A New Mechanistic Scenario for the Origin and Evolution of Vertebrate Cartilage

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Abstract

The appearance of cellular cartilage was a defining event in vertebrate evolution because it made possible the physical expansion of the vertebrate “new head”. Despite its central role in vertebrate evolution, the origin of cellular cartilage has been difficult to understand. This is largely due to a lack of informative evolutionary intermediates linking vertebrate cellular cartilage to the acellular cartilage of invertebrate chordates. The basal jawless vertebrate, lamprey, has long been considered key to understanding the evolution of vertebrate cartilage. However, histological analyses of the lamprey head skeleton suggest it is composed of modern cellular cartilage and a putatively unrelated connective tissue called mucocartilage, with no obvious transitional tissue. Here we take a molecular approach to better understand the evolutionary relationships between lamprey cellular cartilage, gnathostome cellular cartilage, and lamprey mucocartilage. We find that despite overt histological similarity, lamprey and gnathostome cellular cartilage utilize divergent gene regulatory networks (GRNs). While the gnathostome cellular cartilage GRN broadly incorporates Runx, Barx, and Alx transcription factors, lamprey cellular cartilage does not express Runx or Barx, and only deploys Alx genes in certain regions. Furthermore, we find that lamprey mucocartilage, despite its distinctive mesenchymal morphology, deploys every component of the gnathostome cartilage GRN, albeit in different domains. Based on these findings, and previous work, we propose a stepwise model for the evolution of vertebrate cellular cartilage in which the appearance of a generic neural crest-derived skeletal tissue was followed by a phase of skeletal tissue diversification in early agnathans. In the gnathostome lineage, a single type of rigid cellular cartilage became dominant, replacing other skeletal tissues and evolving via gene cooption to become the definitive cellular cartilage of modern jawed vertebrates.

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Introduction

A defining feature of the craniate subphylum (vertebrates and hagfish) is the “New Head”, which has been linked to the evolution of active predation in the vertebrate lineage[1]. The vertebrate head is a composite structure consisting of paired sense organs, an expanded central nervous system, peripheral ganglia, and a muscular, pumping pharynx. These components are physically supported and protected by cellular cartilage, which is replaced by bone in osteichthian vertebrates.

While it can be considered a vertebrate-specific morphological unit, most components of the vertebrate head have clear invertebrate antecedents. For instance, the basic organization of the vertebrate brain has deep roots in the neural tube of invertebrate chordates [2,3], while the paired sense organs and cranial ganglia appear to be derived from evolutionarily ancient cranial placodes and sensory cells[4,5,6]. Similarly, the vertebrate pharynx, though modified for pumping water over the gills and capturing prey, retains the basic respiratory and feeding functions of the pharynx in all deuterostomes.

Although much of the vertebrate head likely evolved via the reorganization and augmentation of simpler precursors, the origin of cellular cartilage is less clear. Invertebrates appear to lack any tissue displaying the combination of morphological and biochemical properties that defines vertebrate cellular cartilage. In addition, comprehensive analysis of gene expression in amphioxus, a basal chordate, suggests that no single invertebrate cell type coexpresses all, or most, of the genes needed to drive cellular cartilage formation[7]. Rather, individual components of the vertebrate cartilage gene regulatory network (GRN) are expressed in different tissues, most of which are mesoderm-derived.

On the other side of the invertebrate/vertebrate transition, all extant vertebrates appear to possess bona fide cellular cartilage, with no tissue constituting an obvious evolutionary intermediate. Consistent with this, work from mouse, zebrafish, and frog suggests that vertebrate cellular cartilage development is mediated by a tightly conserved GRN[8,9,10,11,12,13,14,15,16]. In the head, the vertebrate cartilage GRN is initiated in migrating cranial neural crest cells (CNCC) by the transcription factors SoxE, SoxD, Twist, ifp2, Ets, and Id. After migration, CNCCs activate markers of nascent chondrocytes, including Barx[9,17], Runx[10,18,19,20,21], and Alx/Car1[22,23,24,25]transcription factors. Barx and Runx then work with SoxE and SoxD to drive cartilage
differenciation, partly by activating expression of the structural proteins Col2a1 (fibrillar collagen) [26,27,28] and Aggrecan [29], one of several chondroitin sulfate proteoglycans (CSPGs) expressed in vertebrate cartilage. In the trunk, a similar GRN is activated in mesodermal cells, though its initiation involves the transcription factors Bmp and Pax1/9 [30,31,32]. Conservation of the cartilage GRN in neural crest and trunk mesoderm, and the presence of pharyngeal skeletons in invertebrate deuterostomes and fossil chordates, suggest that cellular cartilage first arose in the pharynx and later expanded into the head and trunk [1,33,34,35,36].

Classical and modern studies suggest that the core features of cellular cartilage development are conserved in the most basal extant vertebrates, the jawless agnathans. The branchial basket cartilage of the agnathan lamprey possesses all of the diagnostic histological and biochemical properties of gnathostome cellular cartilage including stack-of-coins and polygonal morphology, alca-in-blue reactivity and fibrillar collagen expression [37,38,39,40,41,42,43,44]. Modern ablation and vital dye labeling show that both gnathostome and lamprey pharyngeal cartilage is derived from cranial neural crest cells (CNCCs) [45,46]. Furthermore, lamprey CNCCs coexpress many components of the gnathostome cartilage GRN, including *fgfr2*, *Id*, *Twist*, *Eis*, *SoxD*, and *SoxE* [39,47,48,49,50].

While lamprey branchial basket cartilage is likely homologous to definitive gnathostome cellular cartilage, different regions have different properties. The “hard cartilage” [42,43,51] in the dorsal portion of the branchial basket skeleton consists of disorganized polygonal chondrocytes and expresses an elastin-like molecule called Lamprin [37,52,53]. In the branchial and hypobranchial bars, discoidal chondrocytes expressing fibrillar collagen and elastin generate so-called “soft cartilage” [38,42,43,54]. Lamprey also possesses skeletal tissues with no clear relationship to gnathostome cartilage. Lamprey “mucocartilage” is the main skeletal tissue in the ventral pharynx and oral region [42,43,55]. While biochemically similar to definitive cellular cartilage [42,51], mucocartilage is histologically distinct, consisting of scattered mesenchymal cells embedded in a mucopolysaccharide matrix [42,55].

To better understand the evolutionary relationships between agnathan skeletal tissues and gnathostome cartilage, we are analyzing the expression of gnathostome cartilage GRN components in the sea lamprey *Petromyzon marinus*. Here we describe the expression of lamprey homologs of three key regulators of gnathostome cellular cartilage in *Petromyzon marinus*. We observed a lack of *SoxE1* expression at st. 25, and strong expression in the forming branchial bars at st. 26.5, indicating that *SoxE1* is temporarily down-regulated sometime between st. 23 and st. 25 and reactivated at st. 26 [Figure 1A–G]. At st. 28, expression of *SoxE1* in the branchial basket was reduced (Figure 1C,H), in particular in the middle of the vertical branchial bars where cartilage differentiation is first detectable by alcal blue staining [37]. Also at st. 28, we observed broad staining throughout the pharynx including the mesenchyme of the ventral pharynx and oral region. This staining continued until st. 30, when *SoxE1* expression in the branchial bar cartilage was undetectable (Figure 1D).

**Results and Discussion**

SoxE expression, type A fibrillar collagen expression, and alcal blue staining support common evolutionary and developmental origins for mucocartilage and definitive cellular cartilage

In gnathostomes, the *SoxE* transcription factor, *Sox9*, is a marker of CNCC and pre-chondrocytes [56]. Gnathostome Col2a1 is a molecular marker for nascent and differentiated cellular cartilage that is directly regulated by *Sox9* [26,57,58,59]. Alcal blue binds the chondroitin sulfate proteoglycans (CSPGs, i.e. Aggrecan) secreted by differentiated cellular cartilage, and is considered diagnostic for this tissue [60]. Together, the combined expression of these molecules is used to monitor the development of cellular cartilage in gnathostomes. To obtain a more complete view of lamprey cartilage development, we examined *SoxE* expression, type A fibrillar collagen expression, and alcal blue reactivity at several time points during chondrogenesis in lamprey (Figure 1).

Previous reports have shown strong early expression of the lamprey *SoxE* paralogs, *SoxE1*, *SoxE2*, and *SoxE3* in premigratory and early migrating neural crest (Tahara st. 21-23) [61] and expression in the nascent branchial basket (st. 26) [38,39,40,47]. However, expression during chondrogenesis (st. 26.5-30+), and in early post-migratory CNCC (st. 25) has not been reported.

We observed a lack of *SoxE1* expression at st. 25, and strong expression in the forming branchial bars at st. 26.5, indicating that *SoxE1* is temporarily down-regulated sometime between st. 23 and st. 25 and reactivated at st. 26 [Figure 1A–G]. At st. 28, expression of *SoxE1* in the branchial basket was reduced (Figure 1C,H), in particular in the middle of the vertical branchial bars where cartilage differentiation is first detectable by alcal blue staining [37]. Also at st. 28, we observed broad staining throughout the pharynx including the mesenchyme of the ventral pharynx and oral region. This staining continued until st. 30, when *SoxE1* expression in the branchial bar cartilage was undetectable (Figure 1D).

The loss of *SoxE1* in the branchial bar chondrocytes at st. 30 was coincident with their differentiation as revealed by alcal blue staining (Figure 1I). At 35 days post fertilization (approximately 13 days after st. 30), alcal blue staining revealed the deposition of CSPGs in the fully differentiated branchial basket as well as in the mucocartilage of the oral region (upper lip, first pharyngeal arch, lower lip) and ventral pharynx (Figure 1E, J).

Expression of lamprey fibrillar collagen in the forming branchial basket at select time points (st. 25, st. 26) has been described [38,39,62]. We replicated and expanded upon these studies, looking at Col2a1a expression at key stages before, during, and after chondrogenesis. As previously reported, we observed broad Col2a1a expression in post-migratory cranial neural crest cells and somitic mesoderm at st. 25 (Figure 1K). While Col2a1a expression is temporarily downregulated at this stage, cranial neural crest expression of *SoxE1* and *SoxE2* is seen earlier at st. 23 and st. 24, consistent with Col2a1 regulation by *SoxE* in CNCC as seen in gnathostomes. At st. 26.5, strong Col2a1a expression is seen in presumptive mucocartilage in the ventral pharynx, upper and lower lips, first pharyngeal arch, and in dorsal fin mesenchyme (Figure 1L,M). Strong Col2a1a expression is also observed in a horizontal band of cells dorsal to the pharyngeal arches at the position of the nascent subchondral cartilage bars. Negligible expression was seen in the forming branchial bars at this stage. At
st. 28 strong expression of Col2a1a was detected throughout the mucocartilage around the mouth, with weaker expression in branchial basket cartilage (Figure 1N). Similar expression was seen later at 35 d.p.f. (Figure 1O).

Our results show SoxE1 expression is high in post-migratory pharyngeal CNCC, where it is co-expressed with Col2a1, and is then downregulated as these cells differentiate into cellular cartilage. This sequence mirrors the down-regulation of Sox9 seen in differentiating gnathostome cartilages and supports conserved roles for SoxE in lamprey branchial basket cartilage and gnathostome cellular cartilage development as previously proposed [40,47].

In addition to the branchial basket, however, we also observed SoxE1 expression and alcian blue staining throughout the developing mucocartilage of the oral apparatus and ventral pharynx. Furthermore, we found that these tissues express high levels of fibrillar collagen [38,39] as originally suggested by Schaffer[51] and Johnels[42]. Due to its staining properties and mesenchymal morphology, mucocartilage is generally considered a derived form of connective tissue unrelated to cellular cartilage[55]. However, co-expression of fibrillar collagen, SoxE, and CSPGs in mucocartilage suggest some developmental and/or evolutionary relationship between mucocartilage and cellular cartilage. Hardisty[44] proposed that mucocartilage may represent an undifferentiated embryonic tissue that is carried over into the larval stage. An alternate hypothesis is that mucocartilage represents an evolutionary precursor to bona fide cellular cartilage. We decided to further investigate these possibilities by looking at the expression of three other key regulators of vertebrate chondrogenesis; Runx, Barx, and Alx/Cart1, in the lamprey head skeleton.

Runx genes are expressed in lamprey mucocartilage, but not in the branchial basket cartilage

Runx genes are key regulators of both cartilage and bone development in jawed vertebrates. During embryogenesis all three gnathostome Runx paralogs, Runx1,2,3[18] are expressed in presumptive chondrocytes, and Runx2 and Runx3 have been shown to be required for their differentiation into cellular cartilage[10,20,21]. After chondrogenesis, Runx genes regulate the formation of replacement cartilage and the ossification of endochondral bone[63]. Functional studies suggest Runx genes are

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Figure 1. Expression of SoxE1 and fibrillar collagen, and alcian blue staining during chondrogenesis in lamprey larvae. A) SoxE1 expression is not observed in post-migratory CNCC at st. 25. B) Strong, specific expression of SoxE1 in the nascent branchial basket cartilage at st. 26.5. C) Expression of SoxE1 in the branchial basket cartilage and broadly in the head mesenchyme at st. 28. Compared to st. 26.5, SoxE1 expression in the branchial bar cartilage is reduced (arrowhead in inset, showing the pharyngeal arch indicated by the asterisk). D) At st. 30, SoxE1 expression is detectable throughout the head mesenchyme, but has been downregulated in the branchial basket cartilage. E) Alcian blue staining at 35 days post-fertilization (d.p.f) in the branchial basket and the mucocartilage of the upper lip, oral region, and ventral pharynx as well as the dorsal fin. Strong staining is also seen in the notochord (arrowhead). F) Frontal section at the level of f in B showing SoxE1 expression in the nascent branchial basket cartilage and weakly in the CNCC medial to the pharyngeal mesoderm (arrow). G) Ventral view of B showing expression in the mucocartilage around the mouth. H) Ventral view of C. I) Alcian blue staining at st. 30 in the differentiated branchial basket cartilage, notochord, and weakly in the nascent mucocartilage (arrow). J) Ventral view of E showing alcian blue reactivity in the mucocartilage of the ventral pharynx. K) Expression of the lamprey fibrillar collagen gene Col2a1a at st. 25. Expression is seen in the somites (arrow) and post-migratory neural crest cells in the pharynx (arrowhead). L) Col2a1a expression at st. 26.5 throughout the head mesenchyme including presumptive mucocartilage in the ventral pharynx and around the mouth, and in the presumptive subchordal cartilages(arrow). Minimal expression is seen in the vertical branchial bars. Col2a1a mRNA is also present in the dorsal fin (arrowhead) M) Ventral view of L showing mucocartilage expression. N) Broad expression of Col2a1a throughout the pharynx and oral region including presumptive mucocartilage and branchial basket cartilage at st. 30. O) At 35 d.p.f. Col2a1a is still broadly expressed the head skeleton.

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regulated by both Sox9 and Barx genes and are downstream of these factors in the cartilage GRN[9,64].

We performed an exhaustive search of *P. marinus* pre-assemble genome and identified several contigs with similarity to the four most highly conserved deuterostome Runx exons. For three of these exons, we found two distinct aligning lamprey sequences encoding different proteins, suggesting the presence of two lamprey Runx genes (Figure S1, S2). While it is possible that the preassembly *P. marinus* genome does not include all Runx genes, the level of coverage, and the presence of two Runx genes in hagfish, a related agnathan, suggest this is the full complement of lamprey Runx genes. These genes were designated RunxA and RunxB. RunxA expression was first observed at Tahara st. 25 in the presumptive cranial ganglia and mesenchyme flanking the mouth (Figure 2A). By st. 26.5, the onset of chondrogenesis, expression was seen in the pharyngeal endoderm, cranial ganglia, lateral oral mesenchyme, and intermediate first arch (Figure 2B,E,F). Spots of expression were also observed in the neural crest core of the pharyngeal arches (Figure 2E). Expression in the cranial ganglia, upper lip, first arch, pharyngeal endoderm and pharyngeal mesoderm was still detectable at st. 27, when cartilage is histologically identifiable by alcian blue staining (Figure 2C,G). By st. 30, when most of the branchial basket cartilage has differentiated, RunxA was still detectable in the pharyngeal endoderm and cranial ganglia (Figure 2D,H). RunxB deployment was much less extensive at early larval stages, marking bilateral spots in the anterior neural tube and cranial ganglia at st. 25 and 26.5 (Figure 2L,J). At st. 27 additional expression was observed in the dorsal fin mesenchyme, around the heart, and in the endosteal (Figure 2K). At st. 30 strong expression was observed in the dorsal fin and around the heart, with reduced expression in the brain (Figure 2L). By 35 d.p.f. weak expression of both RunxA and RunxB was seen in the pharyngeal endoderm. (Figure S5).

We then examined Runx, SoxE1, and Col2a1a expression in pre-metamorphic ammocoete larvae and recently metamorphosed juvenile lampreys, to determine if these genes are activated in cellular cartilage around the time of metamorphosis. While we did see expression of Col2a1a in the branchial bars (Figure 3B), we observed no specific signal in any pharyngeal tissue at these stages with either the RunxA (not shown) or RunxB riboprobes (Figure 3G). Similarly, SoxE1 expression above background levels was not seen in pre- or post-metamorphic branchial basket cartilage, though specific expression was seen in the gills, notochord, and a subset of cells in the spinal cord (Figure 3C,H).

Taken together, we detected no lamprey RunxA or RunxB expression in branchial basket cartilage at any stage, either before or after metamorphosis. While it is formally possible that an unidentified lamprey Runx paralog is expressed in lamprey branchial basket cartilage, we view this as unlikely. The apparent lack of Runx expression in lamprey cartilage contrasts with gnathostomes where Sox9 and all three Runx genes are broadly co-expressed and interact extensively to regulate chondrogenesis. Assuming RunxA and RunxB represent the full complement of lamprey Runx genes, this difference implies either that Runx lost its ancestral function in cartilage development in the lamprey lineage, or that Runx genes were not part of the ancestral vertebrate cartilage GRN.

Recent work using qPCR on adult tissue suggests enrichment of Runx transcripts in the cartilage of adult hagfish, leading to speculation that Runx genes drive skeletogenesis in these agnathans[65]. Hagfish have historically been considered basal jawless craniates, but modern molecular phylogenies support their grouping with lampreys in a single agnathan clade[66]. If Runx is in fact required for chondrogenesis in hagfish, it would support the lineage-specific loss of Runx from the lamprey cartilage GRN.

Alternately, Runx expression in adult hagfish could reflect a function in adult tissue maintenance or metabolism unrelated to cartilage specification during development. In jawed vertebrates, Runx genes perform a wide range of functions in embryonic and adult tissues. Gnathostome Runx genes are downregulated in differentiated adult cartilage and upregulated in a variety of other tissues including skin, liver, intestine, thyroid and blood where they

![Figure 2. Expression of RunxA and RunxB in lamprey larvae.](image-url)

A) Localized expression of RunxA at st. 25 in oral mesenchyme and nascent cranial ganglia. B, C) Expression of RunxA in cranial ganglia (arrow), the intermediate first arch mesenchyme (arrowhead), pharyngeal endoderm, pharyngeal mesoderm, and oral mesenchyme at st. 26.5 and st. 27. D) Expression of RunxA in pharyngeal endoderm at st. 30. E) Section at the level of e in B showing RunxA expression in the pharyngeal pouch endoderm (arrowhead), pharyngeal mesoderm (arrow), and intermediate first arch mesenchyme (black arrowhead). F) Ventral view of B showing strong expression in the mesenchyme flanking the mouth. G) Ventral view of C. H) Ventral view of D. I) A spot of RunxB expression is seen in the nascent cranial ganglia at st. 25. J,K) Expression of RunxB in the brain and cranial ganglia at st. 26.5 and st. 27. At st. 27, weak expression is also visible around the heart, in the endostyle (arrowhead), and in the dorsal fin. L) At st. 30, strong expression of RunxB is seen in the dorsal fin mesenchym and around the heart, with weaker staining in the brain and pharyngeal endoderm.
regulate cell division and stem cell quiescence [19,67,68,69]. Consistent with a general role in adult tissue maintenance, hagfish Runx transcripts were found in every hagfish tissue examined, with slightly higher levels in hard cartilage[65]. In either case, the lack of detectable enrichment of lamprey Runx mRNA in branchial basket cartilage indicates Runx genes are dispensable for the development of histologically discernable cellular cartilage. We propose this reflects the basal vertebrate state, but it could formally represent the presence of a derived chondrogenic GRN specific to lampreys. Runx expression during cartilage development in hagfish embryos would help distinguish between these two scenarios.

While lamprey Runx expression was not observed in the definitive cellular cartilage of the branchial basket, strong, localized Runx1 expression was observed in subpopulations of nascent mucocartilage around the mouth and in the intermediate portion of the first arch. This restricted expression suggests lamprey Runx genes may be performing tissue-specific functions in the development of particular kinds of mucocartilage, rather than acting as general drivers of skeletogenesis as they are in gnathostomes.

Other domains of Runx expression included the brain, cranial ganglia, and pharyngeal endoderm in early larvae (st. 25–26.5) and the heart, and dorsal fin mesenchyme in late larvae (st. 27–30). Expression of Runx genes is observed in the cranial ganglia of all gnathostomes examined, indicating a deeply conserved role for Runx in the development of these structures[18,21]. Expression in the dorsal fin may be related to a skeletogenic function as these cells also express SoxD and fibrillar collagen (Figure 1)[58], and react with with alcian blue (Figure 1E).

An endodermal specification function for Runx genes appears to be evolutionarily ancient as gnathostomes, amphioxus, sea urchin, and C. elegans all express Runx in the nascent gut, and Runx is required for gut formation in C. elegans [7,67,70]. Runx expression in the pharyngeal endoderm of adult amphioxus has been interpreted as evidence of a rudimentary skeletogenic program in this tissue[63]. Given the pleiotropic nature of Runx function, and the pan-metazoan expression of Runx genes in endoderm, it seems equally likely that Runx genes are performing some other function in this tissue.

**Lamprey Barx** is expressed in lower lip mucocartilage and CNCC in the medial pharyngeal arches, but not in developing branchial bar cartilage

Recent work has shown that Barx is required for the differentiation of CNCC-derived cartilage in the zebrafish head where it is downstream of FGF signaling and regulates Runx expression[9]. Similar broad expression of Barx in the CNCC of chick and mouse suggest this role is conserved among all gnathostomes[17,71]. In addition, functional studies in mouse have demonstrated an essential role for Barx in the chondrogenesis of the mesoderm-derived appendicular skeleton[27].

We isolated a lamprey Barx ortholog, and described its expression in the first pharyngeal arch at st. 26.5 in a previous study[72]. Here we detail lamprey Barx expression from embryonic through late larval stages in all tissues. Barx transcripts are first weakly detectable in a stream of CNCCs migrating into the region of the first pharyngeal arch at st. 24 (data not shown). At st. 25, Barx expression is seen in the lower lip and the forming pharyngeal arches (Figure 4A). At st. 26.5 and 27, strong Barx expression is seen in the lower lip, extending dorsally into the intermediate region of the first pharyngeal arch as previously described (Figure 4B,F,G). Expression is also seen in presumptive CNCC-derived mesenchyme positioned medial to the pharyngeal arch mesoderm in the posterior arches (Figure 4E).

These domains of expression persist into st. 30 when chondrogenesis of the branchial bars is complete (Figure 4D,H). Similar expression is still apparent at 35 d.p.f (Figure S5). No Barx expression was seen in the pharynx of pre-metamorphic ammocoete larvae (Figure 3E) or post-metamorphic juveniles (not shown).

As with Runx, we noted an absence of Barx expression in the laterally-positioned pharyngeal CNCCs which give rise to the
We found multiple contigs corresponding to a single Alx gene in
the P. marinus genome (Figure S1, S2), suggesting it is the only
lamprey Alx homolog. We then examined its expression in
embryos and larvae during cartilage development. Lamprey Alx
expression was first seen at st. 23.5 in a spot of mesenchyme in the
forming upper lip (data not shown). This expression expanded and
intensiﬁed to ﬁll the mesenchyme of the upper lip at st. 25
(Figure 5A). Additional expression was seen in the dorsal and
ventral aspects of the forming pharyngeal arches and in the
forming dorsal ﬁn. By st. 26.5, expression was seen in the
mesenchyme of the dorsal and ventral pharyngeal arches, upper
lip, and dorsal ﬁn (Figure 5B,F). Expression was excluded from the
lower lip mesenchyme and the intermediate portion of the
pharyngeal arches. This expression resolved into distinct dorsal
and ventral spots in each arch at st. 27 (Figure 5C,G). By st. 30, Alx
expression in the pharynx had diminished, but was still detectable
in the ﬁrst arch and subchondral chondrocytes (Figure 5D,H). This
expression pattern persisted until 35 d.p.f. (Figure S3), though no
Alx expression was detectable in the pharynx of ammocoetes
(Figure 3D) or metamorphosed juveniles (not shown).

In gnathostomes, SoxE and Alx genes are co-expressed in CNCC-
derived chondrogenic mesenchyme[25]. While no regulatory
relationship between the two factors has been demonstrated, Sox9
and Alx3/4 double mutant mice have similar hypoplastic skeletal
phenotypes, suggesting they operate within the same cartilage

definitive cellular cartilage of the gill bars. Thus, like Runx, Barx is
likely not directly involved in the development of lamprey cellular
cartilage, whereas it is required for deﬁnitive cartilage formation in
gnathostomes.

Although Barx expression did not mark CNCC in the lateral
pharynx, we did observe Barx expression in CNCC medial to the
mesodermal core of each pharyngeal arch. These cells express
other markers of CNCC including fgf2, Dlx, and SoxE, as well as
fibrillar collagen[39,72]. However, they do not react with alcian
blue or correspond to histologically recognizable mucocartilage. In
lamprey, this region of the pharyngeal arches gives rise to the
smooth muscle of the branchial veins and arteries [43], tissue
which is CNCC-derived in gnathostomes. We speculate that
lamprey Barx expression in the medial portion of the pharyngeal
arches marks CNCC fated to form the branchial vasculature.

Lamprey Alx marks upper lip mesenchyme and portions of
the branchial basket cartilage.

Gnathostomes possess three Alx paralogs, Alx3, Alx4, and Car1.1.
The expression and function of these factors during cartilage
development have been studied almost exclusively in mouse,
where all three paralogs mark chondrogenic mesenchyme in the
frontonasal mass, pharyngeal arches, and limb buds[25,73].
Consistent with their expression patterns, double knock-outs of
Alx3 and Alx4 result in severe reductions in the craniofacial and
appendicular skeletons[22]. Among invertebrate deuterostomes,
Alx is required for skeletal development in sea urchin[74], and
amphioxus Alx is expressed in pharyngeal mesoderm thought to
secrete acellular cartilage[7], suggesting an ancient role for Alx
genes in skeletogenesis.

Figure 4. Expression of Barx in lamprey larvae. A) Barx transcripts
at st. 25 in the mesenchyme of the lower lip. B–D) Barx expression at st.
26.5, st. 27, and st. 30 in the lower lip (ventral ﬁrst arch), intermediate
ﬁrst arch (arrowhead), and in the posterior pharyngeal arches. E) Frontal
section at the level of e in B reveals Barx expression in the pharyngeal
arches is restricted to the CNCC medial to the pharyngeal mesoderm.
Barx transcripts are also detected in the mesenchyme of the lower lip
and the intermediate ﬁrst arch (arrowhead). F–H) Ventral views of B, C,
and D, showing expression in the mucocartilage of the lower lip and in
the medial aspect of the pharyngeal arches.
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Figure 5. Expression of Alx in lamprey larvae. A) Strong Alx
expression in the upper lip at st. 25, with weaker expression in the
dorsal (arrowhead) and ventral aspects of the forming pharyngeal
arches. Expression is also seen in the forming dorsal ﬁn (arrow). B)
Expression of Alx in upper lip and strongly in the dorsal and ventral
aspects of the posterior (3rd–8th) pharyngeal arches. In the ﬁrst and
second arches, Alx expression is only seen dorsally (arrowheads).
Expression is also seen in the dorsal ﬁn (arrow). C) At st. 27, expression
in the dorsal and ventral aspects of the pharyngeal arches, upper lip,
and dorsal ﬁrst arch (arrowhead). Expression in the dorsal ﬁn has been
lost. D) At st. 30, Alx expression is seen in the dorsal ﬁrst arch and
weakly in the subchondral chondrocytes. E) A frontal section at the level
of e in B showing Alx expression in CNCC-derived upper lip
mesenchyme surrounding the anteriormost neural tube (arrowhead).
Expression is also seen in the dorsal portions of the ﬁrst arch (arrow)
and the posterior arches (double arrowheads). F) Ventral view of B
showing expression in the upper lip and the nascent hypobranchial
chondrocytes. G) Ventral view of C. H) Ventral view of D.
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GRN[22, 75]. Unlike their gnathostome counterparts, lamprey SoxE and Alx genes are not broadly co-expressed in nascent cellular cartilage. While lamprey SoxE marks the entire branchial basket cartilage, lamprey Alx is restricted to the dorsal and ventral aspects, and is excluded from the vertical branchial bar cartilage. Recent work has shown that the lamprey branchial basket consists of morphologically and molecularly distinct cell types arranged along its dorso-ventral (DV) axis[37, 72]. In the central portion of the pharyngeal gill bars, vertically stacked branchial bar chondrocytes display a highly ordered discoidal “stack-of-coins” morphology. Dorsally, subchordal, parachordal and trabecular chondrocytes are disorganized and polygonal in shape. Ventrally, the horizontal hypobranchial bars have a semi-ordered pseudo-discoidal morphology. Alx expression in the pharynx corresponds to the forming subchordal and hypobranchial chondrocytes. Both subchordal and hypobranchial chondrocytes likely serve as rigid structural elements, in contrast to the central discoidal chondrocytes, which form flexible bars that bend and recoil during pharyngeal pumping. It is possible that Alx expression in the lamprey branchial basket identifies a particular kind of rigid structural cartilage homologous to the cellular cartilage that comprises the bulk of the gnathostome head skeleton. Similarly, Alx expression in the upper lip mesenchyme suggests Alx may also specify a type of mucocartilage. Historical descriptions classify the skeletal tissue in the upper lip, lower lip, first pharyngeal arch, and ventral pharynx as a single kind of generic mucocartilage[42, 43, 51]. However, at the stages we examined, the skeletal tissue around the larval lamprey mouse appears compact, while the presumptive mucocartilage of the ventral pharynx forms a loose mesenchyme. Alx expression in the upper lip may confer some unique physical properties that distinguish this skeletal tissue from the loose mucocartilage in the ventral pharynx. Like Runx, and Col2a1a, we also observed Alx transcripts in the dorsal fin mesenchyme, a tissue that reacts with alcin blue (Figure 1) and is at least partially neural crest-derived [15]. Coexpression of Runx and Alx is not seen in any skeletal tissue in the head, suggesting lamprey dorsal fin mesenchyme may represent a mucocartilage-like tissue unique to the trunk.

Summary of SoxE1, Col2a1a, CSPG, Runx, Barx, and Alx expression in lamprey skeletal tissues

In sum, our results show expression of SoxE1, Col2a1a, and staining with alcin blue (a proxy for CSPG expression) in all mucocartilage and cellular cartilage of the lamprey head at early larval stages (st. 26.5–st. 30). During this period, RunxA expression was seen in a small subpopulation of mucocartilage flanking the mouth, while RunxB was observed in dorsal fin mesenchyme. Barx expression was observed in a portion of the mucocartilage in the lower lip/first arch, while Alx expression was detected in upper lip mucocartilage, two subpopulations of cellular cartilage, and dorsal fin mesenchyme. No significant overlap of RunxA, RunxB, Barx or Alx expression was seen cellular cartilage (Figure 6), or any other skeletal tissue except in the intermediate first arch (Barx and Runx) the dorsal fin mesenchyme (RunxB and Alx) (Figure 7). However, RunxA, Barx and Alx were all expressed with SoxE1 and Col2a1a in various skeletal tissues and at some point between st. 26.5 and st. 30. In pre-metamorphic ammocoete larvae and post-metamorphic juvenile lampreys, only expression of Col2a1a was detectable above background levels in branchial basket cartilage.

A hypothetical scenario for the origin and evolution of vertebrate cartilage

We are taking a molecular approach to examine possible evolutionary relationships between invertebrate skeletal tissue, gnathostome cartilage, and the skeletal tissues of lamprey. Our data show that, despite similarities with gnathostome cellular cartilage, the cellular cartilage of the lamprey branchial basket deploys a rudimentary differentiation program that does not incorporate Runx or Barx. Furthermore, we find that the flexible “stack of coins” chondrocytes of the branchial basket do not express Alx genes and are thus molecularly distinct from the rigid polygonal subchordal and hypobranchial chondrocytes (Figure 6). Finally, we find that lamprey mucocartilages, though morphologically very different from gnathostome cellular cartilage, express various combinations of genes involved in gnathostome chondrogenesis (Figures 6, 7).

Taken together, our data reveal an array of histologically and molecularly distinct skeletal tissue types in the larval lamprey head, each displaying a subset of the features seen in gnathostome cellular cartilage (Figure 7). This contrasts with the gnathostome condition in which all embryonic cellular cartilage appears to deploy a single tightly conserved chondrogenic GRN. Assuming
that the lamprey head skeleton approximates the basal vertebrate condition, our results have two significant evolutionary implications. First, they suggest that the evolution of the gnathostome head skeleton involved a marked reduction in skeletal tissue diversity. This implies that the basal vertebrate state is the possession of multiple cartilage-like tissue types, while gnathostomes display a derived dependence on a single kind of cellular cartilage. Second, our results suggest that this loss of skeletal tissue

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### Table: The Diversity of Skeletal Tissues in the Larval Lamprey Head

| Tissue type                      | Location                   | Cellular morphology | Alcian blue | Fibr. Coll. | SoxD | SoxE | Runx | Barx | Axl |
|----------------------------------|----------------------------|---------------------|-------------|-------------|------|------|------|------|-----|
| Gnathostome Cartilage            | All elements               | Discoidal, polygonal| +           | +           | +    | +    | +    | +    | +   |
| Gnathostome joint tissue         | Joints                     | Mesenchymal         | +           | ?           | ?    | +    | +    | +    | ?   |
| Cellular Cartilage (soft)        | Branchial bars 3rd-8th arches | Discoidal          | +           | +           | +    | +    | +    | +    | +   |
|                                  | Hypobranchial bars 3rd-8th arches | Irregular discoidal | +           | +           | +    | +    | +    | +    | +   |
| Cellular Cartilage              | Subchordal 3rd-8th arches  | Polygonal           | +           | +           | +    | +    | +    | +    | +   |
| Mucocartilage                   | Dorsal 1st & 2nd arches    | Mesenchymal         | +           | +           | +    | +    | +    | +    | +   |
|                                  | Intermediate 1st arch      | Mesenchymal         | +           | +           | +    | +    | +    | +    | +   |
|                                  | Ventral 1st arch (lower lip) | Mesenchymal        | +           | +           | +    | +    | +    | +    | +   |
|                                  | Intermediate & ventral 2nd arch | Mesenchymal      | +           | +           | +    | +    | +    | +    | +   |
|                                  | Ventral pharynx            | Loose mesenchyme   | +           | +           | +    | +    | +    | +    | +   |
|                                  | Upper lip                  | Mesenchymal         | +           | +           | +    | +    | +    | +    | +   |
|                                  | Lateral mouth              | Mesenchymal         | +           | +           | +    | +    | +    | +    | +   |
| Undescribed (smooth muscle?)    | Medial pharyngeal arches (2nd-8th) | Mesenchymal     | +           | +           | +    | +    | +    | +    | +   |
| Dorsal fin skeleton              | Dorsal fin                 | Mesenchymal         | +           | +           | +    | ?    | +    | +    | +   |

Figure 7. The diversity of skeletal tissues in the larval lamprey head. Based on morphology and gene expression, we speculate that lamprey larvae may possess as many as 11 distinct types of skeletal tissue. This includes 3 kinds of cellular cartilage, 7 kinds of mucocartilage, and a mucocartilage-like skeletal tissue unique to the dorsal fin. The diagram shows the first 6 pharyngeal arches. The upper lip mesenchyme is derived from the premandibular cranial neural crest (pm). For reference, cellular morphology and gene expression in gnathostome cellular cartilage and joint tissue are shown in the shaded rows.

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duality was coincident with the consolidation of several rudimentary skeletogenic GRNs into a single chondrogenic GRN. Thus, during gnathostome evolution, gene programs operating primitively in different types of skeletal tissue became co-expressed in a single type of cellular cartilage.

We previously proposed that genetic cooption of mesodermal gene networks by the evolving cranial neural crest drove the origin of vertebrate cellular cartilage[7]. Our current results suggest similar cooption events occurred during the evolution of gnathostome cellular cartilage. Below we integrate our current and previous data and propose a stepwise mechanistic scenario for the origin and evolution of vertebrate cellular cartilage (Figure 8).

**Step 1: The origin of skeletogenic CNCC**

Our current results, and previous work, reveal a set of core features shared by all skeletogenic neural crest cells, including gnathostome cellular cartilage, lamprey cellular cartilage, and lamprey mucocartilage. These include; 1) differentiation from a mesenchymal precursor cell, 2) persistent expression of Twist, Ets, Id, and SoxE after migration into the pharynx[39,47], 3) secretion of CSPGs[37], and 4) expression of fibrillar collagen. Notably, this list does not include expression of Runx or Barx, which our data show are not general markers of neural crest-derived skeletal tissue.

Using these criteria as a minimal definition of vertebral neural crest-derived skeletal tissue, we can then ask if any protochordate tissue possesses all, or most, of these features. As has been shown previously, Twist, Ets, Id, Alx, and fibrillar collagen are coexpressed in the pharyngeal mesoderm of amphioxus larvae[7,50]. Furthermore, this tissue displays mesenchyme-like cell morphology and may give rise to acellular cartilage bars composed of CSPGs[7,50]. Thus, skeletogenic mesoderm with most attributes of skeletogenic neural crest cells, but lacking SoxE expression, was likely present in the first chordates.

The existence of migratory neuroblasts in a range of invertebrates [76,77,78] suggests that the vertebrate ancestor also possessed some kind of migratory neural tube cell. Assuming that co-expression of Twist, Ets, Id, Alx, and fibrillar collagen reflects a conserved GRN, it is possible that skeletogenic neural crest cells evolved via activation of this GRN in non-skeletogenic migratory neural tube cells. The lack of SoxE expression in amphioxus pharyngeal mesoderm further suggests this cooption involved the integration of SoxE into the evolving skeletogenic GRN. How these changes occurred is unclear. Aside from their function in skeletogenesis, vertebrate SoxE paralogs are required for the specification and differentiation of all neural crest cell lineages, as well as for the development of glia and the otic placode[79]. In addition, amphioxus SoxE is expressed throughout the nascent nervous system in early larvae, but not in any obviously skeletogenic tissues aside from a few cells in the notochord [80]. These data imply that the ancestral function of chordate SoxE was in neural development, rather than skeletogenesis, as has been proposed [65,81]. Furthermore, these results suggest that migratory neural tube cells could have acquired SoxE simply by maintaining SoxE expression after leaving the CNS. Based on these observations, we propose that skeletogenic neural crest cells evolved by activation of a mesodermal skeletogenic GRN in migratory neural tube cells expressing SoxE. (Figure 8A,B). A role for SoxE genes in skeletogenesis then evolved later in the vertebrate lineage as SoxE was integrated into this skeletogenic GRN.

The cartilage GRN in vertebrates is induced and maintained by signals secreted by the pharyngeal endoderm and ectoderm, including Hedgehogs and FGFs[82,83]. Provocatively, FGF and Hedgehog ligands are expressed in the amphioxus pharynx at larval stages when skeletogenesis is likely initiated[7,84]. We further speculate that the activation of mesodermal skeletogenic gene programs could have evolved as proto-CNCC cells gained responsiveness to these signals.

Based on Runx and SoxE co-expression in the foregut of adult amphioxus, it has been proposed that cooption of an endodermal skeletogenic GRN(5), incorporating these factors drove the appearance of skeletogenic CNCC[65,81]. However, expression of lamprey SoxE1 and Runx in non-skeletogenic pharyngeal endoderm and gills (Figure 2, Figure 3B) suggests these factors perform non-skeletogenic functions in chordate endoderm. Furthermore, our data showing that Runx is not expressed in lamprey cellular cartilage implies it is not a core feature of chordate skeletal development. Finally, the early role of SoxE genes in the initial formation of all neural crest cells, suggests its ancestral function was in neural crest cell specification rather than chondrogenesis[79]. Thus, cooption of Runx and SoxE from endoderm was likely not involved in the origin skeletogenic neural crest cells. Instead, our data support later recruitment of Runx by SoxE-expressing skeletogenic neural crest cells in the gnathostome lineage (see below).

**Step 2: The diversification of CNCC-derived skeletal tissues in early jawless vertebrates**

The “New Head” hypothesis suggests that the original function of vertebrate cartilage was to provide structural support for pharyngeal pumping[1]. The concurrent expansion of the CNS, anterior sense organs, and cranial ganglia also likely required additional skeletal support, as did the novel structural demands on the oral region of early vertebrate predators. In the first vertebrates, we posit that these various functions were performed by a generic CNCC-derived skeletal tissue expressing Twist, Id, Ets, Alx, SoxE, and fibrillar collagen, and secreting CSPGs (Figure 8B). Based on gene expression in lamprey, we further speculate that this generic CNCC-derived head skeleton then diversified, with different regions taking on specialized properties in response to particular structural demands. During this phase of vertebrate skeletal evolution, Runx, and Barx, were recruited to different parts of the CNCC-derived skeleton, and Alx, which was initially expressed in all skeletogenic CNCC, was lost from some regions. These changes in gene expression drove the establishment of several different skeletal tissue types with distinct physical properties (Figure 8C). Among these were compact tissues composed of cells with polygonal and discoidal morphologies that would be histologically identifiable as proper “cellular cartilage”, and several types of mesenchymal skeletal tissues in the head and dorsal fin. This primitive diversity of CNCC-derived skeletal cell types is thus preserved in lamprey, where Runx, Barx, and Alx genes mark different subsets of cartilage and mucocartilage around the mouth and in the pharynx (Figure 7). Localized expression of these factors would also require some level of underlying anteroposterior (AP) and dorsoventral (DV) patterning in the oro-pharyngeal region. Consistent with this, recent work suggests sophisticated gnathostome-like AP and DV patterning systems were in place before the divergence of jawed and jawless vertebrates[72,85].

**Step 3: The evolution of gnathostome cartilage**

While chondrogenic gene expression in lamprey identifies several molecularly distinct skeletal tissues (Figure 7), the ubiquitous coexpression of chondrogenic GRN components in the gnathostome head skeleton suggests the presence of only one major cartilage type. If lamprey represents the basal state, then the evolution of the gnathostome head skeleton was associated with a reduction in skeletal tissue diversity. A hallmark of the lamprey
head skeleton is the preponderance of soft, flexible skeletal tissues such as the mucocartilage of the oral region and ventral pharynx, and the discoidal “soft” cartilage of the branchial basket. This contrasts with the gnathostome condition, where uniformly rigid cartilage elements articulate at foci of soft joint tissue. Rigid “hard” cartilages have been described in lamprey based on their staining properties [42,43,51]. Interestingly, these cartilages have the polygonal morphology typical of most gnathostome cartilages and express Alx. We speculate that a similar type of specialized, polygonal, Alx-positive cartilage was present in the first vertebrates where it constituted a small portion of the head skeleton. In the gnathostome lineage, this rigid cartilage proliferated, becoming the dominant component of the head skeleton (Figure 8D). The broad expression of Runx and Barx in the gnathostome head skeleton suggests this transition also involved the expanded expression of these factors. We posit that Runx and Barx may have initially conferred novel structural properties upon this tissue. The transition to rigid structural cartilage may have been driven by the novel physical requirements of holding and processing increasingly large prey items and supporting and protecting a large CNS and sense organs. Similar selective pressures may have driven the subsequent evolution of bone.

In addition to rigid cellular cartilage, gnathostomes also possess soft joint tissue between cartilaginous elements in the head. Like cellular cartilage, these cells are CNCC-derived and initially express SoxE, Twist, Ets, and Id and stain with alcian blue [86]. However, unlike cellular cartilage, this tissue remains mesenchymal and does not express Barx and Runx [9,60]. Based on...
morphology and Gdf5/6/7 expression we previously proposed an evolutionary relationship between gnathostome joint tissue and lamprey mucocartilage in the ventral pharynx[72]. Our current results showing that mucocartilage in the ventral pharynx does not express Runx and Barx lend support this hypothesis. We speculate that gnathostome joints may have evolved by the redeployment of a primitive mucocartilage-like skeletal tissue to the regions between rigid cellular cartilage condensations (Figure 8D).

Materials and Methods

Ethics Statement

All methods were reviewed and approved by the University of Colorado, Boulder IACUC protocol 08-07-MED-02. We performed an exhaustive search of the 5.9X coverage, preassembly Petromyzon marinus genome for homologs of gnathostome Runx, Barx and Axl genes by repeated BLAST[87] searching with gnathostome, amphioxus, and sea urchin protein sequences. We then designed exact-match PCR primers for the two Runx paralogs (RunxA and RunxB), and the single Barx and Axl genes. Runx and Barx exons were PCR amplified from adult lamprey genomic DNA according to standard methods. Axl was isolated from embryonic cDNA using the GeneRacer kit (Invitrogen). A SoxE1 fragment corresponding to nucleotides 211-619 of the published SoxE1 nucleotide sequence[47] was amplified from embryonic cDNA for use as a riboprobe template.

Embryos and early larvae for in situ hybridization were obtained from adult spawning phase sea lampreys (Petromyzon marinus) as previously described[88]. 5–10 cm pre-metamorphic amnionooe larvae, and newly metamorphosed juvenile lampreys were collected from streams feeding Lake Huron in the Fall with a backpack electroshocker and kept in chilled holding tanks until fixed in MEMFA (MOPS buffer, EGTA, MgSO4, Formaldehyde). Thick cross sections of 100–200 microns were then cut through the pharynx using a vibrotome. 

In situ hybridization on embryos, larva, and vibratome sections of juveniles, was performed with 300–500 bp riboprobes against coding regions and/or 3' untranslated regions using a high-stringency hybridization protocol [49,69]. Key parameters of this protocol include post-hybridization washes at 70°C, with stringent hybridization protocol [49,89]. Key parameters of this protocol include post-hybridization washes at 70°C, with stringent hybridization protocol [49,89]. Key parameters of this protocol include post-hybridization washes at 70°C, with stringent hybridization protocol [49,89].

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Author Contributions

Conceived and designed the experiments: DMM. Performed the experiments: MC SL RC DMM. Analyzed the data: RC DMM. Wrote the paper: DMM.

Supporting Information

Figure S1 Phylogenetic analysis of lamprey Runx and Axl genes. Lamprey RunxA and RunxB group with deuterostome Runx/Runt homologs with high confidence values using either the Neighbor-Joining (A) or Maximum Likelihood (B) methods. Lamprey Axl groups with deuterostome Axl/Cart homologs with high confidence values using either the Neighbor-Joining (C) or Maximum Likelihood (D) methods. The related homeobox gene Rx (retinal homeobox) from Drosophila melanogaster serves as an outgroup. Gene names are prefixed with the initials of their respective species names. Bf, Branchiostoma floridae, Pm, Petromyzon marinus, Mm, Mus Musculus, Sc, Scyllium carinicauda, Dn, Danio rerio, Dm, Drosophila melanogaster, Sp, Strongylocentrotus purpuratus, Pt, Paracentrotus lividus, Ce, Caenorhabditis elegans, Mg, Myxine glutinosa.

(TIF)

Figure S2 Clustal alignments of Runx (A) and Axl (B) genes used to generate the trees in Figure S1. Only the sequences spanning the highly conserved DNA binding domains are shown for each alignment.

(TIF)

Figure S3 Expression of RunxA, RunxB, Barx, and Axl in larvae at 35 days post-fertilization. Expression patterns are essentially the same as those seen earlier at st. 30. Side view (A) and ventral view (B) of RunxA mRNA distribution showing weak expression in pharyngeal endoderm. Side view (C) and ventral view (D) of RunxB mRNA distribution showing weak expression in pharyngeal endoderm. Side view (E) and ventral view (F) of Barx mRNA distribution showing persistent expression in the medial aspect of the posterior pharyngeal arches (arrowhead) and the ventral portion of the first pharyngeal arch (arrow). Side view (G) and ventral view (H) of Axl expression in the dorsal and ventral aspects of the branchial basket (asterisk), dorsal fin (arrowhead), and dorsal first arch (arrow).

(TIF)

References

1. Gans C, Northcutt RG (1983) Neural Crest and the Origin of Vertebrates - a New Hypothesis. Science 220: 268–274.
2. Wada H, Saiga H, Satoh N, Holland PWH (1998) Tripartite organization of the amphioxus Pax-2/5/8, Hox and Otx genes. Development 125: 1113–1122.
3. Takahashi T, Holland PW (2004) Amphioxus and ascidian Dmbx homeobox domain protein. Development 131: 3283–3294.
4. Meulemans D, Bronner-Fraser M (2007) The amphioxus SoxB family: implications for the evolution of vertebrate placodes. Int J Dev Biol 51: 359–364.
5. Maier F, Hauss J, Milloz J, Millet J, Graham A, et al. (2005) Molecular evidence from Ciona intestinalis for the evolutionary origin of vertebrate sensory placodes. Dev Biol 282: 494–508.
6. Baslam S, Postlethwait JH (2005) The evolutionary history of placodes: a molecular genetic investigation of the larvacean urochordate Oikopleura dioica. Development 132: 4259–4272.
7. Meulemans D, Bronner-Fraser, M (2007) Insights from Amphioxus into the Evolution of Vertebrate Cartilage. PLoS ONE 2: e767.
8. Yan YL, Miller CT, Nisen RM, Singer A, Liu D, et al. (2002) A zebrafish son9 gene required for cartilage morphogenesis. Development 129: 5065–5079.
9. Specker SM, David BB (2006) barx1 is necessary for ectomesenchyme proliferation and osteochondroprogenitor condensation in the zebrafish pharyngeal arches. Developmental Biology (Orlando) 321: 101–110.
10. Kerney R, Gross JB, Harker J (2007) Zebrafish runx2 is essential for larval hyobranchial cartilage formation in Xenopus laevis. Developmental Dynamics 236: 1650–1662.
11. Ishii M, Merrill AE, Chan YS, Gitelman I, Rice DP, et al. (2003) Msx2 and Twist cooperatively control the development of the neural crest-derived skeletogenic mesenchyme of the murine skull vault. Development 130: 6131–6142.
12. Schorle H, Meier P, Buchert M, Jaenisch R, Mitchell PJ (1996) Transcription factor AP-2 essential for cranial closure and craniofacial development. Nature 381: 235–238.
13. Wenke AK, Rothhammer T, Moser M, Bosscherhoff AK (2006) Regulation of integrin alpha 10 expression in chondrocytes by the transcription factors AP-2 epsilon and Ets-1. Biochemical and Biophysical Research Communications 345: 495–501.
42. Johnels AG (1948) On the development and morphology of the skeleton of the head of Petromyzon. Acta Zoologica (Stockholm) 29: 149–270.

43. Gaskell WH (1908) The Origin of Vertebrates. London: Longmans, Green, and Co.

44. Haidasz MW (1979) Biology of the Cyclostomes. London: Chapman and Hall.

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

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

47. McIndoe DW, Bronner-Fraser M (2006) Importance of SoxE in neural crest development and the evolution of the pharynx. Nature 441: 750–752.

48. Nikitina N, Sauka-Spengler T, Bronner-Fraser M (2008) Dissecting early regulatory relationships in the lamprey neural crest gene network. Proceedings of the National Academy of Sciences of the United States of America 105: 20083–20088.

49. Meulmans D, Bronner-Fraser M (2002) Amphioxus and lamprey AP-2 genes: implications for neural crest evolution and migration patterns. Development 129: 4953–4962.

50. Meulmans D, McIndoe D, Bronner-Fraser M (2003) Id expression in amphioxus and lamprey highlights the role of gene cooption during neural crest evolution. Dev Biol 264: 430–442.

51. Schaffer J (1986) Über das knorpelige skelett von Ammocoetes brachialis nebst bemerkungen über das knorpelgewebe in allgemeinern. Z Wiss Zool 61: 606–686.

52. Rodrigo I, Bovolenta P, Mankoo BS, Imai K (2004) Meox homeodomain proteins are required for Bapx1 gene expression to repress Runx2 in chondrocytes. Exp Cell Res 315: 2231–2240.

53. McBurney KM, Keeley FW, Kibenge FS, Wright GM (1996) Spatial and temporal distribution of lamprin mRNA during chondrogenesis of trabecular cartilage in the sea lamprey. Anat Embryol (Berl) 193: 419–426.

54. Wright GM, Armstrong LA, Moya J, Youson JH (1998) Tribocartilinicular, branchial, and parietal cartilages in the sea lamprey, Petromyzon marinus: fine structure and immunohistochemical detection of elastin. Am J Anat 192: 1–15.

55. Wright GM, Youson JH (1982) Ultrasound of microcartilage in the larval anomuran sea lamprey, Petromyzon marinus L. Am J Anat 165: 79–91.

56. Zhao Q, Eberspaecher H, Seldin MF, de Crombrugghe B (1994) The gene for the homedomain-containing protein Carp-1 is expressed in cells that have a chondrogenic potential during embryonic development. Mech Dev 49: 235–254.

57. Bevendean A, Bevendean A, Eberspaecher H, Seldin MF, de Crombrugghe B (1997) Expression patterns of group-I aristless-related genes during craniofacial and limb development. Mechanisms of Development 157: 165–175.

58. Bell DM, Leung KKH, Wheatley SC, Ng LJ, Zhou S, et al. (1997) SOX9 directly regulates the type-II collagen gene. Nature Genetics 16: 174–178.

59. Meche R, Edelman DB, Jones FS, Makarenkova HP (2005) The homeobox transcription factor BarX2 regulates chondrogenesis during limb development. Development 132: 2153–2164.

60. Simoes B, Concerio N, Viegas CS, Pinto JP, Gaviao JP, et al. (2006) Identification of a promoter element within the zebrafish colXalpha1 gene responsive to runx2 isoforms Osf2/Cbfa1 and il-1 but not to pebp2alphaA. Gene 376: 79–230. 244.

61. Lefebvre V, Behringer RR, de Crombrugghe B (2001) L-Ssox5, Ssox6 and Ssox9 control essential steps of the chondrocyte differentiation pathway. Osteoarthritis Cartilage 9(Suppl A): 809–87.

62. Rodrigue I, Hill RD, Balling R, Munsterberg A, Inui K (2003) Pax1 and Pax9 activate Barx1 to induce chondrogenic differentiation in the sclerotome. Development 130: 473–482.

63. Rodeiro I, Bovolenta P, Manooch BS, Inui K (2004) MoxE homedomain proteins are required for Barx1 expression in the sclerotome and activate in transcription by direct binding to its promoter. Molecular and Cellular Biology 24: 2757–2766.

64. Yamashita S, Andoh M, Ueno-Kudoh H, Sato T, Misyak S, et al. (2009) Sox9 directly promotes Barx1 gene expression to repress Runx2 in chondrocytes. Exp Cell Res 315: 2231–2240.

65. Mallant J, Chen JY (2005) Fossil sister group of craniates: Predicted and found. Journal of Morphology 258: 1–31.

66. Mallant J (1990) Ventilation and the origin of jawed vertebrates: A new mouth. Zoological Journal of the Linnæan Society 117: 329–404.

67. Chen JY, Huang DY, Li CW (1999) An early Cambrian craniate-like chordate. Nature 402: 518–522.

68. Martinez-Morales JR, Henrich T, Ramialison M, Wittbrodt J (2007) New genes during craniofacial and limb development. Developmental Biology (Orlando) 295: 95–108.

69. Mallatt J (1996) Ventilation and the origin of jawed vertebrates: A new mouth. Zoological Journal of the Linnæan Society 117: 329–404.
74. Ettensohn CA, Illies MR, Oliveri P, De Jong DL (2003) Alx1, a member of the Cart1/Alx3/Alx4 subfamily of Paired-class homeodomain proteins, is an essential component of the gene network controlling skeletogenic fate specification in the sea urchin embryo. Development 130: 2917–2928.
75. Mori-Akiyama Y, Akiyama H, Rowitch DH, de Crombrugghe B (2003) Sox9 is required for determination of the chondrogenic cell lineage in the cranial neural crest. Proceedings of the National Academy of Sciences of the United States of America 100: 9360–9365.
76. Jeffery WR, Strickler AG, Yamamoto Y (2004) Migratory neural crest-like cells form body pigmentation in a urochordate embryo. Nature 431: 696–699.
77. Kee Y, Hwang BJ, Sternberg PW, Bronner-Fraser M (2007) Evolutionary conservation of cell migration genes: from nematode neurons to vertebrate neural crest. Genes & Development 21(990-996): 6.
78. Manni L, Lane NJ, Sorrentino M, Zaniolo G, Burighel P (1999) Mechanism of neurogenesis during the embryonic development of a tunicate. Journal of Comparative Neurology 412: 527–541.
79. Haldin CE, LaBonne C (2010) SoxE factors as multifunctional neural crest regulatory factors. Int J Biochem Cell Biol 42: 441–444.
80. Meulemans D, Bronner-Fraser M (2007) Insights from amphioxus into the evolution of vertebrate cartilage. PLoS ONE 2: e767.
81. Rychel AL, Swalla BJ (2007) Development and evolution of chordate cartilage. J Exp Zoolog B Mol Dev Evol 308: 323–335.
82. Wahl J, Mason I (2003) Fgf signalling is required for formation of cartilage in the head. Developmental Biology 264: 522–536.
83. Schwend T, Ahlgren SC (2009) Zebrafish con/disp1 reveals multiple spatiotemporal requirements for Hedgehog-signaling in craniofacial development. BMC Dev Biol 9: 59.
84. Shimeld SM (1999) The evolution of the hedgehog gene family in chordates: insights from amphioxus hedgehog. Development Genes and Evolution 209: 40–47.
85. Takio Y, Kuraku S, Murakami Y, Pasqualetti M, Rijli FM, et al. (2007) Hox gene expression patterns in Lethenteron japonicum embryos--insights into the evolution of the vertebrate Hox code. Developmental Biology (Orlando) 308: 600–620.
86. Kimmel CB, Miller CT, Kruze G, Ullmann B, BreMiller RA, et al. (1998) The shaping of pharyngeal cartilages during early development of the zebrafish. Developmental Biology 203: 245–263.
87. Perriere G, Gouy M (1996) WWW-query: An on-line retrieval system for biological sequence banks. Biochimie 78: 364–369.
88. Nikitina N, Bronner-Fraser M, Saula-Spengler T (2009) Microinjection of RNA and morpholino oligos into lamprey embryos. CSH Protoc 2009: pdb prot5123.