Late Development of Hagfish Vertebral Elements

KINYA G. OTA1*, SATOKO FUJIMOTO2, YASUHIRO OISI2,3, AND SHIGERU KURATANI2,3

1Laboratory of Aquatic Zoology, Marine Research Station, Institute of Cellular and Organismic Biology, Academia Sinica, Yilan, Taiwan
2Laboratory for Evolutionary Morphology, RIKEN Center for Developmental Biology, Kobe, Japan
3Department of Biology, Graduate School of Science, Kobe University, Kobe, Japan

ABSTRACT

It has been demonstrated recently that hagfishes, one of two groups of extant jawless vertebrates, have cartilaginous vertebral elements. Embryological and gene expression analyses have also shown that this group of animals develops a sclerotome, the potential primordium of the axial skeleton. However, it has not been shown unequivocally that the hagfish sclerotome truly differentiates into cartilage, because access to late-stage embryos and information about the cartilaginous extracellular matrix (ECM) are lacking for these animals. Here we investigated the expression patterns of the biglycan/decorin (BGN/DCN) gene in the inshore hagfish, Eptatretus burgeri. The homologue of this gene encodes the major noncollagenous component of the cartilaginous ECM among gnathostomes. We clearly identified the expression of this gene in adult vertebral tissues and in embryonic mesenchymal cells on the ventral aspect of the notochord. Taking into account that the sclerotome in the gnathostomes expresses BGN/DCN gene during the chondrogenesis, it is highly expected the hagfish BGN/DCN-positive mesenchymal cells are derived from the sclerotomes. We propose that hagfishes and gnathostomes share conserved developmental mechanisms not only in their somite differentiation, but also in chondrogenesis of their vertebral elements. J. Exp. Zool. (Mol. Dev. Evol.) 320B:129–139, 2013. © 2013 Wiley Periodicals, Inc.

How to cite this article: Ota KG, Fujimoto S, Oisi Y, Kuratani S. 2013. Late development of hagfish vertebral elements. J. Exp. Zool. (Mol. Dev. Evol.) 320B:129–139.

Vertebral elements, namely cartilaginous or bony segmental nodules associated with the notochord, represent one of the crucial morphological characteristics that define the vertebrates (Janvier, ’96; Liem et al., 2001; Kardong, 2011). The status of this feature has been a central issue in the evolutionary origin of the vertebrates (Goodrich, ’30; Lovtrup, ’77; Forey and Janvier, ’93; Janvier, ’96). The hagfishes constitute one of two groups of extant jawless vertebrates (cyclostomes), which until recently had been believed to lack vertebral elements (Cole, ’05; Forey and Janvier, ’93; Liem et al., 2001; Kardong, 2011) (Fig. 1A). Although the monophyly of the cyclostomes is supported by molecular data from various sources (Stock and Whitt, ’92; Mallatt and Sullivan, ’98; Kuraku et al., ’99; Takezaki et al., 2003; Heimberg et al., 2010), the hagfishes are still placed basal to the other vertebrates in some textbooks, in which the absence of vertebral elements in the hagfishes is considered plesiomorphic, rather than the derived condition (Kardong, 2011; Liem et al., 2001).

According to the classical schema presented by Gadow (1895, ’33), the gnathostome vertebra consists of two dorsal and two ventral elements (see also Goodrich, ’30; Janvier, ’96). Of these, only the dorsal elements are present in the lamprey...
(Tretjakoff, ’26), but none of them were known in the hagfish species, although a few early researchers investigated their skeletal elements (Müller, 1834; Parker, 1883; Cole, ’05; Robson et al., 2000; Wright et al., 2001; see also Ota and Kuratani, 2010). An exceptional description was given by Ayers and Jackson (’00), who showed several anteroposteriorly arranged cartilaginous nodules on the ventral aspect of the caudal notochord of *Bdellostoma* more than a century ago (Fig. 1B). These cartilaginous structures had not been reexamined until our previous report (Ota et al., 2011).

In 2011, we reported that the Japanese inshore hagfish, *Eptatretus burgeri*, has vertebral elements (Ota et al., 2011) (Fig. 1C). These elements arise as small cartilaginous nodules on the ventral aspect of the notochord, reminiscent of the ventral elements of the gnathostomes (Gadow, ’33; Janvier, ’96). Our histological observations on embryos also suggested that the ventromedial part of the hagfish somite is transformed into mesenchyme that resembles the gnathostome sclerotome, the primordium of the vertebra (Christ et al., 2000; Ota et al., 2011). We also showed that the putative sclerotome in the hagfish expresses the *Twist* and *Pax1/9* genes encoding transcription factors involved in the differentiation of the sclerotome in the early and late pharyngular stages, respectively. These findings indicate that these cartilages are developmentally homologous with the vertebral elements of the gnathostomes. Thus, our observations

![Figure 1. Caudal skeletons of three hagfish species. (A) Illustration of the caudal skeleton of *M. glutinosa* by Cole (’05). Median dorsal and ventral bars are indicated in blue. A single isolated cartilaginous nodule is identified (white arrowhead). (B) Illustration of the caudal skeleton of *Bdellostoma* species by Ayers and Jackson (’00). Cartilaginous nodules are identified on the ventral aspect of the notochord (black arrowheads). (C) Lateral view of the Alcian blue-stained caudal region of *E. burgeri*. At the postcloacal level, small cartilaginous nodules are located on the ventral side of the notochord (black arrowheads). In all these species, the median dorsal bar is attached to the notochord by the intermediate cartilaginous nodule (red arrowhead). Abbreviations: cl, cloaca; dfr, dorsal fin radials; mdb, median dorsal bar; mu, mucus gland; mvd, median ventral bar; nt, notochord; vfr, ventral fin radials. Scale bar, 1 mm.](image-url)
suggest that the molecular mechanisms underlying early vertebral development are conserved between the hagfish and gnathostomes and that their evolutionary origins must date back 500 million years (Ota et al., 2011). However, some questions remain to be resolved: whether these putative sclerotomal cells truly form vertebral cartilage and whether their later developmental processes are also conserved between the gnathostomes and hagfishes. To answer these questions, it is necessary to study the late embryonic expression patterns of the genes encoding for the hagfish cartilaginous extracellular matrix (ECM).

The Col2A1 genes encode the major representative protein components of the cartilaginous ECM. The genes have been thoroughly investigated and strong expression patterns in cartilaginous tissues and their primordia in the gnathostomes and lampreys have been reported (Zhang et al., 2006; see also Ota and Kuratani, 2009). However, none of the investigated hagfish Col2A1 genes shows strong expression patterns in the cartilaginous tissues (Zhang and Cohn 2006; Ota and Kuratani, 2010), as observed in the lampreys and gnathostomes (Zhang et al., 2006; Ota and Kuratani, 2009). In addition, the noncollagenous cartilaginous ECM proteins are largely unknown in the hagfish. These data suggest that none of the known cartilaginous ECM protein-encoding genes are expressed at high levels in the hagfish; consequently, there are no available marker genes with which to investigate the processes of chondrogenesis in the hagfish vertebral elements.

Recent progress in our study of the developmental biology of the hagfishes has provided us with the sequence of the hagfish cartilaginous ECM gene. An embryonic cDNA library has been constructed, an expressed sequence tag (EST) project has been completed and the EST sequences are now freely available from a database (Takechi et al., 2011). Because this cDNA library was derived from a late-stage hagfish embryo, it is expected that candidate ECM genes that are expressed in the hagfish vertebral cartilaginous tissues will be found in this database. Moreover, we have successfully incubated a hagfish embryo for more than 200 days after egg deposition (Ota et al., 2011). This prolonged incubation has provided the prehatching stage of the hagfish embryo, which allowed us to investigate how the hagfish ECM genes are expressed during chondrogenesis in the embryonic vertebral tissues (Fig. 2).

In this study, we screened the EST database and found a candidate gene encoding the major protein component of the hagfish cartilaginous ECM, biglycan/decorin (BGN/DCN). The BGN and DCN genes are known members of the class I small leucine-rich proteoglycan (SLRP) gene family and are expressed in the cartilaginous tissues of gnathostomes (Schaefer and

![Figure 2. Prehatching stage of the E. burgeri embryo. (A) Ventral view of the 242-dpd embryo of the hagfish. The stage of this embryo is equivalent to that in Dean’s figure 58 (Dean, 1899). (B) Lateral view of excised and cleared embryo. (C) Lateral view of the head (right side is anterior). (D) Higher magnification of the caudal area in (B). Abbreviations: dfr, dorsal fin radials; dpd, day postdeposition; mvb, median ventral bar; mu, mucus gland; op, opercular ring; te, tentacle; vfr, ventral fin radials; yo, yolk. Scale bars, 1 mm (A); 5 mm (B); 500 μm (C,D).](image-url)
The Eb_eW_009_K05 sequence was resequenced between the and FY414270, respectively. To increase the number of informa-
biglycan (Eb_eW_009_K05). The NCBI accession numbers for
three matching genes that encode epiglycan (Eb_eW_004_E04,
ESTs, four sequences showed similarity to proteoglycan genes:
Cartilaginous, muscular and other morphological elements
are mainly reported using Cole’s nomenclature (Ayers and
Jackson, ’00; Cole, ’05; see also Brodal and Fänge, ’63; Jørgensen
et al., ’98).

RESULTS
Prehatching Hagfish Embryos
Among the 42 fertilized eggs obtained in our previous work (Ota et al., 2011), one was incubated successfully for 242 days after its
deposition (Fig. 2A). The blood vessels and embryonic structures
were visible through the egg membrane (Fig. 2A). The head of this
embryo was hanging over the yolk and the anterior quarter of the
embryo could be seen from the ventral aspect (Fig. 2A). Many
vitelline vessels were observed on the ventral side of the yolk
(Fig. 2A). This embryo was close to the stage illustrated in Dean’s
Figure 58 (Dean, 1899).
Figure 3. Phylogenetic tree of class I SLRP genes. Numbers at each node indicate the bootstrap (ML) and posterior probabilities (BI) (left and right numbers, respectively). The clades of the cyclostome class I SLRP genes are indicated with bold lines. The *EbBGN/DCN* gene is boxed. The vertical order of the sequences in the phylogenetic tree corresponds to that in the multiple-sequence alignment (Supplemental Fig. S1).
Under the microscope, tentacles were observed around the mouth (Fig. 2B, C) and the mucus glands and segmental structure of the myoseptum were recognizable in the trunk region (Fig. 2D). Caudally, the dorsal and ventral cartilaginous fin radials and the median ventral bar could be observed with transmitted light (Fig. 2D). Although the vertebral elements could not be identified in our observation of the whole embryo—based on adult morphology—we expected that the cartilaginous nodules of the vertebral elements should develop on the ventral aspect of the notochord, along the anteroposterior axis between the median ventral bar and the yolk (Figs. 1C and 2D). It is also expected that chondrocytes or mesenchymal cells expressing cartilaginous ECM proteins can be detected in the same part of the caudal region of this embryo.

**Hagfish BGN/DCN Genes**

Our survey of the hagfish EST database identified a clone (Eb_eW_009_K05) containing a class I SLRP gene. The insert of the clone comprised 1,176 base pairs of the coding region and the predicted amino acid sequence was 61.3% and 65.0% identical to two protein fragments encoded by SLRP genes of the lamprey (Supplementary Fig. S1); these two lamprey SLRP genes have been designated “biglycan-like genes 1 and 2” (Shintani et al., 2000). To determine the phylogenetic position of the hagfish class I SLRP gene, we constructed a molecular phylogenetic tree of class I SLRP genes, including ASPN, BGN, and DCN (Fig. 3).

Although the cyclostome SLRP genes showed affinity to the DCN genes of the gnathostomes (Fig. 3), we could not determine whether these cyclostome SLRP genes were orthologous to BGN or DCN. To improve the quality of the phylogenetic trees, we conducted molecular phylogenetic analyses with different datasets. However, these did not resolve the position of the clade containing the cyclostome SLRP genes. Therefore, we designated this newly isolated hagfish class I SLRP gene EbBGN/DCN and the SLRP genes of the lamprey PmBGN/DCN1 and PmBGN/DCN2 (Fig. 3).

In all our phylogenetic analyses, EbBGN/DCN was located basal to the PmBGN/DCN clade with high supporting value (Fig. 3). EbBGN/DCN did not cluster with PmBGN/DCN1 or PmBGN/DCN2 on any phylogenetic tree. According to a previous report (Shintani et al., 2000), duplication of BGN occurred independently in the lamprey lineage after the divergence of DCN and BGN. Our phylogenetic analysis suggested the same scenario, with higher probability. Furthermore, although these three cyclostome BGN/DCN genes show relatively long branches, the supporting values at their nodes are relatively high (Fig. 3). Moreover, the clade of the cyclostome genes is isolated from the other BGN and DCN genes of the gnathostomes. This probably reflects the long period since the divergence of the hagfishes, lampreys and gnathostomes (Kuraku and Kuratani, 2006; Kuraku et al., 2009).

**Expression Patterns of the EbBGN/DCN Genes in the Hagfish Vertebral Elements**

Although BGN and DCN are known to be the major protein components of the cartilaginous ECM in the gnathostomes (Schaefer and Iozzo, 2008; Kalamajski and Oldberg, 2010), it is still unclear whether the EbBGN/DCN proteins and their encoding mRNA are expressed in the hagfish vertebral cartilaginous tissues. Therefore, we investigated the expression patterns of the EbBGN/DCN gene in adult specimens (Fig. 4A–C).

First, we used conventional histology to analyze the postcloacal region of the adult specimens. In transverse views of HE- and Alcian blue-stained sections, five cartilaginous nodules were observed (Fig. 4A): one pair of elements attached to the ventral side of the notochord, another pair located on the lateral sides of the dorsal aorta and a single median element occupying a position between the dorsal aorta and posterior cardinal vein (Fig. 4A). These cartilaginous nodules were separated from each other by eosinophilic noncartilaginous connective tissues (Fig. 4B). At higher magnification, the chondrocytes in each nodule were seen to be surrounded by a thin Alcian blue-positive layer of ECM, indicating the presence of mucopolysaccharides (Fig. 4B). These results are consistent with anatomical observations of Alcian blue-stained whole-mount specimens of the adult hagfish (Fig. 1C).

We next conducted an in situ hybridization analysis of transverse sections at the same postcloacal level in adult specimens using an EbBGN/DCN riboprobe (Fig. 4C). We detected high levels of EbBGN/DCN transcripts inside the adult chondrocytes (Fig. 4C). Although subtle signals were also detected in the noncartilaginous connective tissues surrounding the cartilaginous nodules, the intensity of the signals clearly differed between the chondrocytes and these other connective tissues (Fig. 4C). Thus, the EbBGN/DCN protein is very probably one of the major ECM proteoglycans in the hagfish vertebral cartilage, providing a tool to investigate further the developmental processes of the hagfish vertebral elements in late embryos.

Histological sections of the prehatching-stage embryo were prepared to track back the development of the hagfish vertebral elements. Condensation of the mesenchymal cells of the dorsal and ventral fin radials was observed at the caudal level of the sections (Fig. 4D). Mesenchymal cells also occupied the ventral aspect of the notochord, surrounding the dorsal aorta and posterior cardinal vein (Fig. 4E). We also analyzed the expression pattern of the EbBGN/DCN gene at the same levels in prehatching embryos (Fig. 4F). The mesenchymal cells ventral to the notochord showed strong expression of the gene (Fig. 4F, G). The intensity of EbBGN/DCN expression in the mesenchyme around the dorsal aorta and posterior cardinal vein was also distinguishable from that in the other mesenchymal cells (Fig. 4G). Because the distribution of the EbBGN/DCN transcripts was consistent with that of the hagfish vertebral elements, these cells are very likely to differentiate into EbBGN/DCN-positive cartilage in the adult (Fig. 4C, F, G).
DISCUSSION

The results presented here strongly suggest that EbBGN/DCN is one of the major protein components of the ECM in the hagfish vertebrae, as in gnathostomes (Schaefer and Iozzo, 2008; Kalamajski and Oldberg, 2010). Our data also indicate that a number of embryonic mesenchymal cells on the ventral aspect of the notochord express the EbBGN/DCN gene strongly (Fig. 4F, G). In this study, we could not completely exclude the possibility that the hagfish vertebral elements are derived from other than sclerotomal cells. However, considering that the sclerotome in the gnathostomes produces a thick layer of cartilaginous ECM (Christ et al., 2000), it is reasonable to assume that the EbBGN/DCN-positive chondrocytes in the adult hagfish also derive from the EbBGN/DCN-positive mesenchyme in the late embryo (Fig. 4C, F, G). Based on this evidence, we will discuss the complete developmental processes of the hagfish vertebral elements (Fig. 5).

In previous studies, we have shown that the hagfish embryonic somite differentiates into three somitic derivatives: the dermomyotome, myotome, and sclerotome (Ota et al., 2007, 2011). These compartments are quite similar to those of the gnathostomes in...
their gene expression patterns (Christ et al., 2000; Buckingham and Vincent, 2009). In fact, strong expressions of Pax3/7 on the dorsal side, MyoD on the medial side and Pax1/9 and Twist on the ventromedial side of the somite have been detected (Ota et al., 2007, 2011). Considering the expression pattern of these genes, the developmental scenario of the hagfi et al., 2007, 2011). Considering the expression pattern of these genes, the developmental scenario of the hagfish vertebral elements can be summarized as follows. First, the ventral aspect of the notochord and express the cartilaginous extracellular matrix genes, including BGN/DCN. (C) The mesenchymal cells differentiate into the cartilaginous nodules, continuously expressing the BGN/DCN gene. Abbreviations: ao, dorsal aorta; m, myotome; n, neural tube; nt, notochord; som, somite.

Figure 5. Scheme of the development of the vertebral elements of the hagfish. (A) Medial ventral somites differentiate into mesenchymal cells (blue) in the early pharyngular embryo. These mesenchymal cells express the Twist and Pax1/9 genes. (B) A proportion of the mesenchymal cells migrate into the ventral aspect of the notochord and express the cartilaginous extracellular matrix genes, including BGN/DCN. (C) The mesenchymal cells differentiate into the cartilaginous nodules, continuously expressing the BGN/DCN gene. Abbreviations: ao, dorsal aorta; m, myotome; n, neural tube; nt, notochord; som, somite. processes of vertebral development are shared fundamentally by the hagfishes and gnathostomes.

Except in the early phase of development, the secreted ECM proteins in the vertebral tissues differ between the hagfishes and the other vertebrates. As mentioned above, strong expression patterns of Col2A1 are observed in the cartilaginous tissues of the lampreys and gnathostomes (Zhang et al., 2006; Ota and Kuratani, 2009), but not of the hagfishes (Ota and Kuratani, 2010). Given the long period since the divergence of the hagfishes, lampreys, and gnathostomes, the difference of the expression patterns of the Col2A1 between the hagfishes and other vertebrates can be explained by the following hypotheses. First, Col2A1 was probably a major ECM component in the common ancestor of the vertebrates. Second, this common ancestral state has likely remained unchanged in the gnathostomes and lampreys, but Col2A1 expression has decreased in the hagfish lineage. Finally, in the hagfish, the major ECM component probably changed from Col2A1 to noncollagenous ECM proteins, such as BGN/DCN (Fig. 6). To test these hypotheses, more detailed comparative analyses of the expression patterns of the ECM proteins and chondrogenesis-related transcription factors in the hagfish and other vertebrates are required, while simultaneously considering the evolution of the gene regulatory networks (Meulemans and Bronner-Fraser, 2007; Rychel and Swalla, 2007).

We also hypothesize that the common ancestor of the vertebrates was equipped with a complete set of two dorsal and two ventral vertebral elements, but that these degenerated secondarily in the lineage of the hagfishes and lampreys (Ota et al., 2011). This scenario is consistent with the fossil record, in that a 380-million-year-old fossil agnathan, Euphanerops longaevus, had vertebral elements on both the dorsal and ventral aspects of the notochord, although its phylogenetic position is still contentious (Janvier and Arsenault, 2007; Janvier, 2011). Furthermore, the cartilaginous nodule, whose morphology is reminiscent of that of the dorsal vertebral elements of the gnathostomes, is located at the most caudal level of the hagfish (Ayers and Jackson, ’00; Cole, ’05; Ota et al., 2011) (Fig. 1). Little is known about the molecular and cellular mechanisms underlying the degeneration of the dorsal vertebral element in the hagfish mid-trunk region. An expression analysis of Msx, which is expressed in the dorsally migrated sclerotomes in the gnathostomes, is required to resolve this problem (Monsoro-Burq et al., ’94; Christ et al., 2000).

Our study and the original description of skeletal elements by Ayers and Jackson (’00) indicate that Eptatretus has vertebral elements. However, this finding simultaneously raises the question of why no hagfish vertebral elements have been found in Myxine glutinosa (Miller, 1834; Robson et al., 2000). In fact, in the illustration from Cole shown in Figure 1A (Cole, ’05), a single isolated cartilaginous nodule is depicted on the anterior aspect of the ventral median plate of M. glutinosa, but no multiple nodules can be seen. This exceptional morphology of the caudal skeleton
of *M. glutinosa* can be explained by repression of the development of segregated cartilaginous nodules in the postcloacal regions (Figs. 1 and 6). We propose that the expression pattern of the late *Hox* genes was evolutionarily modified in the hagfish lineage, causing the degeneration of the precloacal vertebral elements in *Eptatretus* species and also of the postcloacal vertebral elements of *Myxine* species (see Coates, '94; Coates and Cohn, '98; Burke et al., '95).

Our previous and present studies have shown that the absence of hagfish vertebral elements probably represents a secondarily degenerate rather than an ancestral condition (Ota et al., 2011). Other morphological characters of the hagfish—such as the absence of a closed vascular system and multiple semicircular canals—that have long been recognized as plesiomorphic in cladistic analyses (Forey and Janvier, '93; Shu et al., 2003; Gess et al., 2006), also seem to be secondarily degenerate characters (Kuratani and Ota, 2008). We hope that these evo-devo-based interpretations of the morphological character states of the hagfish will complement cladistic approaches, providing further insight into the evolutionary processes of the early vertebrates and their morphological features.

**ACKNOWLEDGMENTS**

We are grateful to Captain Osamu Kakitani, the members of the Fishery Association in Gotsu City and Kiyomi Kayano (Director, Sekikatsu, Inc.) for their assistance in collecting hagfish specimens. We also thank Fumiaki Sugawara, Noritaka Adachi, and Hiroki Higashiyama in the Laboratory for Evolutionary Morphology, CDB, RIKEN for maintaining the aquariums, and the members of the Marine Station, ICOB, Academia Sinica for their assistance with administrative support. This research was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan and NSC grant 101-2311-B-001-001-MY2 from the National Science Council of Taiwan.
LITERATURE CITED

Ayers H, Jackson C. 1900. Morphology of the myxinoidae. I. Skeleton and musculature. J Morph 17:185–226.

Brodal A, Fange R, editors. 1963. The biology of Myxine. Universitetsforlaget, Oslo.

Buckingham M, Vincent SD. 2009. Distinct and dynamic myogenic populations in the vertebrate embryo. Curr Opin Genet Dev 19:444–453.

Burke AC, Nelson CE, Morgan BA, Tabin C. 1995. Hox genes and the evolution of vertebrate axial morphology. Development 121:333–346.

Christ B, Huang R, Wilting J. 2000. The development of the avian vertebral column. Anat Embryol 202:179–194.

Coates MI. 1994. The origin of vertebrate limbs. Dev Suppl 169–180.

Coates MI, Cohn MJ. 1998. Fins, limbs, and tails: outgrowths and axial patterning in vertebrate evolution. BioEssays 20:371–381.

Cole FJ. 1933. The evolution of the vertebral column. Cambridge University Press.

Dean B. 1899. On the embryology of Bdellostoma stouti. A genera account of myxinoid development from the egg and segmentation to hatching. Festschrift zum 70ten Geburtstag Carl von Kupffer 220–276.

Forey P, Janvier P. 1993. Agnathans and the origin of jawed vertebrates. Nature 361:129–134.

Gadow H. 1895. On the evolution of the vertebral column of fishes. Philos Roy Soc B 56:163–221.

Gadow H. 1933. The evolution of the vertebral column. Cambridge: Cambridge University Press.

Gess RW, Coates MI, Rubidge BS. 2006. A lamprey from the Devonian period of South Africa. Nature 443:981–984.

Goodrich ES. 1930. Studies on the structure and development of vertebrates. London: Macmillan.

Heimberg AM, Cowper-Sal-lari R, Semon M, Donoghue PC, Peterson KJ. 2010. microRNAs reveal the interrelationships of hagfish, lampreys, and gnathostomes and the nature of the ancestral vertebrate. Proc Natl Acad Sci USA 107:19379–19383.

Janvier P. 1996. Early vertebrates. Oxford: Clarendon Press.

Janvier P. 2011. Comparative anatomy: all vertebrates do have vertebrae. Curr Biol 21:R661–R663.

Janvier P,Arsenault M. 2007. The anatomy of Euphanerops longaeus Woodward, 1900, an anaspid-like jawless vertebrate from the Upper Devonian of Miguasha, Quebec, Canada. Geodiversitas 29:143–216.

Lomholt JP, Weber RE, Malte H, editors. 1998. The biology of hagfishes. Cambridge: Chapman and Hall Ltd.

Kalamajski S, Oldberg A. 2010. The role of small leucine-rich proteoglycans in collagen fibrillogenesis. Matrix Biol 29:248–253.

Kardong K. 2011. Vertebrates: comparative anatomy, function, and evolution. New York: McGraw-Hill.

Kumar S, Nei M, Dudley J, Tamura K. 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 9:299–306.

Kuraku S, Kuratani S. 2006. Time scale for cyclostome evolution inferred with a phylogenetic diagnosis of hagfish and lamprey cDNA sequences. Zool Sci 23:1053–1064.

Kuraku S, Hoshiyama D, Kato H, Suga H, Miyata T. 1999. Monophyly of lampreys and hagfishes supported by nuclear DNA-coded genes. J Mol Evol 49:729–735.

Kuraku S, Meyer A, Kuratani S. 2009. Timing of genome duplications relative to the origin of the vertebrates: did cyclostomes diverge before or after? Mol Biol Evol 26:47–59.

Kuratani S, Ota KG. 2008. Hagfish (cyclostomata, vertebrata): searching for the ancestral developmental plan of vertebrates. BioEssays 30:167–172.

Liem KF, Bemis WE, Walker WF, Kabbe G. 2001. Functional anatomy of the vertebrates: an evolutionary perspective. Belmont CA: Brooks Cole-Thomson Learning.

Løvtrup S. 1977. The phylogeny of vertebrata. New York: Wiley.

Mallatt J, Sullivan J. 1998. 28S and 18S rDNA sequences support 220–226.

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

Monsoro-Burq AH, Bontoux M, Teillet MA, Le Douarin NM. 1994. Heterogeneity in the development of the vertebra. Proc Natl Acad Sci USA 91:10435–10439.

Müller J. 1834. Vergleichende Anatomie der Myxinoideen. Berlin: K Akad der Wissenschaften.

Ota KG, Kuratani S. 2006. The history of scientific endeavors towards understanding hagfish embryology. Zool Sci 23:403–418.

Ota KG, Kuratani S. 2008. Developmental biology of hagfishes, with a report on newly obtained embryos of the Japanese inshore hagfish, Eptatretus burgeri. Zool Sci 25:999–1011.

Ota KG, Kuratani S. 2009. Evolutionary origin of bone and cartilage in vertebrates. In: Pourquie O, editor. The skeletal system. New York: Cold spring harbor laboratory press. p 1–18.

Ota KG, Kuratani S. 2010. Expression pattern of two collagen type 2 alpha1 genes in the Japanese inshore hagfish (Eptatretus burgeri) with special reference to the evolution of cartilaginous tissue. J Exp Zool B (Mol Dev Evol) 314:157–165.

Ota KG, Kuraku S, Kuratani S. 2007. Hagfish embryology with reference to the evolution of the neural crest. Nature 446:672–675.

Ota KG, Fujimoto S, Oisi Y, Kuratani S. 2011. Identification of vertebral-like elements and their possible differentiation from sclerotomes in the hagfish. Nat Commun 2:373.

Parker KW. 1883. On the skeleton of the Marsipobranch fishes. Part I. The Myxinoids (Myxine, and Bdellostoma). Philos T Roy Soc B 174:373–409.

Robson P, Wright GM, Keeley FW. 2000. Distinct non-collagen based cartilages comprising the endoskeleton of the Atlantic hagfish, Myxine glutinosa. Anat Embryol 202:281–290.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574.
Rychel AL, Swalla BJ. 2007. Development and evolution of chordate cartilage. J Exp Zool B (Mol Dev Evol) 308:325–335.

Schaefer L, Iozzo RV. 2008. Biological functions of the small leucine-rich proteoglycans: from genetics to signal transduction. J Biol Chem 283:21305–21309.

Shintani S, Sato A, Toyosawa S, O’HUigin C, Klein J. 2000. Biglycan-like extracellular matrix genes of agnathans and teleosts. J Mol Biol 51:363–373.

Shu DG, Morris SC, Han J, Zhang ZF, Yasui K, Janvier P, Chen L, Zhang XL, Liu JN, Li Y, Liu HQ. 2003. Head and backbone of the Early Cambrian vertebrate Haikouichthys. Nature 421:526–529.

Stock DW, Whitt GS. 1992. Evidence from 18S ribosomal RNA sequences that lampreys and hagfishes form a natural group. Science 257:787–789.

Takechi M, Takeuchi M, Ota KG, et al. 2011. Overview of the transcriptome profiles identified in hagfish, shark, and bichir: current issues arising from some nonmodel vertebrate taxa. J Exp Zool B (Mol Dev Evol) 316:526–546.

Takezaki N, Figueroa F, Zaleska-Rutczynska Z, Klein J. 2003. Molecular phylogeny of early vertebrates: monophyly of the agnathans as revealed by sequences of 35 genes. Mol Biol Evol 20:287–292.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Tretjakoff D. 1926. Die Wilbeläule des Neunauges. Anat Anz 61:387–396.

Wright GM, Keeley FW, Robson P. 2001. The unusual cartilaginous tissues of jawless craniates, cephalochordates and invertebrates. Cell Tissue Res 304:165–174.

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

Zhang G, Miyamoto MM, Cohn MJ. 2006. Lamprey type II collagen and Sox9 reveal an ancient origin of the vertebrate collagenous skeleton. Proc Natl Acad Sci USA 103:3180–3185.