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Development and Evolution of Chordate Cartilage

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ABSTRACT Deuterostomes are a monophyletic group of animals containing vertebrates, lancelets, tunicates, hemichordates, echinoderms, and xenoturbellids. Four out of these six extant groups—vertebrates, lancelets, tunicates, and hemichordates—have pharyngeal gill slits. All groups of deuterostome animals that have pharyngeal gill slits also have a pharyngeal skeleton supporting the pharyngeal openings, except tunicates. We previously found that pharyngeal cartilage in hemichordates and cephalochordates contains a fibrillar collagen protein similar to vertebrate type II collagen, but unlike vertebrate cartilage, the invertebrate deuterostome cartilages are acellular. We found *SoxE* and fibrillar collagen expression in the pharyngeal endodermal cells adjacent to where the cartilages form. These same endodermal epithelial cells also express *Pax1/9*, a marker of pharyngeal endoderm in vertebrates, lancelets, tunicates, and hemichordates. In situ experiments with a cephalochordate fibrillar collagen also showed expression in pharyngeal endoderm, as well as the ectoderm and the mesodermal coelomic pouches lining the gill bars. These results indicate that the pharyngeal endodermal cells are responsible for secretion of the cartilage in hemichordates, whereas in lancelets, all the pharyngeal cells surrounding the gill bars, ectodermal, endodermal, and mesodermal may be responsible for cartilage formation. We propose that endoderm secretion was primarily the ancestral mode of making pharyngeal cartilages in deuterostomes. Later the evolutionary origin of neural crest allowed co-option of the gene network for the secretion of pharyngeal cartilage matrix in the new migratory neural crest cell populations found in vertebrates. *J. Exp. Zool. (Mol. Dev. Evol.) 308B:325–335, 2007.*

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Two separate groups of invertebrate deuterostomes, Hemichordata and Cephalochordata (lancelets), have cartilages supporting the pharyngeal gills (Fig. 1) (Schaeffer, '87; Cameron, 2002; Smith et al., 2003; Cole and Hall, 2004a; Rychel et al., 2006). Members of the invertebrate chordate group Tunicata do not have a pharyngeal skeleton despite having pharyngeal slits (Pennachetti, '84). Vertebrates also have cartilage supporting their pharyngeal gills (Fig. 1), and this tissue is secreted by chondrocytes of neural crest origin (Noden, '78; Kimmel et al., 2001). A major difference, however, is that vertebrate pharyngeal cartilage is a cellular tissue while the hemichordate and lancelet pharyngeal cartilages are acellular (Cole and Hall, 2004a; Rychel et al., 2006). Since lancelets and hemichordates lack chondrocytes in their pharyngeal cartilage, it is not obvious which cells are responsible for secreting the cartilage matrix. In this paper, we use collagen and *SoxE* expression to show that the gill bar cartilages of hemichordates and lancelets are secreted by the pharyngeal epithelia surrounding them, which is endodermal in hemichordates and a combination of endoderm, ectoderm, and mesoderm in lancelets.

Cartilage tissue in vertebrates contains an extensive and characteristic extracellular matrix (ECM). Chondrocytes, cells that produce cartilage

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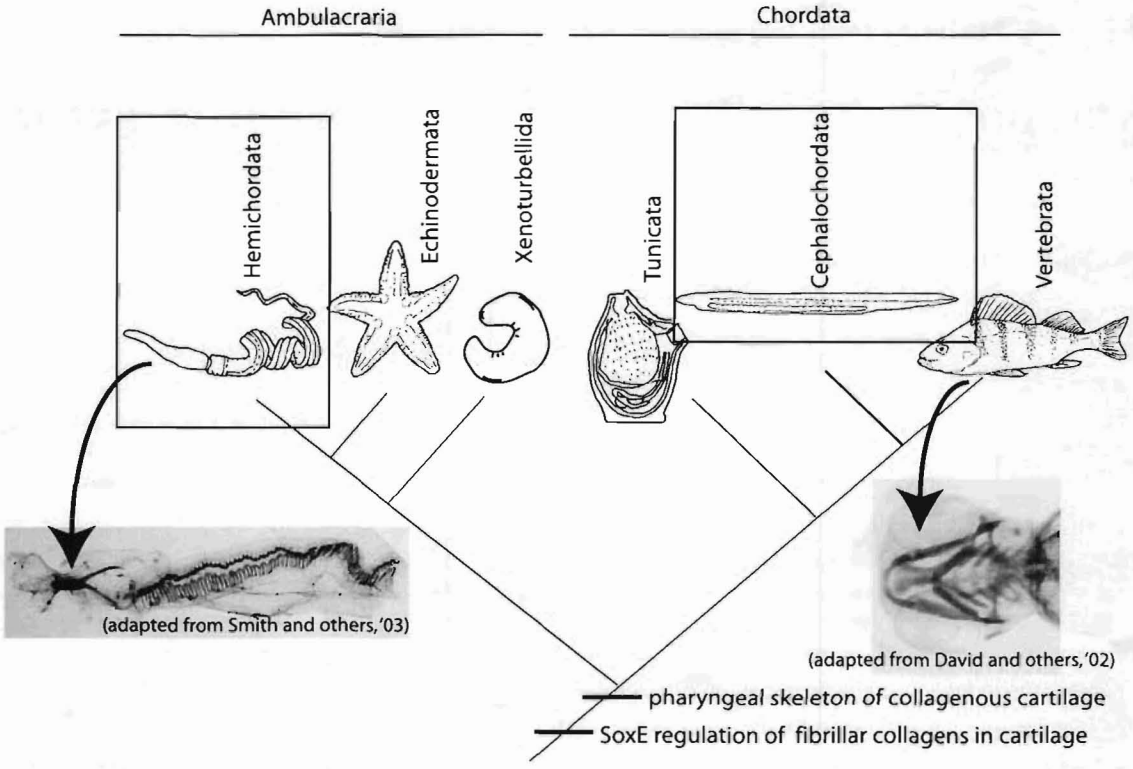

Fig. 1. Deuterostome phylogeny. Deuterostomes are composed of two major clades, Ambulacraria (Hemichordata, Echinodermata, and Xenoturbellida) and Chordata (invertebrates Tunicata, Cephalochordata, and Vertebrata). For this study, *Pax1/9*, fibrillar collagen and *SoxE* expression was examined in hemichordates and cephalochordates (boxed) that, although distantly related invertebrate deuterostomes, share a similar gill skeleton structure. A whole mount alcian blue prep of a hemichordate is shown below the large arrow from Hemichordata (Smith et al., 2003). A whole mount alcian blue prep from a zebrafish is shown beneath the large arrow drawn from Vertebrata (David et al., 2002).

matrix, differentiate from mesodermal mesenchyme cells, or are derived from neural crest cells in the vertebrate head (Goldring et al., 2006). Developmentally, prechondrogenic mesenchyme cells have a mesodermal origin, and neural crest cells have an ectodermal origin. Most connective tissues are known and understood best in vertebrates, but many invertebrates from diverse phyla also have extensive connective tissues, including cartilage (Cole and Hall, 2004a,b). Many of the invertebrate cartilages described by Cole and Hall (2004a,b) are cellular, like vertebrate cartilage, but their developmental origins are largely unknown. However, the acellular cartilages found in hemichordates and cephalochordates are peculiar in that they resemble thickened basal lamina (Hyman, '59; Rychel et al., 2006). Basal laminae do not typically contain the fibril-forming collagens found in vertebrate cartilage, instead they contain mesh-forming collagens, like type IV collagen. Also, basal laminae have abundant structural proteins such as laminin and nidogen that are not in vertebrate cartilages (Dziadek, '95), whereas cartilage is characterized by a specific sulfated proteoglycan, chondroitin sulfate, in the ECM (Hall, 2005).

SoxE (Sox8/9/10) is the invertebrate ortholog to three vertebrate Sox gene duplicates: *Sox8*, *Sox9*, and *Sox10* (Bowles et al., 2000). The expression of these genes is often overlapping and there is some, but not total functional redundancy between *Sox8*, *Sox9*, and *Sox10* (Kellerer et al., 2006; O'Donnell et al., 2006). In vertebrates—both agnathans and gnathostomes—pharyngeal cartilage development is characterized by the early expression of *Sox9* in the neural crest cells that migrate into the pharyngeal pouches (Yan et al., 2002; McCauley and Bronner-Fraser, 2006; Zhang et al., 2006). *Sox9* is responsible for directly activating transcription of several genes coding for proteins that compose vertebrate cartilage ECM. One of the most abundant proteins specific to vertebrate cartilage ECM is type II collagen (*Col2a1*). Both *Sox9* and *Sox10* can directly activate *Col2a1* (Bell

et al., '97; Bi et al., '99; de Crombrugghe et al., 2000; Sekiya et al., 2000; Suzuki et al., 2006) and in *Xenopus*, both *Sox8* and *Sox9* can directly activate *Sox10* (O'Donnell et al., 2006). Prechondrogenic mesenchymal cells derived from neural crest as well as differentiated non-hypertrophic chondrocytes express *Sox9*, *Sox10*, and *Col2a1* in the pharyngeal arches (Ng et al., '97; Zhao et al., '97; Suzuki et al., 2006). In vertebrate cartilage development, chondrocyte cells are trapped within the ECM they secrete, creating a cellular cartilage. Placodes and neural crest-like cells have not been described in hemichordates and are lacking in lancelets, even though tunicates clearly contain placodes and may have neural crest-like cells (Swalla, 2007 for review). Yet both hemichordates and lancelets have fibrillar collagen in their pharyngeal cartilages as seen by transmission electron microscope (TEM) and immunohistochemistry (Rahr, '82; Pardos and Benito, '88; Rychel et al., 2006), even though the gill bar cartilages are acellular (Cole and Hall, 2004b; Rychel et al., 2006).

We have presented evidence previously that gill slits in hemichordates, lancelets, and vertebrates are homologous (Rychel et al., 2006; Swalla, 2007) and that gill bars in hemichordates and cephalochordates are composed of a protein similar to vertebrate type II collagen (Rychel et al., 2006). Now we present new data that show that the pharyngeal endodermal cells in hemichordates and lancelets express a fibrillar collagen and the pharyngeal endoderm in hemichordates also expresses the invertebrate homolog to vertebrate *Sox8/9/10*, *SoxE*. Lancelets also show collagen expression in the ectodermal pharyngeal epithelia and mesodermal coelom lining the atrial surface of the gill bars. These data indicate that the evolutionary origin of a collagenous pharyngeal cartilage was near the time of deuterostome diversification, not at the base of the vertebrates. Our results show pharyngeal cartilage is made using a similar genetic pathway as in vertebrates but rather than neural crest cells secreting the matrix, the role of matrix secretion lies in the endoderm for hemichordates, and partially in the endoderm in lancelets.

MATERIALS AND METHODS

Tissue collection and preparation

Enteropneust hemichordates *Saccoglossus bromophenolosus* were collected at Bay View State Park, WA, during low tide by digging in the mud in locations where small fecal casts are seen. *Saccoglossus kowalevskii* was collected at Smithsonian Marine Station at Fort Pierce, FL, by digging in the sand to the right of the SMS dock. *Branchiostoma floridae* was ordered from Gulf Specimen Marine Laboratories, Inc. in Panacea, FL. *Branchiostoma virginiae* was collected by dredging sand with the Sunburst at the Smithsonian Marine Station in Fort Pierce, FL.

Animals were fixed overnight in 4% paraformaldehyde in 0.5 M NaCl, 0.1 M MOPS, pH 7.5 buffer. The following day they were either transferred to 50% then 80% ethanol and stored at −20°C or dehydrated through a 30, 50, 80, 100% ethanol series and embedded in polyester wax. Sections of 7 μm were made on a Spencer 829 microtome and mounted on gelatin-subbed slides.

In situ hybridization

Slides were deparaffinized in 100% ethanol, and rehydrated through an alcohol series into PBT. Next, the slides were digested with 1 μg/ml proteinase K for 10 min at 37°C. Then the slides were transferred to a 2 mg/ml glycine PBT solution, and post fixed in 4% paraformaldehyde in PBS for 1 hr. Slides were subsequently dipped into triethanolamine and then treated with triethanolamine with 0.25% anhydrous acetic acid. Hybridization with a DIG-labeled RNA probe was carried out at 45°C overnight for *Pax1/9* and collagen and 37°C for *Sb-SoxE*. Slides were washed extensively in 4X SSC, 0.1% Tween-20, 2X SSC, 0.1% Tween-20, and then transferred to Solution A (4 M NaCl, 1 M Tris, pH 8, 0.5 M EDTA, 0.1% Tween-20). Slides were treated with 1 μg/ml RNAse for 20 min at 37°C, transferred back into Solution A, then 2X SSC, 0.1% Tween-20, and 1X SSC, 0.1% Tween-20, followed by PBT. A 0.1% blocking reagent (Roche, Indianapolis, Indiana) in PBT was added for 30 min, and a 1:2000 dilution of a DIG-AP antibody was applied to the slides overnight. Slides were then extensively washed in PBT and transferred into Buffer 3 pH 8 (100 mM Tris pH 8, 100 mM NaCl, 50 mM $MgCl_2$) and then into Buffer 3 pH 9.5 (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$). An AP detection solution containing Buffer 3 pH 9.5, 200 mM levamisole, and NBT/BCIP (Roche, Indianapolis, Indiana) was then applied to the slides and the color reaction was allowed to proceed for approximately 3–5 hrs. The reaction was stopped in PBS, and then the slides were dehydrated, counterstained with

Eosin-Orange G, transferred into xylene, and mounted with Permount (Fisher, Houston, Texas).

Cloning Sb-SoxE with degenerate primers

The following degenerate primers were designed using an amino acid alignment of the HMG domain of SoxE genes from several vertebrates and invertebrates. SoxE F: CCNATGAAYGCNT TYATG, and SoxE R: TCNGGRTGRTTYTTRTG. These primers amplified an approximately 700 bp fragment using genomic DNA from *S. bromophenolosus* using the following program: 94°C for 4 min, then 35 cycles of 94°C for 30 sec, 42°C for 30 sec, 72°C for 1 min, with a final extension of 72°C for 10 min. The PCR reaction contained 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM each primer, and 0.75 U of Taq polymerase. Bands were cut out and purified using Sephaglas (Amersham; Piscataway, NJ), and cloned into a pCR II plasmid (Invitrogen; Carlsbad, CA). The clone's identity was confirmed with a BLAST search and gene tree analysis. The *Sb-SoxE* sequence has been deposited in Genbank with the accession number EF156377.

Fibrillar collagen clones and gene trees

A fibrillar collagen clone containing a partial portion of the triple helical domain and the complete C terminal domain from *S. kowalevskii* was obtained from John Gerhart and Marc Kirschner. Two RNA probes were made from this clone: one full length and one with only C terminal domain sequences. The C terminal probe was made by linearizing the plasmid cDNA with the restriction enzyme *Pst I*, which cut at a unique site 100 nucleotides 5′ of the C terminal domain. This probe contains 100 nucleotides 5′ of the C terminal domain, 700 nucleotides in the C terminal domain, and 1,040 nucleotides in the 3′ UTR. Sense and antisense probes were synthesized using a DIG RNA labeling kit SP6/T7 (Roche, Indianapolis, Indiana). Several clones from a single *B. floridae* fibrillar collagen gene were obtained from Georgia Panopoulou (Panopoulou et al., 2003). The six clones represented the sequence from a partial triple helical domain and the complete C terminal domain. Clones from both the triple helical domain and C terminal domain were used for in situ hybridization. The parsimony gene tree was constructed as previously reported (Rychel et al., 2006) and two new type II collagen sequences from lamprey (Zhang et al., 2006) were added to the dataset. Briefly, complete or partial amino acid sequences from the fibrillar collagen triple helical domains were aligned in Clustal X (Jeanmougin et al., '98). Parsimony heuristic searches with 1,000 random additions were performed in PAUP* 4.0b10 (Swofford, '99) and bootstrapping was done with 1,000 pseudoreplicates.

RESULTS

Fibrillar collagen expression was examined in two groups of invertebrate deuterostomes for this study: an enteropneust hemichordate and a cephalochordate (lancelet) (boxes; Fig. 1). Hemichordates and lancelets were chosen for this study because they are the only invertebrate deuterostomes that have an acellular pharyngeal cartilage. These are considered cartilages because of the fact that they resist KOH digestion (Smith et al., 2003), stain blue green using a Milligan's trichrome histology stain, and also stain with a vertebrate type II collagen antibody (Rychel et al., 2006). The main component of vertebrate pharyngeal cartilage is fibrillar collagen, so we examined collagen expression in the pharyngeal cartilages of juvenile and adult hemichordates and lancelets. Gill bars are continuously added at the posterior so it is possible to look at developing gill bars in adults.

Fibrillar collagens are best characterized in vertebrates, but so far only a single fibrillar collagen has been found in hemichordates and cephalochordates (Fig. 2). Trace lancelet sequences in GenBank were blasted using the conserved C terminal domain of collagen and the sequences retrieved were all highly similar to the clone we report here, suggesting lancelets have a single fibrillar collagen gene. Expressed sequence tags from several different development stages of *S. kowalevskii* have suggested a single fibrillar collagen gene in hemichordates, as well (John Gerhart, personal communication). Gene trees built with all collagen genes known from several invertebrates, including mosquitos and sea urchins, show three types of ancestral fibrillar collagens in animals, the A, B, and C clades (Aouacheria et al., 2004; Rychel et al., 2006; Wada et al., 2006). The fibrillar collagens found in hemichordates and cephalochordates belong to the A clade of fibrillar collagens (Fig. 2) (Rychel et al., 2006). This clade also contains vertebrate type I (*Col1a1*) and type II (*Co2a1*) collagens. Hemichordates and cephalochordates have at least one A clade collagen, whereas echinoderms (sea urchins) have at least three closely related copies,

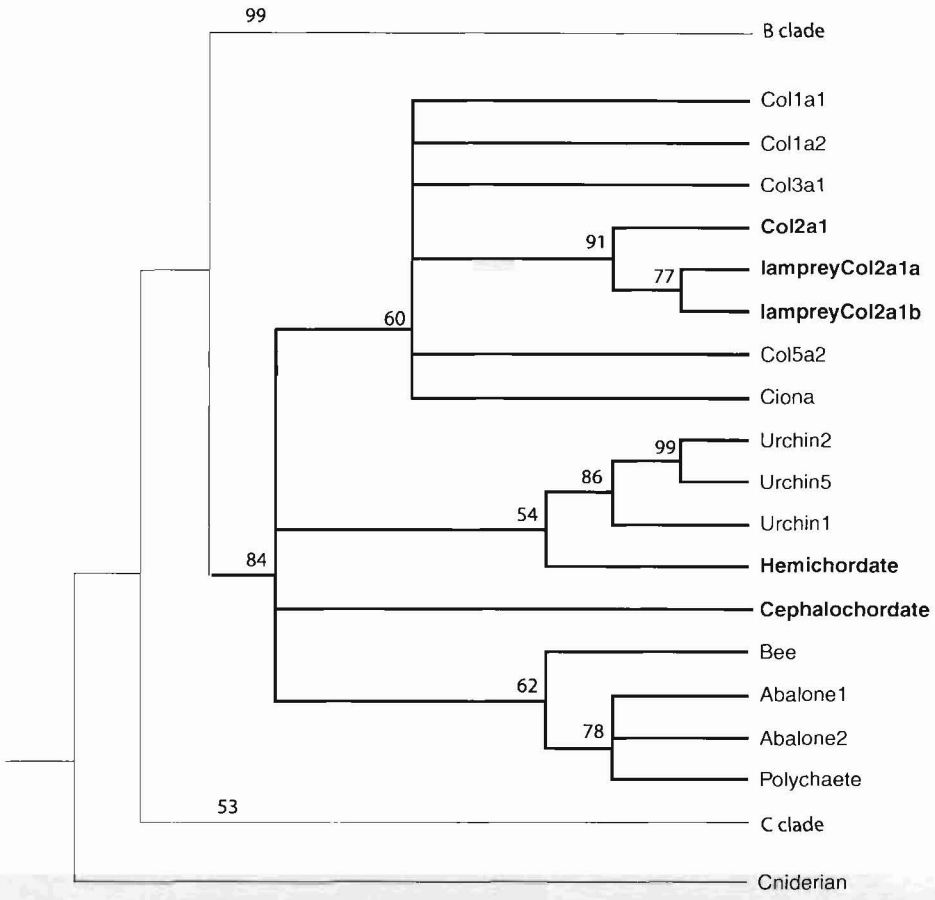

Fig. 2. Fibrillar collagen gene tree. Parsimony analysis yielded a tree with three clades (A–C), but only the A clade is shown here in detail with bold branches. Bootstrap values greater than 50 are shown on the tree, and clades with bootstrap values with less than 50% were collapsed. The A clade contains the vertebrate collagens, *Col1a1*, *Col1a2*, *Col3a1*, *Col2a1*, *Col5a2* as well as several invertebrate collagens from members of the Lophotrochozoa (abalone and polychaete) and Ecdysozoa (bee). Type II collagens from humans and lampreys and the fibrillar collagens from hemichordates and cephalochordates are shown in bold. Invertebrate deuterostome sequences from cephalochordates, hemichordates, and sea urchins group with the vertebrate deuterostome sequences, but not strongly with a particular copy. Multiple copies of sea urchin (*2*, *5*, *1*), abalone (*1*, *2*), and lamprey type II collagens (*Col2a1a* and *Col2a1b*) group together, suggesting independent gene duplications in these groups.

urchin1, 2, and 5 (Fig. 2). Sea urchins also have both B and C clade collagen (Aouacheria et al., 2004). The sea urchin genome has been completed (Sea Urchin Genome Sequencing Consortium, 2006), whereas the hemichordate and cephalochordate genomes have not, so it is possible that more collagens may be identified through genome analysis.

We examined the expression of fibrillar collagen in two harrimaniid hemichordates, *S. kowalevskii* and *S. bromophenolosus*, by in situ hybridization. A diagram of a hemichordate is shown in Figure 3A, with area sectioned for in situ hybridization indicated with a red line. A lancelet is shown in Figure 3B, also with the area sectioned for in situ hybridization indicated with a red line. *Pax1/9*, a marker for pharyngeal endoderm (Ogasawara et al., '99), is expressed in *S. kowalevskii* in the pharyngeal region of the young adult worm (Fig. 3C and D). A section similar to Figure 3C and D was hybridized with the hemichordate fibrillar collagen (Fig. 3F and G) and *Sb-SoxE* (Fig. 3I and J). The gill bar cross sections in Figure 3I and J are a different shape than those in Figure 3C–G because they were located slightly more ventral in the animal. Figure 3F and G show a positive purple signal for fibrillar collagen RNA in the pharyngeal endoderm in the same cells that express *Pax1/9*, shown in the panel above. Most of the RNA is localized in the endodermal epithelium that is closest to the skeletal elements (Fig. 3D and G). Results were similar in the closely related species *S. bromophenolosus* (data not shown). We

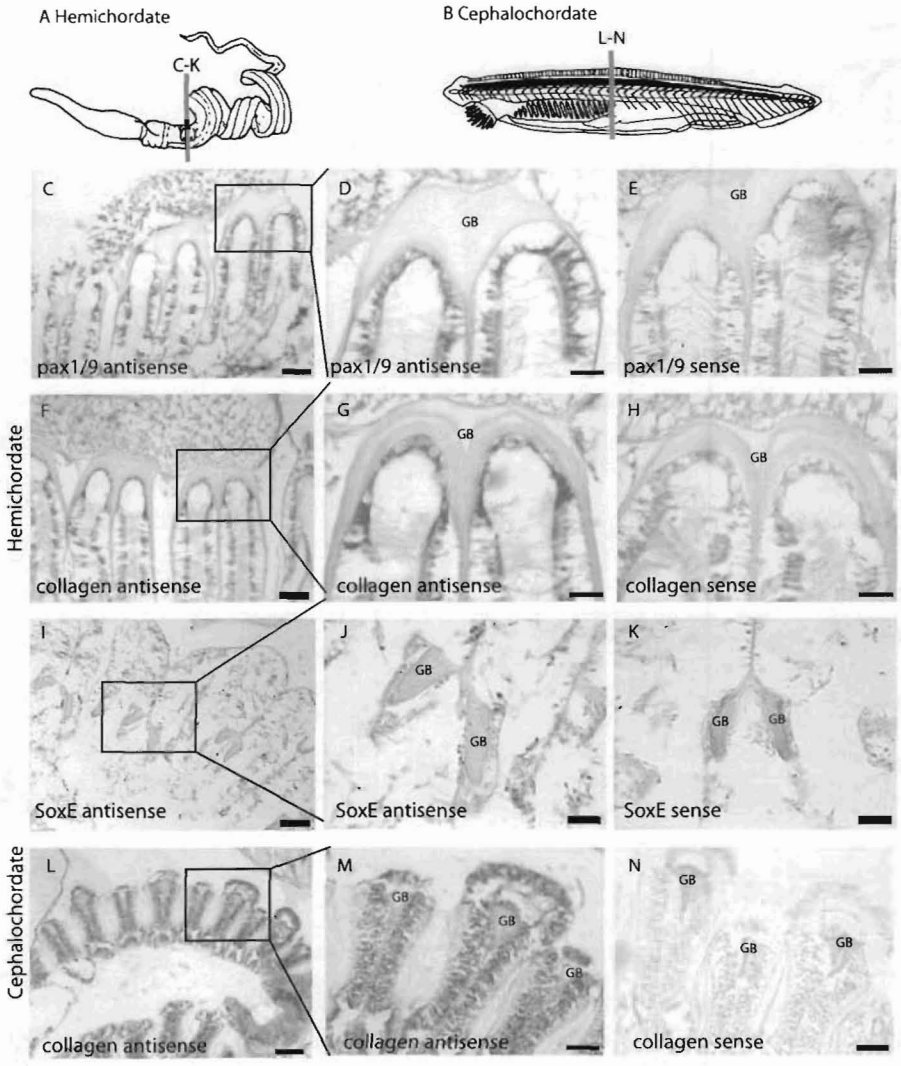

Fig. 3. Fibrillar collagen, *SoxE*, and *Pax1/9* expression. (**A**) Diagram of a hemichordate worm with the areas sectioned and examined in (**C–K**) indicated with a red line. (**B**) Diagram of a cephalochordate with the areas sectioned and examined in L–N indicated with a red line. (C) Lower magnification in situ for *Pax1/9* collagen around hemichordate (*S. kowalevskii*) gill bars. (D) Higher magnification of boxed area in (C) showing the purple signal for *Pax1/9* RNA within the cells surrounding the eosin stained gill bar (GB). (E) Higher magnification of a control section treated with a sense probe for hemichordate *Pax1/9* and stained with eosin. There is no purple label in the cells surrounding the gill bars. (F) Lower magnification in situ for *Pax1/9* around hemichordate (*S. kowalevskii*) gill bars. (G) Higher magnification of the boxed area shown in F. The purple signal for fibrillar collagen RNA expression is within the cells surrounding the gill bar, which is counterstained with eosin pink. (H) Higher magnification of a control section treated with a sense probe for hemichordate fibrillar collagen. There is no purple label in the cells surrounding the gill bars. (I) Lower magnification in situ for *SoxE* around hemichordate (*S. bromophenolosus*) gill bars. (J) Higher magnification of the boxed area shown in (I). (J) The purple signal for *SoxE* is present within the cells around the gill bar. (K) Higher magnification of a control section treated with a sense probe for *SoxE*. (K). (**L**) Lower magnification in situ for fibrillar collagen around cephalochordate (*B. virginiae*) gill bars. (**M**) Higher magnification of the boxed area in (I) showing the purple signal for fibrillar collagen within the cells surrounding the eosin stained gill bar. (**N**) Higher magnification of a control section treated with a sense probe for cephalochordate fibrillar collagen and stained with eosin. There is no purple label in the cells surrounding the gill bars. GB indicates gill bar matrix. Scale bars = 50 μm (C, F, I); 20 μm (D–E, G, H, J, K, M, N).

also examined the expression of *Sb-SoxE* in *S. bromophenolosus*, and expression is seen in the pharyngeal endodermal cells, similar to *Pax1/9* and fibrillar collagen (Fig. 3I and J). Additionally, strong *Sb-SoxE* expression is seen in oocytes (not shown).

In the adult lancelets *B. floridae* and *B. virginiae*, fibrillar collagen expression was very

strong in the pharyngeal endodermal cells, the pharyngeal ectoderm and the mesodermal coelomic pouches on the atrial side of the gill bars (Fig. 3L and M).

The acellular gill bar cartilage of hemichordates appears to form as a result of *SoxE* and collagen expression in the endoderm, followed by secretion of collagens into the ECM. The acellular cartilage of lancelets is also formed by the secretion of fibrillar collagen by the pharyngeal endoderm along with the pharyngeal ectoderm and mesoderm. Collectively, these results indicate that the deuterostome ancestor also was likely to have had a pharyngeal cartilage composed of fibrillar collagen that may have been secreted by endoderm.

DISCUSSION

Until very recently, it was thought that only jawed vertebrates had type II collagen in their pharyngeal cartilage, since lampreys were understood to have head and pharyngeal cartilages composed of a novel protein, lamprin (Robson et al., '93; Wright et al., 2001). However, recent studies have shown that lamprey embryos do express type II collagen in pharyngeal cartilage, and that collagen expression is dependent on the expression of the upstream transcription factor *SoxE* (McCauley and Bronner-Fraser, 2006; Zhang et al., 2006). Now, similar overlapping expression patterns have been shown for hemichordates with a fibrillar collagen and *SoxE*, indicating that this shallow gene network has been conserved in the formation of pharyngeal cartilage since the stem deuterostomes.

The viscerocranium or pharyngeal region of the vertebrate head skeleton was hypothesized to have evolved from the pharyngeal gill skeleton of an invertebrate ancestor similar to the lancelet pharyngeal skeleton (De Beer, '37). This hypothesis was later refuted in light of data that indicated that the pharyngeal skeleton of lancelets, hagfish, and lampreys did not contain collagen structural proteins (Wright et al., 2001). TEM studies and our own antibody studies suggest that cephalochordates as well as hemichordates have collagenous pharyngeal cartilages (Rahr, '82; Pardos and Benito, '88; Rychel et al., 2006) and lend stronger support to the idea that the viscerocranium evolved from ancestral pharyngeal cartilages like those found in hemichordates and lancelets. Despite their phylogenetic distance from each other (Fig. 1), the pharyngeal gill cartilages of hemichordates and cephalochordates are remarkably similar in morphology (Schaeffer, '87; Smith et al., 2003) and structure (Rychel et al., 2006). Furthermore, as in vertebrates the cartilage of hemichordate worms is resistant to KOH digestion and stains with alcian blue dye, (Fig. 1) (Smith et al., 2003). Fibrillar collagen expression in hemichordate and lancelet pharyngeal cartilage cells suggests that pharyngeal collagenous cartilages evolved well before the vertebrate ancestor diverged from more basal deuterostomes.

We explored the evolutionary origins of pharyngeal skeletons by examining the invertebrate deuterostomes (lancelets and hemichordates) that have an elaborate pharyngeal skeleton (Smith et al., 2003). Both groups live in the sand or mud and filter water in a burrow (Kardong, 2006). In contrast, tunicates live on hard substrates, and have an extremely divergent adult body plan, but are classified as chordates based on larval characteristics (Swalla, 2007). Traditionally cephalochordates have been placed as the invertebrate chordate sister group to vertebrates because of certain aspects of the genome organization (*Hox* genes) as well as the presence of somites (Garcia-Fernandez and Holland, '94; Ikuta et al., 2004; Passamaneck and Di Gregorio, 2005). In recent genome phylogenetic analyses, tunicates were moved from the most basal member of the chordates to the closest invertebrate relative to the vertebrates (Blair and Hedges, 2005; Delsuc et al., 2006). Although this hypothesis poses even stronger evidence for a worm-like deuterostome ancestor (Cameron et al., 2000), these results should be interpreted with caution since mitochondrial genome analyses place cephalochordates as the sister group to vertebrates (Bourlat et al., 2006). Increased numbers of echinoderm and hemichordate sequences were recently added to nuclear gene analyses, giving a tree with a well-supported clade that includes vertebrates, cephalochordates, and tunicates, but the relationships within are still unresolved (Bourlat et al., 2006).

Wada et al. (2006) found fibrillar collagen expression in the floor plate nerve cells of the neural canal in larval cephalochordates, and we found similar expression in lancelet adults. Type II collagen (*Col2a1*) is known to be expressed in a variety of vertebrate embryonic neural tubes (Kosher and Solursh, '89; Cheah et al., '91; Yan et al., '95; Ng et al., '97), yet its function there has never been explored. The expression of a lancelet type A fibrillar collagen in many of the same places

as the vertebrate type II collagen (*Col2a1*) lends strength to the speculation that the ancestral clade A fibrillar collagens are functionally similar to vertebrate type II collagen. A recent study mapped expression patterns in vertebrate bone, cartilage, and notochord on a deuterostome fibrillar collagen gene tree, and concluded that the recruitment of fibrillar collagen genes to each of these tissues occurred independently in the three major clades (A, B, and C) (Wada et al., 2006). An alternative explanation that we favor is that an ancestral *SoxE* gene regulated fibrillar collagen expression (A, B, and C) and that the regulatory module was selectively retained in cartilage tissues in the deuterostome ancestor (Fig. 1). The two deuterostomes that have independently lost their pharyngeal cartilages (Swalla, 2006), echinoderms and tunicates, have fibrillar collagens that may have evolved new expression patterns. In echinoderms, there are independent duplications of A clade fibrillar collagens. These and other copies of fibrillar collagens in the B and C clades (Aouacheria et al., 2004) may have been co-opted into a role in biomineralization of the calcium carbonate endoskeleton (Livingston et al., 2006). It is possible that the echinoderm collagens coevolved with other genes involved in biomineralization of the echinoderm calcium carbonate endoskeleton, such as spicule matrix proteins (Benson et al., '87; George et al., '91; Katoh-Fukui et al., '91; Harkey et al., '95; Killian and Wilt, '96; Lee et al., '99; Illies et al., 2002).

Hemichordate and cephalochordate pharyngeal cartilage is acellular and appears to be wholly or partially secreted from endodermally derived epithelia. This type of ECM formation is rare in vertebrates, but is comparable with how acellular bone forms in the vertebral column of teleost fish (Ekanayake and Hall, '87, '88; Fleming et al., 2004). This sort of acellular bone found in modern teleosts is secondarily derived, but acellular bone preceded cellular bone in vertebrate gnathostome evolution (Donoghue et al., 2006), so acellular matrix secretion was likely to be common among stem group jawed vertebrates.

The deuterostome ancestor has been hypothesized to be a filter feeding benthic worm with gill slits and an acellular cartilaginous pharyngeal skeleton (Rychel et al., 2006). This hypothesis suggests that there have been evolutionary losses in the deuterostomes. Tunicates have lost a gill skeleton and echinoderms and xenoturbellids have lost both gill bars and gill slits. Echinoderms evolved a calcium carbonate endoskeleton and a water vascular system independently of hemichordates (Swalla, 2007). The tunicate innovation was the evolution of a cellulose tunic that serves to protect the animal and its gill pharynx (Zeng and Swalla, 2005). Tunicates also filter feed, and like some crinoids, are often attached to a hard substrate, whereas others are planktonic (Zeng and Swalla, 2005). An alternative hypothesis to homology between cephalochordate and hemichordate pharyngeal cartilages is that they are independently derived. We feel this hypothesis is unlikely since the type of gill skeleton found in hemichordates and cephalochordates is similar to the pharyngeal skeleton preserved in stem group vertebrates like the fossil Yunnanozoon (Chen et al., '95). Like hemichordates and cephalochordates, Yunnanozoon and similar stem vertebrates were thought to be marine benthic filter feeders, yet they had an enlarged head region that included eyes and a brain (Chen et al., '95).

We are investigating evolutionary relationships between the skeletal elements found in hemichordates and lancelets and pharyngeal cartilages in vertebrates. Mayor et al. ('99) considered lancelets as completely lacking head skeleton and crafted a hypothesis that describes how cranial neural crest cells developing from the ectoderm gained the potential to make cartilage. In their model, mesoderm cells first had the genetic program for cartilage production, and this program was transferred to the ectoderm—this is a transfer of chondrogenic potential from mesenchyme to epithelia. Our results suggest the opposite, that the endodermal epithelial cells transferred the ability to make cartilage to mesenchymal neural crest cells and head mesoderm.

In the context of the pharyngeal skeleton and the transition from invertebrate chordates to vertebrates, we speculate that the task of making cartilage and the gene networks required for cartilage formation were transferred from the pharyngeal epithelium (mostly endoderm) to the neural crest cells. The acellular manner of making pharyngeal cartilage in hemichordates and cephalochordates is likely the ancestral mode of constructing pharyngeal cartilage in the deuterostomes. Next, in vertebrates, neural crest cells invaded this matrix and may have been influenced by it. Finally, endoderm evolved reduced matrix secretion, but still continued to pattern neural crest migration (Fig. 4) (Graham et al., 2005). We will continue to test and refine these evolutionary hypotheses experimentally with extant animals.

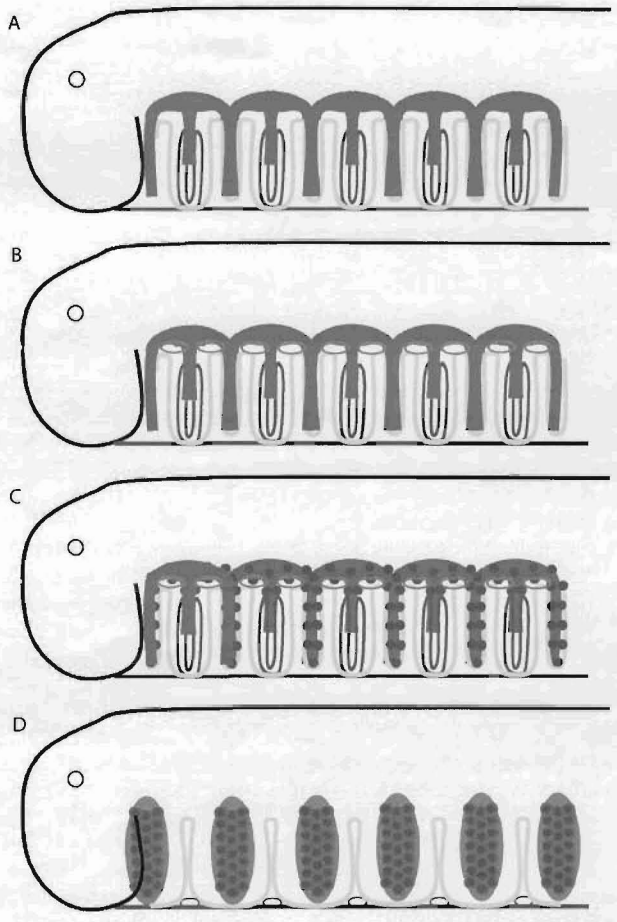

Fig. 4. Evolutionary model for the evolution of pharyngeal cartilage in deuterostomes. (**A**) In hemichordates, the phyaryngeal endoderm (yellow) is the tissue layer responsible for secretion of the acelluar cartilage (green) in between the gill slits. (**B**) The situation is similar in lancelets as in A, except the pharyngeal epithelium has an ectodermal component on the atrial side and a mesodermal component in the gill bar coelom that is involved in matrix section in addition to the pharyngeal endoderm. (**C**) The hypothetical intermediate is an animal that also has endodermal (yellow), ectodermal (blue), and mesodermal (red) components to matrix secretion like lancelets, except this animal also has migratory neural crest cells (blue circles) invading the pharynx that become influenced by the extracellular matrix (green). (**D**) The current vertebrate situation of pharyngeal cartilage formation, where the migratory neural crest cells (blue circles) themselves secrete the extracellular matrix that forms the cartilages. The mesoderm (red) in the pharyngeal pouch does not participate in pharyngeal cartilage formation, but does form musculature of the pharynx.

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