

Expression Pattern of Two Collagen Type 2 $\alpha 1$ Genes in the Japanese Inshore Hagfish (*Eptatretus burgeri*) With Special Reference to the Evolution of Cartilaginous Tissue

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ABSTRACT Collagen type 2 $\alpha 1$ (Col2A1) protein is a major component of the cartilaginous extracellular matrix (ECM) in vertebrates. Over the past two decades, the evolutionary origin of Col2A1 has been studied at the biochemical and molecular levels in extant jawless vertebrates (hagfishes and lampreys). Although these studies have contributed to our understanding of ECM protein evolution, the expression profile of the *Col2A1* gene in hagfishes has not been fully described. We have performed molecular cloning and analyzed the gene expression pattern of the *Col2A1* gene in the Japanese inshore hagfish (*Eptatretus burgeri*). We succeeded in isolating two *Col2A1* genes, *EbCol2A1A* and *EbCol2A1B*, in which *EbCol2A1A* was expressed in the noncartilaginous connective tissues whereas *EbCol2A1B* was detected in some cartilaginous elements. Based on these results, we discuss the evolutionary history of *Col2A1* genes in early vertebrates. *J. Exp. Zool. (Mol. Dev. Evol.)* 312B, 2009. © 2009 Wiley-Liss, Inc.

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Vertebrates are characterized by highly organized skeletal elements that contain a number of different types of extracellular matrix (ECM) proteins, including proteoglycans and fibrillar collagens (Iozzo, '98; Watanabe et al., '98; Knudson and Knudson, 2001; Kiani et al., 2002; Boot-Handford et al., 2003a,b; Hall, 2005; Cohen, 2006). Among these ECM proteins, collagen type 2 $\alpha 1$ (Col2A1), which belongs to the clade A fibrillar collagen, has been studied from an evolutionary aspect because of the conservation of its expression pattern among vertebrate species, especially in cartilaginous tissues, and the absence of the corresponding protein in invertebrate species (Aouacheria et al., 2004, 2006; Wada et al., 2006; Zhang and Cohn, 2006; Zhang et al., 2006; Eames et al., 2007; Meulemans and Bronner-Fraser, 2007). These observations suggested that the *Col2A1* gene represents an evolutionary innovation in the vertebrate lineage. This idea

has prompted examination of the expression profile of the *Col2A1* gene, which has been compared with various vertebrate species and their relatives (Wright et al., '84, '88, 2001; Robson et al., '93, 2000; Yan et al., '95; Aouacheria et al., 2004, 2006; Wada et al., 2006; Zhang and Cohn, 2006; Zhang et al., 2006; Eames et al., 2007; Meulemans and Bronner-Fraser, 2007; Rychel and Swalla, 2007; Ohtani et al., 2008). Of these animal species, the living jawless vertebrates (agnathans),

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whose divergence from the jawed vertebrates (gnathostomes) is estimated to have taken place 500 million years ago, have been recognized as the most crucial animals for understanding the origin of the *Col2A1* gene (Wright et al., '84, '88, 2001; Robson et al., '93, 2000; Kuraku and Kuratani, 2006; Zhang and Cohn, 2006; Zhang et al., 2006; Kuraku et al., 2009; see also Ota and Kuratani, 2009).

Hagfishes are one of the only two extant agnathans and are clustered with lampreys (the other agnathan) into the group cyclostomes; this group has been placed as the sister group of the gnathostomes by molecular phylogenetic analysis (Stock and Whitt, '92; Mallatt and Sullivan, '98; Kuraku et al., '99; Delarbre et al., 2001, 2002; Furlong and Holland, 2002; Takezaki et al., 2003; Kuraku and Kuratani, 2006). Although the monophyly of cyclostomes has now been confirmed by a large amount of molecular data (Furlong and Holland, 2002; Takezaki et al., 2003; Kuraku and Kuratani, 2006, 2009, 2009; Kuraku, 2008), hagfishes have been and remain placed basal to all the vertebrates in the phylogenetic trees reconstructed from morphological characteristics (Løvtrup, '77; Forey and Janvier, '93; Shu et al., 2003; Gess et al., 2006; see also Janvier, '96, 2007). This discrepancy between the molecular and morphological data is interpreted to reflect the extremely degenerated morphology of the hagfishes reported by modern researchers who are familiar with molecular evolutionary studies and is now being examined from the approach of evolutionary developmental studies (Kuraku and Kuratani, 2006; Ota et al., 2007; Kurtani and Ota, 2008a,b; see also Holland, 2007). We also accept the monophyletic relationship between hagfishes and lampreys, the so-called cyclostome theory (Ota and Kuratani, 2006, 2008). However, the apparent primitive morphology of the hagfishes is still a matter of debate among modern scientists, as historically this topic attracted the attention of early zoologists and morphologists (see Janvier, '96, 2007).

The skeletal morphology of the hagfish was investigated intensively from the middle 19th to early 20th centuries by a few researchers (Müller, 1839; Parker, 1883; Studnicka, 1896; Schaffer, 1897; Ayers and Jackson, '00; Cole, '05). These early researchers also studied the histology of the skeletal elements. For example, Müller (1839) first described the detailed anatomy of this animal (Fig. 1A) and reported a variety of cartilaginous elements in the Atlantic hagfish (*Myxine glutinosa*). Müller distinguished two types of cartilages

(“yellowish” and “gray”) in the animal, and his observations were confirmed by subsequent researchers (Parker, 1883; Studnicka, 1896; Schaffer, 1897; Cole, '05; see details in Fig. 1). The most recent to report on the hagfish, Cole ('05) reviewed the reports by earlier researchers and made detailed histological observations of *M. glutinosa*. Based on the staining characteristics and respecting the nomenclature provided by Parker (1883), he classified hagfish cartilage into “soft cartilage” and “hard cartilage” with fine illustrations (Fig. 1D, E). These comprehensive illustrations have proven to be invaluable in a number of evolutionary studies of the vertebrate skeletal elements; the biochemical and molecular studies of hagfish cartilages conducted during the past two decades are based on Cole's descriptions (Wright et al., '84, '88; Robson et al., '93, 2000; Cole, '05; Zhang and Cohn, 2006).

Biochemical studies have shown that the major component of the cartilages differs between cyclostomes and gnathostomes: cartilages of the cyclostomes are resistant to solubilization with cyanogen bromide, and the major component of lamprey and hagfish cartilage are encoded by unique genes (“lamprin” and “myxinin,” respectively) (Wright et al., '84, '88, 2001; Robson et al., '93, 2000). These observations led to the hypothesis that the evolution of the collagenous cartilages emerged in the common ancestor of the gnathostomes rather than the ancestor of all vertebrates (Wright et al., 2001; Hall, 2005).

Recent molecular studies, however, have cast doubt on the validity of this hypothesis. Zhang and Cohn isolated two *Col2A1* genes from the sea lamprey (*Petromyzon marinus*) and a single *Col2A1* gene from the Atlantic hagfish (*M. glutinosa*). This suggests that a collagenous skeleton is present in the common ancestor of all vertebrates (Zhang and Cohn, 2006; Zhang et al., 2006). However, these two works raise two further questions. First, whether hagfishes have multiple *Col2A1* genes remains to be elucidated. A recent phylogenetic analysis of *Col2A1* genes implies the occurrence of gene duplication of the *Col2A1* gene in the common ancestor of cyclostomes (Zhang and Cohn, 2006). This also implies that there may still be uncloned genes in hagfish species. The second question relates to the distribution pattern of *Col2A1* protein and the expression of the isolated *Col2A1* gene in the Atlantic hagfish. Immunohistochemical analysis reported by the authors showed the cross-hybridization of an antibody against human *Col2A1* to the soft cartilages of hagfishes, demonstrating that

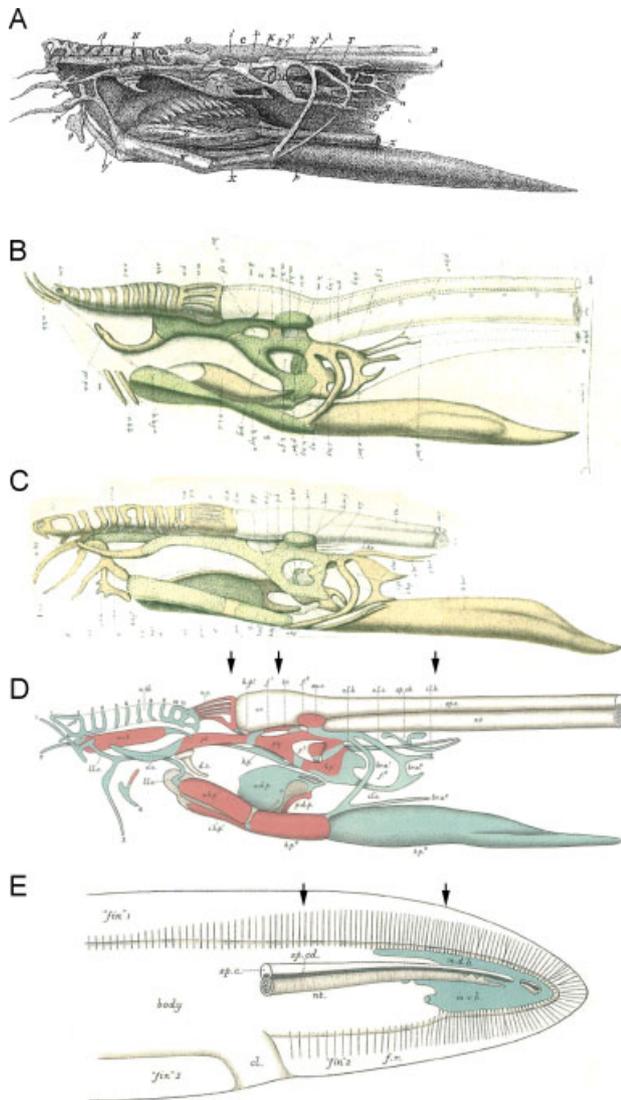


Fig. 1. Illustrations by researchers from the middle 19th to early 20th centuries. The first detailed illustrations were made by Müller (1839) (A). Parker (1883) drew the skeletal elements of the Myxinoidea and Eptatidae species, and he used green and yellow to represent hard and soft cartilages, respectively (B, C). Parker's soft and hard cartilages correspond to Müller's yellowish and gray cartilages, respectively (see text). In Cole's figures ('05), hard, soft, pseudo-hard, and pseudo-soft cartilages were depicted by four color patterns: red, blue, blue with dots, and white with oblique lines, respectively (D, E). According to Cole ('05), differences between these cartilages are based on the staining characteristics of the matrix and the ratio of cells to ECM. The hard cartilage comprises more and the soft cartilage less of the ECM. The hard cartilage is large and the soft cartilage is less in the ECM. Exceptionally, the posterior segment of the basal plate and the thick pad at the cephalic end of the basal plate stain negatively with eosin and were categorized as pseudo-cartilages. The levels of the sections in our histological analysis and in situ hybridization are shown by arrows on Cole's figures (D, E).

Col2A1 protein is expressed in the soft cartilage. However, the expression pattern of *Col2A1* mRNA has not been observed directly (Zhang and Cohn, 2006), which raises the question whether the *Col2A1* gene isolated earlier from the Atlantic hagfish is expressed in the soft cartilages.

To address these two questions, we performed molecular cloning of the *Col2A1* gene, conventional histological analysis, and in situ hybridization in the Japanese inshore hagfish (*Eptatretus burgeri*), focusing on the expression pattern of the soft cartilages. We also discuss the evolutionary history of *Col2A1* genes and their expression pattern in the cyclostome lineage.

MATERIALS AND METHODS

Molecular cloning and identification of the isolated genes

Total RNA was extracted from the embryos of *E. burgeri* (embryo designation Eb060417-3; see Ota et al., 2007) using TRIZOL reagent (Invitrogen, Japan). Degenerate RT-PCR was performed to amplify fragments of cDNA encoding the *Col2A1* gene. The 5' and 3' ends were amplified with a GeneRacer kit (Invitrogen, Japan) and isolated using TOPO TA Cloning Kit Dual Promoter (Invitrogen, Japan). These amplified fragments were sequenced using an ABI 3130XL automated sequencer (Applied Biosystems, Japan). These sequence data were submitted to the DDBJ database (accession numbers AB051358 and AB501359). To identify the orthologous genes of the isolated fragments, comparable sequence data were surveyed from the NCBI protein database using a BLAST search and multiple sequence alignments were generated using the CLUSTALW multiple alignment program (Thompson et al., '94). The highly variable amino acid sites and motifs, which provide information to distinguish the *Col2A1* gene from the other clade A fibrillar collagen genes (*Col1A1*, *Col1A2*, *Col5A2*, and *Col3A1*), were searched from the multiple alignments. A part of the alignment is shown in Figure 2A.

To understand the evolutionary relationship among *Col2A1* genes, phylogenetic analysis was performed using three methods: the neighbor-joining (NJ), maximum likelihood (ML), and Bayesian inference (BP) methods. The alignment for the reconstruction of the trees has 415 amino acid sites, which contained 191 of the major triple helix regions and 224 C-terminal noncollagenous domains, and was derived from 18 vertebrate species (three amniotes, four amphibians, six

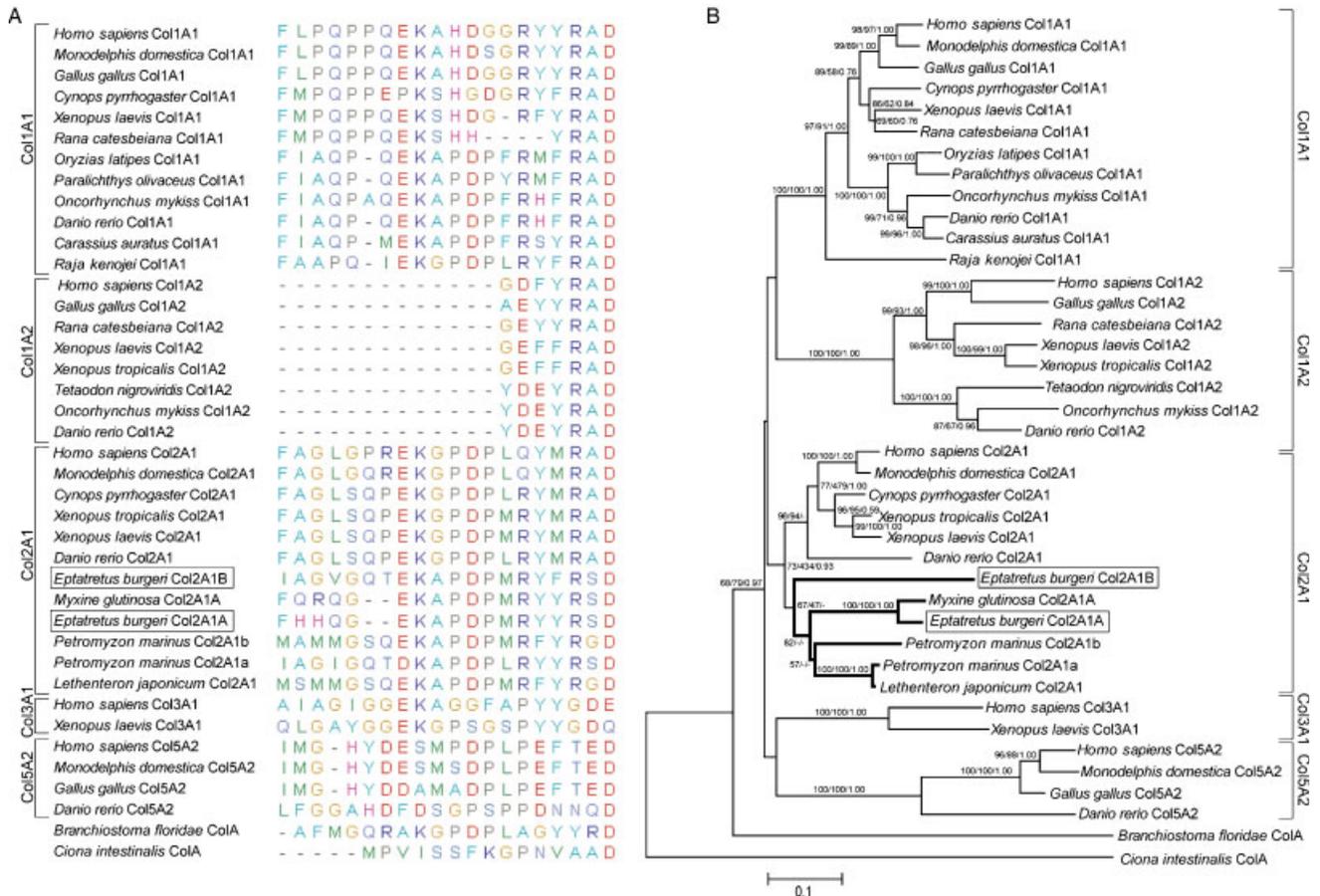


Fig. 2. Identification of the *EbCol2A1A* and *EbCol2A1B* genes. A part of the alignment of fibrillar collagen genes of vertebrate species shows that both *EbCol2A1A* and *EbCol2A1B* belong to *Col2A1* genes and that *EbCol2A1A* and *MgCol2A2* are related closely to each other (A). A phylogenetic tree among fibrillar collagen genes of representative vertebrates and their relatives was reconstructed by the NJ method (B). Numbers at each node indicate the bootstrap probability (NJ and ML) and posterior probabilities of BP (left, middle, and right numbers, respectively). The clade of the cyclostome *Col2A1* genes is indicated with bold lines. The *EbCol2A1A* and *EbCol2A1B* genes are boxed. The vertical order of the sequences in the multiple alignment (A) corresponds to that in the phylogenetic tree (B).

teleosts, one cartilaginous fish, two lampreys, and two hagfishes) and two invertebrate species (ascidian and tunicate) as outgroups. The NJ and ML trees were reconstructed using the 1,000-times bootstrap test; the NJ tree is shown in Figure 2B. JTT and WAG models were applied to reconstruct the NJ and ML trees, respectively. The BP analysis was based on two independent runs of two million generations with samples taken from every 100 generations; each run consisted of one cold and three heated chains. The NJ, ML, and BP analyses were performed using MEGA (Kumar et al., 2008), PhyML (Guindon and Gascuel, 2003), and MrBayes (Ronquist and Huelsenbeck, 2003), respectively. The following protein sequences were retrieved from the GenBank and JGI databases for our gene identification and phylogenetic analyses:

Branchiostoma floridae ColA, ABG36969; *Ciona intestinalis* ColA, ci0100150759; *Cynops pyrrhogaster* Col1A1, Q9YIB4.1; *C. pyrrhogaster* Col2A1, BAA82043.1; *Danio rerio* Col1A1, NP_954684.1; *D. rerio* Col1A2, NP_892013.2; *D. rerio* Col2A1, AAI67477.1; *D. rerio* Col5A2, CAD58730.1; *Gallus gallus* Col1A1, P02457.3; *G. gallus* Col1A2, NP_001073182.2; *G. gallus* Col5A2, XP_421846.2; *Homo sapiens* Col2A1, NP_000080.2; *H. sapiens* Col1A1, NP_000079.2; *H. sapiens* Col5A2, AAL13166.1; *H. sapiens* Col2A1, NP_001835.3; *H. sapiens* Col3A1, P02461.4; *H. sapiens* Col5A2, P20908.3; *Lethenteron japonicum*, BAG67025.1; *Monodelphis domestica* Col1A1, XP_001367735.1; *M. domestica* Col5A2, XP_001378478.1; *M. domestica* Col2A1, XP_001378056.1; *M. glutinosa* Col2A1, ABG36940.1; *Oncorhynchus mykiss* Col1A1, NP_001117649.1; *O. mykiss* Col1A1, BAA33380.1;

O. mykiss Col3A1, BAA33381.1; *O. mykiss* Col1A2, O93484.2; *Oryzias latipes* Col1A1, NP_001116390.1; *Paralichthys olivaceus* Col1A1, BAD77968.1; *Petromyzon marinus* Col2A1b, ABB53638.2; *Petromyzon marinus* Col2A1a, ABB53637.2; *Raja kenoei* Col1A1, BAD98524.1; *Rana catesbeiana* Col1A1, BAA29028.1; *R. catesbeiana* Col1A2, O42350.1; *Tetraodon nigroviridis* Col1A2, CAG11117.1; *Xenopus tropicalis* Col2A1, NP_989220.1; *X. tropicalis* Col1A2, NP_001072718.1; *Xenopus laevis* Col1A1, NP_001080821.1; *X. laevis* Col2A1b, AAH44962.1; *X. laevis* Col2A1, NP_001080727.1.

Conventional histological analysis and in situ hybridization

Juvenile hagfish specimens (total length less than 15 cm) were anesthetized by MS-222, fixed by immersion in Serra's fixative, processed for paraffin sectioning by standard methods, sliced at 6–8 μ m, and stained with Alcian blue, hematoxylin, and eosin. The cartilaginous elements were categorized according to Cole's description ('05). The hard cartilages contain a large amount of ECM and are hematoxylin negative, whereas the soft cartilages have less ECM and are hematoxylin positive. The probes for the in situ hybridization were prepared from the PCR fragments of the 5' and 3' ends of the isolated *Col2A1* genes. These fragments were labeled using a DIG labeling kit (Roche, Japan). In situ hybridization was performed in a Ventana automated machine (Ventana Medical Systems, Inc., Japan). To detect the signals and counterstaining, a BlueMap NBT/BCIP substrate kit and a nuclear Fast Red equivalent reagent ISH RED were applied (Ventana Medical Systems, Inc.). Several pilot experiments of the in situ hybridization were performed to select the appropriate DIG-labeled probes. The gene expression patterns of the *Col2A1* genes were observed in the sectioned tissues at the levels shown in Figure 1D and 1E. Cartilaginous elements were designated mainly using Cole's nomenclature (Cole, '05; see also Brodal and Fänge, '63; Jørgensen et al., '98) (Fig. 3). The list of abbreviations is as follows:

<i>adp</i>	anterior dental plate cartilages
<i>bp1</i>	anterior segment of the basal plate
<i>chc</i>	chordal cells forming the notochordal "jelly"
<i>chep</i>	chordal epithelium
<i>dep</i>	epithelial cells
<i>diep</i>	irregular epithelial cells
<i>dhc</i>	horn cap
<i>dm</i>	dermis
<i>dp</i>	dental plate

<i>edm</i>	epidermis
<i>elb</i>	external lateral velar bar
<i>fr</i>	Fin ray
<i>ht</i>	horn teeth
<i>ilb</i>	internal lateral velar bar
<i>mdb</i>	median dorsal bar of the caudal fin
<i>mvb</i>	median ventral bar of the caudal fin
<i>nc</i>	nasal cartilages
<i>nt</i>	notochord
<i>ntsh</i>	notochordal sheath
<i>olfep</i>	olfactory epithelium
<i>olff</i>	olfactory fold
<i>olfnb</i>	olfactory nerve bundles
<i>orm</i>	oral mucosa
<i>orca</i>	oral cavity
<i>pb</i>	"palatine" bar cartilage
<i>pc</i>	perichondrium
<i>phep</i>	pharyngeal epithelial cells
<i>pokc</i>	pokal cell cone
<i>sdm</i>	subdermis
<i>spc</i>	membranous spinal cord
<i>vel.scr</i>	velar scroll
:	

RESULTS

Identification of two *Col2A1* genes in hagfish

We obtained two different *Col2A1* genes from *E. burgeri* by RT-PCR and sequencing, which we designated as *EbCol2A1A* and *EbCol2A1B*. We recovered cDNA fragments of 1,808 bp (*EbCol2A1A*) and 4,560 bp (*EbCol2A1B*), which contained the region between the major triple helix and the C-propeptide domains. *EbCol2A1A* was 93.1% identical to the amino acid and 98.8% to the DNA sequences of *M. glutinosa Col2A1* (*MgCol2A1*), suggesting that *EbCol2A1A* is the ortholog of *MgCol2A1A* (Zhang et al., 2006). The *EbCol2A1B* gene was categorized as a *Col2A1* gene because of the conserved peptide motifs in the C-terminal region, as shown in Figure 2A, although no orthologous gene was found following databases searches.

To resolve the position of these two *EbCol2A1* genes in the phylogenetic tree of the fibrillar collagen genes, we performed NJ, ML, and BP analysis. The *EbCol2A1A* gene clustered with the *MgCol2A1* gene in the clade of the *Col2A1* genes of the cyclostomes, and this clustering was highly significant in all reconstructed phylogenetic trees. Although the *EbCol2A1B* gene was located on the basal position of the clade of the *Col2A1* genes of the cyclostomes in the NJ and ML trees, the bootstrap value at the node was not high (Fig. 2B). In addition, the clade of *EbCol2A1B*, the other *Col2A1* of cyclostomes, and all of the *Col2A1* of

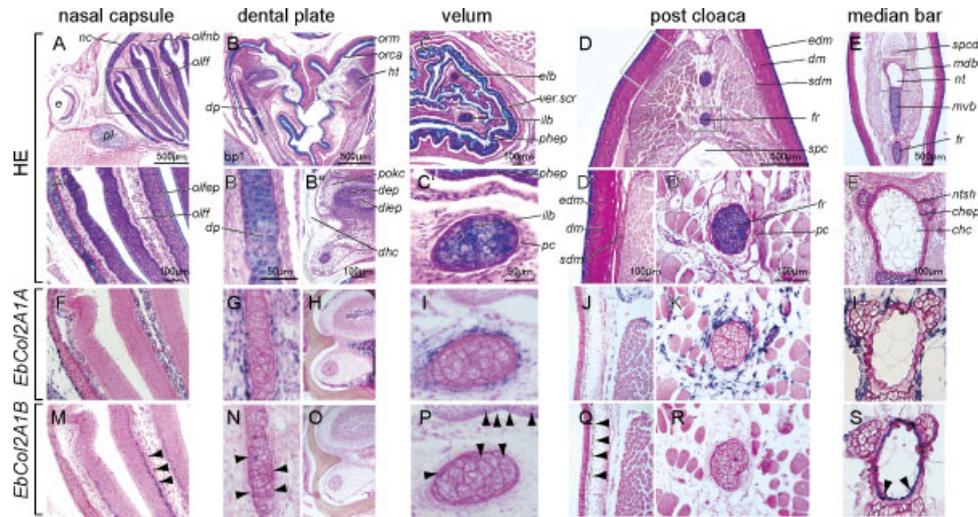


Fig. 3. Histological analysis and expression patterns of *EbCol2A1A* and *EbCol2A1B*. Transverse sections of an adult specimen of *E. burgeri* at the levels of the nasal capsule (A, A', F, M), dental plate (B, B', B', H, H, N, O), velum (C, C', L, P), postcloaca (D, D', D', J, K, Q, R), and median bar (E, E', L, S). The upper two rows show sections stained with eosin, hematoxylin, and Alcian blue (A–E'). The boxed areas in (A)–(E) are enlarged in (A')–(E'). The lower two rows show the sections hybridized with the DIG-labeled probe of *EbCol2A1A* (F–L) and *EbCol2A1B* (M–S). Arrowheads indicate the cells expressing *EbCol2A1B*.

gnathostomes formed a trichotomy in the BP analysis. The resolution of the phylogenetic position of *EbCol2A1B* was not improved even after different multiple alignments were applied (data not shown).

We also note that the phylogenetic relationship between *Col2A1b* of *P. marinus* and the other genes was not resolved with high accuracy. These results seem to reflect the scarcity of effective phylogenetic signals in sequences and the closely related timing of the duplication in this lineage, as reported earlier (Morvan-Dubois et al., 2003; Aouacheria et al., 2004; Wada et al., 2006; Zhang and Cohn, 2006; Zhang et al., 2006; note the inconsistencies between phylogenetic trees in these reports).

Histological observations and expression patterns of the two *Col2A1* genes

To elucidate the expression pattern of the *EbCol2A1A* and *EbCol2A1B* genes in the cartilages and other connective tissues, we performed in situ hybridization on paraffin sections at five transverse levels: olfactory bulb, dental plate, velum, postcloacal, and median bar regions (see Fig. 1D and 1E, and Fig. 3). Based on the staining characteristics defined by Cole ('05), *pb* and *bp1* are categorized into hard cartilages; and *nc*, *adp*, *elb*, *fr*, *mdb*, and *mvb* are identified as soft cartilages. Except for *nc*, these histological observations correspond to Cole's descriptions.

Although *nc* was noted as a hard cartilage by Cole ('05), this cartilage was categorized as soft cartilage in our analysis because the nucleus was stained with hematoxylin and the ECM was stained less (Fig. 3A). Because of the fragile nature of *pb* and *bp1*, the gene expression pattern of these cartilages could not be analyzed. We succeeded in observing the expression pattern in four soft cartilages (*nc*, *adp*, *elb*, and *fr*), notochord (*nt*), three cells in the horn teeth (*pokc*, *dep*, and *diep*), and the other connective tissues surrounding the cartilages and muscles (Fig. 3A–E').

The expression pattern of the *EbCol2A1A* gene can be characterized by the broad expression in the noncartilaginous connective tissues. This gene was expressed in the connective tissues surrounding cartilages and in the notochordal sheath (Fig. 3F–L). In addition, strong signals were detected in the connective tissues in the olfactory bulb and horn teeth (Fig. 3F, H). Although we repeated the experimental procedure after changing the length of the probes and the concentration, no convincing expression of the gene was observed in either soft or hard cartilages.

EbCol2A1B is distinguished from *EbCol2A1A* in the expression patterns observed. The expression of *EbCol2A1B* was limited to the notochord, many epithelial cells, and cartilaginous tissues. Three different layers of notochord were distinguished by our histological observation: *ntsh*, *chep*, and *chc*. Among these notochordal tissues, *chep*, which

is surrounded by *ntsh*, strongly expressed this gene (Fig. 3S). The epithelial cells in the olfactory bulb, pharyngeal epithelial, and epidermal cells also expressed this gene (Fig. 3M, P, Q). Although the signals were faint, the expression of this gene was detected in a number of cells in two soft cartilaginous elements (*dp* and *ilb*). The signals of the *EbCol2A1B* gene in the *dp* and *ilb* were observed in repeated experiments. In the other cartilages (*nc*, *fr*, *mdb*, and *mub*), specific signals of the gene were not detected.

These expression patterns suggested that EbCol2A1A protein acts as the major component of the ECM proteins in the noncartilaginous connective tissues rather than in the skeletal cartilaginous tissues. In contrast, EbCol2A1B protein is present in the ECM of several soft cartilaginous tissues. No tissues expressing both *EbCol2A1A* and *EbCol2A1B* genes were detected, providing evidence of the exclusive expression pattern of each of these two genes.

DISCUSSION

In this study, two different *Col2A1* genes were obtained from the Japanese inshore hagfish, *E. burgeri*. One was the ortholog to the previously reported *MgCol2A1* gene from the Atlantic hagfish (Zhang et al., 2006) and the other was a newly cloned gene that has not been reported. These results seem to answer our initial question about the presence of multiple numbers of *Col2A1* genes in the hagfishes and imply the independent evolution of the *Col2A1* genes in the lineage of the cyclostomes or hagfishes. Moreover, the analysis of the expression patterns indicated that these hagfish *Col2A1* genes have roles that differ from those involved in formation of the skeletal elements in the hagfish.

Cyclostome lineage-specific divergence of the Col2A1 genes

Although the visual inspection in the multiple alignment showed that the *EbCol2A1A* gene is the ortholog of *MgCol2A1* and that *EbCol2A1B* is a *Col2A1* gene, the phylogenetic position of *EbCol2A1B* has not been resolved. All reconstructed trees (NJ, ML, and BP trees) showed a naked and isolated long branch of *EbCol2A1B* gene with a low bootstrap value (Fig. 2B). This naked, long branch implies that *EbCol2A1B* is not a recently duplicated gene from the other *Col2A1* genes of cyclostomes, including two *Col2A1* genes of lampreys, *MgCol2A1* and *EbCol2A1A*. To explain

the naked long branch of *EbCol2A1B* located on the basal position of the cyclostome clade, as shown in Figure 2B, it must be assumed that the gene duplication occurred at least before the divergence between hagfishes and lampreys.

In relation to the gene duplication, it is worth considering the genome organization of the genes linked with *Col2A1* in the vertebrates. It is known that the *Hox* and *Dlx* cluster genes are linked together with five fibrillar collagen genes (*Col1A1*, *Col1A2*, *Col2A1*, *Col3A1*, and *Col5A2*) in the vertebrates. These linked genes, so-called “core Hox paralogon” seem to be derived from large-scale gene duplications (Morvan-Dubois et al., 2003; Zhang and Cohn, 2008; Lynch and Wagner, 2009). Because of the lack of information about the loci of these Hox paralogon genes in the cyclostomes, it is uncertain whether the multiple *Col2A1* genes were derived from tandem or nontandem gene duplications. In the case of the latter, the question remains whether the *Hox* and *Dlx* cluster genes were also duplicated by the cyclostome-specific gene duplication, as proposed earlier (Fried et al., 2003; see also Kuraku, 2008; Kuraku et al., 2009). To address this question, genomic approaches will be required to study the hagfishes and lampreys.

The expression pattern of these Col2A1 genes of hagfish

Our gene expression analysis demonstrated that the *EbCol2A1A* gene was expressed in some noncartilaginous connective tissues, whereas the *EbCol2A1B* gene was detected in two soft cartilages (Fig. 3). These two genes were observed in different tissues exclusively in our analyses. This nonoverlapping expression pattern seems to be explained by the subfunctionalization of *Col2A1* genes. Because of the difficulty in reconstructing the phylogenetic relationship between *Col2A1* genes in cyclostomes, the evolutionary process of the subfunctionalization of the *EbCol2A1* genes is still uncertain. However, the totally different expression patterns of these two genes seem to reflect the divergence of the amino acid sequences.

Although the immunohistochemical analysis conducted by Zhang and Cohn (2006) showed that a human antibody against the highly specific N-terminal regions of *Col2A1* cross-hybridized specifically with the hagfish soft cartilages, this result is inconsistent with our analysis of the gene expression patterns. Our analysis demonstrated that the *EbCol2A1A* gene is strongly expressed in

the noncartilaginous connective tissues surrounding cartilage. Consequently, it is reasonable to assume that a great amount of EbCol2A1A protein is also translated and should be detectable using immunohistochemical techniques. However, the earlier study (Zhang and Cohn, 2006) found no significant antibody signals in the noncartilaginous connective tissues of the Atlantic hagfish.

This inconsistency can be explained by the following possibilities: (i) the antibody to human Col2A1 specifically recognizes EbCol2A1B in the soft cartilage; (ii) the antibody has high affinity with an unknown Col2A1 protein expressed only in the soft cartilage; or (iii) the antibody did not work as a specific marker of the Col2A1 protein in the hagfish. The first option does not fit with our observed expression pattern of the *EbCol2A1B* gene; the antibody against human Col2A1 labeled the tail cartilage, but the expression of the *EbCol2A1B* gene was not observed in the tail cartilage, including *fr*, *mdb*, and *mub*, in our experiments. Moreover, if one assumes that the antibody against human Col2A1 has a high affinity for Col2A1 proteins of various vertebrate species ranging from humans to cyclostomes, as postulated by Zhang and others (Zhang and Cohn, 2006; Zhang et al., 2006), it is improbable that the antibody has specific affinity for any hagfish Col2A1 protein without cross-hybridization; this suggests that the first two assumptions are unrealistic. Because of the absence of information on the specificity of the human antibody against each Col2A1 protein of the hagfish, we cannot reject the first two assumptions completely. However, the ambiguous signals of the immunohistochemical analysis provided by the authors tend to support the third assumption (Zhang and Cohn, 2006).

The heterogeneous expression pattern of the *EbCol2A1B* gene also has implications for the developmental background of the hagfish cartilages. In our study, the expression of *EbCol2A1B* was observed in some cranial cartilages but not in the others. Considering that the cartilages of gnathostomes can be divided into two groups based on their developmental origin (mesoderm- and neural crest-derived cartilages), it is possible that the soft cartilages of the hagfishes can also be categorized similarly into two cartilages (Couly et al., '93; Christ et al., 2000; McCauley and Bronner-Fraser, 2006). Two branchial skeletons of hagfishes (*elb* and *ilb*), which are expected to be derived from neural crest cells, express the *EbCol2A1B* gene, whereas the caudal fin (e.g.,

mdb and *mub*), which seems to develop from the paraxial mesoderm, are negative for the expression of the gene (Kuratani and Ota, 2008a; Ota and Kuratani, 2008). This circumstantial evidence has led us to propose that the neural crest-derived soft cartilages are positive and the paraxial mesoderm-derived cartilages are negative for the expression of the *EbCol2A1B* gene. However, we are cautious in proposing this because of the paucity of detailed investigations of chondrogenesis in the hagfish embryo. Further developmental studies in the hagfish are awaited.

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