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Development of the Chondrocranium in Hagfishes, with Special Reference to the Early Evolution of Vertebrates

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Recent molecular phylogenetic analyses have shown that the modern jawless vertebrates, hagfishes and lampreys, are more closely related to each other than to the other vertebrates, constituting a monophyletic group, the cyclostomes. In terms of their developmental morphology as well, it is possible to identify an embryonic pattern in hagfish embryos that is common to cyclostomes but not shared by jawed vertebrate embryos. On the basis of this pan-cyclostome embryonic pattern, we describe the developmental sequence of the chondrocranium and associated structures in the hagfish species *Eptatretus burgeri* and *E. atami*. Our aim was to establish homologies of the skeletal elements among cyclostomes by comparison of the developmental patterns with a lamprey, *Lethenteron reissneri*, to characterize further the cyclostome morphotype and its diversification in early vertebrate evolution. We show that the hagfish and lamprey chondrocrania can be compared perfectly at the level of modules corresponding to the craniofacial primordia constituting the cyclostome morphotype. In the adult anatomy, however, there are many instances in which homology cannot be established at the level of single skeletal elements, mainly because of the apparently highly apomorphic nature of the hagfish cranium. Even at the craniofacial modular level, the chondrocrania of cyclostomes and those of jawed vertebrates display very few primary homologies and are therefore very difficult to compare. We also discuss the problem of the homology of a neurocranial element, the trabecula.

Key words: agnathans, craniofacial development, cyclostomes, embryo, evolution, hagfish

INTRODUCTION

The morphology of vertebrate embryonic skulls has attracted the attention of a number of comparative morphologists, as the developmental pattern of the skull is believed to reflect the developmental architecture of the vertebrate head and body plan and the evolutionary origins of these structures (reviewed by Holmgren and Stensiö, 1936). Typically, this category of descriptive study, as concisely summarized by the monumental work of De Beer (1937), has tried to address, *inter alia*, the issues of the basic segmental pattern of the neurocranium and viscerocranium, the number of vertebral segments incorporated into the occipital region, and the presence or absence of premandibular pharyngeal arches, all of which may be observable in undifferentiated forms in the embryonic head mesenchyme. In this context, cyclostomes have always been an enigmatic group among vertebrates, primarily because of the difficulty researchers have encountered in comparing their cranial morphology with that of gnathostomes. As summarized by Gee (1996), our understanding of elasmobranch embryos has strongly influenced the comparative morphology of vertebrates: as a result of this "elasmobranch worship," the embryonic development of cyclostomes has often been described as fitting the scheme obtained from shark embryos (Koltzoff, 1901; Damas, 1944).

Among cyclostomes, the crania of larval and adult lampreys are well studied (Parker, 1883b; Kaensche, 1890; Nestler, 1890; Bujor, 1891; Schaffer, 1897; Sewertzoff, 1897; Gaskell, 1908; Marinelli and Strenger, 1954; reviewed by De Beer, 1923, 1937). Embryonic development and metamorphosis of the lamprey cranium have also been documented by Johnels (1948). In hagfish, the adult cranium was described by Müller (1834, 1839), Parker (1883a), Ayers and Jackson (1900), Cole (1905), Marinelli and Strenger (1954), and Holmgren and Stensiö (1936). It has often been emphasized that the anatomic patterns of the cranium differ substantially between the lamprey and hagfish (Fürbringer, 1897; Stensiö, 1927, 1932) (Fig. 1, Supplementary Figure S1 online); the findings of such studies support the concept that the living agnathans fall into two major lineages: the lampreys and the hagfishes. Comparative embryology of the cyclostomes is important for precise homologization of the skeletal elements of these two cyclostome groups (Holmgren and Stensiö, 1936; Holmgren, 1946;

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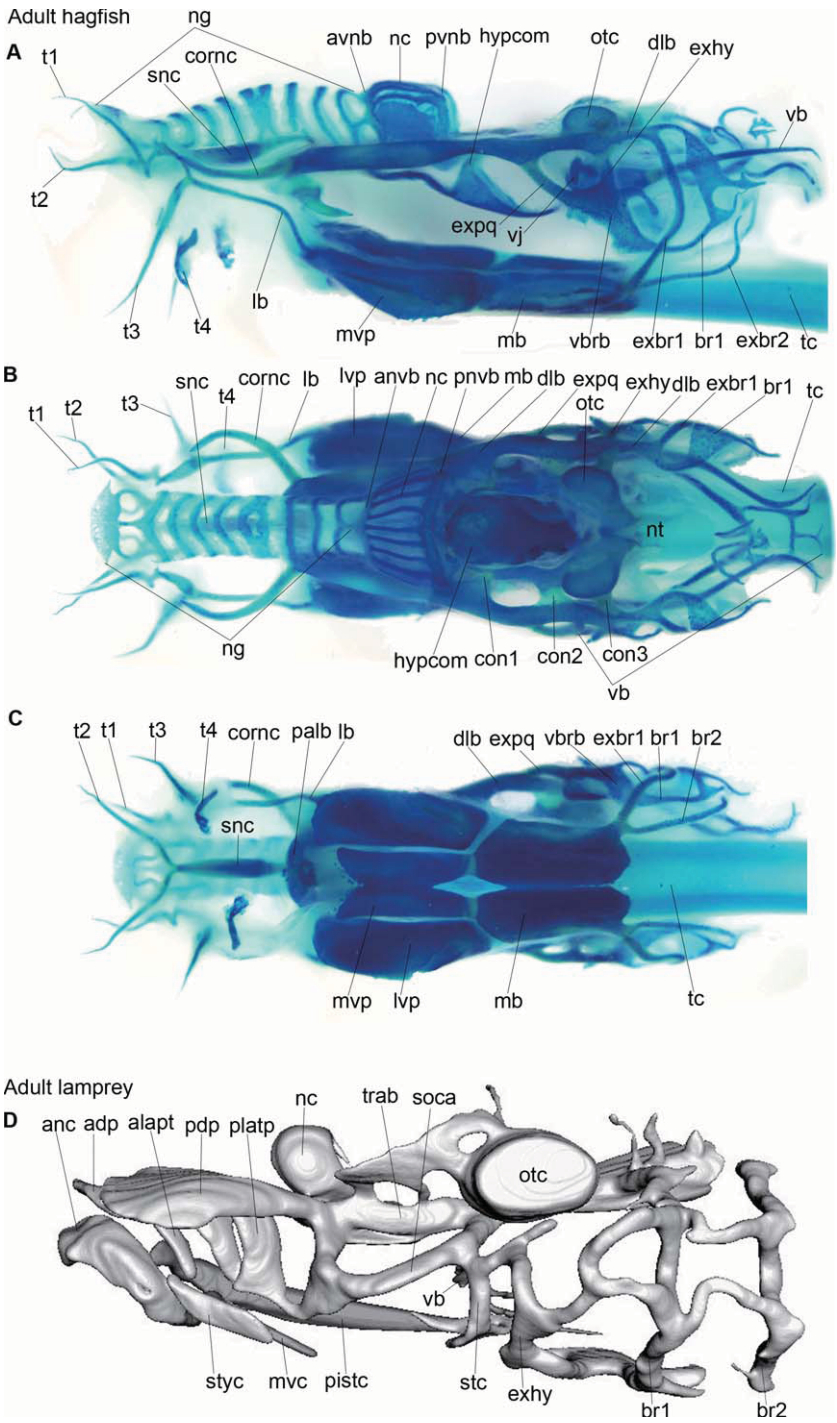

Fig. 1. Adult chondrocrania of cyclostomes. Lateral **(A)**, dorsal **(B)**, and ventral **(C)** views of an Alcian-blue-stained whole-mount chondrocranium of *Eptatretus burgeri*, and 3D reconstructed model of the cranium in an adult lamprey, *Lethenteron reissneri* **(D)**. See the list in the text for abbreviations.

Johnels, 1948).

Unlike in the case of the lamprey, our knowledge of the development of the hagfish cranium is extremely poor. The embryonic development of the hagfish cranium has been reported by only a pair of researchers, and their descriptions have been based on only a few embryonic stages (Neumayer, 1938; Holmgren, 1946). As we have shown in our previous study (Oisi et al., 2013), the most conserved stage of cyclostome development is the pharyngula stage, well before chondrification of the cranium. To identify and compare the origin of each skeletal element, we need to use a whole series of staged embryos, ranging from the stage of initial chondrification (just after acquisition of the cyclostome-specific morphotype) to the establishment of the basic cranial architecture that prefigures the adult morphology.

Information on hagfish development is limited mainly due to a lack of embryonic materials: the fish's deep-sea habitat makes such materials difficult to access (reviewed by Ota and Kuratani, 2006). In 2006, however, we succeeded in obtaining embryos of the inshore hagfish *Eptatretus burgeri* (Ota et al., 2007); since then, we have collected a complete series of embryos that can be used to describe cranial development on the embryological and morphological levels. In addition, we have obtained a prehatching-stage embryo of a closely related species, the brown hagfish *E. atami*, in which we observed the adult configuration of the cranium.

In developmental studies of *E. burgeri* conducted to date, we have reported the development of the neural crest, the differentiation of somites, and the appearance of putative vertebrae in the caudalmost part of the trunk (Ota et al., 2007, 2011). We have also reported the conservation of the embryonic pattern of head development between hagfishes and lampreys during their embryogenesis, and this finding has enabled the homologization of some structures. This paper is based on our new dataset on hagfish craniogenesis—and especially on the pan-cyclostome pattern that we identified previously in embryos (Oisi et al., 2013)—and is intended to present a new interpretation of the cyclostome cranium and its significance in the early evolution of vertebrates. This is also the first description of hagfish chondrocranial development based on a complete series of developing embryos.

MATERIALS AND METHODS

Sample collection

E. burgeri embryos were collected as described previously (Ota et al., 2007), staged according to the method of Dean (1899), and fixed with Serra's fixative or 4% paraformaldehyde.

Collection of lamprey samples

Ammocoete and adult lampreys, *Lethenteron reissneri*, a brook lamprey species closely related to *L. japonicum*, were collected from the rivers in Nagano Prefecture and from Lake Biwa in Saga Prefecture by the Kurosawa Inc. (Saku, Sinsyu, Japan) and the

Biwako Satellite-area Research Group, Japan. They were fixed in Serra's fixative overnight at 4°C and then gradually dehydrated in a graded series of methanol and phosphate-buffered saline (PBS). Finally, they were stored in 100% methanol at –20°C.

Histology

To avoid distortion of histologic sections, and especially to preserve the cartilage, we used Kawamoto's Film Method and a Paraffin Section Preparation Kit (Section Lab Co. Ltd.; see http://section-lab.jp/English.htm). The adhesive side of the paraffin transfer film (fine type) was attached firmly to the cut surface of the paraffin block, and each section was cut slowly at 6 to 8 mm thick with a microtome. One side of the cut "film-sections" was firmly placed onto a glass slide with the sectioned side down, and the slide was bathed in water at 45°C for 10 s. The films were then pressed onto, and attached to, the glass slide with a rubber roller and dried on a hot plate at 50°C for 24 h to transfer the embryonic tissues onto the slides. The adhesive compound and paraffin were removed by treatment with xylene for 1 to 3 days and then used in histological, immunohistochemical, and in situ hybridization procedures. Images were recorded with a DP70 digital camera (Olympus Inc., Tokyo, Japan) attached to a light microscope and reconstructed with a computer graphics program Avizo (Visualization Sciences Group).

In situ hybridization

In situ hybridization was performed either by using a manual standard protocol or a Ventana automated instrument (Roche, Japan). In the standard protocol, serial sections were fixed for 10 min in 4% paraformaldehyde in PBS at room temperature, washed twice in PBS, treated with proteinase K in 0.01 M Tris buffer for 10 min, and then fixed again for 10 min in 4% paraformaldehyde at room temperature. After rinsing twice in PBS, the sections were incubated with 0.25% acetic anhydride and 0.1 M triethanolamine (pH 8), washed in PBS, air dried, and hybridized with riboprobes at 51°C for 16 to 20 h. The sections were then washed in 5 × saline sodium citrate (SSC) buffer at 55°C, treated with 50% formamide in 2 × SSC at 60°C for 20 min, then washed once in 2 × SSC and twice in 0.2 × SSC, at 60°C each, for 20 min each. After being blocked with 1.5% blocking reagent (Roche) in 0.1 M Tris buffer with 0.15 M NaCl (pH 7.6), the sections were incubated with alkaline-phosphatase-conjugated anti-digoxigenin (DIG) antibody (Roche). After final washes of the sections with Tris buffer, positive cells were stained purple with nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate toludinium salt (BCIP). With the Ventana instrument, signals were detected and counterstaining performed by using a BlueMap NBT/BCIP substrate kit (Roche) and a nuclear fast red-equivalent reagent, ISH RED, (Roche), as described previously (Ota et al., 2007).

Immunohistochemistry and histochemistry

Histologic observations were made on hematoxylin- and eosin-stained sections (thickness, 6 to 8 μm), some of which were stained further with 0.1% Alcian blue to show the cartilage in older embryos of hagfish and adult lamprey and the mucocartilage in ammocoetes. To detect axon bundles, anti-acetylated tubulin was applied to sections after *in situ* hybridization or Alcian blue staining. Anti-mouse IgG1 was used as the secondary antibody. All histologic images were recorded with a DP70 digital camera (Olympus) attached to a light microscope.

Table of nomenclature:

adp, anterior dorsal plate in the lamprey
ah, adenohypophysis
alapt, anterior lateral apical cartilage in the lamprey
anc, annular cartilage in the lamprey
anp, anterior nasal process (ANP)
avnb, anterior vertical nasal bar
br1–2, internal branchial arch 1–2 cartilage (the 3rd pharyngeal arch is counted as br1)
con1, rostral commissure of dlb
con2, middle commissure of dlb
con3, posterior commissure of dlb
cornc, cornual cartilage
dp, dental plate primordium
dlb, dorsal longitudinal bar
e, eye
en, external nostril
exbr1, extrabranchiale 1
exbr2, extrabranchiale 2
exhy, extrahyal
expq, extrapalato-quadrate
gp4, 4 pharyngeal-pouch-derived gill pouch
gs4, 4 pharyngeal-pouch-derived gill slit
hy, hyoid arch
hypcom, hypophyseal commissure
lb, labial cartilage
lp, lingual plate
lvp, latero-rostral part of basal plate ("*bas1l*" of Holmgren 1946)
ma, mandibular arch
mb, medial part of basal plate ("*bas2*" of Holmgren 1946)
mm, mandibular mesoderm
mo, mouth
mphp, PHP-derived mesoderm
mvc, medio-ventral cartilage in the lamprey
mvp, medio-rostral part of basal plate ("*bas1m*" of Holmgren 1946)
nc, nasal capsule
ng, nasal duct cartilages
nhd, nasohypophyseal duct
nhp, nasohypophyseal plate
ne, nasal epithelium
nt, notochord
oc, oral cavity
onc, oronasohypophyseal cavity
ot, otocyst
otc, otic capsule
p1–9, pharyngeal pouches 1 to 9
pa3, pharyngeal arch 3
palb, palatine bar
pch, parachordals
pdp, posterior dorsal plate in the lamprey
ph, pharynx
php, posthypophyseal process (PHP)
pistc, piston cartilage in the lamprey
platp, posterior lateral plate in the lamprey
pom, periotic mesenchyme
ptr, posterior trabecula of hagfish (parachordal of Neumayer 1938)
pvnb, posterior vertical nasal bar
rtr, rostral trabecula
snc, subnasal cartilage
soca, subocular arch in the lamprey
stc, styliform cartilage in the lamprey
styc, stylet cartilage in the lamprey
tc, tongue cartilage ("*bas3*" of Holmgren 1946)
t1–3, cartilaginous support for tentacles
trab, trabecula
vb, velar bar
vbrb, ventral branchial bar
vch, velum chamber
vj, joint caput for velum
vm, velum mesoderm and muscle
V, trigeminal nerve

RESULTS

Stages 40 to 50

In vertebrate embryos, neural crest-derived ectomesenchyme occupies primarily the rostroventral portion of the head, including the pharyngeal arches, whereas mesodermal mesenchyme occupies the dorsal and caudal perineural portions of the head (reviewed by Noden, 1988). To obtain insights into the distribution pattern of neural-crest-derived ectomesenchyme in the prechondogenic stages of *E. burgeri* embryos, we first observed the expression patterns of four Dlx genes (*EbDlx2/3/5B*, *EbDlx2/3/5C, EbDlx1/4/6A*, *EbDlx1/4/6C*[not shown]; Fujimoto et al., 2013) in stage 45 *E. burgeri* embryos, corresponding to the mid-pharyngula stage of this animal. *Dlx* genes, which are homeobox-containing regulatory genes homologous to *Distal-less* in *Drosophila*, are ectomesenchymal markers in the region including, and caudal to, the mandibular arch (see Kuratani et al., 2012). The transcripts of all the Dlx genes showed similar distribution patterns: Dlx-positive ectomesenchyme was found in the mandibular and posterior pharyngeal arches, as well as in the caudal and lateral regions of the posthypophyseal process (PHP), which is a cyclostome-specific craniofacial primordium (Oisi et al., 2013). The mesenchyme in the anterior nasal process (ANP), which is mainly of premandibular neural crest-origin in the lamprey (Kuratani, 2012), did not express any Dlx genes (Fig. 2A–G). Notably, the periotic mesenchyme was also negative for Dlx gene expression (Fig. 2A–C).

As noted previously (Shigetani et al., 2002), the premandibular ectomesenchyme (the ectomesenchyme distributed rostral to the mandibular arch) is specifically Dlx-negative in jawed vertebrate embryos. In contrast, in the lamprey embryo, part of the premandibular ectomesenchyme destined to form the upper lip of the ammocoetes larva expresses Dlx genes as specifiers of the oral apparatus (Shigetani et al., 2002). However, this phenomenon can be ascribed to the secondary migration of mandibular arch crest cells into the premandibular (including PHP) domain covering the premandibular ectomesenchyme laterally, as implied by cell lineage studies (Kuratani, 2012; Kuratani et al., 2012). Thus, the presence of Dlx-positive and -negative mesenchyme in the hagfish PHP is consistent with observations of the lamprey.

To identify mesodermal mesenchyme in the hagfish embryos, we also observed the expression patterns of *EbTbx1/10A* (the hagfish homolog of *Tbx1/10*, the marker gene for prochondrogenic head mesoderm; Oisi et al., 2013) and of *EbSoxE* (a *Sox10* homolog) in *E. burgeri* (Fig. 2H–M; Ota et al., 2007). At stage 40 there was no sign of chondrification, as detected by *SoxE* expression in the embryonic head (Fig. 2H). Instead, a clear accumulation of mesenchyme expressing *EbTbx1/10A* was seen to surround the otocyst, prefiguring the future otic capsule (Fig. 2I). This mesenchyme appeared to correspond to the periotic mesenchyme found in gnathostome embryos (Ladher et al., 2005; Monks and Morrow, 2012). At stage 50, the above-noted *EbTbx1/10A*-positive mesenchyme could be followed rostrally, as a longitudinal strand of cells, toward the ventral aspect of the eye (Figs. 2J, K, 3), possibly prefiguring trabeculae of this animal (Fig. 3C, D).

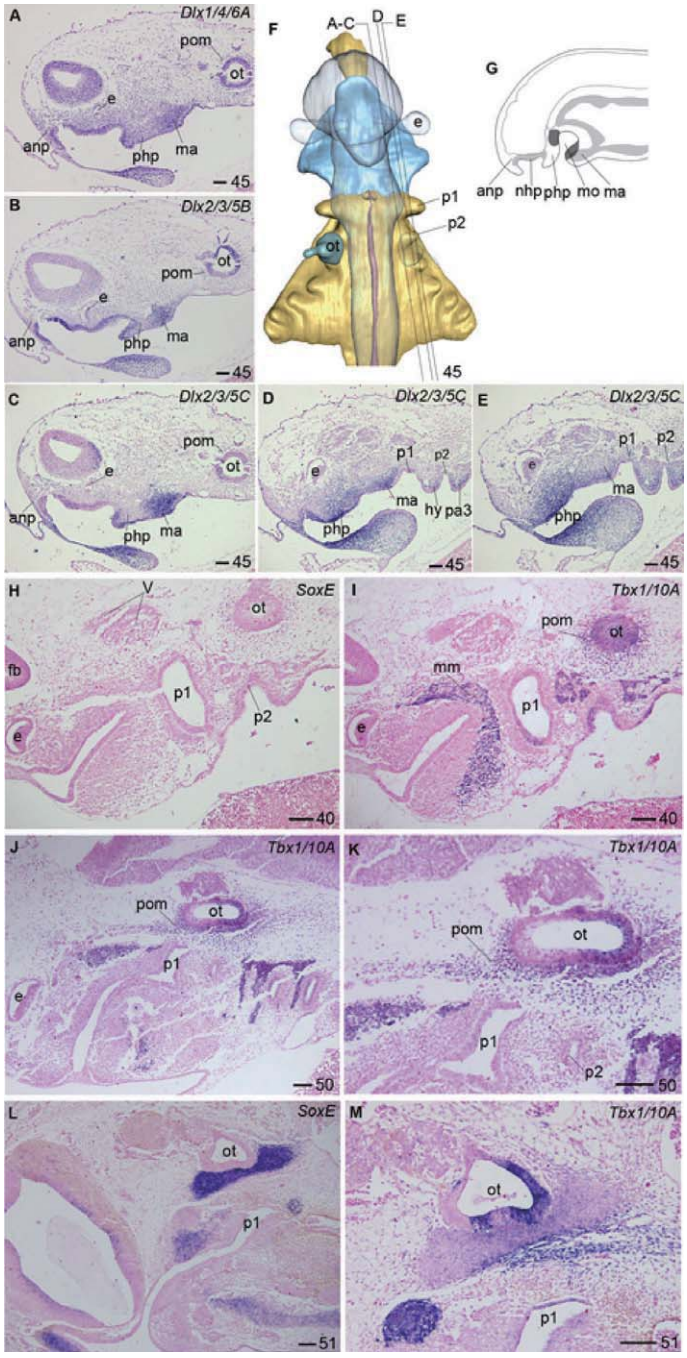

Fig. 2. Embryonic expression of genes for craniofacial structure in *Eptatretus burgeri*. Histologic sections of *E. burgeri* at stages 45 **(A–E)**, 40 **(H, I)**, 50 **(J, K)**, and 51 **(L, M)**, hybridized with *Dlx1/4/6A* **(A)**, *Dlx2/3/5B* **(B)**, *Dlx2/3/5C* **(C–E)**, *Tbx1/10A* **(I–K, M)**, or *SoxE* **(H, L)** riboprobes. Also dorsal view of reconstruction **(F)** and schematic diagram of Dlx genes expression in the mid-sagittal region **(G)** of a stage 45 embryo (dark gray). At stage 40 **(I)**, *Tbx1/10A*, the mesodermal neurocranial marker gene, is expressed in the pharyngeal arch muscle anlage as well as in the mesenchyme surrounding the otocyst, representing the otic capsule anlage. By stage 50 **(J, K)**, the mandibular arch muscle anlage has differentiated into tentacular and lingual muscle primordia, and the periotic prochondrogenic mesenchyme has grown rostrally to form the common anlage for trabeculae and the dorsal longitudinal bar. **(L)** *SoxE* expression at stage 51 depicts a rostrally growing longitudinal prochondrogenic anlage, continuous with the otic capsule. Abbreviations: anp, anterior nasal process; e, eye; mm, mandibular mesoderm; ot, otocyst; p1, 2, pharyngeal pouches 1, 2; php, posthypophyseal process; pom, periotic mesenchyme. See the list in the text for other abbreviations. Bars = 100 μm.

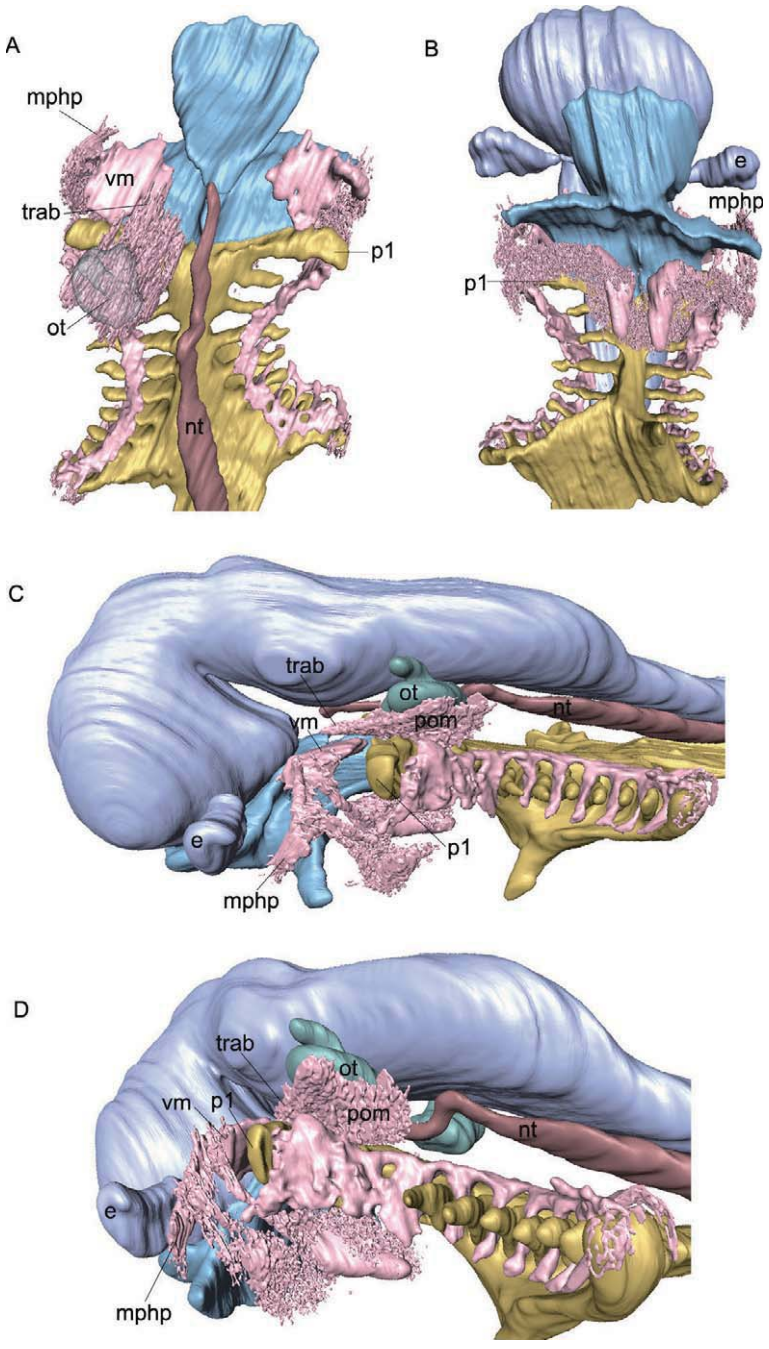

Fig. 3. Mesenchymal cranial primordium of a stage 50 *Eptatretus burgeri* embryo. 3D reconstructions were based on sectioned specimens hybridized with *Tbx1/10A* (A–D) riboprobes. **(A)** Dorsal view. **(B)** Ventral view. **(C)** Left lateral view. **(D)** Oblique posterior view. The ectodermal oronasohypophyseal cavity is colored light blue, the pharyngeal endoderm is colored yellow, and the *Tbx1/10A* positive-mesoderm colored pink. mphp, muscle primordia in the php; vm, velum muscle; nt, notocord; trab, trabecula. See the list in the text for other abbreviations.

Comparison of this pattern of expression with that of the Dlx genes at stage 45 suggested that this mesenchyme represented either the head mesodermal derivative or the premandibular ectomesenchyme (Fig. 2A–G).

Stage 51

By stage 51, when *EbSoxE* was being upregulated in the otic capsule, *EbTbx1/10A* had begun to be downregulated in the above-noted periotic cartilage (Fig. 2L, M). Stage 51 was the youngest stage at which reconstruction of the prechondrogenic primordial cranium could be performed on the basis of the expression of both *EbSoxE* and *EbTbx1/10A* (Fig. 4). This stage of *E. burgeri* appeared to resemble “Stadium I” (i.e., stage I) of the “*Bdellostoma*” (*Eptatretus stouti*) embryo described by Neumayer (1938) and was younger than the *Myxine* embryo described by Holmgren (1946). Comparison of Neumayer’s Stadium I and our stage 51, however, was not simple solely on the basis of cranial morphologic examination, as our observation was based on *EbSoxE*-positive mesenchyme, part of which was not recognized as a cartilage precursor by these previous authors.

By this stage, the anterior nasal process of the *E. burgeri* embryo had developed a transverse cartilage primordium rostral to the nasohypophyseal plate (nhp), the common placode that differentiates into the nasal epithelium and adenohypophysis (Kupffer, 1899, 1900). From its position relative to the forebrain, this cartilage appeared to correspond to a common precursor of the future “anterior and posterior vertical nasal bars” (avnp and pvnb) of Holmgren (1946) and the “cartilago nasalis posterior et anterior” of Neumayer (1938) (Fig. 4A, F).

The mesenchyme ventral to the otocyst formed an overt primordium of the otic capsule (otc), which had a large dorsal fenestra and in which the otocyst was embedded (Fig. 4A, B). This cartilaginous capsule was medially continuous with the cranial floor on both sides of the notochord (Fig. 4A, B). This cranial base may have contained the skeletal elements generally called the parachordals; Neumayer (1938) thought that the medial wall of this capsule represented the parachordals. Holmgren (1946), on the other hand, described an independent primordium for the parachordals as a separate element medial to the otic capsule. However, we were unable to detect any independent anlage—even by the expression of *EbSoxE* in the prochondrogenic mesenchyme—that would have implied the separate origin of parachordals distinct from the otic capsule.

From the anterolateral aspect of the otic capsule, a thick longitudinal prochondrogenic mesenchymal bar grew rostrally toward the area ventral to the eye primordium and toward the ventrolateral aspect of the forebrain (Fig. 4A–C); this bar corresponded to the common anlage for the trabecula and the dorsal longitudinal bar described in the hagfish (Holmgren, 1946; see below).

In the ventral part of the mandibular arch, which differentiates into the tongue apparatus of this animal (Yalden, 1985), an extensive sheet-like prochondrogenic primordium was observed on the oral floor, representing the early development of the lingual plate (lp; Fig. 4D, F). The position of

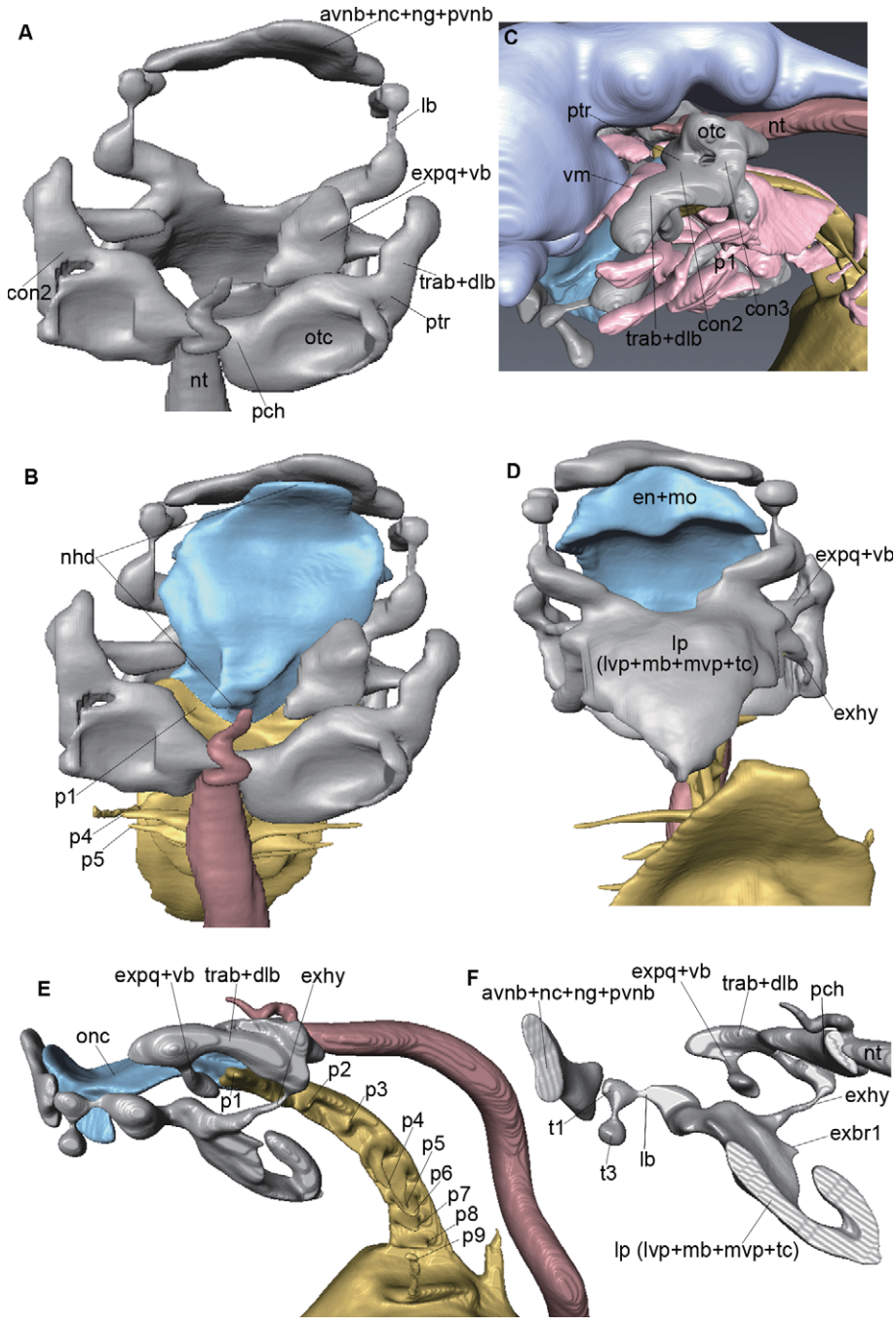

Fig. 4. Prochondrogenic cranial primordium of a stage 51 *Eptatretus burgeri* embryo. 3D-reconstructions were based on *SoxE* and *Tbx1/10A* expression. **(A, F)** Reconstructions of the cranial primordium. **(B–E)** Reconstructions with epithelial structures (ectodermal oronasohypophyseal cavity, light blue; pharyngeal endodermal lining, yellow; *Tbx1/10A*-positive head muscle primordium, pink). **(A, B)** Dorsal views. **(D)** Ventral views. **(E, F)** Left lateral views. lp, lingual plate; otc, otic capsule; t1 and t3, common anlage for tentacular cartilages I and III; trab + dlb, common anlage for trabeculae and the dorsal longitudinal bar; vb, velar bar. See the list in the text for other abbreviations.

this prochondrogenic mesenchyme corresponded to the site occupied by the ventral plate of mucocartilage (ventrolateral plate) in the ammocoete larva of the lamprey (Supplementary Figure S1 online; Gaskell, 1908; Holmgren and Stensiö, 1936). Curiously, this plate in the hagfish is turned upward to form a hook at this stage (Fig. 4E, F). Also, there was an arch-like connection—the extrahyal (exhy) of Holmgren (1946)—linking the lateral aspect of the lingual plate (Fig. 4D–F) and the above-noted longitudinal bar at the level of the hyoid arch and representing a hyoid arch element (Fig. 4E).

Rostrally, the lingual plate continued into a pair of prochondrogenic nodules below and posterior to the nasal cartilage primordium noted above. Situated in the rostral part of the early PHP, these nodules appeared to differentiate into tentacular cartilages, most probably corresponding to those for tentacles 1 and 3 (Fig. 4F; see below).

As a mandibular arch element, a ventral process grew medially from the common anlage of the trabecula and the dorsal longitudinal bar (Fig. 4A, C), close to the junction of the ectodermal oronasohypophyseal cavity and the pharyngeal endoderm (Fig. 4E). This mesenchymal process represented the extrapalatoquadrate of Holmgren (1946) and was associated with the primordium of the velar bar (*vb* in Fig. 4C, E, F).

Stage 53

From this stage onward, reconstruction of the hagfish embryos was based on cartilaginous tissues, which are easily detected in histologic sections (Figs. 5 and 6). As described previously (Oisi et al., 2013), the PHP-derived oronasohypophyseal septum had grown completely, separating the nasohypophyseal duct and oral cavity dorsoventrally by this stage (Figs. 5A, D and 6A, C). Some authors (e.g., Stensiö, 1927) also used the term “palato-subnasal lamina”.

The overall morphology of the chondrocranium of stage 53 *E. burgeri* embryo (Figs. 5 and 6) had a conspicuous resemblance to that of the *Myxine* embryo described by Holmgren (1946). By this stage, the nasal epithelium had differentiated to acquire the characteristic zigzag, comb-like morphology (ne; Fig. 6B–D). On the anterior and the posterior aspect of this cavity, two transverse cartilaginous bars had appeared (compare Figs. 5B, D to 6B–D). The posterior bar corresponded to the “posterior vertical nasal bar (pvnb)” and the anterior one to the “anterior vertical nasal bar” (avnb) of Holmgren (1946), or to the “Vordere Nasalknorpel” (anterior nasal cartilage;

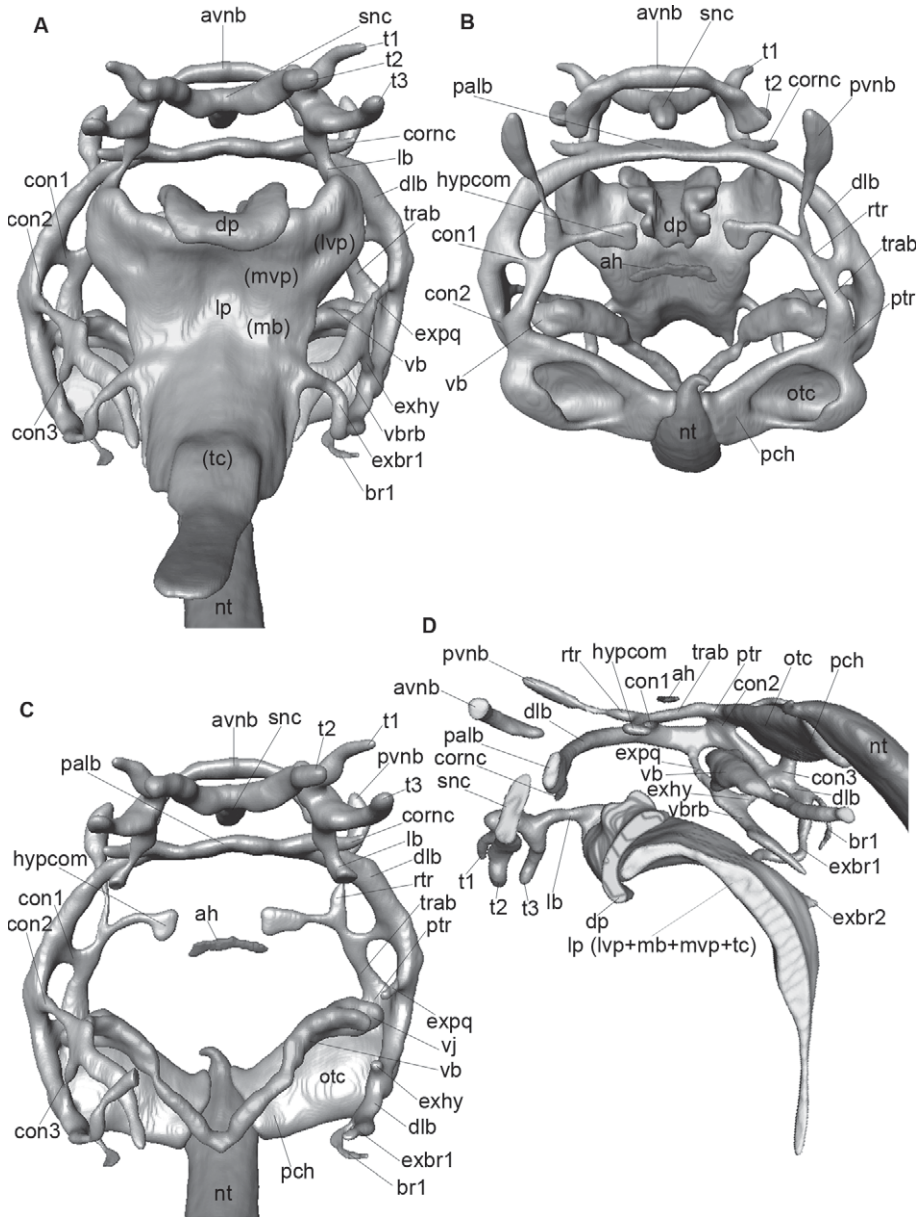

Fig. 5. Chondrocranium of a stage 53 *Eptatretus burgeri* embryo. Ventral **(A, C)**, dorsal **(B)**, and left lateral **(D)** views of a 3D-reconstructed model. Lingual cartilage is removed in **(C)**. See the list in the text for abbreviations.

Neumayer, 1938). As noted above, these cartilages appeared to have been derived from the single common anlage observed in the ANP of the previous stage (Fig. 4A, F). Unlike the posterior bars, which remained separated bilaterally, the anterior bar by this stage had formed a complete arch rostral to the nasal cavity (Figs. 5B, 6B, D).

The anterior part of the oronasohypophyseal septum—the derivative of PHP—contained three pairs of rod-like cartilages, corresponding to the supporting skeletons for tentacles 1 to 3 (t1 to t3 in Figs. 5A, D and 6A, C, D; the tentacular blastema of Holmgren, 1946). The cartilage bars in the second tentacle were fused posteriorly to form a median longitudinal rod, or subnasal cartilage (snc), which is also situated within the oronasohypophyseal septum, below the nasohypophyseal duct (compare Figs. 5C, D to 6A, C, D). The cartilaginous rods for tentacles 1 and 3, on the other hand, were posteriorly united with each other and further connected posteriorly, by means of a string of labial cartilage (lb), with the lingual cartilage complex located clearly on the oral floor or in the ventral part of the mandibular arch (Fig. 5A, D).

By this stage, trabeculae ("trab" in the definition of Holmgren, 1946) were seen as conspicuous longitudinal cartilages separated from the more ventrally located dorsal longitudinal bar (dlb; see below) and had become a pair of rods, posterior parts of which developed in a similar plane as that of the notochord. At the level of the adenohypophysis the rod sank below the nasohypophyseal duct and grew a medially oriented commissure that would later unite with its counterpart to form the "hypophyseal commissure" (hypcom; Holmgren, 1946; Figs. 5B–D and 6B–D). This commissure was found slightly rostral to the adenohypophysis (ah) (Figs. 5B, C and 6B, C). Rostral to the above commissure, the trabeculae grew more rostrally to unite with the posterior vertical nasal bar described above (Figs. 5B–D and 6B–D).

In addition to the trabecula, there was another longitudinal cartilaginous rod lateral and slightly ventral to the trabecula and the otic capsule (Figs. 5B–D). This rod corresponded to what was called the "dorsal longitudinal bar" by Holmgren (1946), although this is not the dorsalmost cartilage in the hagfish chondrocranium. By this stage, the dorsal longitudinal bar connected to the trabecula and otic capsule by means of cartilaginous communications at three places: with the rostral part of the trabecula (con1), with the posterior part of the trabecula (con2), and with the posteroventral part of the otic capsule (con3) (Figs. 5B–D and 6B–C′). The major part of the dorsal longitudinal bar was located lateral and ventral to the nasohypophyseal duct; rostrally it continued into a cartilage called the "cornual cartilage" (Holmgren, 1946) and into a palatine bar, indicating that this cartilage, like the above-noted tentacular cartilages, develop in the derivative of the PHP of the earlier embryo (Oisi et al., 2013). From the Dlx-expression patterns of earlier embryos (Fig. 2A–G), as well as from the topographic position of the dorsal longitudinal bar, this cartilage appeared to have been derived either from the dorsal part of the original mandibular arch ectomesenchyme that had migrated rostrally into the lateral part of the PHP (for its pos-

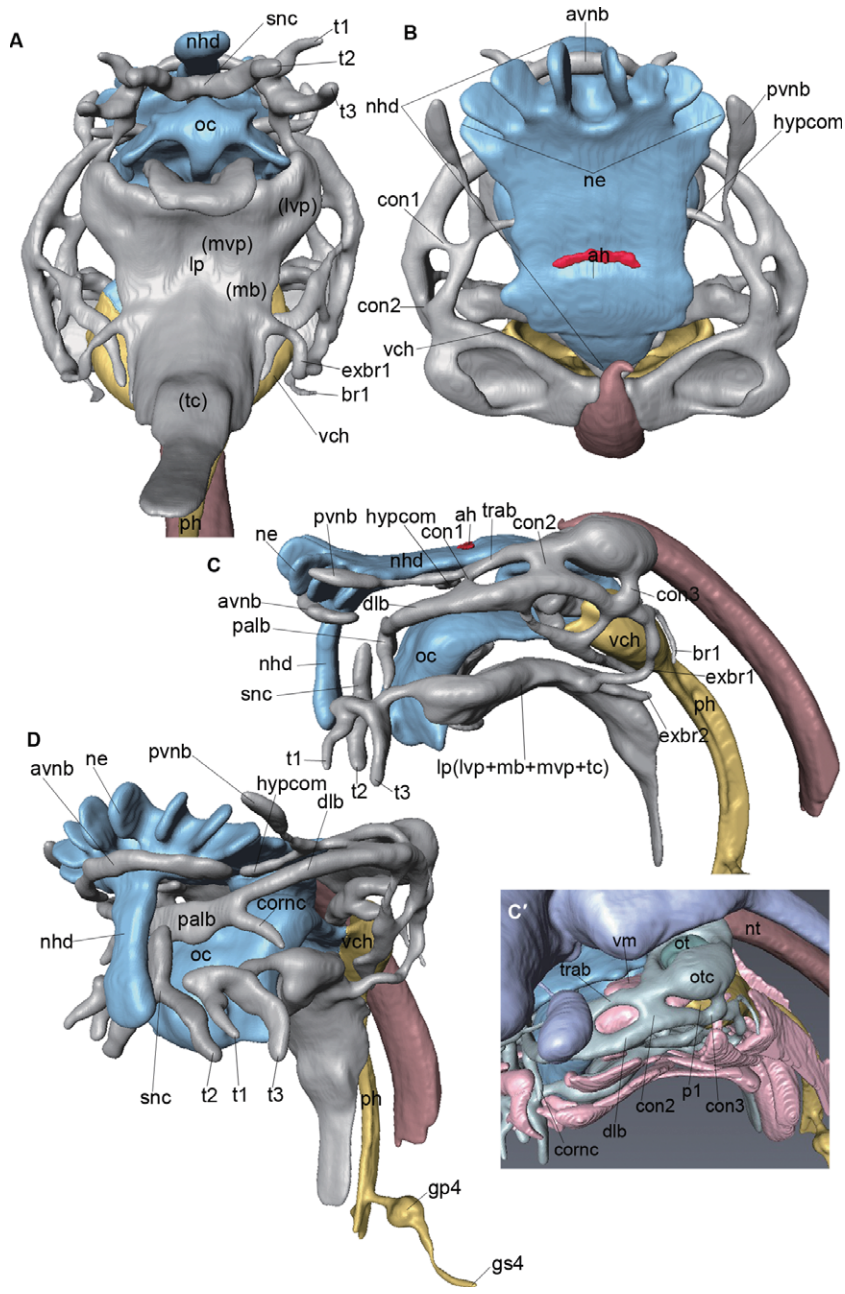

Fig. 6. The same chondrocranium as shown in Fig. 5, reconstructed with endodermal (yellow) and ectodermal (light blue) linings associated with the cranium. The *Tbx1/10A*-positive head muscle primordium is coloured pink.Ventral **(A)**, dorsal **(B)**, lateral **(C, C′)**, and oblique anterior **(D)** views of a 3D-reconstructed model. Note in the lateral view **(C)** that most of trabeculae (trab) lies below the nasohypophyseal duct, or within the oronasohypophyseal septum (space between nasohypophyseal duct and oral cavity). The lingual plate lies below the oral cavity, or within the ventral part of the mandibular arch. See the list in the text for abbreviations.

sible similarity to the gnathostome palatoquadrate, see the Discussion) or from the more medially located mesenchyme. As observed in the patterns of *EbTbx1/10A* and *EbSoxE* expression, however, it became clear that the above-noted trabecula and the dorsal longitudinal bar differentiated from a common longitudinal prochondrogenic anlage observed at stage 51 (compare Fig. 4C to Fig. 6C′). Namely, the trabecula and the dorsal longitudinal bar were dorsolaterally separated from each other by fenestration out of this mesenchyme. There were two sites of fenestration; therefore, the above-noted three communications do not represent secondarily established commissure cartilages but instead the remnant of the original common anlage that has not been absorbed (Fig. 6C′).

Beneath the oral cavity, in the ventral mandibular arch domain that will differentiate into the lingual apparatus, the anlage of the lingual cartilages could still be identified as an undivided single sheet of cartilage (Figs. 5A, D and 6A, C). This cartilage appeared to contain two types of cartilaginous primordia. The rostral paired cartilage corresponded to the primordium of the "latero-rostral part of the basal plate (lvp)" (Holmgren, 1946). Holmgren labeled this structure at this embryonic stage "bas1l". Medial and dorsal to these cartilages, the primodium of the dental plate (dp) was developing (dental cartilage of Marinelli and Strenger, 1954). Holmgren referred to the latter as the "medio-rostral part of the basal plate (mvp)" ("bas1m" of Holmgren, 1946). Caudal to the lvp, there was another pair of longitudinal plates labeled "bas3" by Holmgren (1946). According to Holmgren, there was another element at the junction of lvp and bas3, called the "medial part of the basal plate" ("bas2" of Holmgren, 1946); this was not discerned in our embryo at this stage.

Rostrally, the rostrolateral part of the lvp developed a rod-like communicating cartilage that grew laterally and rostrally to invade the PHP domain and establish a connection with tentacular cartilages 1 and 3 (Figs. 5D and 6C). This communicating cartilage, therefore, represents a commissure between two different craniofacial modules in the hagfish embryo.

Another group of cartilages to be noted at this stage is the pharyngeal arch-associated cartilages of the hagfish. First, there are two pairs of thin cartilaginous arches connecting the dorsal longitudinal bar and the lingual cartilage primordium. They correspond to "extrabranchiale I (exbr1)" and "extrabranchiale II (exbr2)" of Holmgren (1946), belonging to the third and fourth pharyngeal arches, respectively (Figs. 5A, C, D and 6A, C). Anterior to these cartilages, another vertical cartilage had developed, connecting the dorsal longitudinal bar at the level of the posterior commissure dorsally with another newly formed ventral cartilage that had developed ventral to the rostralmost part of the pharynx (Figs. 5A, C, D and 6A, C). The former is called the extrahyal (exhy) and the latter the ventral branchial bar (vbrv) by Holmgren (1946). The topographic relationship

between the main branch of the facial nerve, often called the hyomandibular, has been investigated to determine the second arch origin of the extrahyal (Neumayer, 1938); this was also ascertained by our study here (not shown).

Stage 60

The *E. burgeri* embryo at stage 60 particularly resembles "Stadium II" of *E. stouti* by Neumayer (1938); however, the rostrum of the latter chondrocranium appears to be somewhat compressed as a result of secondary distortion. Our reconstructions (Fig. 7) appear to reflect the normal proportion of the hagfish embryonic head at this stage. Although the morphology of the chondrocranium of *E. burgeri* at stage 60 was highly complex, it could readily be derived from that of the previous stage.

The most conspicuous change was the appearance of the nasal duct cartilages (ng), which developed rostral to the anterior vertical nasal band as a series of inverted U-shaped cartilaginous bands along the nasohypophyseal duct (Fig. 7A, E, F, H). Apparently, these cartilages support the hagfish-specific elongated nasohypophyseal duct, and they are functionally and morphologically reminiscent of the tracheal rings of amniotes. In the lamprey, the nasohypophyseal duct of which does not elongate during development, no equivalent cartilages are present. The above-noted chondrocranium in *E. stouti* does not possess nasal duct cartilages, although the specimen examined may have been at a slightly younger stage. Alternatively, as another possibility,

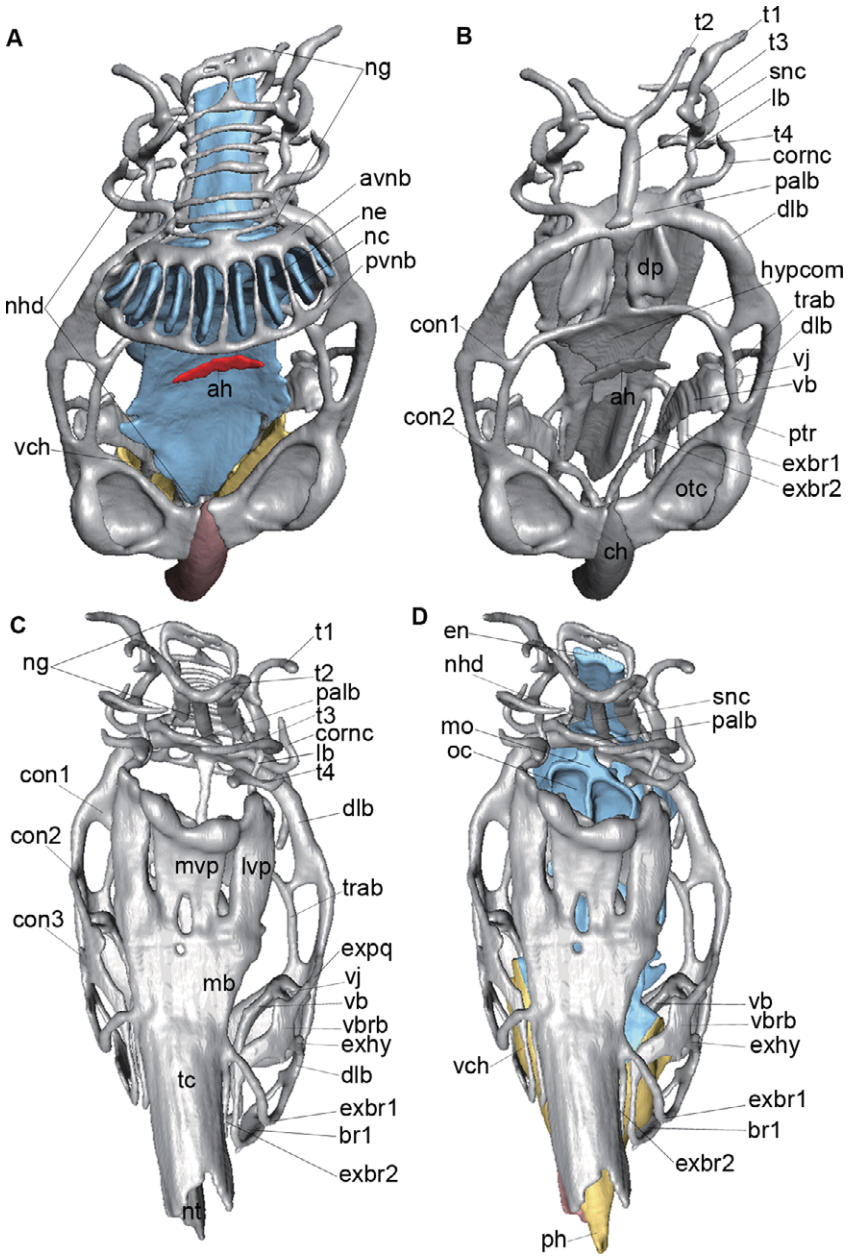

Fig. 7. Continued.

the late development of the nasal duct cartilage may represent a species-specific heterochronic variation, because this cartilage in *E. stouti* is again only poorly developed in "Stadium III" (Neumayer, 1938), the overall chondrocranial morphology of which resembled that of the prehatching stage of *E. atami*.

Rostrally, the supporting cartilage appeared as an independent cartilaginous rod for the fourth tentacle (Fig. 7B, C, F, G). Nasal capsule had appeared as several longitudinal cartilaginous rods along the sulci of the nasal epithelium (Fig. 7A, E, H). In the velum, the velar bar had now grown along the longitudinal axis with the change in orientation of the velum characteristic to the hagfish (Fig. 7F).

Prehatching stage

For the embryonic stage close to hatching (about six months after stage 60) we used *E. atami*, a species closely related to *E. burgeri* (Figs. 8 and 9). This specimen was one of a number of specimens donated by the Kasai Marine Aquarium, Tokyo, that were still in their shells; its siblings hatched in the laboratory. As described below, the morphology of the embryo examined was entirely consistent with the adult morphology of *E. burgeri*, and we considered that it represented the equivalent developmental stage in that species.

The snout of *E. atami* at this stage had lifted upward to form a longitudinally elongated head characteristic of the

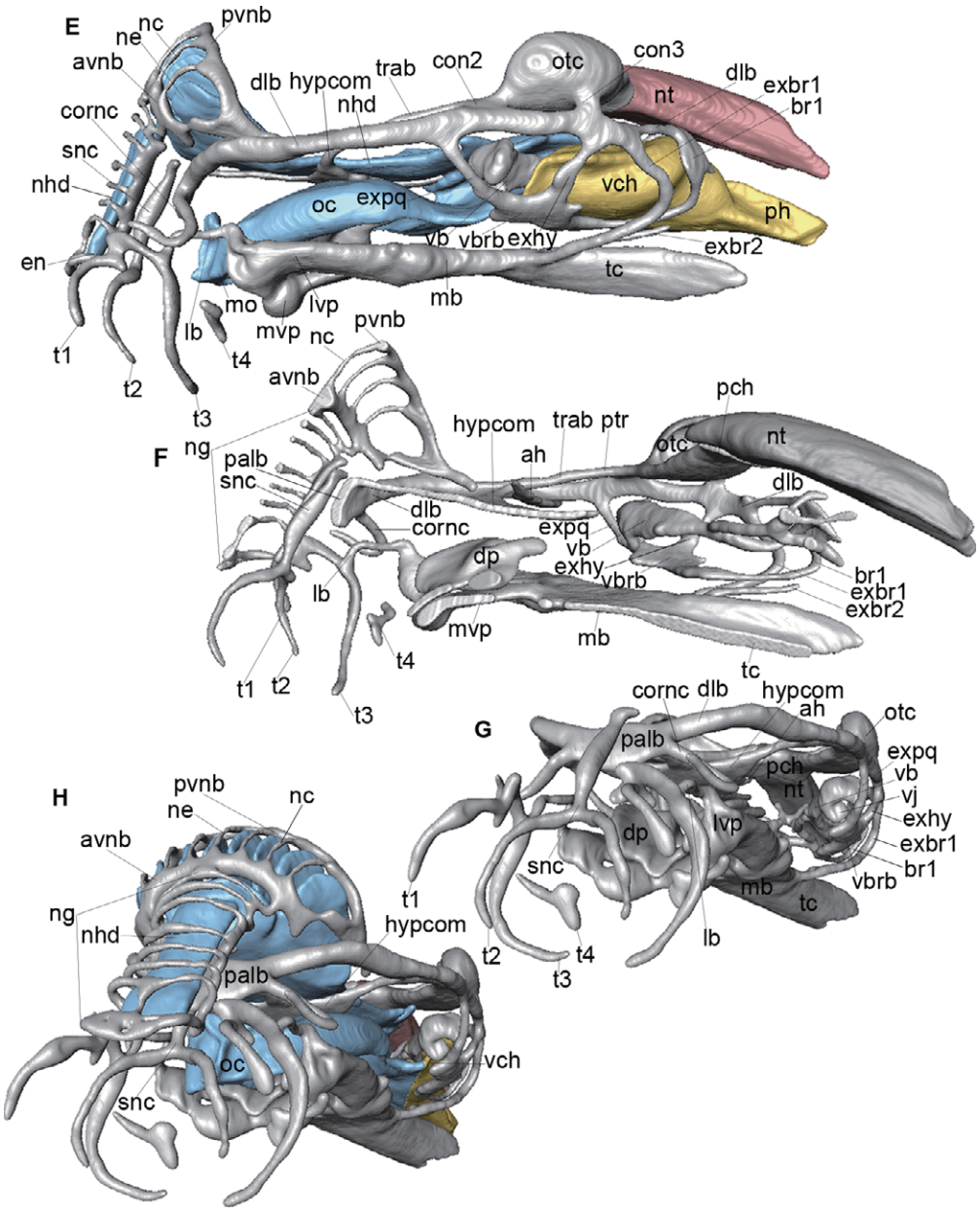

Fig. 7. Chondrocranium of a stage 60 *Eptatretus burgeri* embryo. **(B, C, F, G)** Reconstruction of the chondrocranium. **(A, D, E, H)** Reconstruction with epithelial structures (ectodermal oronasohypophyseal cavity, light blue; pharyngeal endodermal lining, yellow). **(A, B)** Dorsal views. **(C, D)** Ventral views. **(E, F)** Left lateral views. **(G, H)** Oblique anterior views. See the list in the text for abbreviations.

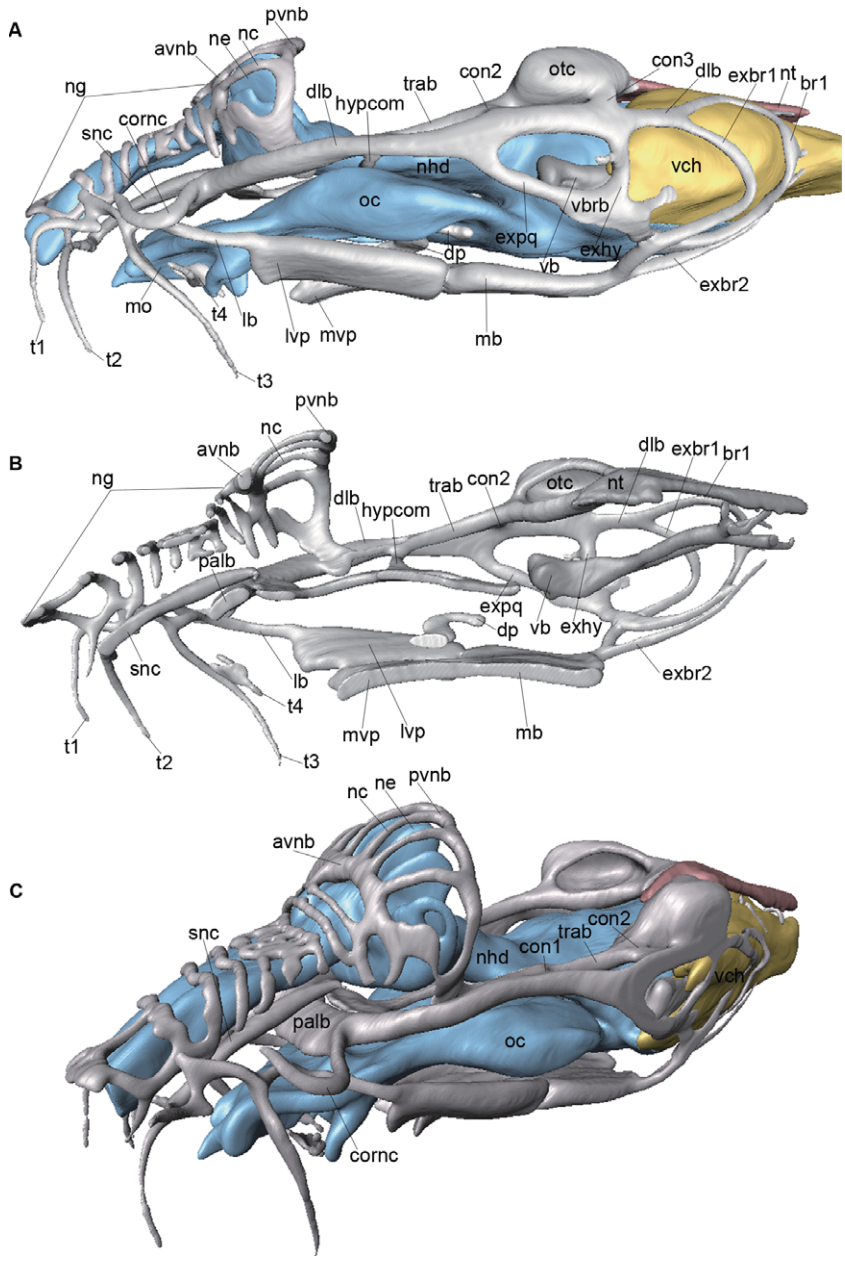

Fig. 8. Chondrocranium of a prehatching-stage *Eptatretus atami* embryo. **(B)** Reconstruction of the chondrocranium. **(A, C)** Reconstruction of the chondrocranium with epithelial structures (ectodermal oronasohypophyseal cavity, light blue; pharyngeal endodermal lining, yellow). **(A. B)** lateral views. **(C)** Oblique anterior view. See the list in the text for abbreviations.

adult hagfish described by Müller (1834, 1839) and Parker (1883a, 1883b) (Figs. 8 and 9). In every aspect, the configuration of the chondrocranium at this stage was nearly identical to that of the adult *E. burgeri* (Fig. 1). Owing to the secondary shift in head morphology, the position of the dorsal longitudinal bar had also been lifted to reach a level approximately identical to the horizontal level of the trabecula, giving the impression that these longitudinal cartilages together form the lateral wall of the neurocranium of this animal, similar to the orbital cartilage in the adult lamprey or the taenia marginalis, spheno-orbital commissure, and orbitotemporal commissure of jawed vertebrates (De Beer, 1937). Marinelli and Strenger (1954) have also called this cartilage the taenia dorsolateralis. However, the above-mentioned cartilages do not appear to correspond to the neurocranial lateral wall; instead, they more likely to represent an ectomesenchymal derivative (see below).

Another cartilage that first appeared at this stage was the subnasal cartilage found as a single median cartilage beneath the nasohypophyseal duct (and therefore within the oronasohypophyseal septum) and continuing rostrally to bifurcate bilaterally into the supporting cartilage for tentacle 2 (Figs. 8 and 9). As suggested by our own previous study (Oisi et al., 2013), this cartilage appears to correspond to the dorsomedial supporting tissue including rostral dorsal plate in the ammocoete larva of the lamprey (Supplementary Figure S1 online).

Between the anterior and posterior vertical nasal bars, along the longitudinal depressions of the nasal cavity, at this stage there were several longitudinal cartilage rods, connecting the two vertical bars to form the nasal basket (Figs. 8 and 9). Curiously, some of the longitudinal cartilages are reported to develop in “Stadium II” (stage II) of *E. stouti* (Neumayer, 1938), implying that there are heterochronic variations in chondrogenesis among members of the hagfish group (Hypetotreti, or Myxiniformes).

DISCUSSION

Here, we have described, for the first time, the entire developmental sequence of the cranium of two *Eptatretus* species at mainly morphologic and histologic levels. The monophyly of cyclostomes has become generally accepted thanks to recent molecular phylogenetic analyses (Mallatt and Sullivan, 1998; Kuraku et al., 1999; Takezaki et al., 2003; Kuraku, 2008; Heimberg et al., 2010). However, in terms of the morphological and anatomical divergence between the two cyclostome groups, chondrocranial morphology has not been compared well between hagfish and lampreys, let alone between cyclostomes and jawed vertebrates (De Beer, 1937).

The assumption that the hagfish represents more basal lineages than lampreys was ascribable partly to the secondary loss of structures in the hagfish (Forey and Janvier, 1993; Gess et al., 2006; Khonsari et al., 2009; also see Oisi et al., 2013), even involving traits used to define vertebrates, such as the eye lens and other eye-associated structures (Stockard, 1909; reviewed by Jørgensen et al., 1998); it can also be explained in terms of the existence of hagfish-specific traits such as posterior shift of the caudal pharyngeal arches (Stockard, 1906; Holmgren, 1946) and secondary opening of the nasohypophyseal duct into the pharynx (Oisi et al.,

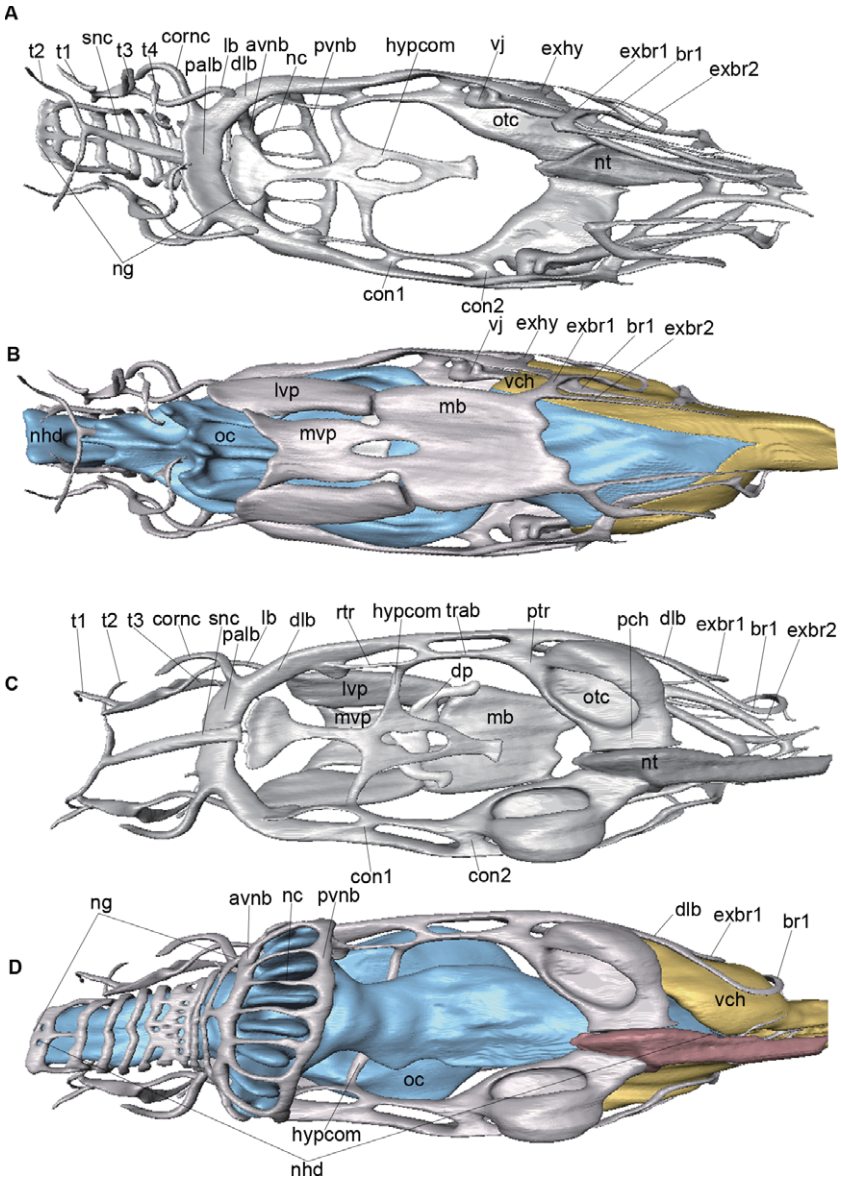

Fig. 9. Chondrocranium of a prehatching-stage *Eptatretus atami* embryo. **(A, C)** Reconstruction of the chondrocranium. **(B, D)** Reconstruction of the chondrocranium with epithelial structures (ectodermal oronasohypophyseal cavity, light blue; pharyngeal endodermal lining, yellow). **(A, B)** ventral views (lingual plate is removed in **(A)**). **(C , D)** dorsal views. See the list in the text for abbreviations.

2013). Some of these traits potentially represent autoapomorphies of hagfish, as lampreys and gnathostomes (outgroup) used to be placed more closely to each other, at morphological level, for the absence of these traits (reviewed by Oisi et al., 2013). Notably, apparently shared morphological traits led Stensiö (1927) to conclude that hagfish anatomy is similar to that of pteraspids, a group of fossil, jawless stem gnathostomes (also see Holmgren, 1946). Stensiö's view was also based on the presence of the characteristic head shield in late hagfish embryos, which was reminiscent of that in pteraspids. Our observation of staged hagfish embryos, however, suggested that this shield represents skin folds made by compression of the egg shell (data not shown). Taken together, close affinity of hagfish and lamprey is consistent with the embryonic similarity between these animals, leading to an expectation that thorough morphological comparison should be possible between their chondrocrania as well.

Most previous comparisons between lamprey and hagfish crania have been performed at anatomical levels for each skeletal element, with consideration of the topographic relationships with cranial muscles and nerves (Neumayer, 1938; Holmgren, 1946; De Beer, 1937; also see Strahan, 1960 for theoretical comparison). These observations have often resulted in appropriate homologization of cartilage elements. A very successful example was the comparative musculoskeletal anatomy of the lingual apparatus in the hagfish and lamprey (Yalden, 1985). Also notable was the growth and transformation of the PHP in lamprey development, showing that the upper lip that pushes the nasohypophyseal opening (nostril) to the dorsal aspect of the head in the ammocoete larva (Sewertzoff, 1901; Goodrich, 1909; De Beer, 1923; Damas, 1944). This process does not occur in hagfish embryos (Oisi et al., 2013).

When the evolutionary divergence is extensive enough to obliterate one-to-one comparisons (especially between distantly related animals), it often becomes necessary to consider the basic embryonic architecture shared by closely related animals. Another problem associated with the classical comparative method is that, especially in comparing cyclostomes and jawed vertebrates, one should rely on the assumption that all the vertebrates share the same ancestral developmental plan, without which one-to-one homologies of skeletal elements would not be expected between these two taxa. In this respect, many past comparisons were influenced by the elasmobranch worship (Gee, 1996). However, many of the patterns seen in crown gnathostomes, such as division of the mandibular arch into upper and lower jaw components, are likely to have occurred after the divergence of the cyclostomes. Thus cyclostome and crown gnathostome chondrocrania should be compared at deeper levels of homology than would be the case in comparisons among gnathostome species.

In a previous study, we elucidated the rise of the pan-cyclostome embryonic pattern (i.e., an embryonic morphotype shared by cyclostomes, but not by crown gnathostomes) by comparing staged developing embryos of the lamprey and hagfish, consistent with the molecular-based suggested affinity of these animals (Oisi et al., 2013). This pattern is characterized by possession of a nasohypophyseal plate (a single median placode that yields the nasal epithelium and adenohypophysis) bordered by an ANP and a PHP (Fig. 2A–G). Together with the ventral part of the mandibular

arch, these processes serve as craniofacial primordia in cyclostomes—much like the nasal prominences and maxillomandibular processes in jawed vertebrates. In both the hagfish and the lamprey the ANP later differentiates into the posterodorsal margin of the nasohypophyseal duct (external nostril). The PHP, on the other hand, differentiates into the upper lip in the ammocoetes larva or the anterolateral part of the oral funnel in the adult lamprey, whereas it becomes the oronasohypophyseal septum in the hagfish (Oisi et al., 2013; also see Heintz, 1963).

The dorsal portion of the mandibular arch mesoderm secondarily shifts rostrally to reside in the PHP and its derivatives (Kuratani et al., 2004). The mid-portion of the arch transforms into the velum, and the ventral part of the arch differentiates into the tongue apparatus (Kuratani, 2012). This tripartite pattern of mandibular arch differentiation is common to the lamprey and hagfish, consistent with the branching pattern of the trigeminal nerves in these animals. However, this pattern is not shared by crown gnathostomes (Oisi et al., 2013).

Thus, the first step in establishing skeletal homologies between the hagfish and the lamprey would be to identify the domains, or modular structures, of the chondrocranial portions corresponding to the developmental components of the above-mentioned pan-cyclostome pattern. It would also be possible to compare pre-metamorphosing larval elements, including ammocoete-specific mucocartilages, at the level of mesenchymal distribution in these craniofacial modules. Thereafter, comparison with the gnathostome pattern may be only partially possible at the deeper levels of basic embryogenetic architecture—for example of the undifferentiated mandibular arch before taxon-specific compartmentalization.

The above-mentioned craniofacial components in cyclostome embryos, like the craniofacial primordia in jawed vertebrates, appear to contain cephalic neural-crest-derived ectomesenchyme (Horigome et al., 1999; Kuratani et al., 1999; Shigetani et al., 2002; Oisi et al., 2013). The initial migratory patterns of the crest cells, their anteroposterior specification along the anteroposterior axis of the premigratory neural crest, and the regulatory gene expression patterns in the crest cells of lamprey embryos are reminiscent of those in jawed vertebrates (Horigome et al., 1999; McCauley and Bronner-Fraser, 2003). The only difference between the lamprey and the jawed vertebrate embryo is that, in the lamprey, the hyoid arch stream of the crest cells adhering proximally on the fourth rhombomere is found medial to the otocyst (Horigome et al., 1999). This lamprey-specific topographic relationship is shared by the hagfish (Oisi et al., 2013), suggesting its cyclostome-specific nature. Thus, the position of the otocyst is slightly more rostral with respect to the hyoid arch in the cyclostomes than in jawed vertebrate embryos.

Apart from the above-noted cyclostome-specific traits, it is highly plausible that the basic ectomesenchymal distribution and skeletogenic properties are also very similar to those in jawed vertebrates, suggesting that a craniofacial skeleton including pharyngeal arch components and prechordal neurocranial elements is also found in the cyclostomes. Although little is known about the head mesoderm of cyclostome embryos, chordal (mesodermal) cranial elements are apparent in both the hagfish and lampreys, similar to that of gnathostomes. The distribution of the head mesoderm in early lamprey embryos resembles that of jawed vertebrate embryos (Kuratani et al., 1999; Adachi and Kuratani, 2012; Adachi et al., 2012).

The problem of hagfish and lamprey trabeculae

Trabeculae of the hagfish arise as dorsal parts of the common, prochondrogenic mesenchymal anlage for the trabeculae and the dorsal longitudinal bar, which grows from the rostral aspect of the otic capsule. In the developmental context the nature of the so-called trabeculae in the lamprey chondrocranium is important. In terms of morphology (a pair of rod-like cartilages surrounding the adenohypophysis), this cartilage has often been compared with trabeculae in jawed vertebrates (Damas, 1944; reviewed by De Beer, 1937). As is now well established experimentally, these cartilage are neural crest-derived prechordal cranial elements (Couly et al., 1993; Wada et al., 2011). The lamprey trabeculae, however, were observed by Koltzoff (1901) and Filatoff (quoted by Sewertzoff, 1916) to differentiate from the head mesodermal element (reviewed by De Beer, 1937). Morphologically, as well, the cyclostome trabeculae have been compared with the parachordals of crown gnathostomes (Sewertzoff, 1916; Neumayer, 1938). Johnels (1968) also supported the mesodermal origin of these elements from a morphologic and embryologic viewpoint (the earliest primordium appears dorsal to the first aortic arch, lateral to the notochord). The position adjacent to the notochord is more suited to mesodermal neurocranial elements that require notochordally derived signals to chondrify (Couly et al., 1993). A cell-labeling study using a vital dye has revealed that the greater part of the lamprey trabeculae is derived from the mandibular mesoderm (Kuratani et al., 2004; but see also Newth, 1956; Langille and Hall, 1988). Thus the trabeculae in the lamprey appears to be a misnomer and are more likely to represent mesodermally derived parachordals that extended anteriorly beyond the level of the hypothalamus (Kuratani et al., 2004).

From its position in the embryonic head, the trabeculae of the hagfish, described here, do not appear entirely equivalent to those of the lamprey. First, as described above, in the hagfish components corresponding to the lamprey trabeculae are composites of the trabecula and the dorsal longitudinal bar (Fig. 4C). Previously, only the dorsal component of this complex, lying rostral to the rostral tip of the notochord, was defined as hagfish trabeculae. The portion of the cranial base associated with the notochord was called the hagfish “parachordals” (Neumayer, 1938; Holmgren, 1948). Unlike the lamprey trabeculae, the posterior part of which is found adjacent to the notochord, most of the hagfish trabeculae is located rostral to the notochord in later development (Fig. 4). Importantly, however, the putative paraxial mesodermal component of this cartilage is found lateral to the notochord in the prechondrogenic stages (Fig. 3A, C). Therefore, rostral extension of the parachordals is likely to take place in the hagfish. At least the posterior portion of the hagfish trabeculae that grow from the rostral part of the otic capsule and lies slightly dorsal to the nasohypophyseal duct would correspond to the parachordals in jawed vertebrates, as a large part of the lamprey trabeculae do. The rostral halves of the hagfish trabeculae, on the other hand, may be

more similar, if not entirely homologous, to trabeculae of jawed vertebrates (see below). Its position within the PHP appears to be consistent with this assumption.

Second, the hagfish trabeculae are, as a whole, widely separated from each other and do not appear to have originated from paraxial mesoderm in the head, although the possibility remains that the hagfish trabeculae correspond to the rostral, widely expanded portion of the trabeculae in the lamprey.

Third, especially in terms of the position of the hypophyseal commissure and the longitudinal commissure connected to the posterior nasal cartilage, the rostral portion of the hagfish trabeculae more closely resemble the typical prechordal cranium in jawed vertebrates. Moreover, as inferred from the embryology of the lamprey, the homolog of the jawed vertebrate trabeculae in the hagfish should be sought in cartilages derived from the hagfish PHP or from the oronasohypophyseal septum (Kuratani et al., 2004; Oisi et al., 2013; see below). Thus, although the neural crest origin of this cartilage needs to be demonstrated, it is possible that rostral parts of the hagfish trabeculae+dorsal longitudinal bars are similar to the crown gnathostome trabeculae. As noted above, examining the Dlx gene expression pattern will not answer this question, and further labeling studies are needed to determine the nature of the hagfish trabeculae.

The problem of the cyclostome pharynx and the mandibular arch

Crown gnathostomes are characterized by differentiation of the mandibular arch into upper and lower jaws. This dorsoventral division and morphological specification are shared by all jawed vertebrates; the cartilaginous primordium of the dorsal moiety is generally called the palatoquadrate, and the ventral moiety is called Meckel's cartilage (reviewed by Goodrich, 1930 and by De Beer, 1937).

Recent molecular genetic studies have shown that the identity of the mandibular arch as the rostralmost element of branchiomerism (the metameric arrangement of pharyngeal arches) is specified by the absence of Hox gene transcription in this arch (Hox code-default state; Rijli et al., 1993), which is shared by the lamprey and gnathostomes (Takio et al., 2004). Thus, the morphological identity of the mandibular arch appears to be universal among vertebrates. For the more caudally located pharyngeal arch skeletons in hagfish, the previous homologizations between hagfishes and lampreys performed by several authors are mostly correct in terms of cranial nerve innervation patterns (Lindstrom, 1949; Homma, 1978; Koyama et al., 1987; Nishizawa et al., 1988; Ronan, 1988; Song and Boord, 1993) and the distribution of pharyngeal arch muscles described by Marinelli and Strenger (1954, 1956); both of which were confirmed by our anatomic studies of adult hagfish and lamprey (Oisi et al., 2013). Division of the mandibular arch into upper and lower elements, on the other hand, would require careful treatment.

It was also molecular genetic studies that elucidated the basic mechanism specifying dorsoventral polarity of the mandibular arch in gnathostomes. Dlx genes are expressed in a dorsoventrally nested pattern in the pharyngeal arch ectomesenchyme (Beverdam et al., 2002; Depew et al., 2002; Minoux and Rijli, 2010; Gillis et al., 2013; reviewed by Takechi et al., 2013). *Dlx5* and *Dlx6* in the mouse are specifically expressed in the ventral half of the mandibular arch, and their simultaneous disruption leads to the transformation of lower jaw morphology into that of the upper jaw (maxillary process derivatives) (Depew et al., 2002). Gain of function of their upstream gene, *Ednra*, in the upper jaw domain, on the other hand, transforms the upper jaw morphology into that of the lower (Sato et al., 2008). Thus the dorsoventrally nested expression pattern of Dlx genes (i.e., the Dlx code) parallels morphological specification of the pharyngeal arch skeleton.

In the lamprey, the presence of the Dlx code is enigmatic. There are at least six Dlx genes (*DlxA to -F*) in the lamprey, five of which are specifically expressed in an ubiquitous fashion in the pharyngeal ectomesenchyme, including in the mandibular arch (Kuraku et al., 2010; also see Neidert et al., 2001). To date, there have been no reports from which we can infer dorsoventrally nested expression, although a dorsoventrally symmetrical nested pattern of expression around the gill pores has been suggested (Cerny et al., 2010). Moreover, *LjBapxA*, a homolog of *Bapx1*, the specifier of the jaw joint in gnathostomes (Miller et al., 2003), is not expressed in the mandibular arch of the lamprey (Cerny et al., 2010; Kuraku et al., 2010), suggesting that gnathostome-type basic topographic specification is absent in the lamprey. Although the expression pattern of *dHand* cognate, a ventral pole specifier, suggests the presence of dorsoventral polarity in the lamprey, the apparently unpolarized expression of Dlx genes in this taxon is consistent with the dorsoventrally symmetrical morphology of its posterior pharyngeal arch skeletons.

In the hagfish, too, dorsoventral polarity is not apparent from our preliminary analyses (Fig. 2A–G), although the morphologic pattern of the pharyngeal arch skeleton is less clear than that in the lamprey. This may be due partly to the secondary posterior shift of the posterior portion of the pharynx, which takes place in the late phase of organogenesis in the hagfish (Stockard, 1906; Oisi et al., 2013). The only conspicuous differentiation along the dorsoventral axis in the hagfish visceral skeleton is that of the lingual apparatus, which is derived from the ventral portion of the mandibular arch. As noted above, homology of this structure to that of the lamprey is well established at the morphologic level (Yalden, 1985). This skeletomuscular complex, however, does not appear to depend on any localized expression of Dlx genes; instead, it may develop through a different mechanism. Therefore, although this structural complex has often been homologized with Meckel's cartilage in gnathostomes, the developmental mechanisms in the two taxa would not be identical at the molecular level.

No homolog of the lower jaw elements therefore seems to exist in the cyclostomes. Upper jaw homology requires different consideration, because in the jawed vertebrates the upper jaw is specified by *Dlx1* and *Dlx2*, which are expressed ubiquitously in the pharyngeal arch ectomesenchyme. Thus, the upper jaw in gnathostomes is likely to represent the default state of the Dlx code. Even if dorsoventrally nested expression is lacking in the cyclostomes, the default specification mechanism may have been acquired before the lower jaw specification program. This question, however, will require consideration of another candidate palatoquadrate homolog

("pq" in the hagfish; Holmgren, 1946).

Homology of the velum in lamprey and hagfish has been called into doubt by several authors (Goodrich, 1909; Strahan, 1958; Janvier, 1981, 1996). However, we have shown that this structure arises in a very similar pattern in both animals: the velum in both animals arises in the mid-part of the mandibular arch, between the oral ectoderm and rostral endodermal wall of the first pharyngeal pouch (von Kupffer, 1895; Oisi et al., 2013).

Comparison of chondrocrania between the hagfish and the lamprey: developmental architecture of the cyclostome cranium

The chondrocranium of the hagfish consists largely of the following elements: the nasal capsule cartilages, neurocranial base, otic capsule, lingual cartilages, other pharyngeal arch cartilages, and premandibular cartilages, including tentacle-supporting cartilages. One of the earliest skeletal elements to chondrify is the otic capsule (Fig. 2H–M), as is the case in the lamprey embryo (reviewed by De Beer, 1937). Quite interestingly, in the presumed Late Devonian stem lamprey (or stem cyclostome) *Euphanerops*, the otic capsule has also been suggested to be the first skeletal element to calcify in the developmental series (Janvier and Arsenault, 2007). This element in the hagfish, along with the trabecula and the dorsal longitudinal bar, is most likely to represent, possibly entirely, the mesodermally derived neurocranial elements.

In the more ventral part of the chondrocranium, we can establish homologies on the basis of the pan-cyclostome embryonic pattern (Oisi et al., 2013). We can compare components derived from the anterior nasal process, namely the dorsal wall posterior to the nostril in the lamprey head and the supranasal region in the hagfish. This domain in the hagfish contains nasal duct cartilages and many cartilaginous elements constituting the nasal capsule. In the lamprey chondrocranium, part of the nasal capsule will correspond to these components.

Components derived from the PHP will also contain cartilages derived from both the mandibular arch and the premandibular crest cells. To this category belong all of the tentacular cartilages (although there remains a problem as to the nature of the T4 cartilage that develops between the oronasohypophyseal septum and the lower mandibular arch; this T4 cartilage is similar to the cornual cartilage) and the subnasal cartilage of the hagfish chondrocranium, as well as the palatine bar, hypophyseal commissure, and possibly the rostral part of the dorsal longitudinal bar and the trabecula.

In the lamprey chondrocranium, the mucocartilage in the upper lip and possibly the rostral part of the trabeculae appear to arise from the equivalent anlage; in the hagfish, all of the above PHP-derived cartilage elements combined will be homologous with the upper lip (rostral dorsal plate and lateral wall of the upper lip) and trabecula, as well as a part of the nasal capsule. More precisely, the distribution of the trigeminal nerve branches suggests that the upper lip in the ammocoete will further be subdivided into a dorsal median portion (rostral dorsal plate) innervated predominantly by the ophthalmic nerve and a lateral wall innervated by the rostral branch of V2, 3 (Oisi et al., 2013). On the

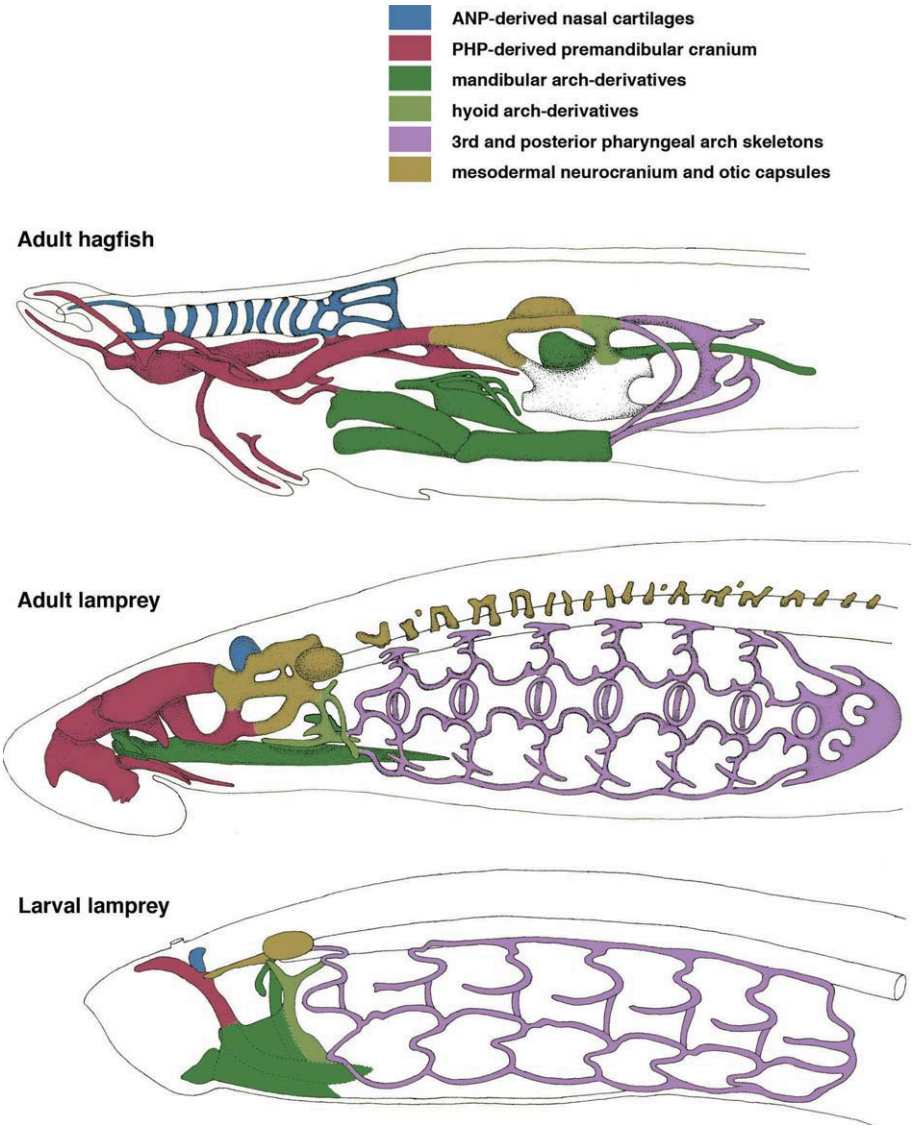

Fig. 10. Homology of chondrocranial elements in cyclostomes. Hagfish and lamprey chondrocrania were compared on the basis of our results. Hagfish chondrocranium was redrawn from the work of Holmgren and Stensiö (1936), and those of the lamprey from the work of Marinelli and Strenger (1954) and Fontaine (1958).

Table 1. Comparison of chondrocranial elements in cyclostomes.

developmental origins	Chondrocranial elements	
	Hagfish	Lamprey
nasal-capsule	nasal capsule	nasal capsule
	anterior vertical nasal bar	
	posterior vertical nasal bar	
	nasal duct cartilages	
mandibular-arch-derivatives	velar cartilage	velar cartilage
	dorsal longitudinal bar (anterior)?	ventro-lateral plate (in larva)
	extra-mandibular	ventro-medial longitudinal bar (in larva)
	(extrapalato-quadrate)	dental plate (in adult)
	dental plate	lingual cartilages (in adult)
	lingual cartilages	anterior lateral apical cartilage
	basal plate	medial apical cartilage
	tongue cartilage	piston cartilage
PHP-derivatives	rostral trabecula	trabecular commissure?
	and trabecular commissure?	lateral mouth plate (in larva)
	palatine bar	rostro-dorsal plate (in larva)
	(rostral connection of	styliform cartilage (in adult)
	longitudinal bar)	posterior lateral plate (in adult)
	cornual cartilage	posterior dorsal plate (in adult)
	labial cartilage	anterior dorsal plate (in adult)
	subnasal cartilage	anterior lateral plate (in adult)
		anterior dorsal plate (in adult)
		annular cartilage (in adult)
		medio-ventral cartilage (in adult)
		stylet cartilage (in adult)
hyoid-arch-derivatives	extrahyal	extrahyal
mesodermal neurocranium	auditory capsule	auditory capsule
	parachordals	parachordals
	posterior trabecula?	trabecula?
	dorsal longitudinal bar (posterior)?	subocular arch (in adult)
posterior arch-derivatives	extrabranchiale 1	branchiale 1
	branchiale 1	
	extrabranchiale 2	branchiale 2
	branchiale 2	

basis of the similarity of the innervation pattern in the hagfish to that in the lamprey, the lateral wall in the lamprey may correspond to the T1, T3 and T4 cartilages in the hagfish, whereas the T2 cartilage may be more similar to the dorsal roof. At any rate, the questionable cornual cartilage in the hagfish is located in such a way as to divide the cutaneous innervation area into a domain innervated by V1 and one innervated by the dorsal V2, 3 branch (Marinelli and Strenger, 1956; Oisi et al., 2013). On the basis of the above discussion, we summarized the homologous relationships between the lamprey and hagfish crania (Fig. 10, Table 1).

There are a number of cyclostome-specific traits, namely differentiation of the lingual apparatus in the ventral mandibular arch region; differentiation of the velum in the mid-portion of the mandibular arch; absence of an intertrabecula homolog; absence of occipital vertebrae; close association between the otic capsule and parachordals; presence of lateral (external) pharyngeal arch skeletons; and presence of skeletal elements in the PHP derivatives. Some of these features will be counted as cyclostome synapomorphies, but others will potentially represent plesiomorphies of vertebrates established by the common ancestor of cyclostomes and gnathostomes but secondarily lost in gnathostome lineages (see Oisi et al., 2013). To describe the evolutionary sequence of craniogenesis, it will be crucial to analyze the crania of gnathostome stems, the embryology of which remains unknown even in the best-studied cephalaspids (Osteostraci) and galeaspids (Galeaspida). Further collaborations spanning paleontology and evolutionary developmental biology, along with comparative embryology, will be needed to further our understanding of cranial evolution.

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REFERENCES

Adachi N, Kuratani S (2012) Development of head and trunk mesoderm in a dogfish, *Scyliorhinus torazame*. I. Embryology and morphology of the head cavities and related structures. Evol Dev 14: 234–256

Adachi N, Takechi M, Hirai T, Kuratani S (2012) Development of the head and trunk mesoderm in the dogfish, *Scyliorhinus torazame*. II. Comparison of gene expressions between the head mesoderm and somites with reference to the origin of the vertebrate head. Evol Dev 14: 257–276

Ayers H, Jackson CM (1900) Morphology of the myxinoidei. I. Skeleton and musculature. J Morph 17: 185–226

Beverdam A, Merlo GR, Paleari L, Mantero S, Genova F, Barbieri O,

Janvier P, Levi G (2002) Jaw transformation with gain of symmetry after *Dlx5*/*Dlx6* inactivation: Mirror of the past? Genesis 34: 221–227

Bujor MP (1891) Contribution a l'étude métamorphose de l'ammocoetes branchialis en *Petromyzon planeri*. Rev Biol N Fr: 3474–3486 (in French)

Cerny R, Cattell M, Sauka-Spengler T, Bronner-Fraser M, Yu F, Medeiros DM (2010) Evidence for the prepattern/cooption model of vertebrate jaw evolution. Proc Nat Acad Sci USA 107: 17262–17267

Cole FJ (1905) A monograph on the general morphology of myxinoid fishes, based on a study of *Myxine*. Part 1. The anatomy of the skeleton. Trans R Soc Edin XLI (Part III): 749–791

Couly GF, Coltey PM, Le Douarin NM (1993) The triple origin of skull in higher vertebrates: A study in quail-chick chimeras. Development 117: 409–429

Damas H (1944) Recherches sur le développement de *Lampetra fluviatilis* L. – contribution à l'étude de la cephalogénèse des vertébrés. Arch Biol Paris 55: 1–289

De Beer GR (1923) Some observations on the hypophysis of *Petromyzon* and of *Amia*. Quart J Microsc Sci 67: 257–292

De Beer GR (1937) The Development of the Vertebrate Skull. Oxford University Press, London

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 Geburststag Carl von Kupffer, Gustav Fischer, pp 220–276

Depew MJ, Lufkin T, Rubenstein JL (2002) Specification of jaw subdivisions by *Dlx* genes. Science 298: 371–373

Fontaine M (1958) Classe des cyclostomes. In "Traité de Zool. Anatomie, Systématique, Biologie Tome XIII.: Agnathes et Poissons" Ed by Grassé PP, Masson et Cie Éditeurs, Paris, pp 13–425

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

Fujimoto S, Oisi Y, Kuraku S, Ota K, Kuratani S (2013) Non-parsimonious evolution of hagfish *Dlx* genes. BMC Evol Biol 13: 15

Fürbringer M (1897) Über die spino-occipitalen Nerven der Selachier und Holocephalen und ihre vergleichende Morphologie. Festschr für Carl Gegenbaur, pp 349–788

Gaskell WH (1908) "On the Origin of Vertebrates" Longmans, Green, London and New York

Gee H (1996) "Before the Backbone: Views on the Origin of the Vertebrates" Chapman and Hall, London

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

Gillis JA, Modrell MS, Baker CVH (2013) Developmental evidence for serial homology of the vertebrate jaw and gill arch skeleton. Nat Commun 4: 1436

Goodrich ES (1909) Vertebrata Craniata. First Fascicle, Cyclostomes and Fishes. Adam and Charles Black, London

Goodrich ES (1930) Studies on the Structure and Development of Vertebrates. McMillan, London

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

Heintz A (1963) Phylogenetic aspect of myxinoids. In "The Biology of Myxine" Ed by A Brodal, R Fänge, Universitetsforlaget, Oslo, pp 9–21

Holmgren N (1946) On two embryos of *Myxine glutinosa*. Act Zool 27: 1–90

Holmgren N, Stensiö E (1936) Kranium und Visceralskelett der Akranier, Cyclostomen und Fische. In: "Handbuch der vergleichenden Anatomie der Wirbeltiere" Ed by Bolk L, Göppert E, Kallius E, Lubosch W, Urban & Schwarzenberg, Berlin und Wien, Bd 4, pp 233–499

Homma S (1978) Organization of the trigeminal motor nucleus before and after metamorphosis in lampreys. Brain Res 140: 33–42

Horigome N, Myojin M, Hirano S, Ueki T, Aizawa S, Kuratani S (1999) Development of cephalic neural crest cells in embryos of *Lampetra japonica*, with special reference to the evolution of the jaw. Dev Biol 207: 287–308

Janvier P (1981) The phylogeny of the Craniata, with particular reference to the significance of fossil "agnathans". J Vert Paleontol 1: 121–159

Janvier P (1996) "Early Vertebrates" Clarendon Press, Oxford

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

Jørgensen J, Lomholt JP, Weber RE, Malte H (1998) "The biology of hagfishes" Chapman and Hall, Cambridge

Johnels AG (1948) On the development and morphology of the skeleton of the head of *Petromyzon*. Acta Zool 29: 140–279

Kaensche CC (1890) Beiträge zur Kenntniss der Metamorphose des *Ammocoetes branchialis* in *Petromyzon.* 2 Heft, in Sgchneider's Zool, Beitr

Khonsari RH, Li B, Vernier P, Northcutt RG, Janvier P (2009) Agnathan brain anatomy and craniate phylogeny. Acta Zool 90: 52–68

Koltzoff NK (1901) Entwicklungsgeschichte des Kopfes von *Petromyzon planeri*. Bull Soc Nat Moscou 15: 259–289

Koyama H, Kishida R, Goris RC, Kusunoki T (1987) Organization of sensory and motor nuclei of the trigeminal nerve in lampreys. J Comp Neurol 264: 437–448

Kupffer C von (1895) Ueber die Entwickelung des Kiemenskelets von Ammocoetes und die organogene Bestimmung des Exoderms. Ver Anat Ges 10: 105–123

Kupffer C von (1899) Zur Kopfentwicklung von *Bdellostoma*. Sitzungsber Ges Morphol Physiol 15: 21–35

Kupffer C von (1900) "Studien zur vergleichenden Entwicklungsgeschichte des Kopfes der Kranioten. 4 Heft: Zur Kopfentwicklung von *Bdellostoma*" Verlag von JF Lehmann, München und Leipzig

Kuraku S (2008) Insights into cyclostome phylogenomics: pre-2R or post-2R. Zool Sci 25: 960–968

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

Kuraku S, Takio Y, Sugahara F, Takechi M, Kuratani S (2010) Evolution of oropharyngeal patterning mechanisms involving Dlx and endothelins in vertebrates. Dev Biol 341: 315–323

Kuratani S (2012) Evolution of the vertebrate jaw from developmental perspectives. Evol Dev 14: 76–92

Kuratani S, Horigome N, Hirano S (1999) Developmental morphology of the cephalic mesoderm and re-evaluation of segmental theories of the vertebrate head: evidence from embryos of an agnathan vertebrate, *Lampetra japonica*. Dev Biol 210: 381–400

Kuratani S, Murakami Y, Nobusada Y, Kusakabe R, Hirano S (2004) Developmental fate of the mandibular mesoderm in the lamprey, *Lethenteron japonicum*: comparative morphology and development of the gnathostome jaw with special reference to the nature of trabecula cranii. J Exp Zool (Mol Dev Evol) 302B: 458–468

Kuratani S, Adachi N, Wada N, Oisi Y, Sugahara F (2012) Developmental and evolutionary significance of the mandibular arch and prechordal/premandibular cranium in vertebrates: revising the heterotopy scenario of gnathostome jaw evolution. J Anat 222: 41–55

Ladher RK, Wright TJ, Moon AM, Mansour SL, Schoenwolf GC (2005) FGF8 initiates inner ear induction in chick and mouse. Genes Dev 19: 603–613

Langille RM, Hall BK (1988) Role of the neural crest in development of the trabeculae and branchial arches in embryonic sea lamprey, *Petromyzon marinus* (L). Development 102: 301–310

Lindstrom T (1949) On the cranial nerves of the cyclostomes with special reference to N. trigeminus. Act Zool Stockh 30: 315–458

Mallatt J, Sullivan J (1998) 28S and 18S rDNA sequences support the monophyly of lampreys and hagfishes. Mol Biol Evol 15: 1706–1718

Marinelli W, Strenger A (1954) Vergleichende Anatomie und Morphologie der Wirbeltiere. 1. *Lampetra fluviatilis*. Franz Deuticke, Wien

Marinelli W, Strenger A (1956) Vergleichende Anatomie und Morphologie der Wirbeltiere. 2. *Myxine glutinosa*. Franz Deuticke, Wien

McCauley DW, Bronner-Fraser M (2003) Neural crest contributions to the lamprey head. Development 130: 2317–2327

Miller CT, Yelon D, Stainier DY, Kimmel CB (2003) Two endothelin 1 effectors, hand2 and bapx1, pattern ventral pharyngeal cartilage and the jaw joint. Development 130: 1353–1365

Minoux M, Rijli FM (2010) Molecular mechanisms of cranial neural crest cell migration and patterning in craniofacial development. Development 137: 2605–2621

Monks DC, Morrow BE (2012) Identification of putative retinoic acid target genes downstream of mesenchymal *Tbx1* during inner ear development. Dev Dyn 241: 563–573

Müller J (1834) Vergleichende Anatomie der *Myxinoiden*, der Cyclostomen mit durchbohrtem Gaumen. Osteologie und Myologie. Abhandl königl Akad Wiss, Berlin, pp 65–340

Müller J (1839) Vergleichende Anatomie der *Myxinoiden*. Dritte Fortsetzung. Über das Gefässystem. Abhandl königl Akad Wiss: 175–303

Neidert AH, Virupannavar V, Hooker GW, Langeland JA (2001) Lamprey *Dlx* genes and early vertebrate evolution. Proc Natl Acad Sci USA 98: 1665–1670

Nestler K (1980) Anatomie und Entwicklungsgeschichte von *Petromyzon planeri*. Arch Naturgesch Jahrg 1: 81–112

Neumayer L (1938) Die Entwicklung des Kopfskelettes von *Bdellostoma*. St. L. Arch Ital Anat Embryol 40 Suppl: 1–222

Newth DR (1956) On the neural crest of the lamprey embryo. J Embryol Exp Morphol 4: 358–375

Nishizawa H, Kishida R, Kadota T, Goris RC (1988) Somatotopic organization of the primary sensory trigeminal neurons in the hagfish, *Eptatretus burgeri*. J Comp Neurol 267: 281–295

Noden DM (1988) Interactions and fates of avian craniofacial mesenchyme. Development 103: 121–140

Oisi Y, Ota KG, Fujimoto S, Kuratani S (2013) Craniofacial development of hagfishes and the evolution of vertebrates. Nature 493: 175–180

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

Ota KG, Kuratani S (2006) History of scientific endeavours towards the hagfish embryology. Zool Sci 23: 403–418

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

Parker KW (1883a) On the skeleton of the marsipobranch fishes. Part I. The Myxinoids (*Myxine*, and *Bdellostoma*). Phil Trans R Soc Lond 174: 373–409

Parker KW (1883b) On the skeleton of the marsipobranch fishes. Part II. *Petromyzon*. Phil Trans R Soc Lond 174: 411–457

Rijli FM, Mark M, Lakkaraju S, Dierich A, Dollé P, Chambon P (1993) Homeotic transformation is generated in the rostral branchial region of the head by disruption of *Hoxa-2*, which acts as a selector gene. Cell 75: 1333–1349

Ronan M (1988) The sensory trigeminal tract of Pacific hagfish. Brain Behav Evol 32: 169–180

Sato T, Kurihara Y, Asai R, Kawamura Y, Tonami K, Uchijima Y, et al. (2008) An endothelin-1 switch specifies maxillomandibular identity. Proc Nat Acad Sci USA 105: 18806–18811

Schaffer J (1897) Bemerkungen über die Histologie und Histogenese des Knorpels der Cyclostomen. Archiv Mikroskop Anat 50: 170–188

Sewertzoff A (1897) Beitrag zur Entwickelungsgeschichte des Wirbeltierschädels. Anat Anz 13: 409–425

Sewertzoff AN (1901) Entwicklungsgeschichte des Kopfes von *Petromyzon planeri*. Bull Soc Imp Nat Moscou 15: 259–589

Sewertzoff AN (1916) Etudes sur l'évolution des Vertébrés inferieurs. I Arch Russ Anat Hisol Embryol I: pp 1–104

Shigetani Y, Sugahara F, Kawakami Y, Murakami Y, Hirano S, Kuratani S (2002) Heterotopic shift of epithelial-mesenchymal interactions in vertebrate jaw evolution. Science 296: 1316–1319

Song J, Boord RL (1993) Motor components of the trigeminal nerve and organization of the mandibular arch muscles in vertebrates. Phylogenetically conservative patterns and their ontogenetic basis. Act Anat 148: 139–149

Stensiö EA (1927) "The Downtonian and Devonian Vertebrates of Spitsbergen. Part 1: Family Cephalaspidea" Arno Press, New York

Stensiö EA (1932) "The cephalaspids of Great Britain" British Museum (Natural History), London

Stockard CR (1906) The development of the mouth and gills in *Bdellostoma stouti*. Am J Anat 5: 481–517

Stockard CR (1909) The embryonic history of the lens in *Bdellostoma stouti* in relation to recent experiments. Am J Anat 6: 511–515

Strahan R (1958) The velum and the respiratory current of *Myxine*. Act Zool 39: 1–14

Strahan R (1960) Speculations on the evolution of the agnathan head. In "Proceedings of the Centenary and Bicentenary Congress of Biology" Ed by RD Purchon, Malaya University Press, Singapore, pp 83–94

Takechi M, Adachi N, Hirai T, Kuratani S, Kuraku K (2013) The Dlx genes as clues for vertebrate genomics and craniofacial evolution. Sem Cell Dev Biol 24: 110–118

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

Takio Y, Pasqualetti M, Kuraku S, Hirano S, Rijli FM, Kuratani S (2004) Lamprey *Hox* genes and the evolution of jaws. Nature OnLine 429:1 p following 262; http://www.nature.com/cgi-taf/DynaPage.taf?file=/nature/journal/v429/n6989/full/nature02616_fs.html

Wada N, Nohno T, Kuratani S (2011) Dual origins of the prechordal cranium in the chicken embryo. Dev Biol 356: 529–540

Yalden DW (1985) Feeding mechanisms as evidence for cyclostome monophyly. Zool J Linn Soc 84: 291–300

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