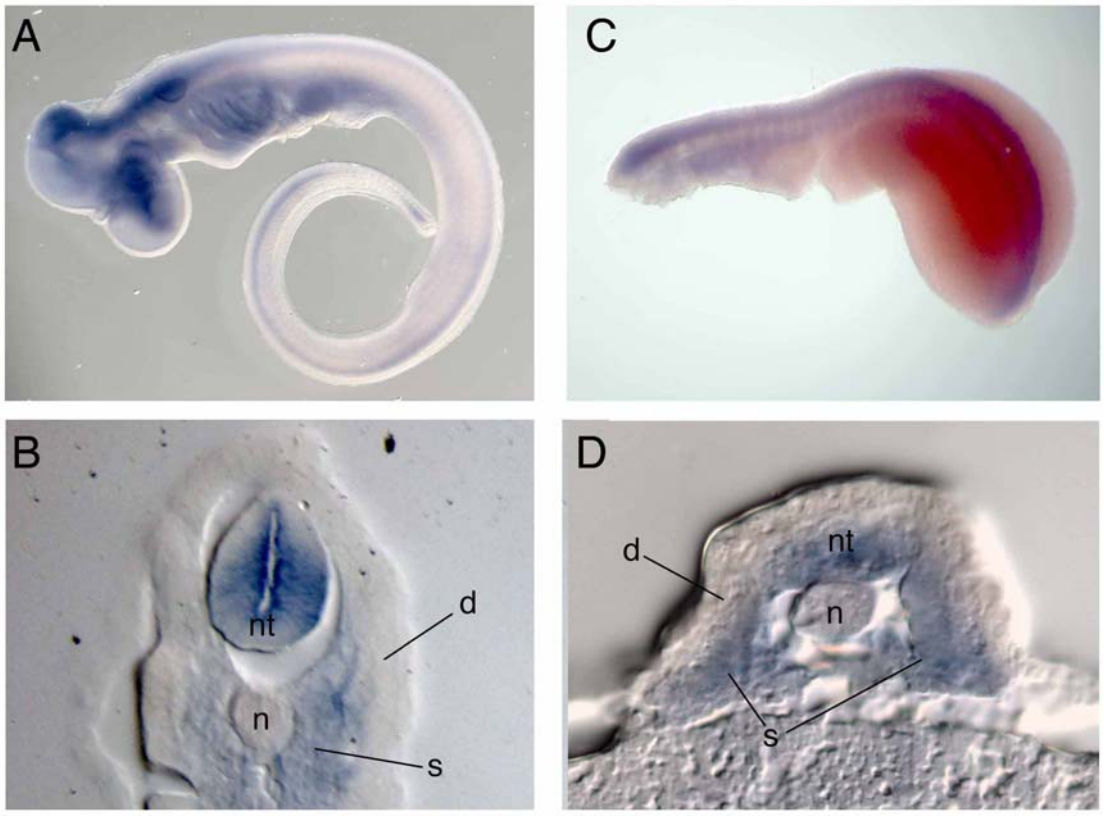

Figure 4-11. *Gli* gene expression in catshark and lamprey. Catshark *Gli2* is evident in the pharyngeal arches and somites at stage 26 (A). The transverse section showing that catshark *Gli2* is expressed in the neural tube and somite, but is excluded from the floor plate (B). The lamprey *Gli1/2/3* is found in the pharyngeal arches and somites at stage 23 (C). It is evident at the neural tube and sclerotome (D). All the sections are with dorsal to the top. *d*, dermomyotome; *n*, notochord; *nt*, neural tube; *s*, sclerotome.

CHAPTER 5
GENERAL DISCUSSION

Evolution of Collagen Genes in Chordates

Collagens are the main components of animal extracellular matrix (Expositio JY, 2002). Thus far, 27 types collagen genes have been identified (Pace et al., 2003). The collagens are divisible into two major groups: fibril and non-fibril collagens. The fibril collagens are further divisible into clades A, B and C (Aouacheria, A. et al. 2004). Clade A fibrillar collagens are the major fibril-forming collagen, including types I, II, III and V (Aouacheria, A. et al. 2004).

The identification of type II collagen in lamprey and hagfish suggests that the differentiation of clade A fibrillar collagen genes predated the split of agnathans and gnathostomes. An independent duplication of the *Col2a1* gene then occurred in the lamprey lineage. The expression patterns of *Col2a1a* and *Col2a1b* suggest that subfunctionalization followed this duplication of the ancestral *Col2*a1 gene, with the ancestral expression pattern being partitioned between *Col2a1a* and *Col2a1b*. Force and colleagues proposed a mechanism by which duplicated genes are preserved during evolution by subfunctionalization. The proposal states that members of the pair undergo reduction of their activity and expression patterns until together the pair of genes equal that of their single ancestral gene (Force et al., 1999; Lynch et al., 2001). Our finding that the expression domains of *Col2a1a* and *Col2a1b* in lampreys correspond to that of the single *Col2a1* gene in jawed vertebrates supports their duplication-degeneration-complementation (DDC) model.

The hagfish *Col2a1* was clustered with lamprey *Col2a1a,* with lamprey *Col2a1b* falling out as a sister branch. This topology suggests that *Col2a1* was present in the common ancestor of agnathans and gnathostomes, and that an additional duplication gave rise to *Col2a1a* and *Col2a1b* in the cyclostome (lampreys + hagfishes) lineage. The results predict that there should

be another *Col2a1* gene in hagfish. Efforts to locate the hagfish *Col2a1* were unsuccessful. Either we did not isolate it due to the limitation of degenerative PCR method, or it was lost in the hagfish lineage during evolution. The isolation of *Col2a1* genes also suggests that other clade A fibrillar collagen genes may be present in the two agnathans due to the duplications of clade A fibrillar collagen genes. Further studies are needed to prove these conjectures.

The presence of an ancestral state of clade A fibrillar collagen gene, *AmphiColA,* in lancets suggests that duplication of the ancestral *ColA,* the precursor of the clade A fibrillar collagen multi-gene family, occurred in the vertebrate lineage after the divergence of lancelets. The five types of clade A fibrillar collagen are limited to vertebrates. Our findings on lancelet *AmphiColA* gene were further supported by the recent report of several deuterostome collagen genes, including the similar genes in Chinese lancelet (Wada et al., 2006). Wada and his collagues also isolated one copy of clade a fibrillar collagen gene named *BbCol1*. Whether *AmphiColA* and *BbCol1* is the same gene or they are paralogous genes, requires further investigation.

Ascidians also possess one copy of clade fibrillar collagen gene and this gene also can not be clustered with the five types of vertebrate collagens (Wada et al., 2006), and this finding further supports my hypothesis that the duplication event happened in the vertebrate stem group, before the split of the agnathans and gnathostomes. The sea urchin was reported to have three different undifferentiated clade A fibrillar collagen genes (Aouacheria et al., 2004; Wada et al., 2006), which may suggest that they evolved independently in the echinoderm lineage. Very recently, one copy of clade a fibrillar collagen was isolated from hemichordate, specifically the acorn worm (Rychel and Swalla, 2007). Their analysis of hemichordate *ColA* gene and other invertebrate sequences proved that all the invertebrate *ColA* genes do not cluster with the

vertebrate collagen genes and verified that *ColA* gene underwent the extensive duplications in vertebrate lineage. After the duplication events, the type, I, II, III and V differentiated from the ancestral *ColA* gene.

Regulatory Relationships of *SoxE* and *ColA* Genes May Have Been Established Before the Origin of Vertebrates

The Sox family of transcription factors consists of at least ten subgroups (A-H) that are characterized by a specific 79-amino acid DNA-binding region, termed the high-mobility-group (HMG) box (Bowles et al., 2000). *SoxE* subgroup includes three genes, *Sox8, Sox9* and *Sox10*. It was well established that *Sox9* is required for *Col2a1* expression and for chondrogenesis in jawed vertebrates (Bi et al., 1999; Yan et al., 2005). Expression of *Col2a1* is regulated directly by *Sox9*, which binds to a chondrocyte-specific enhancer to activate *Col2a1* transcription (Bell et al., 1997; Lefebvre et al., 1997). Mutation of *Sox9* in zebrafish disrupts the stacking of chondrocytes and the separation and shaping of individual cartilage elements, and in humans causes campomelic dysplasia (Wagner et al., 1994; Yan et al., 2002).

Although it was reported that the compositions of lamprey and gnathostome cartilage matrices are different (Wright et al., 2001), the genetic pathway that regulates early development of the cranial skeleton (e.g., *Bmp2/4, Dlx, Msx etc.*) is well conserved (Cohn, 2002; McCauley and Bronner-Fraser, 2003; Neidert et al., 2001; Shigetani et al., 2002). Our finding of presence of type II collagen in lamprey cartilage led us to hypothesize that the genetic program to make cartilage in agnathans and gnathostomes is conserved. To test this hypothesis, we isolated one lamprey *Sox9* orthologous genes by degenerate RT-PCR. *In situ* hybridization using the amplicon as a probe revealed that lamprey *Sox9* co-localized with two *Col2a1* genes during catilage development. Therefore, this conservation of regulatory relationship between Sox9 and *Col2a1* was established before the split of agnathans and gnathostomes. Moreover, our isolation

of *Col2a1* genes and localization of *Col2a1* protein in hagfish head cartilage suggested that hagfish might use the same conserved chondrogenic program. Very recently, the isolation and expression of hagfish *Sox9* gene confirmed this conservation (Ota et al., 2007). Thus, it seems likely that in all the vertebrates, *Sox9* can regulate *Col2a1*.

In invertebrate chordates, there are no strict orthologues to *Sox9* and *Col2a1* genes, instead they have *SoxE* and *ColA* genes. The relationship of the *SoxE* and *ColA* is very interesting since *Sox9* is able to regulate *Col2a1* in vertebrates. In the lancelet, we isolated the *ColA* gene and found it is expressed in the floor plate of neural tube and in the notochord. This finding was also supported by *BbCol1* (Wada et al., 2006). Very recently, the lancelet *SoxE* was isolated and it was found in the notochord, and thus it co-localizes with *ColA* (Meulemans and Bronner-Fraser, 2007*)*. Interstingly, the two genes were also found to co-expressed in the lancelet pharyngeal cartilages (Meulemans and Bronner-Fraser, 2007; Rychel et al., 2006; Rychel and Swalla, 2007). The similar co-expression of *SoxE* and *ColA* was also reported in hemichordate cartilages (Rychel et al., 2006; Rychel and Swalla, 2007). This evidence suggests that the relationship between *SoxE* and *ColA* had been established at least in the ancestor of deuterostomes, long before the origin of vertebrates. Considering this conservation, it is not surprising that, in addition to *Sox9*, *Sox10* can also directly activate *Col2a1* gene expression (Suzuki et al., 2006).

Collagenous Skeletons verses Non-Collagenous Skeletons

Vertebrates

The phylogenetic relationships of the vertebrates were set up largely based on anatomical characters, particularly those of the skeleton. The skeletons of jawed vertebrates (gnathostomes) are composed of cartilage and bone, which contain high levels of COL2A1 and COL1A1 proteins, respectively, in their extracellular matrices. By contrast, the cartilaginous skeletons of lampreys and hagfishes, the only extant jawless fishes (agnathans), have been reported to lack

collagen-based cartilage and to contain instead the elastin-like proteins lamprin and myxinin (Janvier and Arsenault, 2002; Wright et al., 2001; Wright and Youson, 1983). This difference in the histological matrices of vertebrate skeletons has led to three ideas: that type II collagen became the major structural component of gnathostome cartilage after the divergence of these two lineages. The collagenous skeleton is a gnathostome synapmorphy, and the earliest vertebrates were presumed to have non-collagen based cartilage (Wright et al., 2001).

Localization of type II collagen mRNA and protein in the lamprey skeleton reveals that a collagenous skeleton is not restricted to the gnathostome lineage, but instead is a character shared by the crown vertebrates. This may provide a molecular explanation for Parker's observation in 1883 that lampreys have hard hyaline cartilage (Parker, 1883). I suggest that the additional cartilage matrix molecules (e.g., lamprin and myxinin) of agnathans may represent derived character states that that were added onto the more ancient collagenous skeleton. Expression of elastin-related molecules in a subset of lamprey cranial cartilages that also express *Col2a1* may underlie the different structural and mechanical properties within the lamprey skeleton.

Furthermore, *Sox9* is expressed in strikingly similar patterns in lamprey and gnathostome embryos. The co-expression of *Sox9* with *Col2a*1 during skeletogenesis in both lineages raises the possibility that the regulatory relationship between these two genes had already been established in their common ancestor. In gnathostomes, SOX9 is a target of parathyroid hormone-related protein (PTHrP), which regulates chondrocyte differentiation through a negative feedback loop with Indian hedgehog (Huang et al., 2001; Vortkamp et al., 1996). Interestingly, PTHrP expression has recently been detected in lamprey cartilage (Trivett et al., 2005). Our discovery of conserved expression of *Sox9* and *Col2a1*, taken together with the

extensive conservation of upstream regulatory genes such as *AP2*, *Dlx*, *Msx*, *Id* and *PTHrP* (Cohn, 2002; McCauley and Bronner-Fraser, 2003; Neidert et al., 2001; Shigetani et al., 2002; Trivett et al., 2005), suggests that the genetic program for chondrogenesis from the initial induction of chondrogenic mesenchyme to the synthesis of collagen matrix was assembled surprisingly early in vertebrate evolution.

We have also found that hagfishes, the sister group to lampreys, also possess a *Col2a1* orthologue, and that COL2A1 protein is localized to their soft cartilage (Chapter 3). Taken together with lamprey COL2A1 data, these results suggest that the common ancestor of all crown-group (the living jawed and jawless) vertebrates had COL2A1-based cartilage. Thus, COL2A1-based cartilage is a synapomorphy of all crown-group vertebrates.

Invertebrates

Presence of an undifferentiated clade A fibrillar collagen in lancelets and tunicates (Wada et al., 2006) suggests that the expansion of the *ColA* gene family occurred in stem vertebrates after the divergence of lancelets and tunicates. The expression of *ColA* in notochord and pharyngeal arches suggested that the lancelet skeleton is collagenous (Meulemans and Bronner-Fraser, 2007; Rychel et al., 2006; Rychel and Swalla, 2007), not non-collagenous as previously proposed (Wright et al., 2001). The expression of *SoxE* and *ColA* genes in hemichordates further supports the idea that the collagenous skeleton evolved in the deuterostomes before the origin of vertebrates. Based on biochemical analysis, the cartilage of cephalopod mollusks was shown to contain two collagen chains, a1 and a2 (Kimura and Karasawa, 1985; Sivakumar and Chandrakasan, 1998; Sivakumar et al., 2003). The evolutionary depth of the origin of collagenous skeletons needs further investigation. Although the completed arthropod genomes indicate that fibrillar collagen was lost, cartilage tissue was reported in the bookgills of horseshoe crab (Libbin et al., 1992; Libbin et al., 1976; Wright et al., 2001). Furthermore,

horseshoe crab cartilage was shown to contain chondroitin sulphate (Sugahara et al., 1996). Whether horseshoe crab cartilage is collagenous or non-collagenous is very interesting due to its pivotal phylogentic position. Further investigations would be useful in probing the evolutionary depth of collagenous skeletons.

Roles of Gene Duplications in Vertebrate Novelties

Ohno first raised the idea that genome duplication played a major role in the evolution of vertebrate complexities (Ohno, 1970). It is now clear that, in fact, vertebrates tend to have more copies of genes than do invertebrates, and that there is extensive variation in gene number among different clades of vertebrates (Dehal and Boore, 2005; Meyer and Schartl, 1999; Panopoulou and Poustka, 2005). Recent studies of 14 different genomes confirmed that the whole genome duplication occurred at the dawn of vertebrate evolution (Blomme et al., 2006). One of the most intensively studied cases of gene family expansion is that of *Hox* genes (Holland et al., 1994; Martinez and Amemiya, 2002; Ruddle et al., 1994a; Ruddle et al., 1994b). *Hox* genes code for homeodomain-containing transcription factors, which tend to occur in tightly linked clusters in the vertebrate genomes. They are involved in patterning the vertebrate body plan (Shashikant et al., 1991). Now it is widely accepted that the *Hox* cluster number reflects the genome duplication events (Panopoulou and Poustka, 2005). These four *Hox* clusters are thought to have evolved in two rounds of whole genome duplication, and the *Hox* cluster duplications gave the vertebrates the opportunity to generate morphological novelties (Holland et al., 1994; Wagner et al., 2003). Based on the 2R (two rounds of duplication) hypothesis, one genome duplication happened at the stem of all vertebrates and the other in the stem lineage of jawed vertebrates, the gnathostomes (Panopoulou and Poustka, 2005). This scenario is also supported by the evolutionary history of non-*Hox* genes, like *Engrailed* and *Otx* (Escriva et al., 2002; Garcia-Fernandez, 2005; Germot et al., 2001; Holland and Williams, 1990).

It is also noteworthy that collagen genes are physically linked to the *Hox* clusters in gnathostomes (Bailey et al., 1997; Morvan-Dubois et al., 2003). In lampreys, there are at least three *Hox* clusters (Force et al., 2002; Fried et al., 2003; Irvine et al., 2002). Our identification of two *Col2a1* genes supports the suggestion that one of their three *Hox* clusters may have arisen by an independent duplication in the lamprey lineage (Force et al., 2002; Fried et al., 2003; Irvine et al., 2002). The presence of *Col2a1* gene in hagfish and *ColA* gene in lancelets further support this idea since lancelet has one *Hox* gene cluster and hagfish has at least 7 *Hox* clusters (Stadler et al., 2004). The skeleton has been proposed to be one of the vertebrate innovations that resulted from the gene number increase in genome (Shimeld and Holland, 2000). The duplications of *Hox* clusters and the linked clade A fibrillar collagen genes may have facilitated the evolution of vertebrate morphological novelties. The *Hox* clusters play major roles in animal body patterning, and the clade A fibrillar collagen genes lay down the connective tissues, including the skeletons.

Origin of Vertebrate Chondrocytes

It has been suggested that the notochord may represent a primitive form of cartilage, based on their many shared structural, cellular and molecular properties, and that vertebrate chondrocytes may have evolved from notochordal cells (Cole and Hall, 2004; Stemple, 2005). In gnathostomes, the notochord and/or notochordal sheath expresses most of the vertebrate clade A fibrillar collagen genes (Dubois et al., 2002; Ghanem, 1996; Yan et al., 1995; Zhao et al., 1997). My finding that *AmphiColA* is expressed in the notochord and notochordal sheath of *Branchiostoma floridae,* taken together with the recently reported data on *Ciona intestinalis CiFCol1* and *Branchiostoma belcheri BbFCol1* (both clade A fibrillar collagens), supports the idea that an ancestral *ColA* gene was expressed in the notochord of stem-group chordates (Robson et al., 2000; Satou et al., 2001; Wada et al., 2006). We suggest that duplication and

divergence of the clade A collagen genes in stem-group vertebrates may have facilitated the evolutionary origin of chondrocytes from notochordal cells. This hypothesis deals specifically with the origin of vertebrate chondrocytes, and, as mentioned above, it is important to note that cartilage is also found in several invertebrates, including cephalopods, snails, and horseshoe crabs (Cole and Hall, 2004; Wright et al., 2001). Future work on the molecular basis of invertebrate chondrogenesis should reveal whether fibrillar collagens also were utilized in these independent evolutionary events.

Origin of Vertebrate Axial Skeletons

The vertebral column is a key defining character of all vertebrates. Lampreys possess some axial skeletal structures named arcualia, which have been suggested to be homologs of vertebral elements. In higher vertebrates, the vertebral columns derive mainly from sclerotome. Almost a century ago, comparative anatomists suggested that lampreys may have a small sclerotome (Brand-Saberi and Christ, 2000; Maurer, 1906; Scott, 1882). However, a recent study on the Japanese lamprey *LjPax9* gene showed that this sclerotome marker is absent in paraxial mesoderm (Ogasawara et al., 2000). This finding suggested that lampreys may lack a sclerotome. In addition, the major matrix of lamprey cartilage was reported to be an elastin-like molecule, named lamprin, instead of COL2A1 protein (Wright et al., 2001). The chondrogenic difference suggested that lamprey cartilage development may use a different genetic program. So, the embryonic origin of arcualia remains puzzling. Here I reported that the lamprey and hagfish cartilage matrix contains COL2A1 proteins and that lamprey chondrocytes also express *Sox9*, a chondrogenic master gene in jawed-vertebrates, which suggests that a chondrogenetic pathway is conserved in all extant vertebrates (cyclostomes and gnathostomes). More importantly, the *Col2a1* and *Sox9* genes in lamprey are also expressed in the arcualia (Zhang et al., 2006).

According to my finding on the conserved chondrogenetic pathway in lamprey and the previously described anatomical structure of axial cartilages from comparative embryology, I hypothesized that lamprey arcualia develop from the sclerotome, as in tetrapods. By studying eight sclerotomal marker gene expression patterns in lamprey and catshark, I confirmed that lamprey and catshark possess a sclerotome at the molecular level. Given the conservation of chondrogenetic pathways, it is very likely the lamprey arcualia develop from the sclerotome. More interestingly, the sclerotomal genes are members of multigene families, making it likely that the origin of the vertebral column was one of the vertebrate novelties that resulted from the large gene or whole genome duplication events that occurred during chordate evolution. Taken together, the results presented in this thesis push back, to an unexpectedly early evolutionary node, the genomic and developmental events that gave rise to the vertebrate skeleton.

LIST OF REFERENCES

Agulnik, S. I., Papaioannou, V. E. and Silver, L. M. (1998). Cloning, mapping, and expression analysis of TBX15, a new member of the T-Box gene family. *Genomics* **51**, 68-75.

Akazawa, H., Komuro, I., Sugitani, Y., Yazaki, Y., Nagai, R. and Noda, T. (2000). Targeted disruption of the homeobox transcription factor Bapx1 results in lethal skeletal dysplasia with asplenia and gastroduodenal malformation. *Genes Cells* **5**, 499-513.

Akiyama, H., Kim, J. E., Nakashima, K., Balmes, G., Iwai, N., Deng, J. M., Zhang, Z., Martin, J. F., Behringer, R. R., Nakamura, T. et al. (2005). Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. *Proc Natl Acad Sci U S A* **102**, 14665-70.

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. et al. (2004). Interactions between Sox9 and beta-catenin control chondrocyte differentiation. *Genes Dev* **18**, 1072-87.

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**, 47711-9.

Aruga, J., Mizugishi, K., Koseki, H., Imai, K., Balling, R., Noda, T. and Mikoshiba, K. (1999). Zic1 regulates the patterning of vertebral arches in cooperation with Gli3. *Mech Dev* **89**, 141-50.

Bagheri-Fam, S., Barrionuevo, F., Dohrmann, U., Gunther, T., Schule, R., Kemler, R., Mallo, M., Kanzler, B. and Scherer, G. (2006). Long-range upstream and downstream enhancers control distinct subsets of the complex spatiotemporal Sox9 expression pattern. *Dev Biol* **291**, 382-97.

Bagheri-Fam, S., Ferraz, C., Demaille, J., Scherer, G. and Pfeifer, D. (2001). Comparative genomics of the SOX9 region in human and Fugu rubripes: conservation of short regulatory sequence elements within large intergenic regions. *Genomics* **78**, 73-82.

Bailey, W. J., Kim, J., Wagner, G. P. and Ruddle, F. H. (1997). Phylogenetic reconstruction of vertebrate Hox cluster duplications. *Mol Biol Evol* **14**, 843-53.

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**, 211-25.

Bairati, A. and Gioria, M. (2004). Collagen fibrils of an invertebrate (Sepia officinalis) are heterotypic: immunocytochemical demonstration. *J Struct Biol* **147**, 159-65.

Ballard, W. W., Mellinger, J. and Lechenault, H. (1993). A series of normal stages for development of *Scyliorhinus canicula*, the Lesser Spotted Dogfish (*Chondricthyes: Scyliorhinidae*). *The Journal of Experimental Zoology* **267**, 318-336.

Balling, R., Helwig, U., Nadeau, J., Neubuser, A., Schmahl, W. and Imai, K. (1996). Pax genes and skeletal development. *Ann N Y Acad Sci* **785**, 27-33.

Beaster-Jones, L., Horton, A. C., Gibson-Brown, J. J., Holland, N. D. and Holland, L. Z. (2006). The amphioxus T-box gene, AmphiTbx15/18/22, illuminates the origins of chordate segmentation. *Evol Dev* **8**, 119-29.

Begemann, G., Gibert, Y., Meyer, A. and Ingham, P. W. (2002). Cloning of zebrafish T-box genes tbx15 and tbx18 and their expression during embryonic development. *Mech Dev* **114**, 137-41.

Bell, D. M., Leung, K. K., Wheatley, S. C., Ng, L. J., Zhou, S., Ling, K. W., Sham, M. H., Koopman, P., Tam, P. P. and Cheah, K. S. (1997). SOX9 directly regulates the type-II collagen gene. *Nat Genet* **16**, 174-8.

Belluoccio, D., Wilson, R., Thornton, D. J., Wallis, T. P., Gorman, J. J. and Bateman, J. F. (2006). Proteomic analysis of mouse growth plate cartilage. *Proteomics* **6**, 6549-53.

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**, 153-72.

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

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

Bhatia-Gaur, R., Donjacour, A. A., Sciavolino, P. J., Kim, M., Desai, N., Young, P., Norton, C. R., Gridley, T., Cardiff, R. D., Cunha, G. R. et al. (1999). Roles for Nkx3.1 in prostate development and cancer. *Genes Dev* **13**, 966-77.

Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R. and de Crombrugghe, B. (1999). Sox9 is required for cartilage formation. *Nat Genet* **22**, 85-9.

Bi, W., Huang, W., Whitworth, D. J., Deng, J. M., Zhang, Z., Behringer, R. R. and de Crombrugghe, B. (2001). Haploinsufficiency of Sox9 results in defective cartilage primordia and premature skeletal mineralization. *Proc Natl Acad Sci U S A* **98**, 6698-703.

Bialek, P., Kern, B., Yang, X., Schrock, M., Sosic, D., Hong, N., Wu, H., Yu, K., Ornitz, D. M., Olson, E. N. et al. (2004). A twist code determines the onset of osteoblast differentiation. *Dev Cell* **6**, 423-35.

Blair, J. E. and Hedges, S. B. (2005). Molecular phylogeny and divergence times of deuterostome animals. *Mol Biol Evol* **22**, 2275-84.

Blomme, T., Vandepoele, K., De Bodt, S., Simillion, C., Maere, S. and Van de Peer, Y. (2006). The gain and loss of genes during 600 million years of vertebrate evolution. *Genome Biol* **7**, R43.

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**, 1222-37.

Bohensky, J., Shapiro, I. M., Leshinsky, S., Terkhorn, S. P., Adams, C. S. and Srinivas, V. (2007). HIF-1 regulation of chondrocyte apoptosis: induction of the autophagic pathway. *Autophagy* **3**, 207-14.

Boot-Handford, R. P. and Tuckwell, D. S. (2003). Fibrillar collagen: the key to vertebrate evolution? A tale of molecular incest. *Bioessays* **25**, 142-51.

Bourlat, S. J., Juliusdottir, T., Lowe, C. J., Freeman, R., Aronowicz, J., Kirschner, M., Lander, E. S., Thorndyke, M., Nakano, H., Kohn, A. B. et al. (2006). Deuterostome phylogeny reveals monophyletic chordates and the new phylum Xenoturbellida. *Nature* **444**, 85-8.

Bowles, J., Schepers, G. and Koopman, P. (2000). Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. *Dev Biol* **227**, 239-55.

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**, 1513-21.

Boyle, W. J., Simonet, W. S. and Lacey, D. L. (2003). Osteoclast differentiation and activation. *Nature* **423**, 337-42.

Brand-Saberi, B. and Christ, B. (2000). Evolution and development of distinct cell lineages derived from somites. *Curr Top Dev Biol* **48**, 1-42.

Brand-Saberi, B., Ebensperger, C., Wilting, J., Balling, R. and Christ, B. (1993). The ventralizing effect of the notochord on somite differentiation in chick embryos. *Anat Embryol (Berl)* **188**, 239-45.

Brent, A. E., Schweitzer, R. and Tabin, C. J. (2003). A somitic compartment of tendon progenitors. *Cell* **113**, 235-48.

Brown, D., Wagner, D., Li, X., Richardson, J. A. and Olson, E. N. (1999). Dual role of the basic helix-loop-helix transcription factor scleraxis in mesoderm formation and chondrogenesis during mouse embryogenesis. *Development* **126**, 4317-29.

Bruckner, P. and van der Rest, M. (1994). Structure and function of cartilage collagens. *Microsc Res Tech* **28**, 378-84.

Brunet, L. J., McMahon, J. A., McMahon, A. P. and Harland, R. M. (1998). Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. *Science* **280**, 1455-7.

Buchberger, A., Schwarzer, M., Brand, T., Pabst, O., Seidl, K. and Arnold, H. H. (1998). Chicken winged-helix transcription factor cFKH-1 prefigures axial and appendicular skeletal structures during chicken embryogenesis. *Dev Dyn* **212**, 94-101.

Burgess, R., Cserjesi, P., Ligon, K. L. and Olson, E. N. (1995). Paraxis: a basic helix-loop-helix protein expressed in paraxial mesoderm and developing somites. *Dev Biol* **168**, 296-306.

Burgess, R., Rawls, A., Brown, D., Bradley, A. and Olson, E. N. (1996). Requirement of the paraxis gene for somite formation and musculoskeletal patterning. *Nature* **384**, 570-3.

Bussen, M., Petry, M., Schuster-Gossler, K., Leitges, M., Gossler, A. and Kispert, A. (2004). The T-box transcription factor Tbx18 maintains the separation of anterior and posterior somite compartments. *Genes Dev* **18**, 1209-21.

Buttitta, L., Mo, R., Hui, C. C. and Fan, C. M. (2003). Interplays of Gli2 and Gli3 and their requirement in mediating Shh-dependent sclerotome induction. *Development* **130**, 6233-43.

Buxton, P. G., Hall, B., Archer, C. W. and Francis-West, P. (2003). Secondary chondrocyte-derived Ihh stimulates proliferation of periosteal cells during chick development. *Development* **130**, 4729-39.

Cameron, R. A., Rowen, L., Nesbitt, R., Bloom, S., Rast, J. P., Berney, K., Arenas-Mena, C., Martinez, P., Lucas, S., Richardson, P. M. et al. (2006). Unusual gene order and organization of the sea urchin hox cluster. *J Exp Zoolog B Mol Dev Evol* **306**, 45-58.

Carpio, R., Honore, S. M., Araya, C. and Mayor, R. (2004). Xenopus paraxis homologue shows novel domains of expression. *Dev Dyn* **231**, 609-13.

Castanon, I. and Baylies, M. K. (2002). A Twist in fate: evolutionary comparison of Twist structure and function. *Gene* **287**, 11-22.

Chimal-Monroy, J., Rodriguez-Leon, J., Montero, J. A., Ganan, Y., Macias, D., Merino, R. and Hurle, J. M. (2003). Analysis of the molecular cascade responsible for mesodermal limb chondrogenesis: Sox genes and BMP signaling. *Dev Biol* **257**, 292-301.

Christ, B., Huang, R. and Scaal, M. (2004). Formation and differentiation of the avian sclerotome. *Anat Embryol (Berl)* **208**, 333-50.

Christ, B., Huang, R. and Scaal, M. (2007). Amniote somite derivatives. *Dev Dyn* **236**, 2382-2396.

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

Cohn, M. J. (2002). Lamprey Hox genes and the origin of jaws. *Nature* **416**, 386-7.

Cole, A. G. and Hall, B. K. (2004a). Cartilage is a metazoan tissue; integrating data from nonvertebrate sources. *Acta Zoologica (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**, 261-73.

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

Cole, F. J. (1905b). 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.

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**, 390-7.

Cotrufo, M., Della Corte, A., De Santo, L. S., Quarto, C., De Feo, M., Romano, G., Amarelli, C., Scardone, M., Di Meglio, F., Guerra, G. et al. (2005). Different patterns of extracellular matrix protein expression in the convexity and the concavity of the dilated aorta with bicuspid aortic valve: preliminary results. *J Thorac Cardiovasc Surg* **130**, 504-11.

Couly, G. F., Coltey, P. M. and Le Douarin, N. M. (1993). The triple origin of skull in higher vertebrates: a study in quail-chick chimeras. *Development* **117**, 409-29.

Dahl, E., Koseki, H. and Balling, R. (1997). Pax genes and organogenesis. *Bioessays* **19**, 755-65.

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**, 739-50.

De Luca, F., Barnes, K. M., Uyeda, J. A., De-Levi, S., Abad, V., Palese, T., Mericq, V. and Baron, J. (2001). Regulation of growth plate chondrogenesis by bone morphogenetic protein-2. *Endocrinology* **142**, 430-6.

Dehal, P. and Boore, J. L. (2005). Two rounds of whole genome duplication in the ancestral vertebrate. *PLoS Biol* **3**, e314.

DeLise, A. M. and Tuan, R. S. (2002). Alterations in the spatiotemporal expression pattern and function of N-cadherin inhibit cellular condensation and chondrogenesis of limb mesenchymal cells in vitro. *J Cell Biochem* **87**, 342-59.

Delsuc, F., Brinkmann, H., Chourrout, D. and Philippe, H. (2006). Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* **439**, 965-8.

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**, 911-21.

Dessau, W., von der Mark, H., von der Mark, K. and Fischer, S. (1980). Changes in the patterns of collagens and fibronectin during limb-bud chondrogenesis. *J Embryol Exp Morphol* **57**, 51-60.

Dietrich, S., Schubert, F. R. and Gruss, P. (1993). Altered Pax gene expression in murine notochord mutants: the notochord is required to initiate and maintain ventral identity in the somite. *Mech Dev* **44**, 189-207.

Dietrich, S., Schubert, F. R. and Lumsden, A. (1997). Control of dorsoventral pattern in the chick paraxial mesoderm. *Development* **124**, 3895-908.

Dobreva, G., Chahrour, M., Dautzenberg, M., Chirivella, L., Kanzler, B., Farinas, I., Karsenty, G. and Grosschedl, R. (2006). SATB2 is a multifunctional determinant of craniofacial patterning and osteoblast differentiation. *Cell* **125**, 971-86.

Dockter, J. and Ordahl, C. P. (2000). Dorsoventral axis determination in the somite: a re-examination. *Development* **127**, 2201-6.

Dockter, J. L. (2000). Sclerotome induction and differentiation. *Curr Top Dev Biol* **48**, 77-127.

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**, 655-64.

Donoghue, P. C., Forey, P. L. and Aldridge, R. J. (2000). Conodont affinity and chordate phylogeny. *Biol Rev Camb Philos Soc* **75**, 191-251.

Donoghue, P. C., Kouchinsky, A., Waloszek, D., Bengtson, S., Dong, X. P., Val'kov, A. K., Cunningham, J. A. and Repetski, J. E. (2006a). Fossilized embryos are widespread but the record is temporally and taxonomically biased. *Evol Dev* **8**, 232-8.

Donoghue, P. C. and Sansom, I. J. (2002). Origin and early evolution of vertebrate skeletonization. *Microsc Res Tech* **59**, 352-72.

Donoghue, P. C., Sansom, I. J. and Downs, J. P. (2006b). Early evolution of vertebrate skeletal tissues and cellular interactions, and the canalization of skeletal development. *J Exp Zoolog B Mol Dev Evol* **306**, 278-94.

Dubois, G. M., Haftek, Z., Crozet, C., Garrone, R. and Le Guellec, D. (2002). Structure and spatio temporal expression of the full length DNA complementary to RNA coding for alpha2 type I collagen of zebrafish. *Gene* **294**, 55-65.

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**, 1025-36.

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L. and Karsenty, G. (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* **89**, 747-54.

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.

Eferl, R., Hoebertz, A., Schilling, A. F., Rath, M., Karreth, F., Kenner, L., Amling, M. and Wagner, E. F. (2004). The Fos-related antigen Fra-1 is an activator of bone matrix formation. *Embo J* **23**, 2789-99.

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**, 261-77.

El-Hodiri, H., Bhatia-Dey, N., Kenyon, K., Ault, K., Dirksen, M. and Jamrich, M. (2001). Fox (forkhead) genes are involved in the dorso-ventral patterning of the Xenopus mesoderm. *Int J Dev Biol* **45**, 265-71.

Erlebacher, A., Filvaroff, E. H., Gitelman, S. E. and Derynck, R. (1995). Toward a molecular understanding of skeletal development. *Cell* **80**, 371-8.

Escriva, H., Manzon, L., Youson, J. and Laudet, V. (2002). Analysis of lamprey and hagfish genes reveals a complex history of gene duplications during early vertebrate evolution. *Mol Biol Evol* **19**, 1440-50.

Eswarakumar, V. P., Monsonego-Ornan, E., Pines, M., Antonopoulou, I., Morriss-Kay, G. M. and Lonai, P. (2002). The IIIc alternative of Fgfr2 is a positive regulator of bone formation. *Development* **129**, 3783-93.

Exposito, J. Y., Cluzel, C., Garrone, R. and Lethias, C. (2002). Evolution of collagens. *Anat Rec* **268**, 302-16.

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**, 2568-74.

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

Fang, J. and Hall, B. K. (1996). In vitro differentiation potential of the periosteal cells from a membrane bone, the quadratojugal of the embryonic chick. *Dev Biol* **180**, 701-12.

Fang, J. and Hall, B. K. (1997). Chondrogenic cell differentiation from membrane bone periostea. *Anat Embryol (Berl)* **196**, 349-62.

Felsenstein, J. (2004). Inferring Phylogenies. Sinauer, Sunderland, MA: Sinauer Associates.

Force, A., Amores, A. and Postlethwait, J. H. (2002). Hox cluster organization in the jawless vertebrate Petromyzon marinus. *J Exp Zool* **294**, 30-46.

Force, A., Lynch, M., Pickett, F. B., Amores, A., Yan, Y. L. and Postlethwait, J. (1999). Preservation of duplicate genes by complementary, degenerative mutations. *Genetics* **151**, 1531-45.

Forey, P. and Janvier, P. (1993). Agnathans and the origin of the jawed vertebrates. *Nature* **361**, 129-134.

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. et al. (1994). Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature* **372**, 525-30.

Franz-Odendaal, T. A., Hall, B. K. and Witten, P. E. (2006). Buried alive: how osteoblasts become osteocytes. *Dev Dyn* **235**, 176-90.

Freitas, R., Zhang, G. and Cohn, M. J. (2006). Evidence that mechanisms of fin development evolved in the midline of early vertebrates. *Nature* **442**, 1033-7.

Fried, C., Prohaska, S. J. and Stadler, P. F. (2003). Independent Hox-cluster duplications in lampreys. *J Exp Zoolog B Mol Dev Evol* **299**, 18-25.

Furumoto, T. A., Miura, N., Akasaka, T., Mizutani-Koseki, Y., Sudo, H., Fukuda, K., Maekawa, M., Yuasa, S., Fu, Y., Moriya, H. et al. (1999). Notochord-dependent expression of MFH1 and PAX1 cooperates to maintain the proliferation of sclerotome cells during the vertebral column development. *Dev Biol* **210**, 15-29.

Gadow, H. and Abbott, E. C. (1895). On the vertebral coumn of Fishes. *Philosophical Transactions of the Royal Society of London, Series B* **186**, 163-221.

Gadow, H. F. (1933). The evolution of the vertebral column: a contribution to the study of the vertebrate phylogeny. Cambridge: Cambridge University Press.

Garcia-Fernandez, J. (2005). The genesis and evolution of homeobox gene clusters. *Nat Rev Genet* **6**, 881-92.

Gemballa, S., Ebmeyer, L., Hagen, K., Hannich, T., Hoja, K., Rolf, M., Treiber, K., Vogel, F. and Weitbrecht, G. (2003). Evolutionary transformations of myoseptal tendons in gnathostomes. *Proc Biol Sci* **270**, 1229-35.

Geoffroy, V., Kneissel, M., Fournier, B., Boyde, A. and Matthias, P. (2002). High bone resorption in adult aging transgenic mice overexpressing cbfa1/runx2 in cells of the osteoblastic lineage. *Mol Cell Biol* **22**, 6222-33.

Gerber, H. P. and Ferrara, N. (2000). Angiogenesis and bone growth. *Trends Cardiovasc Med* **10**, 223-8.

Gerlach, D., Wolf, M., Dandekar, T., Muller, T., Pokorny, A. and Rahmann, S. (2007). Deep metazoan phylogeny. *In Silico Biol* **7**, 0015.

Germot, A., Lecointre, G., Plouhinec, J. L., Le Mentec, C., Girardot, F. and Mazan, S. (2001). Structural evolution of Otx genes in craniates. *Mol Biol Evol* **18**, 1668-78.

Ghanem, E. (1996). Immunohistochemical localization of type I and II collagens in the involuting chick notochords in vivo and in vitro. *Cell Biol Int* **20**, 681-5.

Glass, D. A., 2nd, Bialek, P., Ahn, J. D., Starbuck, M., Patel, M. S., Clevers, H., Taketo, M. M., Long, F., McMahon, A. P., Lang, R. A. et al. (2005). Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Dev Cell* **8**, 751-64.

Goldring, M. B., Tsuchimochi, K. and Ijiri, K. (2006). The control of chondrogenesis. *J Cell Biochem* **97**, 33-44.

Gong, Y., Slee, R. B., Fukai, N., Rawadi, G., Roman-Roman, S., Reginato, A. M., Wang, H., Cundy, T., Glorieux, F. H., Lev, D. et al. (2001). LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell* **107**, 513-23.

Goodrich, E. S. (1930). Studies on the structure and development of the vertebrates. London: Macmillan.

Guindon, S. and Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52**, 696-704.

Guo, X., Day, T. F., Jiang, X., Garrett-Beal, L., Topol, L. and Yang, Y. (2004). Wnt/beta-catenin signaling is sufficient and necessary for synovial joint formation. *Genes Dev* **18**, 2404-17.

Haas, A. R. and Tuan, R. S. (1999). Chondrogenic differentiation of murine C3H10T1/2 multipotential mesenchymal cells: II. Stimulation by bone morphogenetic protein-2 requires modulation of N-cadherin expression and function. *Differentiation* **64**, 77-89.

Haenig, B. and Kispert, A. (2004). Analysis of TBX18 expression in chick embryos. *Dev Genes Evol* **214**, 407-11.

Hall, B. K. (1967). The formation of adventitious cartilage by membrane bones under the influence of mechanical stimulation applied in vitro. *Life Sci* **6**, 663-7.

Hall, B. K. (1970). Cellular differentiation in skeletal tissues. *Biol Rev Camb Philos Soc* **45**, 455-84.

Hall, B. K. (2005). Bone and cartilage: development and evolutionary skeletal biology. San Diego: Elsevire Academic Press.

Hall, B. K. and Jacobson, H. N. (1975). The repair of fractured membrane bones in the newly hatched chick. *Anat Rec* **181**, 55-69.

Hall, B. K. and Miyake, T. (2000). All for one and one for all: condensations and the initiation of skeletal development. *Bioessays* **22**, 138-47.

Hamerman, D. (1989). The biology of osteoarthritis. *N Engl J Med* **320**, 1322-30.

Hardingham, T. E. and Fosang, A. J. (1992). Proteoglycans: many forms and many functions. *Faseb J* **6**, 861-70.

Hartmann, C. (2006). A Wnt canon orchestrating osteoblastogenesis. *Trends Cell Biol* **16**, 151-8.

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**, 341-51.

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**, 261-2.

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

Herbrand, H., Pabst, O., Hill, R. and Arnold, H. H. (2002). Transcription factors Nkx3.1 and Nkx3.2 (Bapx1) play an overlapping role in sclerotomal development of the mouse. *Mech Dev* **117**, 217-24.

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**, 727-38.

Hinoi, E., Bialek, P., Chen, Y. T., Rached, M. T., Groner, Y., Behringer, R. R., Ornitz, D. M. and Karsenty, G. (2006). Runx2 inhibits chondrocyte proliferation and hypertrophy through its expression in the perichondrium. *Genes Dev* **20**, 2937-42.

Holland, L. Z. and Holland, N. D. (1996). Expression of AmphiHox-1 and AmphiPax-1 in amphioxus embryos treated with retinoic acid: insights into evolution and patterning of the chordate nerve cord and pharynx. *Development* **122**, 1829-38.

Holland, P. W., Garcia-Fernandez, J., Williams, N. A. and Sidow, A. (1994). Gene duplications and the origins of vertebrate development. *Dev Suppl*, 125-33.

Holland, P. W. and Williams, N. A. (1990). Conservation of engrailed-like homeobox sequences during vertebrate evolution. *FEBS Lett* **277**, 250-2.

Hornik, C., Brand-Saberi, B., Rudloff, S., Christ, B. and Fuchtbauer, E. M. (2004). Twist is an integrator of SHH, FGF, and BMP signaling. *Anat Embryol (Berl)* **209**, 31-9.

Hou, B., Xu, Z. W., Yang, C. W., Gao, Y., Zhao, S. F. and Zhang, C. G. (2007). Protective effects of inosine on mice subjected to lethal total-body ionizing irradiation. *J Radiat Res (Tokyo)* **48**, 57-62.

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.

Huang, L. F., Fukai, N., Selby, P. B., Olsen, B. R. and Mundlos, S. (1997). Mouse clavicular development: analysis of wild-type and cleidocranial dysplasia mutant mice. *Dev Dyn* **210**, 33-40.

Huang, W., Chung, U. I., Kronenberg, H. M. and de Crombrugghe, B. (2001). The chondrogenic transcription factor Sox9 is a target of signaling by the parathyroid hormone-related peptide in the growth plate of endochondral bones. *Proc Natl Acad Sci U S A* **98**, 160-5.

Ichida, F., Nishimura, R., Hata, K., Matsubara, T., Ikeda, F., Hisada, K., Yatani, H., Cao, X., Komori, T., Yamaguchi, A. et al. (2004). Reciprocal roles of MSX2 in regulation of osteoblast and adipocyte differentiation. *J Biol Chem* **279**, 34015-22.

Iida, K., Koseki, H., Kakinuma, H., Kato, N., Mizutani-Koseki, Y., Ohuchi, H., Yoshioka, H., Noji, S., Kawamura, K., Kataoka, Y. et al. (1997). Essential roles of the winged helix

transcription factor MFH-1 in aortic arch patterning and skeletogenesis. *Development* **124**, 4627-38.

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**, 3561-73.

Inada, M., Yasui, T., Nomura, S., Miyake, S., Deguchi, K., Himeno, M., Sato, M., Yamagiwa, H., Kimura, T., Yasui, N. et al. (1999). Maturational disturbance of chondrocytes in Cbfa1-deficient mice. *Dev Dyn* **214**, 279-90.

Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev* **15**, 3059-87.

Iozzo, R. V. (1998). Matrix proteoglycans: from molecular design to cellular function. *Annu Rev Biochem* **67**, 609-52.

Irvine, S. Q., Carr, J. L., Bailey, W. J., Kawasaki, K., Shimizu, N., Amemiya, C. T. and Ruddle, F. H. (2002). Genomic analysis of Hox clusters in the sea lamprey Petromyzon marinus. *J Exp Zool* **294**, 47-62.

Ishii, M., Han, J., Yen, H. Y., Sucov, H. M., Chai, Y. and Maxson, R. E., Jr. (2005). Combined deficiencies of Msx1 and Msx2 cause impaired patterning and survival of the cranial neural crest. *Development* **132**, 4937-50.

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). *Osteoarthritis Cartilage* **11**, 6-15.

Jackson, A., Vayssiere, B., Garcia, T., Newell, W., Baron, R., Roman-Roman, S. and Rawadi, G. (2005). Gene array analysis of Wnt-regulated genes in C3H10T1/2 cells. *Bone* **36**, 585-98.

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**, 315-28.

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

Jochum, W., Passegue, E. and Wagner, E. F. (2001). AP-1 in mouse development and tumorigenesis. *Oncogene* **20**, 2401-12.

Johnson, J., Rhee, J., Parsons, S. M., Brown, D., Olson, E. N. and Rawls, A. (2001). The anterior/posterior polarity of somites is disrupted in paraxis-deficient mice. *Dev Biol* **229**, 176-87.

Junqueira, L. C., Toledo, O. M. and Montes, G. S. (1983). Histochemical and morphological studies on a new type of acellular cartilage. *Basic Appl Histochem* **27**, 1-8.

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**, 277-89.

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**, 543-8.

Karreth, F., Hoebertz, A., Scheuch, H., Eferl, R. and Wagner, E. F. (2004). The AP1 transcription factor Fra2 is required for efficient cartilage development. *Development* **131**, 5717-25.

Karsenty, G. (2003). The complexities of skeletal biology. *Nature* **423**, 316-8.

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., 2nd, Hartmann, C., Li, L., Hwang, T. H., Brayton, C. F. 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**, 303-14.

Kawakami, Y., Tsuda, M., Takahashi, S., Taniguchi, N., Esteban, C. R., Zemmyo, M., Furumatsu, T., Lotz, M., Belmonte, J. C. and Asahara, H. (2005). Transcriptional coactivator PGC-1alpha regulates chondrogenesis via association with Sox9. *Proc Natl Acad Sci U S A* **102**, 2414-9.

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

Kenner, L., Hoebertz, A., Beil, T., Keon, N., Karreth, F., Eferl, R., Scheuch, H., Szremska, A., Amling, M., Schorpp-Kistner, M. et al. (2004). Mice lacking JunB are osteopenic due to cell-autonomous osteoblast and osteoclast defects. *J Cell Biol* **164**, 613-23.

Kim, I. S., Otto, F., Zabel, B. and Mundlos, S. (1999). Regulation of chondrocyte differentiation by Cbfa1. *Mech Dev* **80**, 159-70.

Kimura, S. and Karasawa, K. (1985). Squid cartilage collagen: isolation of type I collagen rich in carbohydrate. *Comparative Biochemistry and Physiology* **81B**, 361-365.

Kimura, S. and Matsuura, F. (1974). The chain compositions of several invertebrate collagens. *J Biochem (Tokyo)* **75**, 1231-40.

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

Kobayashi, H., Gao, Y., Ueta, C., Yamaguchi, A. and Komori, T. (2000). Multilineage differentiation of Cbfa1-deficient calvarial cells in vitro. *Biochem Biophys Res Commun* **273**, 630-6.

Kobayashi, T. and Kronenberg, H. (2005). Minireview: transcriptional regulation in development of bone. *Endocrinology* **146**, 1012-7.

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 U S A* **102**, 18023-7.

Komori, T. (2002). Runx2, a multifunctional transcription factor in skeletal development. *J Cell Biochem* **87**, 1-8.

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R. T., Gao, Y. H., Inada, M. et al. (1997). Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* **89**, 755-64.

Koob, T. J. and Long, J. H. J. (2000). The Vertebrate Body Axis: Evolution and Mechanical Function. *American Zoologist* **40**, 1-18.

Koopman, P., Schepers, G., Brenner, S. and Venkatesh, B. (2004). Origin and diversity of the SOX transcription factor gene family: genome-wide analysis in Fugu rubripes. *Gene* **328**, 177-86.

Kos, L., Chiang, C. and Mahon, K. A. (1998). Mediolateral patterning of somites: multiple axial signals, including Sonic hedgehog, regulate Nkx-3.1 expression. *Mech Dev* **70**, 25-34.

Koseki, H., Wallin, J., Wilting, J., Mizutani, Y., Kispert, A., Ebensperger, C., Herrmann, B. G., Christ, B. and Balling, R. (1993). A role for Pax-1 as a mediator of notochordal signals during the dorsoventral specification of vertebrae. *Development* **119**, 649-60.

Koyama, E., Leatherman, J. L., Shimazu, A., Nah, H. D. and Pacifici, M. (1995). Syndecan-3, tenascin-C, and the development of cartilaginous skeletal elements and joints in chick limbs. *Dev Dyn* **203**, 152-62.

Kraus, F., Haenig, B. and Kispert, A. (2001). Cloning and expression analysis of the mouse T-box gene Tbx18. *Mech Dev* **100**, 83-6.

Kumar, S., Tamura, K. and Nei, M. (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **5**, 150-63.

Kume, T., Deng, K. and Hogan, B. L. (2000). Murine forkhead/winged helix genes Foxc1 (Mf1) and Foxc2 (Mfh1) are required for the early organogenesis of the kidney and urinary tract. *Development* **127**, 1387-95.

Kume, T., Jiang, H., Topczewska, J. M. and Hogan, B. L. (2001). The murine winged helix transcription factors, Foxc1 and Foxc2, are both required for cardiovascular development and somitogenesis. *Genes Dev* **15**, 2470-82.

Kusserow, A., Pang, K., Sturm, C., Hrouda, M., Lentfer, J., Schmidt, H. A., Technau, U., von Haeseler, A., Hobmayer, B., Martindale, M. Q. et al. (2005). Unexpected complexity of the Wnt gene family in a sea anemone. *Nature* **433**, 156-60.

Kveiborg, M., Sabatakos, G., Chiusaroli, R., Wu, M., Philbrick, W. M., Horne, W. C. and Baron, R. (2004). DeltaFosB induces osteosclerosis and decreases adipogenesis by two independent cell-autonomous mechanisms. *Mol Cell Biol* **24**, 2820-30.

Lammi, M. J., Hayrinen, J. and Mahonen, A. (2006). Proteomic analysis of cartilage- and bone-associated samples. *Electrophoresis* **27**, 2687-701.

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. et al. (1996). PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* **273**, 663-6.

Lee, C. S., Buttitta, L. and Fan, C. M. (2001). Evidence that the WNT-inducible growth arrest-specific gene 1 encodes an antagonist of sonic hedgehog signaling in the somite. *Proc Natl Acad Sci U S A* **98**, 11347-52.

Lee, C. S., Buttitta, L. A., May, N. R., Kispert, A. and Fan, C. M. (2000). SHH-N upregulates Sfrp2 to mediate its competitive interaction with WNT1 and WNT4 in the somitic mesoderm. *Development* **127**, 109-18.

Lee, M. H., Kim, Y. J., Yoon, W. J., Kim, J. I., Kim, B. G., Hwang, Y. S., Wozney, J. M., Chi, X. Z., Bae, S. C., Choi, K. Y. et al. (2005). Dlx5 specifically regulates Runx2 type II

expression by binding to homeodomain-response elements in the Runx2 distal promoter. *J Biol Chem* **280**, 35579-87.

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**, 157-67.

Lefebvre, V., Behringer, R. R. and de Crombrugghe, B. (2001). L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. *Osteoarthritis Cartilage* **9 Suppl A**, S69-75.

Lefebvre, V. and de Crombrugghe, B. (1998). Toward understanding SOX9 function in chondrocyte differentiation. *Matrix Biol* **16**, 529-40.

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**, 2336-46.

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**, 5718-33.

Lengner, C. J., Hassan, M. Q., Serra, R. W., Lepper, C., van Wijnen, A. J., Stein, J. L., Lian, J. B. and Stein, G. S. (2005). Nkx3.2-mediated repression of Runx2 promotes chondrogenic differentiation. *J Biol Chem* **280**, 15872-9.

Lettice, L., Hecksher-Sorensen, J. and Hill, R. (2001). The role of Bapx1 (Nkx3.2) in the development and evolution of the axial skeleton. *J Anat* **199**, 181-7.

Lettice, L. A., Purdie, L. A., Carlson, G. J., Kilanowski, F., Dorin, J. and Hill, R. E. (1999). The mouse bagpipe gene controls development of axial skeleton, skull, and spleen. *Proc Natl Acad Sci U S A* **96**, 9695-700.

Levanon, D., Brenner, O., Negreanu, V., Bettoun, D., Woolf, E., Eilam, R., Lotem, J., Gat, U., Otto, F., Speck, N. et al. (2001). Spatial and temporal expression pattern of Runx3 (Aml2) and Runx1 (Aml1) indicates non-redundant functions during mouse embryogenesis. *Mech Dev* **109**, 413-7.

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**, 128-36.

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**, 423-30.

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. et al. (2003). Runx1/AML1 hematopoietic transcription factor contributes to skeletal development in vivo. *J Cell Physiol* **196**, 301-11.

Libbin, R. M., Hirschman, A., Person, P. and Blumenthal, N. C. (1992). Alkaline phosphatase and peptidase levels in invertebrate cartilage. *Calcif Tissue Int* **51**, 62-6.

Libbin, R. M., Ozer, R., Person, P. and Hirschman, A. (1976). In vitro accumulation of mineral components by invertebrate cartilage. *Calcif Tissue Res* **22**, 67-75.

Linsenmayer, T. F., Gibney, E. and Schmid, T. M. (1986). Segmental appearance of type X collagen in the developing avian notochord. *Dev Biol* **113**, 467-73.

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. 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**, 11-9.

Liu, C. J., Zhang, Y., Xu, K., Parsons, D., Alfonso, D. and Di Cesare, P. E. (2007). Transcriptional activation of cartilage oligomeric matrix protein by Sox9, Sox5, and Sox6 transcription factors and CBP/p300 coactivators. *Front Biosci* **12**, 3899-910.

Liu, W., Toyosawa, S., Furuichi, T., Kanatani, N., Yoshida, C., Liu, Y., Himeno, M., Narai, S., Yamaguchi, A. and Komori, T. (2001). Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. *J Cell Biol* **155**, 157-66.

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**, 12712-8.

Liu, Z., Xu, J., Colvin, J. S. and Ornitz, D. M. (2002). Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18. *Genes Dev* **16**, 859-69.

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

Long, F., Chung, U. I., Ohba, S., McMahon, J., Kronenberg, H. M. and McMahon, A. P. (2004). Ihh signaling is directly required for the osteoblast lineage in the endochondral skeleton. *Development* **131**, 1309-18.

Luvalle, P., Ma, Q. and Beier, F. (2003). The role of activating transcription factor-2 in skeletal growth control. *J Bone Joint Surg Am* **85-A Suppl 2**, 133-6.

Lynch, M., O'Hely, M., Walsh, B. and Force, A. (2001). The probability of preservation of a newly arisen gene duplicate. *Genetics* **159**, 1789-804.

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.

Mallatt, J. and Winchell, C. J. (2007). Ribosomal RNA genes and deuterostome phylogeny revisited: more cyclostomes, elasmobranchs, reptiles, and a brittle star. *Mol Phylogenet Evol* **43**, 1005-22.

Martinez, P. and Amemiya, C. T. (2002). Genomics of the HOX gene cluster. *Comp Biochem Physiol B Biochem Mol Biol* **133**, 571-80.

Maurer, F. (1906). Die Entwickelung des Muskelsystems und der elekrischen Organe. In *Handbuch der Vergleichenden und Experimentellen Entwickelungslehre der Wirbeltiere*, vol. 3 (ed. O. Hertwig), pp. 1-80. Jena: Gustav Fischer.

McBurney, K. M., Keeley, F. W., Kibenge, F. S. and Wright, G. M. (1996a). Spatial and temporal distribution of lamprin mRNA during chondrogenesis of trabecular cartilage in the sea lamprey. *Anat Embryol (Berl)* **193**, 419-26.

McBurney, K. M., Keeley, F. W., Kibenge, F. S. and Wrignt, G. M. (1996b). Detection of lamprin mRNA in the anadromous sea lamprey using in situ hybridization. *Biotech Histochem* **71**, 44-53.

McCauley, D. W. and Bronner-Fraser, M. (2003). Neural crest contributions to the lamprey head. *Development* **130**, 2317-27.

McMahon, J. A., Takada, S., Zimmerman, L. B., Fan, C. M., Harland, R. M. and McMahon, A. P. (1998). Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev* **12**, 1438-52.

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

Meyer, A. and Schartl, M. (1999). Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. *Curr Opin Cell Biol* **11**, 699-704.

Miller, C. T., Yelon, D., Stainier, D. Y. and Kimmel, C. B. (2003). Two endothelin 1 effectors, hand2 and bapx1, pattern ventral pharyngeal cartilage and the jaw joint. *Development* **130**, 1353-65.

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

Minina, E., Kreschel, C., Naski, M. C., Ornitz, D. M. and Vortkamp, A. (2002). Interaction of FGF, Ihh/Pthlh, and BMP signaling integrates chondrocyte proliferation and hypertrophic differentiation. *Dev Cell* **3**, 439-49.

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**, 4523-34.

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

Mo, R., Freer, A. M., Zinyk, D. L., Crackower, M. A., Michaud, J., Heng, H. H., Chik, K. W., Shi, X. M., Tsui, L. C., Cheng, S. H. et al. (1997). Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. *Development* **124**, 113-23.

Monsoro-Burq, A. H., Bontoux, M., Teillet, M. A. and Le Douarin, N. M. (1994). Heterogeneity in the development of the vertebra. *Proc Natl Acad Sci U S A* **91**, 10435-9.

Monsoro-Burq, A. H., Duprez, D., Watanabe, Y., Bontoux, M., Vincent, C., Brickell, P. and Le Douarin, N. (1996). The role of bone morphogenetic proteins in vertebral development. *Development* **122**, 3607-16.

Monsoro-Burq, A. H. and Le Douarin, N. (2000). Duality of molecular signaling involved in vertebral chondrogenesis. *Curr Top Dev Biol* **48**, 43-75.

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 U S A* **100**, 9360-5.

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**, 252-67.

Morvan-Dubois, G., Le Guellec, D., Garrone, R., Zylberberg, L. and Bonnaud, L. (2003). Phylogenetic analysis of vertebrate fibrillar collagen locates the position of zebrafish alpha3(I) and suggests an evolutionary link between collagen alpha chains and hox clusters. *J Mol Evol* **57**, 501-14.

Moss, M. L. (1970). Enamel and bone in shark teeth: with a note on fibrous enamel in fishes. *Acta Anat (Basel)* **77**, 161-87.

Moss, M. L. (1977). Skeletal tissues in sharks. *American zoologist* **17**, 335-342.

Muller, T. S., Ebensperger, C., Neubuser, A., Koseki, H., Balling, R., Christ, B. and Wilting, J. (1996). Expression of avian Pax1 and Pax9 is intrinsically regulated in the pharyngeal endoderm, but depends on environmental influences in the paraxial mesoderm. *Dev Biol* **178**, 403-17.

Mundlos, S., Huang, L. F., Selby, P. and Olsen, B. R. (1996). Cleidocranial dysplasia in mice. *Ann N Y Acad Sci* **785**, 301-2.

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**, 125-32.

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**, 227-33.

Murtaugh, L. C., Zeng, L., Chyung, J. H. and Lassar, A. B. (2001). The chick transcriptional repressor Nkx3.2 acts downstream of Shh to promote BMP-dependent axial chondrogenesis. *Dev Cell* **1**, 411-22.

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**, 1049-58.

Neidert, A. H., Virupannavar, V., Hooker, G. W. and Langeland, J. A. (2001). Lamprey Dlx genes and early vertebrate evolution. *Proc Natl Acad Sci U S A* **98**, 1665-70.

Neubuser, A., Koseki, H. and Balling, R. (1995). Characterization and developmental expression of Pax9, a paired-box-containing gene related to Pax1. *Dev Biol* **170**, 701-16.

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**, 108-21.

Nieto, M. A., Patel, K. and Wilkinson, D. G. (1996). In situ hybridization analysis of chick embryos in whole mount and tissue sections. *Methods Cell Biol* **51**, 219-35.

O'Rourke, M. P. and Tam, P. P. (2002). Twist functions in mouse development. *Int J Dev Biol* **46**, 401-13.

Ogasawara, M., Shigetani, Y., Hirano, S., Satoh, N. and Kuratani, S. (2000). Pax1/Pax9-Related genes in an agnathan vertebrate, Lampetra japonica: expression pattern of LjPax9 implies sequential evolutionary events toward the gnathostome body plan. *Dev Biol* **223**, 399-410.

Ogasawara, M., Wada, H., Peters, H. and Satoh, N. (1999). Developmental expression of Pax1/9 genes in urochordate and hemichordate gills: insight into function and evolution of the pharyngeal epithelium. *Development* **126**, 2539-50.

Ohbayashi, N., Shibayama, M., Kurotaki, Y., Imanishi, M., Fujimori, T., Itoh, N. and Takada, S. (2002). FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. *Genes Dev* **16**, 870-9.

Ohno, S. (1970). Evolution by gene duplication. New York: Springer.

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**, 1446-65.

Ota, K. G., Kuraku, S. and Kuratani, S. (2007). Hagfish embryology with reference to the evolution of the neural crest. *Nature* **446**, 672-5.

Ota, K. G. and Kuratani, S. (2006). The history of scientific endeavors towards understanding hagfish embryology. *Zoolog Sci* **23**, 403-18.

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. et al. (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* **89**, 765-71.

Pace, J. M., Corrado, M., Missero, C. and Byers, P. H. (2003). Identification, characterization and expression analysis of a new fibrillar collagen gene, COL27A1. *Matrix Biol* **22**, 3-14.

Panopoulou, G., Hennig, S., Groth, D., Krause, A., Poustka, A. J., Herwig, R., Vingron, M. and Lehrach, H. (2003). New evidence for genome-wide duplications at the origin of vertebrates using an amphioxus gene set and completed animal genomes. *Genome Res* **13**, 1056-66.

Panopoulou, G. and Poustka, A. J. (2005). Timing and mechanism of ancient vertebrate genome duplications -- the adventure of a hypothesis. *Trends Genet* **21**, 559-67.

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

Pathi, S., Rutenberg, J. B., Johnson, R. L. and Vortkamp, A. (1999). Interaction of Ihh and BMP/Noggin signaling during cartilage differentiation. *Dev Biol* **209**, 239-53.

Pavalko, F. M., Chen, N. X., Turner, C. H., Burr, D. B., Atkinson, S., Hsieh, Y. F., Qiu, J. and Duncan, R. L. (1998). Fluid shear-induced mechanical signaling in MC3T3-E1 osteoblasts requires cytoskeleton-integrin interactions. *Am J Physiol* **275**, C1591-601.

Peignoux-Deville, J., Lallier, F. and Vidal, B. (1982). Evidence for the presence of osseous tissue in dogfish vertebrae. *Cell Tissue Res* **222**, 605-14.

Person, P. and Mathews, M. B. (1967). Endoskeletal cartilage in a marine polychaete, Eudistylia polymorpha. *Biol Bull* **132**, 244-52.

Peters, H., Doll, U. and Niessing, J. (1995). Differential expression of the chicken Pax-1 and Pax-9 gene: in situ hybridization and immunohistochemical analysis. *Dev Dyn* **203**, 1-16.

Peters, H., Neubuser, A., Kratochwil, K. and Balling, R. (1998). Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. *Genes Dev* **12**, 2735-47.

Peters, H., Wilm, B., Sakai, N., Imai, K., Maas, R. and Balling, R. (1999). Pax1 and Pax9 synergistically regulate vertebral column development. *Development* **126**, 5399-408.

Pourquie, O., Coltey, M., Teillet, M. A., Ordahl, C. and Le Douarin, N. M. (1993). Control of dorsoventral patterning of somitic derivatives by notochord and floor plate. *Proc Natl Acad Sci U S A* **90**, 5242-6.

Powell, M. L. K., S. I.; Sower, S. A. (2005). Current knoledge of hagfish reproduction: Implications for fisheries management. *Integr. Comp. Biol.* **45**, 158-165.

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**, 45539-45.

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**, 651-62.

Provot, S. and Schipani, E. (2005). Molecular mechanisms of endochondral bone development. *Biochem Biophys Res Commun* **328**, 658-65.

Provot, S., Zinyk, D., Gunes, Y., Kathri, R., Le, Q., Kronenberg, H. M., Johnson, R. S., Longaker, M. T., Giaccia, A. J. and Schipani, E. (2007). Hif-1alpha regulates differentiation of limb bud mesenchyme and joint development. *J Cell Biol* **177**, 451-64.

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 (Carcharius acutus). *Connect Tissue Res* **12**, 111-8.

Rau, M. J., Fischer, S. and Neumann, C. J. (2006). Zebrafish Trap230/Med12 is required as a coactivator for Sox9-dependent neural crest, cartilage and ear development. *Dev Biol* **296**, 83-93.

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**, 1842-53.

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**, 453-61.

Robledo, R. F., Rajan, L., Li, X. and Lufkin, T. (2002). The Dlx5 and Dlx6 homeobox genes are essential for craniofacial, axial, and appendicular skeletal development. *Genes Dev* **16**, 1089-101.

Robson, P., Wright, G. M. and Keeley, F. W. (2000). Distinct non-collagen based cartilages comprising the endoskeleton of the Atlantic hagfish, Myxine glutinosa. *Anat Embryol (Berl)* **202**, 281-90.

Robson, P., Wright, G. M., Sitarz, E., Maiti, A., Rawat, M., Youson, J. H. and Keeley, F. W. (1993). Characterization of lamprin, an unusual matrix protein from lamprey cartilage. Implications for evolution, structure, and assembly of elastin and other fibrillar proteins. *J Biol Chem* **268**, 1440-7.

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**, 3231-44.

Rodrigo, I., Hill, R. E., Balling, R., Munsterberg, A. and Imai, K. (2003). Pax1 and Pax9 activate Bapx1 to induce chondrogenic differentiation in the sclerotome. *Development* **130**, 473-82.

Romer, A. S. (1985). The Vertebrate Body. Philadelphia: Saunders College Publishing.

Ronquist, F. and Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572-4.

Rose, T. M., Henikoff, J. G. and Henikoff, S. (2003). CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) PCR primer design. *Nucleic Acids Res* **31**, 3763-6.

Ruddle, F. H., Bartels, J. L., Bentley, K. L., Kappen, C., Murtha, M. T. and Pendleton, J. W. (1994a). Evolution of Hox genes. *Annu Rev Genet* **28**, 423-42.

Ruddle, F. H., Bentley, K. L., Murtha, M. T. and Risch, N. (1994b). Gene loss and gain in the evolution of the vertebrates. *Dev Suppl*, 155-61.

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**, 541-9.

Rychel, A. L. and Swalla, B. J. (2007). Development and evolution of chordate cartilage. *J Exp Zoolog B Mol Dev Evol* **308**, 325-35.

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**, 1361-6.

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**, 504-9.

Sasaki, H. and Hogan, B. L. (1993). Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo. *Development* **118**, 47-59.

Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M. and Kondoh, H. (1999). Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. *Development* **126**, 3915-24.

Satokata, I., Ma, L., Ohshima, H., Bei, M., Woo, I., Nishizawa, K., Maeda, T., Takano, Y., Uchiyama, M., Heaney, S. et al. (2000). Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. *Nat Genet* **24**, 391-5.

Satou, Y., Takatori, N., Yamada, L., Mochizuki, Y., Hamaguchi, M., Ishikawa, H., Chiba, S., Imai, K., Kano, S., Murakami, S. D. et al. (2001). Gene expression profiles in Ciona intestinalis tailbud embryos. *Development* **128**, 2893-904.

Sauka-Spengler, T., Meulemans, D., Jones, M. and Bronner-Fraser, M. (2007). Ancient evolutionary origin of the neural crest gene regulatory network. *Dev Cell* **13**, 405-20.

Scaal, M. and Christ, B. (2004). Formation and differentiation of the avian dermomyotome. *Anat Embryol (Berl)* **208**, 411-24.

Scaal, M. and Wiegreffe, C. (2006). Somite compartments in anamniotes. *Anat Embryol (Berl)* **211 Suppl 1**, 9-19.

Schipani, E. (2006). Hypoxia and HIF-1alpha in chondrogenesis. *Ann N Y Acad Sci* **1068**, 66-73.

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

Schipani, E., Lanske, B., Hunzelman, J., Luz, A., Kovacs, C. S., Lee, K., Pirro, A., Kronenberg, H. M. and Juppner, H. (1997). Targeted expression of constitutively active receptors for parathyroid hormone and parathyroid hormone-related peptide delays endochondral

bone formation and rescues mice that lack parathyroid hormone-related peptide. *Proc Natl Acad Sci U S A* **94**, 13689-94.

Schipani, E., Ryan, H. E., Didrickson, S., Kobayashi, T., Knight, M. and Johnson, R. S. (2001). Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. *Genes Dev* **15**, 2865-76.

Schneider, A., Brand, T., Zweigerdt, R. and Arnold, H. (2000). Targeted disruption of the Nkx3.1 gene in mice results in morphogenetic defects of minor salivary glands: parallels to glandular duct morphogenesis in prostate. *Mech Dev* **95**, 163-74.

Schwartz, N. B., Pirok, E. W., 3rd, 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.

Schweitzer, R., Chyung, J. H., Murtaugh, L. C., Brent, A. E., Rosen, V., Olson, E. N., Lassar, A. and Tabin, C. J. (2001). Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development* **128**, 3855-66.

Scott, W. B. (1882). Beitrage zur entwicklungsgeschichte der petromyzonten. *Morphol. Jahrb.* **7**.

Shashikant, C. S., Utset, M. F., Violette, S. M., Wise, T. L., Einat, P., Einat, M., Pendleton, J. W., Schughart, K. and Ruddle, F. H. (1991). Homeobox genes in mouse development. *Crit Rev Eukaryot Gene Expr* **1**, 207-45.

Shigetani, Y., Sugahara, F., Kawakami, Y., Murakami, Y., Hirano, S. and Kuratani, S. (2002). Heterotopic shift of epithelial-mesenchymal interactions in vertebrate jaw evolution. *Science* **296**, 1316-9.

Shimeld, S. M. (1997). Characterisation of amphioxus HNF-3 genes: conserved expression in the notochord and floor plate. *Dev Biol* **183**, 74-85.

Shimeld, S. M. and Holland, P. W. (2000). Vertebrate innovations. *Proc Natl Acad Sci U S A* **97**, 4449-52.

Shimeld, S. M., van den Heuvel, M., Dawber, R. and Briscoe, J. (2007). An amphioxus gli gene reveals conservation of midline patterning and the evolution of hedgehog signalling diversity in chordates. *PLoS ONE* **2**, e864.

Shinohara, M. and Takayanagi, H. (2007). Novel osteoclast signaling mechanisms. *Curr Osteoporos Rep* **5**, 67-72.

Shu, D.-G., Luo, H.-L., Conway Morris, S., Zhang, X.-L., Hu, S.-X., Chen, L., Han, J., Zhu, M., Li, Y. and Chen, L.-Z. (1999). Lower Cambrian vertebrates from south China. *Nature* **402**, 42-46.

Shum, L., Coleman, C. M., Hatakeyama, Y. and Tuan, R. S. (2003). Morphogenesis and dysmorphogenesis of the appendicular skeleton. *Birth Defects Res C Embryo Today* **69**, 102-22.

Singh, M. K., Petry, M., Haenig, B., Lescher, B., Leitges, M. and Kispert, A. (2005). The T-box transcription factor Tbx15 is required for skeletal development. *Mech Dev* **122**, 131-44.

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**, 161-9.

Sivakumar, P., Suguna, L. and Chandrakasan, G. (2003). Similarity between the major collagens of cuttlefish cranial cartilage and cornea. *Comp Biochem Physiol B Biochem Mol Biol* **134**, 171-80.

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. et al. (2005). Overlapping expression of Runx1(Cbfa2) and Runx2(Cbfa1) transcription factors supports cooperative induction of skeletal development. *J Cell Physiol* **203**, 133-43.

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**, 1135-48.

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**, 321-39.

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**, 402-11.

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**, 2072-86.

Stadler, P. F., Fried, C., Prohaska, S. J., Bailey, W. J., Misof, B. Y., Ruddle, F. H. and Wagner, G. P. (2004). Evidence for independent Hox gene duplications in the hagfish lineage: a PCR-based gene inventory of Eptatretus stoutii. *Mol Phylogenet Evol* **32**, 686-94.

Steinke, D., Hoegg, S., Brinkmann, H. and Meyer, A. (2006). Three rounds (1R/2R/3R) of genome duplications and the evolution of the glycolytic pathway in vertebrates. *BMC Biol* **4**, 16.

Stemple, D. L. (2005). Structure and function of the notochord: an essential organ for chordate development. *Development* **132**, 2503-12.

Stickens, D., Behonick, D. J., Ortega, N., Heyer, B., Hartenstein, B., Yu, Y., Fosang, A. J., Schorpp-Kistner, M., Angel, P. and Werb, Z. (2004). Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. *Development* **131**, 5883-95.

Stickney, H. L., Barresi, M. J. and Devoto, S. H. (2000). Somite development in zebrafish. *Dev Dyn* **219**, 287-303.

Stokes, M. D. and Holland, N. D. (1995). Embryos and larvae of a lancelet, *Branchiostoma floridae*, from hatching through metamorphosis: growth in the laboratory and external morphology. *Acta Zool. (Stockh.)* **76**, 105-120.

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

Strachan, T. and Read, A. P. (1994). PAX genes. *Curr Opin Genet Dev* **4**, 427-38.

Streuli, C. H., Schmidhauser, C., Kobrin, M., Bissell, M. J. and Derynck, R. (1993). Extracellular matrix regulates expression of the TGF-beta 1 gene. *J Cell Biol* **120**, 253-60.

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**, 26745-54.

Summers, A. P. and Koob, T. J. (2002). The evolution of tendon--morphology and material properties. *Comp Biochem Physiol A Mol Integr Physiol* **133**, 1159-70.

Suzuki, T., Sakai, D., Osumi, N., Wada, H. and Wakamatsu, Y. (2006). Sox genes regulate type 2 collagen expression in avian neural crest cells. *Dev Growth Differ* **48**, 477-86.

Swofford, D. L. (2002). PAUP: phylogenetic analysis using Parsimony. Sunderland, MA: Sinauer.

Swofford, D. L., Olsen, G. J., Waddell, P. J. and Hillis, D. M. (1996). Molecular systematics. Sunderland, MA: Sinauer.

Tahara, Y. (1988). Normal stages of development in the lamprey, *Lampera reissneri* (Dybowski). *Zoological Science* **5**, 10.

Takatori, N., Hotta, K., Mochizuki, Y., Satoh, G., Mitani, Y., Satoh, N., Satou, Y. and Takahashi, H. (2004). T-box genes in the ascidian Ciona intestinalis: characterization of cDNAs and spatial expression. *Dev Dyn* **230**, 743-53.

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**, 467-81.

Takio, Y., Pasqualetti, M., Kuraku, S., Hirano, S., Rijli, F. M. and Kuratani, S. (2004). Evolutionary biology: lamprey Hox genes and the evolution of jaws. *Nature* **429**, 1 p following 262.

Tanaka, M. and Tickle, C. (2004). Tbx18 and boundary formation in chick somite and wing development. *Dev Biol* **268**, 470-80.

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**, 2024-39.

Tribioli, C. and Lufkin, T. (1999). The murine Bapx1 homeobox gene plays a critical role in embryonic development of the axial skeleton and spleen. *Development* **126**, 5699-711.

Trivett, M. K., Potter, I. C., Power, G., Zhou, H., Macmillan, D. L., John Martin, T. and Danks, J. A. (2005). Parathyroid hormone-related protein production in the lamprey Geotria australis: developmental and evolutionary perspectives. *Dev Genes Evol*, 1-11.

Ueta, C., Iwamoto, M., Kanatani, N., Yoshida, C., Liu, Y., Enomoto-Iwamoto, M., Ohmori, T., Enomoto, H., Nakata, K., Takada, K. et al. (2001). Skeletal malformations caused by overexpression of Cbfa1 or its dominant negative form in chondrocytes. *J Cell Biol* **153**, 87-100.

Valkkila, M., Melkoniemi, M., Kvist, L., Kuivaniemi, H., Tromp, G. and Ala-Kokko, L. (2001). Genomic organization of the human COL3A1 and COL5A2 genes: COL5A2 has evolved differently than the other minor fibrillar collagen genes. *Matrix Biol* **20**, 357-66.

van der Rest, M. and Garrone, R. (1991). Collagen family of proteins. *Faseb J* **5**, 2814-23.

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**, 4209-10.

Vega, R. B., Matsuda, K., Oh, J., Barbosa, A. C., Yang, X., Meadows, E., McAnally, J., Pomajzl, C., Shelton, J. M., Richardson, J. A. et al. (2004). Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. *Cell* **119**, 555-66.

Vickaryous, M. K. and Hall, B. K. (2006). Osteoderm morphology and development in the nine-banded armadillo, Dasypus novemcinctus (Mammalia, Xenarthra, Cingulata). *J Morphol* **267**, 1273-83.

Volk, S. W., Luvalle, P., Leask, T. and Leboy, P. S. (1998). A BMP responsive transcriptional region in the chicken type X collagen gene. *J Bone Miner Res* **13**, 1521-9.

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**, 613-22.

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**, 370-7.

Wagner, E. F. and Karsenty, G. (2001). Genetic control of skeletal development. *Curr Opin Genet Dev* **11**, 527-32.

Wagner, G. P., Amemiya, C. and Ruddle, F. (2003). Hox cluster duplications and the opportunity for evolutionary novelties. *Proc Natl Acad Sci U S A* **100**, 14603-6.

Wagner, T., Wirth, J., Meyer, J., Zabel, B., Held, M., Zimmer, J., Pasantes, J., Bricarelli, F. D., Keutel, J., Hustert, E. et al. (1994). Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell* **79**, 1111-20.

Walker, L. M., Publicover, S. J., Preston, M. R., Said Ahmed, M. A. and El Haj, A. J. (2000). Calcium-channel activation and matrix protein upregulation in bone cells in response to mechanical strain. *J Cell Biochem* **79**, 648-61.

Wallin, J., Wilting, J., Koseki, H., Fritsch, R., Christ, B. and Balling, R. (1994). The role of Pax-1 in axial skeleton development. *Development* **120**, 1109-21.

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**, 1624-36.

Watanabe, Y., Duprez, D., Monsoro-Burq, A. H., Vincent, C. and Le Douarin, N. M. (1998). Two domains in vertebral development: antagonistic regulation by SHH and BMP4 proteins. *Development* **125**, 2631-9.

Weinstein, M., Xu, X., Ohyama, K. and Deng, C. X. (1998). FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. *Development* **125**, 3615-23.

Weir, E. C., Philbrick, W. M., Amling, M., Neff, L. A., Baron, R. and Broadus, A. E. (1996). Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes

chondrodysplasia and delayed endochondral bone formation. *Proc Natl Acad Sci U S A* **93**, 10240-5.

Welch, U., Chiba, A. and Honma, Y. (1998). The notochord. In *The biology of hagfishes*, (ed. J. M. Jorgensen J. P. Lomholt R. E. Weber and H. Malte), pp. 145-159. London: Chapman & Hall.

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

Wezeman, F. H. (1998). Morphological foundations of precartilage development in mesenchyme. *Microsc Res Tech* **43**, 91-101.

Wicht, H. and Northcutt, R. G. (1995). Ontogeny of the head of the Pacific hagfish (Eptatretus stouti, Myxinoidea): development of the lateral line system. *Philos Trans R Soc Lond B Biol Sci* **349**, 119-34.

Williams, E. E. (1959). Gadow's arcualia and the development of tetrapod vertebrae. *Q Rev Biol* **34**, 1-32.

Wilm, B., James, R. G., Schultheiss, T. M. and Hogan, B. L. (2004). The forkhead genes, Foxc1 and Foxc2, regulate paraxial versus intermediate mesoderm cell fate. *Dev Biol* **271**, 176-89.

Winnier, G. E., Kume, T., Deng, K., Rogers, R., Bundy, J., Raines, C., Walter, M. A., Hogan, B. L. and Conway, S. J. (1999). Roles for the winged helix transcription factors MF1 and MFH1 in cardiovascular development revealed by nonallelic noncomplementation of null alleles. *Dev Biol* **213**, 418-31.

Winslow, M. M., Pan, M., Starbuck, M., Gallo, E. M., Deng, L., Karsenty, G. and Crabtree, G. R. (2006). Calcineurin/NFAT signaling in osteoblasts regulates bone mass. *Dev Cell* **10**, 771-82.

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**, 130-47.

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**, 165-74.

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**, 407-24.

Wright, G. M. and Youson, J. H. (1983). Ultrastructure of cartilage from young adult sea lamprey, Petromyzon marinus L: a new type of vertebrate cartilage. *Am J Anat* **167**, 59-70.

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**, 1671-7.

Yan, Y. L., Hatta, K., Riggleman, B. and Postlethwait, J. H. (1995). Expression of a type II collagen gene in the zebrafish embryonic axis. *Dev Dyn* **203**, 363-76.

Yan, Y. L., Miller, C. T., Nissen, R. M., Singer, A., Liu, D., Kirn, A., Draper, B., Willoughby, J., Morcos, P. A., Amsterdam, A. et al. (2002). A zebrafish sox9 gene required for cartilage morphogenesis. *Development* **129**, 5065-79.

Yan, Y. L., Willoughby, J., Liu, D., Crump, J. G., Wilson, C., Miller, C. T., Singer, A., Kimmel, C., Westerfield, M. and Postlethwait, J. H. (2005). A pair of Sox: distinct and overlapping functions of zebrafish sox9 co-orthologs in craniofacial and pectoral fin development. *Development* **132**, 1069-83.

Yang, A. H., Chen, J. Y. and Lin, J. K. (2003). Myofibroblastic conversion of mesothelial cells. *Kidney Int* **63**, 1530-9.

Yang, X. and Karsenty, G. (2002). Transcription factors in bone: developmental and pathological aspects. *Trends Mol Med* **8**, 340-5.

Yang, X. and Karsenty, G. (2004). ATF4, the osteoblast accumulation of which is determined post-translationally, can induce osteoblast-specific gene expression in non-osteoblastic cells. *J Biol Chem* **279**, 47109-14.

Yasutake, J., Inohaya, K. and Kudo, A. (2004). Twist functions in vertebral column formation in medaka, Oryzias latipes. *Mech Dev* **121**, 883-94.

Yoon, B. S., Ovchinnikov, D. A., Yoshii, I., Mishina, Y., Behringer, R. R. and Lyons, K. M. (2005). Bmpr1a and Bmpr1b have overlapping functions and are essential for chondrogenesis in vivo. *Proc Natl Acad Sci U S A* **102**, 5062-7.

Yoshida, C. A., Yamamoto, H., Fujita, T., Furuichi, T., Ito, K., Inoue, K., Yamana, K., Zanma, A., Takada, K., Ito, Y. et al. (2004). Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. *Genes Dev* **18**, 952-63.

Yu, K., Xu, J., Liu, Z., Sosic, D., Shao, J., Olson, E. N., Towler, D. A. and Ornitz, D. M. (2003). Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth. *Development* **130**, 3063-74.

Yusuf, F. and Brand-Saberi, B. (2006). The eventful somite: patterning, fate determination and cell division in the somite. *Anat Embryol (Berl)* **211 Suppl 1**, 21-30.

Zelzer, E., Glotzer, D. J., Hartmann, C., Thomas, D., Fukai, N., Soker, S. and Olsen, B. R. (2001). Tissue specific regulation of VEGF expression during bone development requires Cbfa1/Runx2. *Mech Dev* **106**, 97-106.

Zelzer, E., Mamluk, R., Ferrara, N., Johnson, R. S., Schipani, E. and Olsen, B. R. (2004). VEGFA is necessary for chondrocyte survival during bone development. *Development* **131**, 2161-71.

Zelzer, E. and Olsen, B. R. (2003). The genetic basis for skeletal diseases. *Nature* **423**, 343-8.

Zeng, L., Kempf, H., Murtaugh, L. C., Sato, M. E. and Lassar, A. B. (2002). Shh establishes an Nkx3.2/Sox9 autoregulatory loop that is maintained by BMP signals to induce somitic chondrogenesis. *Genes Dev* **16**, 1990-2005.

Zeng, L. Y. and Swalla, B. J. (2005). Molecular phylogeny of the protochordates: chordate evolution. *Canadian Journal of Zoology-Revue Canadienne De Zoologie* **83**, 24-33.

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 U S A* **103**, 16829-33.

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 U S A* **103**, 3180-5.

Zhang, P., Jimenez, S. A. and Stokes, D. G. (2003). Regulation of human COL9A1 gene expression. Activation of the proximal promoter region by SOX9. *J Biol Chem* **278**, 117-23.

Zhao, Q., Eberspaecher, H., Lefebvre, V. and De Crombrugghe, B. (1997). Parallel expression of Sox9 and Col2a1 in cells undergoing chondrogenesis. *Dev Dyn* **209**, 377-86.

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**, 14989-97.

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 U S A* **103**, 19004-9.

Zhou, R., Bonneaud, N., Yuan, C. X., de Santa Barbara, P., Boizet, B., Schomber, T., Scherer, G., Roeder, R. G., Poulat, F. and Berta, P. (2002). SOX9 interacts with a component of the human thyroid hormone receptor-associated protein complex. *Nucleic Acids Res* **30**, 3245-52.

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**, 2191-203.

BIOGRAPHICAL SKETCH

GuangJun Zhang was born in northeast of China. He entered the Medical College of TongJi University in 1993 and finished clinical medicine training and earned his medical degree in 1998. Immediately after medical school, he started a masters program at the Chinese Academy of Preventive Medicine in late 1998. He learned from Dr. MingYi Xia during the masters training, where his work focused mainly on the phylogeography of human blood fluke parasites, schistosomes, by using ribosomal and mitochondrial gene markers. After that he worked as research associate for two years at the Institute of Neuroscience, Chinese Academy of Science. During this time, he worked predominantly on the signal transduction pathways in cortical neurons. In August of 2003, GuangJun Zhang began his Ph.D. training at the Zoology Department, University of Florida. With Dr. Martin Cohn as his advisor, GuangJun Zhang accomplished his dissertation work on the vertebrate skeletal development and evolution. During his Ph.D. program, he was awarded Grinter Fellowship and Winstar Dissertation Fellowship at the University of Florida.