Expression of Sox and Fibrillar Collagen Genes in Lamprey Larval Chondrogenesis With Implications for the Evolution of Vertebrate Cartilage

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ABSTRACT Lampreys possess unique types of cartilage in which elastin-like proteins are the dominant matrix component, whereas gnathostome cartilage is mainly composed of fibrillar collagen. Despite the differences in protein composition, the *Sox–col2a1* genetic cascade was suggested to be conserved between lamprey pharyngeal cartilage and gnathostome cartilage. We examined whether the cascade is conserved in another type of lamprey cartilage, the trabecular cartilage. We found that *SoxD* and *SoxE* are expressed in both trabecular and pharyngeal cartilages. However, trabecular cartilage shows no clade A fibrillar collagen gene expression, including genes expressed in pharyngeal cartilage of this animal. On the basis of these observations, we propose that lampreys possess an ancestral type of cartilage that is similar to amphioxus gill cartilage, and in this respect, gnathostome cartilage can be regarded as derived for the loss of elastin-like protein as a cartilage component and recruitment of fibrillar collagen, which is included as a minor component in the ancestral cartilage, as the main component. *J. Exp. Zool. (Mol. Dev. Evol.) 310B:596–607, 2008.*

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The evolution of skeletal tissues is one of the major events in vertebrate evolutionary history. In the gnathostomes, differentiation of chondrocytes is controlled by conserved transcription factors, such as Sox9 and Sox5 (Bi et al., '99; Smits et al., 2001). These transcription factors directly regulate expression of genes encoding cartilage matrix proteins, including col2a1, col11a2, and aggrecan (Bell et al., '97; Lefebvre et al., '97, '98; Liu et al., 2000; Sekiya et al., 2000). Coexpression of Sox transcription factors and fibrillar collagen genes is conserved in cartilage of different developmental origins (e.g., pharyngeal chondrocytes from the neural crest, limb chondrocytes from the lateral plate mesoderm, and vertebral chondrocytes from the somitic mesoderm) in several vertebrate species (Healy et al., '99; Yan et al., 2002).

Extant agnatha are known to possess unique types of cartilage. Lampreys possess two types of cartilage: one found in the branchial arch and pericardium, which are subjected to compression and recoil (referred to as recoil cartilage), and the other in the trabeculae and the tongue piston, which are more rigid structures (rigid cartilage) (Wright and Youson, '83; Robson et al., '97).

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Unlike gnathostomes, in which the cartilage contains fibrillar collagen as the main component, the main matrix components of lamprey cartilage are elastin-like proteins (Wright et al., '88). The elastin-like protein of lamprey trabecular cartilage has been identified as lamprin (Robson et al., '93), although that of the pharyngeal cartilage has not been characterized (Robson et al., '97). Another species of extant agnatha, the hagfish, also possesses two types of cartilages. Hagfish type 1 cartilage is more similar to lamprey cartilage, and contains the elastin-like protein called myxinin as the main component. Hagfish type 2 cartilage, on the other hand, consists mainly of collagen and superficially resembles notochord cells (Wright et al., '84). This cartilage variation in vertebrates raises questions about the homology and the evolution of the vertebrate cartilage.

Cartilaginous structures are reported in the protochordates, such as pharyngeal bars and oral cirri of amphioxus. Evolutionary implications of these structures regarding the origin of the vertebrate cartilage were discussed based on gene expression patterns (Zhang and Cohn, 2006; Meulemans and Bronner-Fraser, 2007; Rychel and Swalla, 2007; Hecht et al., 2008), which is discussed in more detail below. In addition, because of expressions of collagen genes, notochord is also suggested to represent a primitive form of cartilage (Zhang and Cohn, 2006). However, cartilaginous tissues are also found in other invertebrates, as seen in the tentacles of brachiopods and polychaetes, and the cuttlefish funnel (Cole and Hall, 2004a,b). These are unlikely to be homologous to the vertebrate cartilage, because they are found in phylogenetically distinct lineages. Thus, cartilages seem to have evolved independently in several times during metazoan evolution, most likely via production of thick extracellular matrix (Cole and Hall, 2004a,b). These examples suggest that several types of cartilage could have evolved also during vertebrate evolution.

There is also another reason to call the homology of lamprey cartilage into question. Type 2 collagen, which is the main component of the gnathostome cartilage, is encoded by col2a1 gene, a member of the clade A fibrillar collagen gene family (Boot-Handford and Tuckwell, 2003; Aouacheria et al., 2004). In gnathostomes, four other genes are assigned in the clade A, such as col1a1, col1a2, and col5a2, which are included in mineralized bone. Col3a1 is also a member of the clade A collagen genes family (Boot-Handford and Tuckwell, 2003; Aouacheria et al., 2004). It is widely accepted that the common ancestors of gnathostomes experienced two rounds of genome duplication and that gnathostomes thus possess four ancestral gene sets (tetralogy) (Furlong and Holland, 2002). The tetralogy is most obviously seen in the four sets of Hox clusters (Holland et al., '94). Clade A fibrillar collagen genes, including *col2a1*, are linked with Hox clusters on chromosomes (Bailey et al., '97); for example, *col2a1* is linked with the HoxD cluster (Bailey et al., '97). This linkage between Hox clusters and collagen genes implies that the clade A fibrillar collagen genes also increased in number via the two rounds of genome duplications (Wada et al., 2006). As lamprey diverged from the gnathostome lineage at around the time of the second genome duplication (Furlong and Holland, 2002), lamprey may not possess an ortholog of *col2a1*, but may instead possess ancestral collagen genes.

Previous studies addressed the homology of lamprey cartilage with those of gnathostomes. Zhang et al. (2006) found that a group E Sox gene is expressed in lamprey branchial cartilage, where a fibrillar collagen gene similar to *col2a1* is also expressed. Therefore, although the main component is not fibrillar collagen, the genetic machinery of SoxE regulation of the fibrillar collagen gene is conserved in lamprey branchial cartilage. Zhang and Cohn (2006) also presented preliminary evidence that the conserved genetic machinery can be found in hagfish cartilage.

Here, we extend these previous analyses to a specific type of cartilage, the trabecular cartilage in a lamprey species *Lethenteron japonicum*. We found coexpression of *SoxD* and *SoxE* in both pharyngeal chondrocytes and trabecular chondrocytes. In contrast, we did not detect expression of any of the clade A fibrillar collagen genes in the trabecular chondrocytes. Our careful examination of fibrillar collagen genes indicates that the ortholog of *Pmcol2a1a*, which has been reported to be expressed in pharyngeal chondrocytes (Zhang et al., 2006), is not expressed in the pharyngeal chondrocytes of *L. japonicum*. Instead, we found that the ortholog of *Pmcol2a1b* is expressed in pharyngeal cartilage. The evolutionary history of vertebrate cartilage is discussed based on the conserved expression of *Sox* genes and the divergent expression patterns of matrix protein genes.

MATERIALS AND METHODS

Animals

Adult lampreys (*L. japonicum*) were collected in the Shiribeshi-Toshibetsu River, Hokkaido,

Japan. Mature eggs were squeezed from females and fertilized in vitro with sperm. Sometimes eggs were squeezed from females anesthetized in ethyl 3-aminobenzoate methanesulfonate (MS-222). Embryos were cultured at 16°C. Developmental stages were determined following the report of Tahara ('88).

Isolation of Lethenteron genes and molecular phylogenetic analyses

Small DNA fragments of *LjSoxE* were isolated by polymerase chain reaction using the following primer set: SoxE-F: ATG GTI TGG GCI CAR GCI GC, SoxE-R: TA RTC IGG RTG RTC YTT YTT. Subsequently, *LjSoxE* was isolated by screening a cDNA library. *LjSoxD*, *LjColA*/*col2a1a*, *LjColB*/*col2a1b*, and *LjColC* were amplified based on genome sequences (http://pre.ensembl.org/Petromyzon_marinus/) using the primers given below: LjSoxD-F: GCG GAG CGC GAG CAC CAG CTC GCC GGG ATG, LjSoxD-R: CC CAC ATT GTA GAA CTG CTT CAT CTC CTG; LjColA-F: C GGA CCT CCT GGG CCT AGT GGC CCT GCT GG, LjColA-R: T GTG GGC TGG CAT AGA CGC AGG TCT CGC CC; LjColB-F: A TCC GCG CGG AGG GCA ACT CAC GCT TCA CC, LjColB-R: A ACG GCA GCC GCG AGG TCT TCT GCG TAC GG; LjColC-F: CGC CCA GGC AAT CCA GGC AAC ATG, LjColC-R: GG GCC AAT TTC GAC GCC AAA CTC TTG. After determining the nucleotide sequences of cDNA clones, molecular phylogenetic analyses were performed using TreePuzzle 5.0 (Schmidt et al., 2002). The molecular phylogenetic trees were constructed under the WAG amino acid substitution model (Whelan and Goldman, 2001) and substitution rate heterogeneity was corrected according to the gamma distribution model. Accession numbers of genes isolated in this study: *LjColA*/*col2a1a*: AB450763, *LjColB*/*col2a1b*: AB450764, *LjColC*: AB450765, *LjSoxD1*: AB450766, *LjSoxE3*: AB450767.

In situ hybridization and histological analyses

In situ hybridization was performed according to Ogasawara et al. (2000) with some minor modifications. Gene expression was examined by observing whole-mount specimens or cryostat sections. Histological analyses were also performed on cryostat specimens after staining with hematoxylin and eosin. Verhoeff elastic-Van Gieson staining was performed according to the method of Cole and Hall (2004b). Cartilage was histologically visualized by Weigert staining (details of the method described elsewhere; Yao et al., in preparation).

RESULTS

Histological analyses of Lethenteron cartilage

To trace gene expression in the trabecular and pharyngeal cartilages of *L. japonicum*, we first examined the developmental scheme of chondrogenesis in this animal. The first sign of chondrogenesis in the pharyngeal arch was observed at stage 25, when the heartbeat commences. At this stage, prechondrogenic cells could be distinguished as tightly packed mesenchymal cells (Fig. 1A). At stage 26, the mesenchymal cells began to be packaged, from the middle part of the pharyngeal arch, into a perpendicular bar of one cell width (Fig. 1B). At stage 27, chondrocytes were uniformly arranged into a single row of layered cells, which was surrounded by clearly visible perichondrium. Differentiation of chondro-

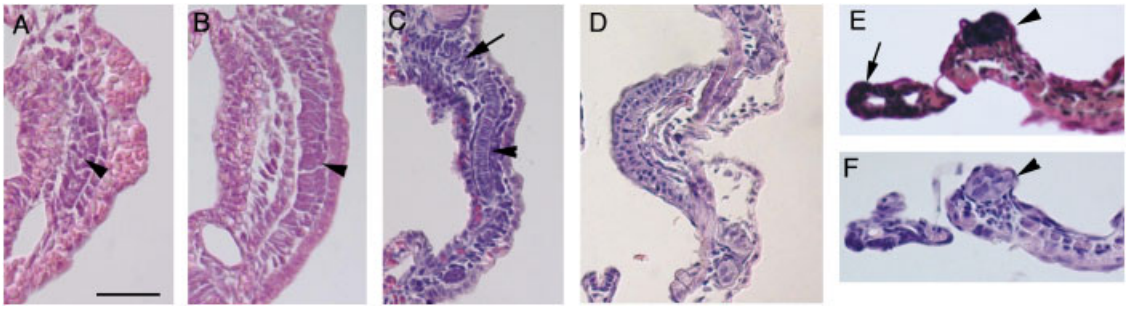

Fig. 1. Differentiation of pharyngeal chondrocytes. (**A**) Chondrocytes began to show packing at stage 25. (**B**) At stage 26, mesenchymal cells accumulated in a column. (**C**) At stage 28, chondrocytes arranged into a single row of layered cells from the middle. In the dorsal region, cells were less organized (arrow). (**D**) Differentiated chondrocytes at stage 29. (**E**) Verhoeff staining of pharyngeal chondrocytes at stage 30. The branchial artery was also stained (arrow). Arrowheads indicate pharyngeal chondrocytes. Transverse sections and horizontal sections are shown in (A–D) and (**E**, **F**), respectively. Scale bar: 20 μm.

cytes always proceeded from the middle of the branchial arch, which was clearly observed at stage 28 (Fig. 1C, D). At this stage, although cells were tightly packed in the middle, those on the dorsal and ventral sides were arranged more loosely (Fig. 1C). At stage 30, chondrocytes, which can be stained by the Verhoeff method, were clearly visible (Fig. 1E, F).

Although chondrogenesis in the branchial arch commences at stage 25, that in the trabeculae was observed at stage 27 or 28. Here, we follow the time course of trabecular chondrogenesis at the level of the eyes along the anterior–posterior axis (Fig. 2D). At stage 27, when pigmentation of the eyecup begins, the mesenchymal cells could be observed on both sides of the notochord (Fig. 2A), which began to accumulate at stage 28 (Fig. 2B). At stage 29, chondrocytes were clearly visible, surrounded by perichondrium (Fig. 2C).

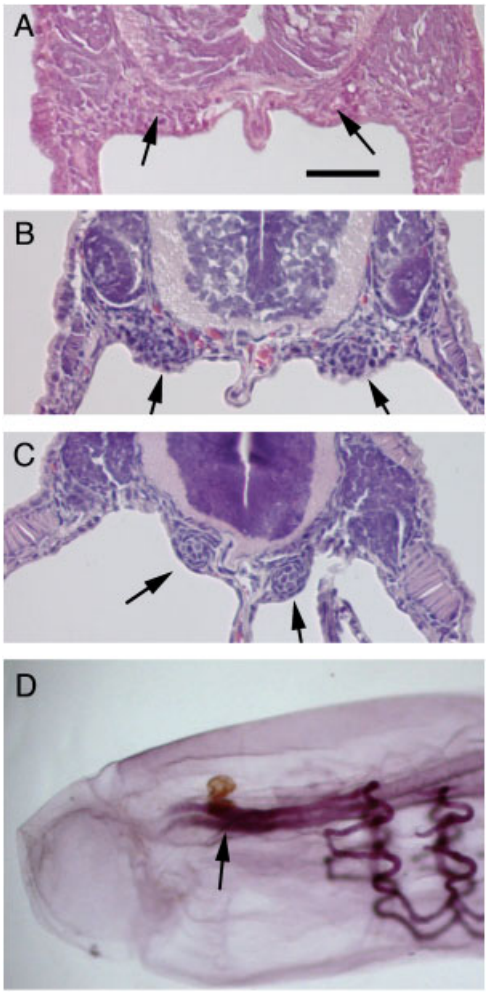

Fig. 2. Differentiation of trabecular cartilage. (**A**) At stage 27, mesenchymal cells accumulated in the region ventral to the neural tube. (**B**) The mesenchymal cells accumulated at stage 28. (**C**) Chondrocytes were tightly packed and surrounded by perichondrium at stage 29. Scale bar: 20 μm. (**D**) Trabecular cartilage at stage 30 visualized by Weigert staining (Yao et al., in preparation). Arrows indicate trabecular cartilage.

Expression of fibrillar collagen genes in Lethenteron cartilage

The most abundant protein in gnathostome cartilage is type 2 collagen that is encoded by *col2a1*. One of the two homologs of *col2a1*, *Pmcol2a1a* is expressed in the pharyngeal cartilage of *Petromyzon marinus*, whereas the other *col2a1* homolog, *Pmcol1a1b*, is expressed predominantly in notochord, but not in pharyngeal arches (Zhang et al., 2006). We isolated orthologs of *Pmcol2a1* from *L. japonicum* and examined their expression. Molecular phylogenetic analyses clearly indicated orthology between *LjColA/col2a1a* and *Pmcol2a1a* and between *LjColB/col2a1b* and *Pmcol2a1b* (Fig. 3A). As it is not yet clear whether these two genes arose by duplication of the ancestral *col2a1* gene (see the Discussion), we hesitate to designate them cognates of the gnathostome *col2a1*.

At stage 25, expression of *LjColA/col2a1a*, the ortholog of *Pmcol2a1a*, was observed in the head mesoderm and somitic mesoderm (Fig. 4A–D). Somitic expression was excluded from the myotome, consistent with the reported *Pmcol2a1a* expression in the dermatome and sclerotome (Fig. 4B, D; Zhang et al., 2006). Kusakabe and Kuratani (2005, 2007) suggested that the lateral thin layer of somites depicted as dermatome by Zhang et al. (2006) might be homologous to the amniote dermomyotome, as they expressed *Pax3/7* *Pax3/7*, a dermomyotome marker. *LjColA/col2a1a* expression in these cells is consistent with the possible homology of the *Pax3/7*-positive cells with dermomyotome (Kusakabe and Kuratani, 2005, 2007). Expression was also observed in the roofplate and the floorplate of the neural tube, and the hypochord (Fig. 4D). As *Pmcol2a1a* was expressed in pharyngeal cartilage (Zhang et al., 2006), *LjColA/col2a1a* was also expressed in the pharyngeal arch and epidermis at stage 27 (Fig. 4E, H, I). However, based on our detailed observations, it was not expressed in any chondrocytes (Fig. 4G, H, I). Comparison of expression with the muscle cell marker *LjMA2* (Fig. 4K; Kusakabe et al., 2004) indicated that *LjColA/col2a1a* was not expressed in muscle cells either. Thus, *LjColA/col2a1a* is specifically expressed in the nonchondrogenic and nonmyogenic mesenchymes of the pharyngeal

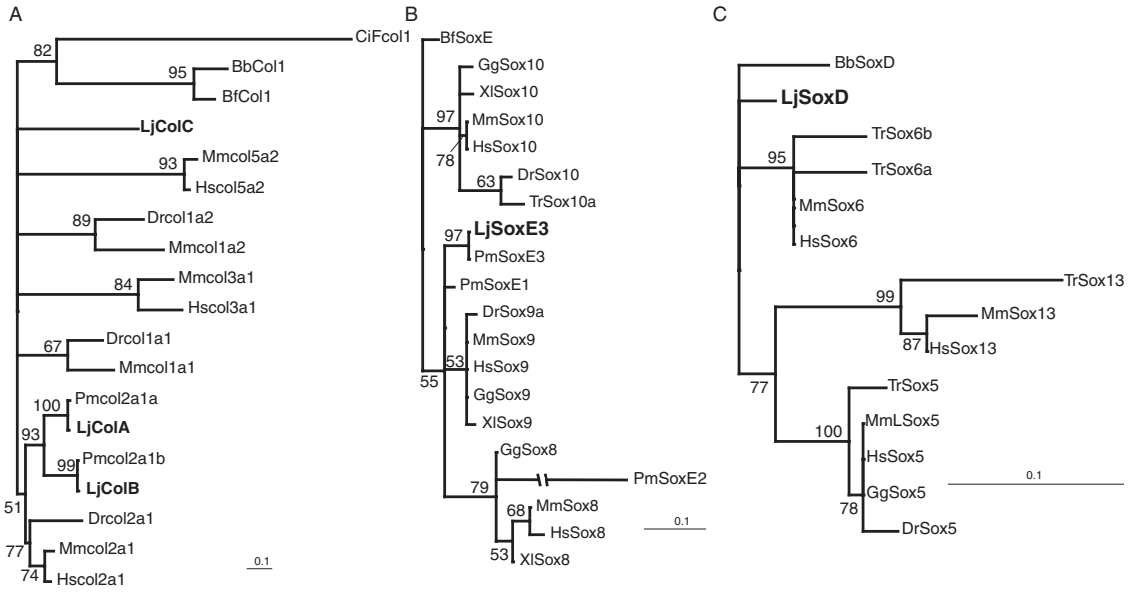

Fig. 3. Molecular phylogenetic analyses of *Lethenteron* genes. (**A**) Molecular phylogenetic tree of clade A fibrillar collagen genes constructed from 302 amino acid sites in the C-terminus noncollagenous domain. Orthology between *LjFcol1*/*col2a1a* and *Pmcol2a1a* and between *LjFcol2*/*col2a1b* and *Pmcol2a1b* was clearly supported, whereas orthology with gnathostome genes was not well resolved. (**B**) Molecular phylogenetic tree of group E Sox genes constructed from 104 amino acid sites in the HMG domain. *LjSoxE3* is an ortholog of *PmSoxE3*, whereas orthology with gnathostome genes was not resolved. (**C**) Molecular phylogenetic tree of group D Sox genes constructed from 102 amino acid sites in the HMG domain. *LjSoxD1* diverged from the node of three gnathostome SoxD genes. Numbers on the nodes indicate confidence values calculated by the quartet puzzling method (Schmidt et al., 2002). Ci, *Ciona intestinalis* (ascidian); Bb, *Branciostoma belcheri* (amphioxus); Bf, *B. floridae* (amphioxus); Dr, *Danio rerio* (zebra fish); Tr, *Takifugu rubripes* (fugu); Xl, *Xenopus laevis* (frog); Gg, *Gallus gallus* (chicken); Mm, *Mus musculus* (mouse); Hs, *Homo sapiens* (human). HMG, high-mobility group.

arch, possibly differentiating into connective tissues. No expression of *LjColA*/*col2a1a* was observed in the trabecular chondrocytes (Fig. 4F). Expression of *LjColA*/*col2a1a* was also observed on the dorsal or ventral sides of the pharynx as well as in the fin mesenchyme (Fig. 4J).

The other fibrillar collagen gene of *L. japonicum*, *LjColB*/*col2a1b*, is an ortholog of *Pmcol2a1b*, which is expressed in the notochord, floorplate, and sclerotome (Zhang et al., 2006). In the early stages, *LjColB*/*col2a1b* expression was restricted to the notochord (Fig. 5A, B). In addition, at stage 27, we observed expression in the pharyngeal arch (Fig. 5C), where no expression was reported for *Pmcol2a1b*. In contrast to *LjColA*/*col2a1a*, the expression of *LjColB*/*col2a1b* was confined to densely packed chondrocytes (Fig. 5D, E). At stage 28, *LjColB*/*col2a1b* expression disappeared from its middle part, where chondrocyte differentiation occurs earlier, and expression was detected only in the dorsal and ventral parts of the cartilage (Fig. 5F). We did not find any *LjColB*/*col2a1b* expression in the trabeculae, whereas some mesenchymal cells abutting the trabecular chondrocytes are positive for *LjColB*/*col2a1b* (Fig. 5G, H). The expression was also detected in the fin and the notochord at the trunk level (Fig. 5I).

We identified another type of clade A fibrillar collagen in the genome sequence of *P. marinus* (http://pre.ensembl.org/Petromyzon_marinus/) and isolated the *Lethenteron* ortholog (designated as *LjColC*). The expression of *LjColC* was quite similar to that of *LjColA*. It was expressed in cells surrounding the somitic myotomes and also in mesenchymal cells of the branchial arch, but not in chondrocytes (Fig. 6A, B, D, E). No *LjColC* expression was detected in the trabeculae (Fig. 6C). *LjColC* was expressed in the fin mesenchyme (data not shown).

Lampreys are known to possess cartilage-like structure, so-called mucocartilage in the upper and lower lips, and in the velum (Wright and Youson, '82). We found that *LjColA* and *LjColC*, but not *LjColB*, show expression in the upper lip and the lower lip (Figs. 4E, 5F, 6B). Although we know little about the developmental process of the mucocartilage, *LjColA* and *LjColC* may encode structural components of the mucocartilage.

In summary, although pharyngeal chondrocytes expressed *LjColB*/*col2a1b*, which is the ortholog of

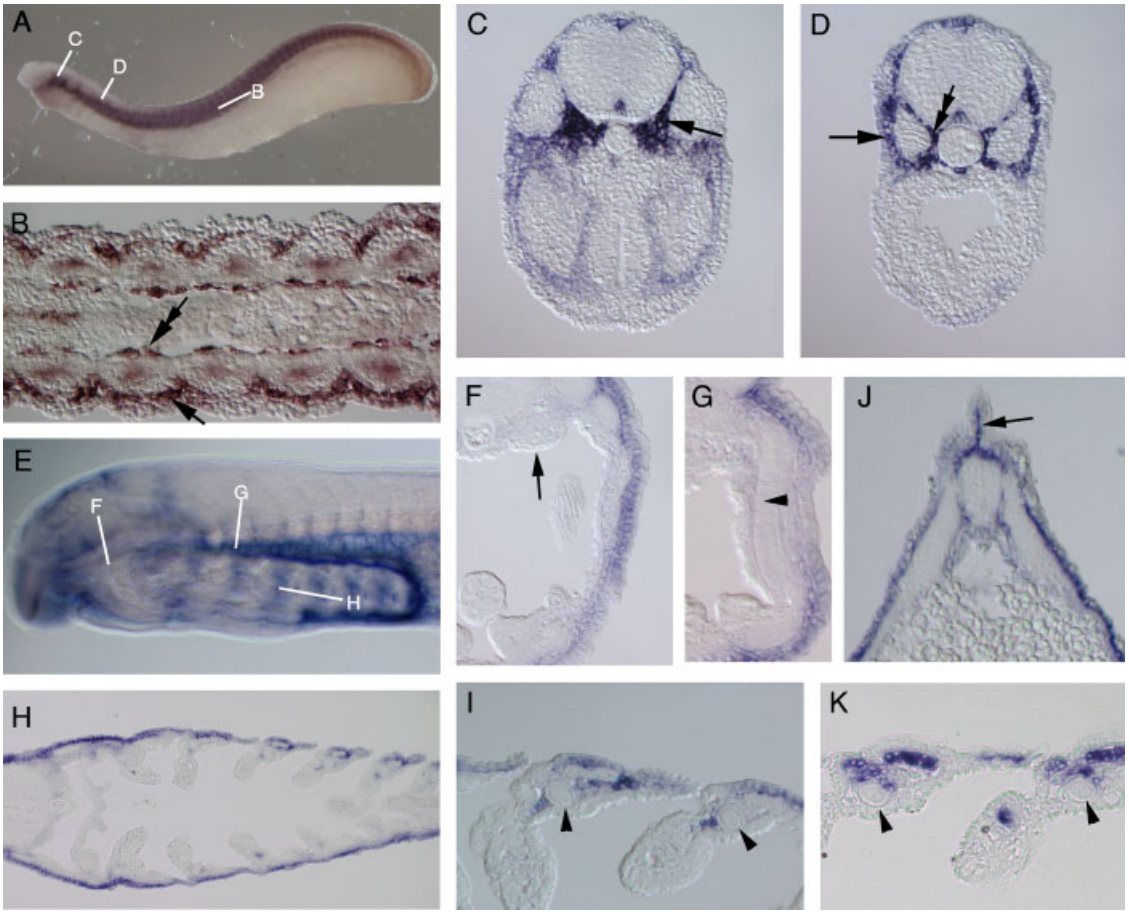

Fig. 4. Expression pattern of *LjColA/col2a1a*. (**A–D**) At stage 25, *LjColA/col2a1a* was expressed in head mesodermal cells (arrow in C) and cells surrounding myotomes (arrows in B and D indicate dermomyotome and double arrows indicate sclerotome). (B–D) Sections shown in (A). (**E–J**) At stage 27, expression was observed in pharyngeal mesenchymal cells but not in chondrocytes (G, I: arrowheads indicate pharyngeal chondrocytes). No expression was detected in trabecular chondrocytes (F: arrow indicates trabecular chondrocytes). Expression was also observed in epidermal cells. (F–H) Sections shown in (E). (I) Expression in pharyngeal chondrocytes (arrowheads) at higher magnification. (J) A transverse section of the trunk region indicating expression in fin mesenchymal cells (arrow). (**K**) Expression of *LjMA2* in pharyngeal muscle.

Pmcol2a1b, neither clade A fibrillar collagen gene was expressed in the trabecular cartilage (Table 1).

Expression of Sox genes in Lethenteron cartilage

Next, we examined the expression of Sox cognates, which, in gnathostomes, regulate *col2a1* expression in chondrocytes (Bell et al., '97; Lefebvre et al., '97). One group E Sox gene was isolated from *L. japonicum*. Our molecular phylogenetic analysis indicated that it is an ortholog of *PmSoxE3* (Fig. 3B) (McCauley and Bronner-Fraser, 2006). The earliest expression of *LjSoxE3* was found in the otic vesicle at stage 22 (data not shown). Slightly later, expression was also detected in the anterior brain and migrating neural crest cells (Fig. 7A). At stage 27, high level of expression was detected in the velum and branchial arches (Fig. 7B, C). Horizontal sections of embryos clearly indicated the expression in chondrocytes as well as in the perichondrium (Fig. 7E). *LjSoxE3* was also expressed in the trabecular chondrocytes (Fig. 7D) and in the fin fold at stage 29.

SoxD family genes, such as Sox5 and Sox6, are also involved in gnathostome chondrogenesis (Smits et al., 2001). A group D Sox gene from *L. japonicum* branched off from the basal node of SoxD genes of gnathostomes (Fig. 3C). *LjSoxD1* expression was detected in the notochord, anterior brain, and otic vesicle during stage 24 (Fig. 8A). At stage 25, expression was also detected in migrating

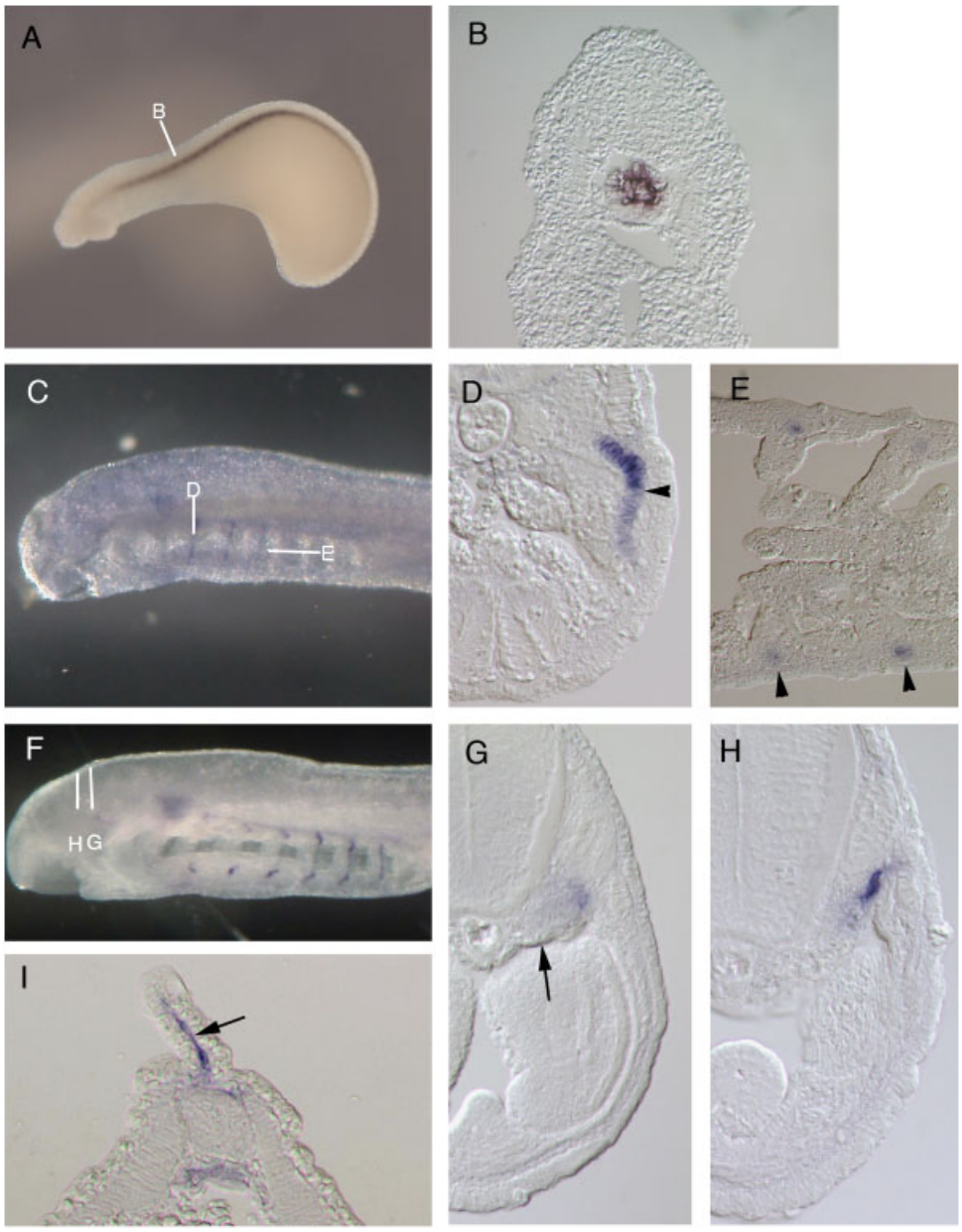

Fig. 5. Expression pattern of *LjColB/col2a1b*. (**A**, **B**) At stage 24, expression was observed in the notochord. (B) Transverse section shown in (A). (**C**–**E**) Expression was observed in pharyngeal chondrocytes at stage 26. (D, E) Sections shown in (C). Arrowheads indicate pharyngeal chondrocytes. (**F**–**I**) At stage 27, expression occurred in pharyngeal chondrocytes, but was not detected in trabecular chondrocytes. Some mesenchymal cells were positive for *LjColB/col2a1b*. These cells were not trabecular chondrocytes based on their more anterior and lateral position (H). The arrow in (G) indicates trabecular cartilage. (I) Expression was also observed in fin mesenchyme cells. Transverse section of the trunk.

neural crest cells and the branchial arch (Fig. 8B). At stage 26, expression was up-regulated in pharyngeal chondrocytes, but was excluded from chondrocytes of perichondrium (Fig. 8C, D). Unlike *LjSoxE3*, expression of *LjSoxD1* was not detected in ectomesenchyme cells in the velum or in the first pharyngeal arch (Fig. 8E, F). Expression in the pharyngeal chondrocytes was maintained until stage 28 (Fig. 8G). At this stage, *LjSoxD1* was also expressed in the trabecular cartilage (Fig. 8H) and fin mesenchyme (Fig. 8I).

DISCUSSION

Cartilage is one of the most important characteristics of vertebrates. Genetic mechanism of

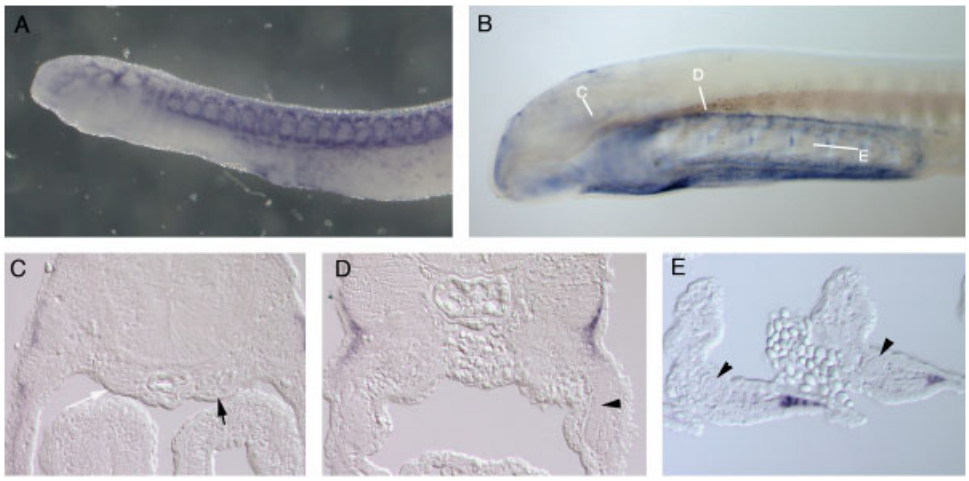

Fig. 6. Expression pattern of *LjColC*. (**A**) At stage 25, expression was observed in cells surrounding the myotome. (**B**–**E**) At stage 27, although pharyngeal expression was observed, it was not in pharyngeal chondrocytes (arrowheads in D, E). No expression was detected in trabecular chondrocytes (arrow in C). (C–E) Section shown in (B).

TABLE 1. Comparison of expression patterns of fibrillar collagen genes in two lamprey species

	P. marinus	*L. japonicum*
ColA/col2a1a	Sclerotome, dermatome floorplate, hypochord, pharyngeal chondrocytes fin mesenchyme	Sclerotome, dermatome, roofplate floorplate, hypochord pharyngeal mesenchyme, epidermis, fin mesenchyme
ColB/col2a1b	Notochord floorplate, hypochord, sclerotome fin mesenchyme	Notochord pharyngeal chondrocytes head mesenchyme, fin mesenchyme

Expressions that were not detected in the other species are underlined.

chondrocyte differentiation is well characterized in gnathostomes where SoxD and SoxE genes directly regulate expression of genes encoding matrix proteins such as col2a1 (Bell et al., '97; Lefebvre et al., '97, '98; Bi et al., '99; Sekiya et al., 2000; Smits et al., 2001). An important question is, thus, how the genetic machinery has been established for chondrogenesis in the ancestors of vertebrates. In this aspect, the genetic mechanism of lamprey chondrogenesis is a critical issue, because lampreys, although they also possess pharyngeal cartilage derived from neural crest cell (Langille and Hall, '88; McCauley and Bronner-Fraser, 2006), possess a unique type of cartilage that is mainly composed of noncollagenous proteins (Wright et al., '88; Robson et al., '97). Zhang et al. (2006) reported that lampreys possess two orthologs of gnathostome *col2a1*; one ortholog is expressed in pharyngeal chondrocytes. In addition, SoxE gene, whose mammalian homolog directly regulates chondrocyte expression of *col2a1*, is expressed in pharyngeal chondrocytes (McCauley and Bronner-Fraser, 2006; Zhang et al., 2006). Their study showed striking similarities between lamprey and gnathostome cartilage, showing that one group E Sox gene is essential for chondrogenesis of lamprey pharyngeal arches (McCauley and Bronner-Fraser, 2006).

This study provides further support for previous findings. We found that in *L. japonicum*, the clade A collagen gene *LjColB/col2a1b* is expressed in pharyngeal chondrocytes (Fig. 5). However, *LjColB/col2a1b* is not an ortholog of *Pmcol2a1a*, which is expressed in *Petromyzon* chondrocytes, but an ortholog of *Pmcol2a1b*, which is expressed in the notochord (Zhang et al., 2006). Other than expression in the pharyngeal cartilage, the expression patterns are similar for each orthologous pair (Table 1). Thus, it is unlikely that we misassigned the orthologous gene pairs here. Therefore, *P. marinus* and *L. japonicum* may utilize distinct orthologs for cartilage. Alternatively, because *LjColA/col2a1a* is also expressed in the pharyngeal arch but not in chondrocytes (Fig. 4), the expression of *Pmcol2a1a* in nonchondrocyte mesenchyme of the pharyngeal arch may have

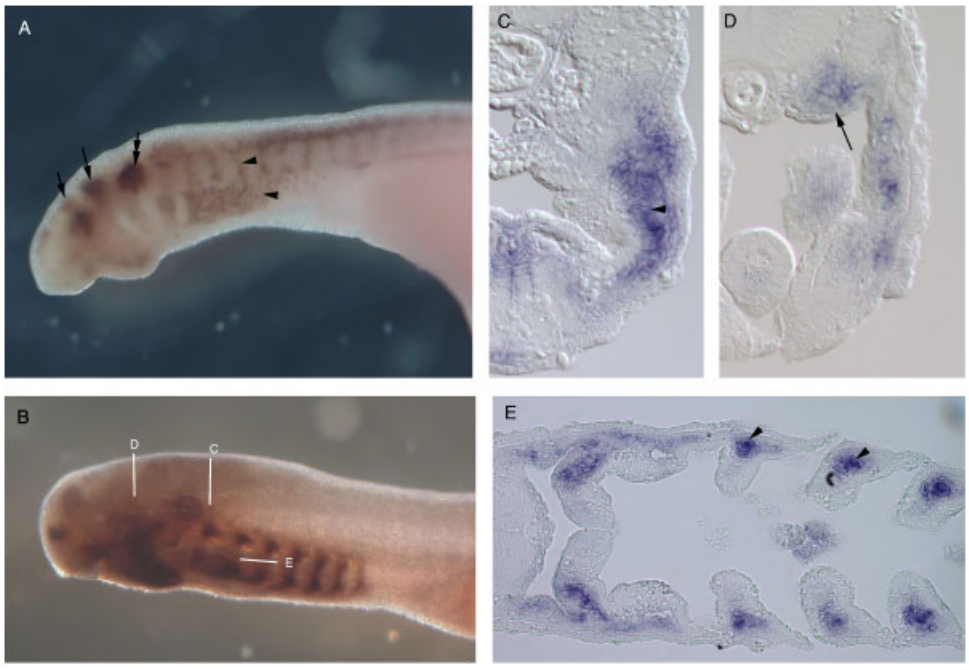

Fig. 7. Expression of *LjSoxE3*. (**A**) At stage 23, expression was in the anterior brain (arrow), otic vesicle (double arrow), and migrating neural crest cells (arrowhead). (**B**–**E**) At stage 27, expression was detected in ectomesenchyme cells, including pharyngeal chondrocytes (arrowheads in C, E). Trabecular chondrocytes were also positive for *LjSoxE3* (arrow in D). (C–E) Sections shown in (B).

been misidentified as chondrocyte expression. Indeed, chondrocyte expression is difficult to determine in whole-mount specimens. In either case, both studies agree that lamprey pharyngeal cartilage contains fibrillar collagen. In addition, two groups of Sox genes (*LjSoxD1* and *LjSoxE3*) are expressed in pharyngeal chondrocytes. Therefore, these studies consistently indicate that there is a genetic cascade for chondrocytes in which group D and group E Sox genes regulate the expression of fibrillar collagen col2a1, and that this cascade is conserved in lamprey pharyngeal chondrocytes (McCauley and Bronner-Fraser, 2006; Zhang et al., 2006).

These results suggest that the common ancestors of vertebrates may have possessed cartilage in which Sox genes regulate the expression of the cartilage fibrillar collagen gene col2a1, as seen in extant agnatha and gnathostomes. However, the evolutionary story may be more complex. Here, we raise two points. First, regarding the orthology of lamprey collagen gene, although Zhang et al. (2006) demonstrated orthology of two *Petromyzon* collagen genes with gnathostome *col2a1*, the evidence should be interpreted more carefully. As clade A fibrillar collagen genes are linked with Hox clusters, as mentioned above, there is little doubt that these genes arose through genome duplication (Wada et al., 2006). It is still unclear whether lamprey experienced the second round of genome duplication and how many Hox clusters agnathans have (Furlong and Holland, 2002). These questions remain to be resolved because molecular phylogenetic analyses based on a small number of genes have produced inconsistent results. Therefore, care should be taken in judging orthology of any lamprey genes.

Second, this study indicated that there is no clade A fibrillar collagen gene expression in trabecular chondrocytes, although we cannot exclude the possibility that another type of fibrillar collagen is expressed in the trabecular chondrocytes. Therefore, even if we accept the orthology of *col2a1* and *Pmcol2a1a–b*/*LjColA–B* (Zhang et al., 2006), lampreys possess a cartilage that is not marked by the expression of *col2a1* cognates. Trabecular cartilage contains lamprin as the main component (McBurney et al., '96), and lamprin gene expression in trabecular cartilage may possibly be regulated by SoxD and SoxE in trabeculae.

In contrast to the divergent expression patterns of fibrillar collagen genes in lamprey larval chondrocytes, the group D and group E Sox genes show consistent expression in lamprey chondrocytes in both the pharyngeal arch and trabeculae.

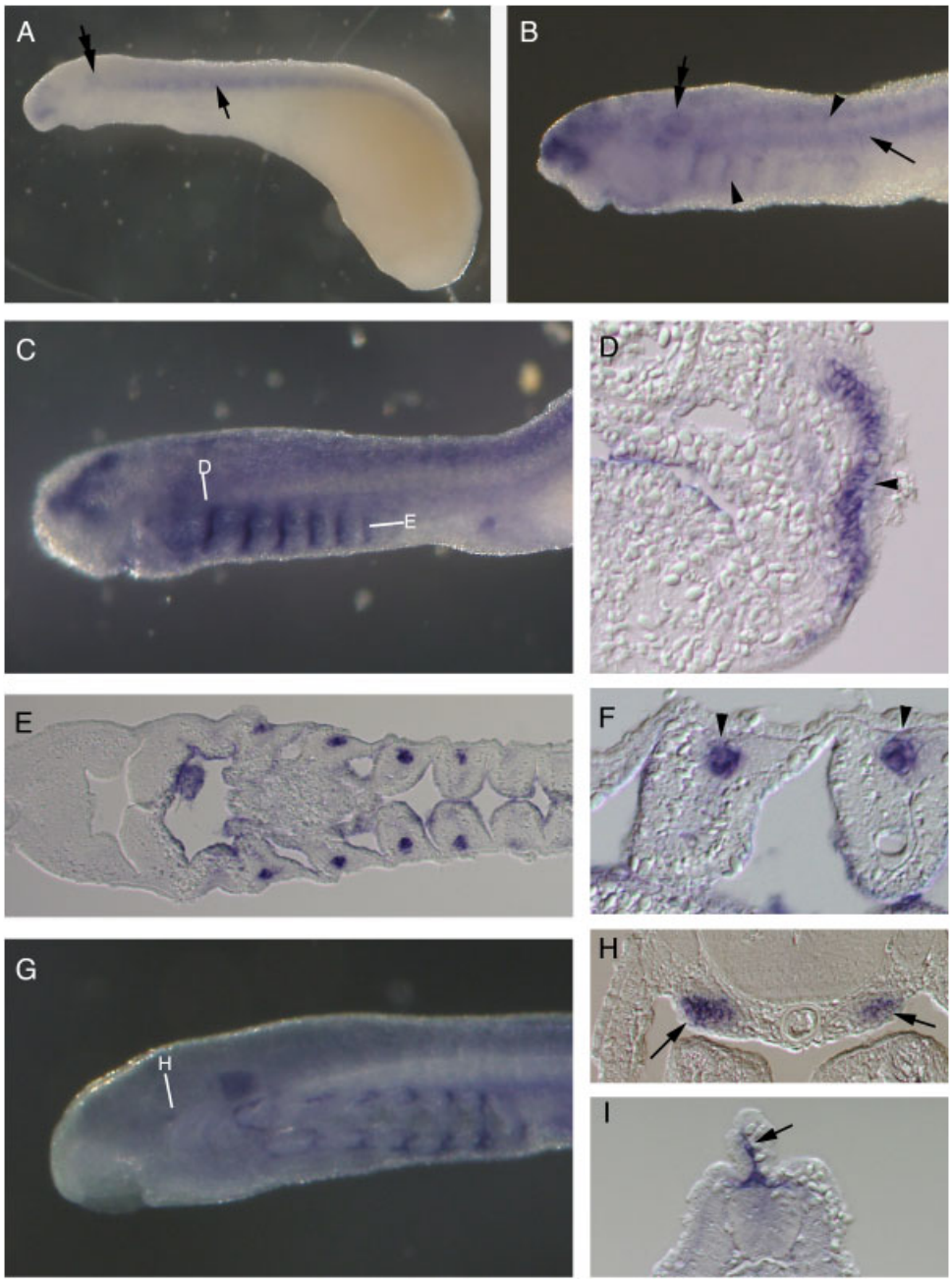

Fig. 8. Expression of *LjSoxD*. (**A**) At stage 24, expression was detected in the notochord (arrow), anterior brain region, and otic vesicle (double arrow). (**B**) Expression was also detected in neural crest cells migrating to the branchial arches (arrowheads) at stage 25, as well as notochord, brain, and otic vescile. (**C**) At stage 26, expression was up-regulated in pharyngeal chondrocytes. (**D**, **E**) Sections shown in (C). (**F**) Higher magnification view of the pharyngeal arches. LjSoxD is specifically expressed in pharyngeal chondrocytes (arrowheads). (**G**–**I**) At stage 28, expression was also detected in trabecular chondrocytes (arrows in G, H) and in fin mesenchyme cells (arrow in I).

The conserved expression of *SoxD* and *SoxE* supports homology among the cartilage of agnathans and gnathostomes. Recently, Rychel and Swalla (2007) found that *SoxE* and fibrillar collagen genes are expressed in pharyngeal endoderm of amphioxus and acorn worms, which may be responsible for chondrogenesis of the gill cartilage. Hecht et al. (2008) also show that *Runx* and *Hh* are expressed in pharyngeal region of the adult amphioxus, suggesting that these two genes might be involved in cartilage differentiation of amphioxus. Although, it is necessary to confirm

that these genes really function in matrix secretion (e.g., Meulemans and Bronner-Fraser, 2007, showed the expression of the fibrillar collagen gene in the pharyngeal mesoderm of larvae and suggested the mesodermal origin of the pharyngeal gill cartilage), this observation suggests that ancestral deuterostomes would have possessed a Sox–collagen genetic cascade for chondrogenesis. Amphioxus cartilage is observed in pharyngeal bars and oral cirri. Although the chemical components of amphioxus cartilage are unknown, Wright et al. (2001) presented an evidence that the main component of amphioxus cartilage is not collagen but cyanogen bromide (CNBr)-resistant fiber. The cartilage matrix proteins of lamprey, such as lamprin, are also found to be CNBr resistant, and thus the amphioxus matrix protein may be similar to those of lamprey cartilage. These studies suggest that amphioxus may possess cartilage regulated by putative Sox–elastin-like protein and Sox–collagen gene cascades.

The vertebrate cartilage may have diverged from the ancestral cartilage similar to that found in amphioxus. This ancestral condition may be more conserved in lamprey cartilage, where elastin-like proteins are the main component and collagen is included as a minor component in one type of cartilage. Gnathostome cartilage may be a more diverged cartilage type because they lost elastin and utilize fibrillar collagen as the main component. These observations indicate that the evolutionary history of chordate cartilage includes various matrix components being recruited and/or lost under the transcriptional regulation of Sox genes.

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