Cartilage Diversification and Modularity Presaged the Evolution of the Gnathostome Head Skeleton

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Abstract

The vertebrate head skeleton has evolved a myriad of forms since their divergence from invertebrate chordates. The connection between novel gene expression and cell types is therefore of importance in this process. The transformation of the jawed vertebrate (gnathostome) head skeleton from oral cirri to jointed jaw elements required a diversity of cartilages. Although lampreys are a sister clade to gnathostomes, they display skeletal diversity with distinct gene expression and histologies, a useful model for addressing joint evolution. Specifically, the lamprey tissue known as mucocartilage has noted similarities with the jointed elements of jawed vertebrates. We thus asked whether the mucocartilage of jawless lampreys is homologous to gnathostome joint tissue. To do this, we characterized new genes that are involved in gnathostome joint formation and characterized the histochemical properties of lamprey skeletal types. We find that most of these genes are minimally found in mucocartilage and are likely later innovations, but we do identify new activity for gdf5/6/7b in both hyaline and mucocartilage, supporting its role as a chondrogenic regulator. Contrary to previous works, our histological assays do not find any perichondrial fibroblasts surrounding mucocartilage, suggesting that mucocartilage is non-skeletogenic tissue that is partially chondrified. Interestingly, we also identify new histochemical features of the lamprey otic capsule that diverge from normal hyaline. Paired with our new insights into lamprey mucocartilage, we propose a broader framework for skeletal evolution in which an ancestral soxD/E and gdf5/6/7 network directs mesenchyme along a spectrum of cartilage-like features.

Introduction

The evolution of vertebrates from invertebrate chordates involved a combination of morphological and genomic changes. Vertebrates collectively underwent one round of whole-genome duplication, and both extant jawed and jawless taxa (gnathostomes and cyclostomes respectively) are thought to have experienced lineage-specific duplications as well (Simakov et al., 2020) (Smith & Keinath, 2015; Van de Peer et al., 2009). These genomic expansions are thought to be connected with novel vertebrate traits, as this extra genetic material would help facilitate morphological diversification (Ohno, 2013). Although invertebrate chordates like amphioxus have a head made partially of cellular cartilage (Jandzik et al., 2015), the skeletal system has been greatly elaborated in vertebrates in the form of the skull, jaw, pharynx, fin, and limbs across taxa. In particular, the evolution of the jaw is thought to have facilitated the diversification of vertebrates by allowing a greater range of feeding styles and niches to occupy (Gans, 1989; Gans & Northcutt, 1983; Northcutt & Gans, 1983). The origins of the vertebrate jaw are still enigmatic, so the role of novel or co-opted genes in its development is an important part of understanding this process.

The vertebrate jaw is characterized by dorso-ventral patterning in the first pharyngeal arch via nested dlx expression and jagged-cdn-bmp signaling (Talbot et al., 2010; Walker et al., 2006), producing an intermediate domain that expresses a suite of genes like gdf5 and nkx3.2 where the future jaw joint (Miller et al., 2003; Thomas et al., 1998). It has been shown that the transcription factor barx1 is involved with positioning this future jaw joint, as its expression is anti-correlated with this tissue, and its
knockdown results in ectopic joint tissue in zebrafish (Nichols et al., 2013; Sperber & Dawid, 2008; Square et al., 2015). Conversely, the transcription factor trps1 is highly correlated with joint tissue and is believed to maintain articular cartilage, with its knockdown resulting in increased hypertrophy (Michikami et al., 2012; Nishioka et al., 2008; Suemoto et al., 2007; Wuelling et al., 2009). Another important process in articular cartilage formation is TGF-β signaling, with TGFβr2 involved in maintaining the future joint interzone (Baffi et al., 2006; Seo & Serra, 2007; Spagnoli et al., 2007; Woronowicz et al., 2018). The role of iroquois proteins in joint formation are still poorly understood, but it has been demonstrated that irx1, irx5, and irx7 have distinct roles in inhibiting chondrocyte maturation and thus some role in joint formation (Askary et al., 2015; Farmer et al., 2021). While joint tissue shares similar ECM expression to other chondrogenic tissues (Chijimatsu & Saito, 2019; Darling et al., 2004), it is surrounded by the viscous liquid of the synovial cavity which contains lubricin/prg4, a glycoprotein with important roles in joint homeostasis (Askary et al., 2016; Kozhemyakina et al., 2015; Takahata et al., 2022). Despite these advances in our knowledge of gnathostome chondrogenesis and joint formation, we still know little about the evolutionary processes by which these genes were co opted into the chondrogenic program.

The jawless lamprey has become an important model organism in our understanding of vertebrate evolution and what skeletal traits may have been present in the common ancestor of cyclostomes and gnathostomes. Despite the differences in morphology between these lineages, both groups shared nested dlx expression and edn signaling within the pharyngeal arches as well as an absence of hox expression in the mandibular arch (Cerny et al., 2010; Kuraku et al., 2010; Takio et al., 2004). These together would mean that much of the patterning of the head skeleton is governed by similar mechanisms. Lamprey furthermore have a diversity of skeletal types throughout their body during development; they have gnathostome-like hyaline in the branchial arches that express master chondrogenic genes like soxD and soxE homologs (Lakiza et al., 2011; Ohtani et al., 2008; Uy et al., 2012), and they also have mucocartilage, a connective tissue interspersed throughout the anterior larval head skeleton and fin [Fig. 1A]. This tissue has puzzled evolutionary biologist for more than a century, as its morphology and histology are different than gnathostome cartilages and even from lamprey branchial cartilage (Root, Gould, et al., 2021). Despite these differences, it expresses a suite of similar genes in common with gnathostome joint tissue including gdf5 (Cerny et al., 2010), lecticans (Root, Jandzik, et al., 2021), and col2a1 / col11a1 homologs among others (Root et al., 2022). These genes are not universally expressed across mucocartilage, and a diversity of subtypes of mucocartilage has been previously noted (Cattell et al., 2011). While these data imply a relationship between mucocartilage and joint tissue, it is unclear whether these cell types could be considered homologous.

To better understand the relationship between lamprey mucocartilage and gnathostome joint tissue, we characterized expression of lamprey homologs of the cartilage developmental regulators gdf5, barx1, trps1, tgfβr2, and irx1,5,7 as well as the extracellular matrix genes prg4 and col9a1 throughout early skeletal development in lamprey (Tahara stages 26–28 (Tahara, 1988). We found that several of these regulator genes and their respective homologs were not expressed in mucocartilage at any developmental time point while others were only temporarily present. This implies that much of the developmental toolkit for cartilage development was only acquired after the divergence of gnathostomes and cyclostomes. We
also compared the histochemical affinities of mucocartilage with multiple staining methods. We found that, despite the differences in gene expression between mucocartilage populations, we can only distinguish two subtypes using these musculoskeletal staining techniques. We did reveal histochemical differences in the cartilage of the otic capsule, having features that diverge from traditional gnathostome hyaline and are more akin to elastic cartilage. Taken together, our results suggest that lamprey mucocartilage is not homologous to gnathostome joint tissue but is still governed by a similar core set of chondrogenic factors, meaning that these fibroblast tissues are partially using a shared cartilage gene regulatory network. Paired with our insights into the lamprey otic cartilage, this would mean that cartilage diversity and thus modularity were likely present in the last common ancestor of vertebrates, an important step in the later evolution and acquisition of jaws.

**Results**

**Expression of prg4 and col9a1**

Previous work with lecticans and fibrillar collagens in lamprey have revealed that these genes are spatially distributed throughout connective tissues such that there is no set of these genes that is uniquely specific to lamprey cartilages (Root et al., 2022; Root, Jandzik, et al., 2021). We were therefore interested in identifying minor ECM components that may be associated with these cell types, predicting that these genes would behave similarly. Type IX collagen is a FACIT collagen that is well established as a cartilage ECM protein that stabilizes other components like lecticans and fibrillar collagens (Aszodi et al., 2001; Dreier et al., 2008; Van Camp et al., 2006), so we decided to characterize the expression of col9a1 as well as the aforementioned prg4.

We did not detect prg4 transcripts until stage T27, and this activity is confined to a small patch along the ventromedial plane [Fig. 2A]. Upon sectioning and further review, this expression corresponds to the anterior streams of the ventral aorta. This expression continues through T28 until T29 when it abrogates and expression is no longer visible [Fig. 2B]. Based on these findings, we conclude that prg4 is not associated with any skeletal development in lamprey, meaning that its deployment in skeletogenesis likely arose after gnathostomes and cyclostomes diverged.

Conversely, we find abundant col9a1 activity in lamprey cartilage. When early skeletogenesis begins in lamprey around stage T26, col9a1 can be visualized in the ventral pharynx, notochord, and otic capsule, with weak expression observed in the nascent pharyngeal arches [Fig. 2C]. By stage 27, col9a1 is found throughout the anterior oral region, otic capsule, and the dorsal and ventral borders of the branchial arches [Fig. 2D]. By this stage, the ambiguous expression across the ventral pharynx is becoming specified anteriorly in the ventromedial longitudinal bar (vmlb) and the mucocartilages surrounding the endostyle. These expression domains are almost identical in stage T28, with less expression in the endostylic cartilages being noted [Fig. 2E]. It is at this stage that col9a1 expression in the anterior oral region also presages the cartilage of the nasal capsule, and we begin to see weak expression in the epibranchial and hypobranchial bars. The specification of the ventral pharynx is nearly complete by this
stage, and we see col9a1 activity in this region confined to the most posterior portion. When compared with previous ECM genes in lamprey skeleton, col9a1 is a fairly reliable marker of traditional hyaline cartilages as well as mucocartilages, with the only col9a1-negative tissues of these being the trabecular and parachordal processes (hyaline) and the ventrolateral plate (mucocartilage).

Our findings for col9a1 and prg4 are in contrast with previous studies of cartilage ECM genes in that they show much less heterogeneity. prg4 is absent from all hyaline and mucocartilage at all developmental stages observed, an interesting finding in the larger context of cartilage evolution. prg4 is important not only in gnathostome joint cartilage but also in the surrounding fibroblasts in the synovium, providing structural support as well as signaling to local macrophages (Alquraini et al., 2015; Schmidt et al., 2007). It is thus possible that prg4 was coopted in gnathostome joint evolution from the vascular system, although this would need to be further explored. In comparison, col9a1 is among the most specific markers for both hyaline and mucocartilage found to date. As a minor component of the chondrocyte ECM, it is a surprise that its expression is more common in skeletal populations than major components like col2a1 or col11a1. We believe that this may be partially due to lineage-specific changes in P. marinus, as it has been demonstrated that the arctic lamprey Lethenteron camtschaticum maintains type II collagen in its branchial arches during the onset of chondrogenesis (Ohtani et al., 2008), meaning that col9a1 is likely an accurate marker of skeletogenic mesenchyme despite the changes that have happened in P. marinus. Taken together, our results improve our understanding of the ancestral vertebrate cartilage ECM by providing clearer examples of its development; col9a1 was an integral part of the ancestral chondrocyte ECM, likely in conjunction with type II and XI collagen, while prg4 was almost certainly coopted later in evolution.

### Expression of Chondrogenic Regulatory Genes

Lamprey have three pro-orthologs of the gnathostome gdf5, gdf6, and gdf7 genes, known collectively as gdf5/6/7a,b, and a newly discovered gdf5/6/7c. Of these, gdf5/6/7b has been the most thoroughly investigated, with previous data corresponding to this gene (Cerny et al., 2010). We designed riboprobes for all three genes, having expanded on previous findings for gdf5/6/7b. Expression of gdf5/6/7a is minimal at Tahara stage 26, having observed a small band running medially along the dorsal top of the body as well as some expression in the presumptive oral endoderm [Fig. 3A]. Transcripts are additionally visible in the heart by stages T27 and T28, although expression in throughout the head and pharynx is ubiquitous [Fig. 3B,C]. Likewise, gdf5/6/7c is indeterminate throughout the head and pharynx at all stages examined, with no particular association with any tissue [Fig. 3D]. In contrast, we see more specific expression of gdf5/6/7b in the skeletal system at these stages. We detect transcripts of gdf5/6/7b at stage T26 throughout the mesenchyme of the upper lip and pharynx as well as the ventral endoderm in this region [Fig. 3E]. By stage 27, we observe additional expression in the endoderm of the pharynx, the anterior oral region, the medial flap, and expression in the ventral pharynx reveals activity in the epithelium of the endostyle [Fig 3F]. Expression at T28 largely mirrors that seen at stage T27, but direct expression in the dorsal and ventral poles of the branchial arches is also observed at this stage [Fig. 3G]. While there is little correlation between gdf5/6/7a and gdf5/6/7c with the lamprey skeleton, our
new *gdf5/6/7b* probe has greatly improved our understanding of its expression, showing new activity throughout the developing skeleton and likely affecting both hyaline and mucocartilage. While previous studies linked its expression to mucocartilage exclusively, *gdf5/6/7b* likely has a role in lamprey chondrogenesis more broadly.

We were next interested in the potential antagonistic relationship between *barx1* and mucocartilage, so we analyzed the expression of *barx* homologs during the aforementioned stages. Previous work has characterized a lamprey *barx* gene (Cattell et al., 2011; Cerny et al., 2010), but we have identified two additional *barx* homologs of interest. We therefore designed three riboprobes to test the expression of all three paralogs in tandem. Our new probe for the previously studied *barx*, henceforth known as *barxA*, corroborates previous findings for this gene, being expressed at stage T26, T27, and T28 in the medial cranial neural crest cells (CNCCs) of the pharynx and the mesenchyme of the lower lip [Fig. 4C,D,E]. At later stages, this expression specifies to pharyngeal arch derivatives, as expression is even observed in the vmlb [Fig. 4E]. In contrast to *barxA*, we see little specific activity in the other *barx* genes. *barxB* expression is weakly expressed throughout the head ectoderm at stage 26 [Fig. 4A], but we identify transcripts in the presumptive CNCCs in the pharynx by T27 and T28 albeit highly unspecific [data not shown]. Likewise, *barxC* expression is weak throughout all stages observed, with only minor activity in the facial ectoderm [Fig. 4B]. Even when paired with new probes and investigating all new genes, we find no anticorrelation between *barx* genes and mucocartilage, confirming previous findings about these genes. The function of *barx* in ancestral vertebrates was most likely the patterning of pharyngeal arch mesenchyme generally, only later acquiring a more specific role in the patterning of the jaw.

Because *irx7* is a teleost-specific duplicate, we focused on the homologs *irx1* and *irx5* for our study, as these genes are most similar to *irx7* (Askary et al., 2015; Farmer et al., 2021). Our transcriptomic analyses reveal that lamprey only have three *irx* homologs, named *irxA*, *irxB*, and *irxC*, so we opted to characterize the expression for all three lamprey genes. The expression of *irxA* is mostly confined to earlier stages before this study, being almost absent by stage 27, but we do observe expression at stage T26 in presumptive migratory cells throughout the head and pharynx, with noted activity in the heart as well [Fig. 4F]. *irxC* does not appear to be associated with any musculoskeletal tissues, as we detect transcripts of *irxC* throughout the facial ectoderm at all observed stages albeit at lower levels [Fig. 4G]. Of the three *irx* genes, *irxB* showed the most relevant activity in the pharynx. We detect transcripts of *irxB* at stage 26 throughout the pharyngeal arches [Fig. 4H]. However, *irxB* is localized specifically in the pharyngeal musculature as confirmed by sectioning by stage T27, its expression overlapping much of the expression of the muscle actin gene *ma2* (McCaulay & Bronner-Fraser, 2006; Yokoyama et al., 2021) [Fig. 4I]. *irxB* expression is more dynamic at stage 28, being identified throughout the pharyngeal mesoderm as well as the medial velum and the anterior oral region [Fig. 4J]. Upon sectioning and further review, this expression corresponds to the musculature of the pharynx and hypobranchial process as well as the ventral pharynx. Taken together, our findings suggest that *irx* genes do not have a significant role in lamprey skeletogenesis and were coopted into skeletogenesis later in vertebrate evolution, their primary role being likely myogenic and neurogenic in nature.
Our transcriptomic analyses identify only one trps homolog with high sequence similarity to that seen in gnathostomes, implying that it is a direct ortholog of trps1. At stage T26, we identify trps1 expression in the pharyngeal arches, brain, and along the dorsal part of the body [Fig. 5A]. At stage 27, trps1 is localized in the upper lip musculature, the mesenchyme of the lower lip and velum as well as the CNCCs and mesoderm throughout the pharynx in a pattern similar to barx1 [Fig. 5B]. By T28, trps1 activity in the velum is confined to the medial region, and most expression in the lower lip region is no longer present [Fig. 5C]. We observe waning expression in the pharyngeal mesoderm along the anterior-posterior axis, with transcripts remaining posteriorly. Despite the importance of trps1 in gnathostome joint tissue, we do not find a similar correlation with the lamprey homolog in mucocartilage. It is thus likely that trps1 was co-opted from a network similar to barx1 that was involved with pharyngeal arch patterning.

We identified one tgfβr2 gene with high sequence similarity to gnathostomes, and we focused primarily on this homolog. At stage 26, we find weak tgfβr2 expression in the mesenchyme of the upper lip and strong expression in the heart [Fig. 5D]. By T27, tgfβr2 is found throughout the pharyngeal mesoderm, the musculature of the upper lip, and the mesenchyme of the external velum [Fig. 5E]. tgfβr2 expression in the velum shifts medially during stage 28, and we notice a similar intensity of expression throughout CNCC derivatives in the pharynx [Fig. 5F]. Expression in the anterior oral region has largely abated by this time, however, but we also observe new activity in the mucocartilage of the hyoid. Taken together, tgfβr2 in lamprey has considerably less roles in skeletogenesis than in gnathostomes, especially so throughout mucocartilage. In the broader context of skeletal evolution, tgfβr2 was highly pleiotropic in the common ancestor of vertebrates and was later co-opted into skeletal development specifically.

Our results show that several genes involved in gnathostome joint formation are almost entirely absent from lamprey mucocartilage at most developmental stages. We notice distinct patterns in these genes, however, being either pharyngeal arch dominant like barx and trps1 or more general throughout the body like irx homologs as well as tgfβr2. These results support the idea that jaw evolution evolved through the cooption of modules both within and outside the pharyngeal arches, suggesting a more complex and stepwise acquisition of these associated genes. We have also found new areas of expression for gdf5/6/7b in the developing skeletal system, further supporting the role of these genes as important regulators of lamprey cartilage. These findings together imply that, although a soxD/E and gdf5/6/7 almost certainly govern lamprey chondrogenesis, any deeper parts of this network will be harder to determine.

**Histological and histochemical properties of lamprey mucocartilage**

We first tested whether lamprey hyaline and mucocartilage could be distinguished from one another under multiple histochemical stains. Previous studies have used toluidine blue (TB) as a useful metachromatic stain when viewing musculoskeletal tissues like cartilage (Bergholt et al., 2019; Williams, 1941), so we used TB staining on different lamprey cartilage sections. By Tahara stage 30, both the hyaline cartilage of the trabecles and branchial arches and mucocartilage stain purple, an indication of high polysaccharides, though mucocartilage tends to stain stronger purple [Fig. 6B,C,D]. A notable
difference between these skeletal types is that the pericellular matrices of lamprey hyaline are visible using TB staining, with clear demarcation between chondrocyte nests. Additionally, we also note differences between the hyaline cartilage of the trabecles and branchial arches with that seen in the otic capsule, the former having an interterritorial matrix staining strongly blue, a feature more which deviates from normal hyaline [Fig. 6E]. We next sought to use the polychromatic stain Masson Trichrome (MT) on paraaffin sections of skeletal tissues to validate our findings with TB. By stage T30, mucocartilage is universally indicated by red staining in the chondrocytes, blue staining in the interterritorial matrix, and no visible pericellular matrix between nests [Fig. 6J,K,L]. In contrast, lamprey hyaline cartilage stains strongly red throughout the matrix, but the pericellular matrix does not seem visible [Fig. 6M]. These results for mucocartilage and hyaline are largely similar in gnathostomes for skeletogenic and non-skeletogenic connective tissues respectively. We last tried RGB Trichrome, a recently developed polychromatic series (Picrosirius Red, Fast Green FCF, and Alcian Blue) which has useful applications in distinguishing musculoskeletal cell types from one another including hyaline, elastic cartilage, and fibrocartilage (Gaytan et al., 2020). By stage T30, there are considerable differences in staining between lamprey hyaline and mucocartilage. Hyaline chondrocytes are visible and stain blue, the pericellular matrix is visible and stained red, and perichondrium is visible and likewise stained red [Fig. 6O]; in contrast, the interterritorial matrix of mucocartilage stains almost exclusively blue with varying degrees of visible fibers, and cells are weakly visible [Fig. 6N,P,Q]. This coincides with the staining affinities of non-skeletogenic connective tissues in gnathostomes (Gaytan et al., 2020). Similar to our TB stains, we see differences in histological staining in the cartilage of the otic capsule compared to normal hyaline with RGB, staining green in a manner similar to that seen in gnathostome elastic cartilage (Gaytan et al., 2020) [Fig. 6O]. Overall, lamprey hyaline and mucocartilage stain different from one another in both metachromatic and polychromatic tests, the later cell type more resembling non-skeletogenic connective tissues, but we still notice differences within lamprey hyaline.

We next asked whether we could use the aforementioned stains to distinguish mucocartilages from one another, as their differences in gene expression would suggest that they are different cell types (Cattell et al., 2011). We were specifically interested in the mucocartilages of the velum, as previous work has suggested that the interior portion of this structure, known as the medial flap, is non-skeletogenic mesenchyme (Yokoyama et al., 2021). Compared to TB staining, we find considerably less differences between mucocartilages using polychromatic stains. No discernable differences are detected using MT, and our findings with RGB are likewise minimal. Using TB staining, we were however able to identify some differences. We first looked at the velum, where the interior and exterior halves of the are clearly separated dorsally by a thin belt of musculature [Fig. 6F]. When analyzing the medial flap, it comparatively stains blue and the pericellular matrix between chondrocyte nests is visible [Fig. 6F,G]. Proceeding ventrally, most of the medial flap is composed of tightly-packed cuboidal cells surrounded by thickened epithelium [Fig. 6G]. The posterior mucocartilages can largely be divided between the vmlb, the endostilic cartilages, and the ventral pharynx. The ventral pharynx and endostilic cartilages stain uniformly in a way similar to other mucocartilages, but the vmlb is more similar to the medial flap, with less purple staining and visible pericellular matrices [Fig. 6H,I]. Overall, we were only able to identify two
distinct mucocartilage types using TB staining, the tissues of the vmlb and medial flap being distinct. The medial flap has been previously considered non-skeletogenic, but we find no evidence that its histological properties should be considered as anything other than mucocartilage, as it shares features with established mucocartilaginous tissues like the vmlb. While further work must be done in larval lamprey to refine MT and RGB methods, our TB stainings show that mucocartilage is histologically more similar than gene expression studies would otherwise imply.

We lastly asked whether we could conclusively identify perichondrial tissues which surround the mucocartilage. Electron microscopy studies reported on perichondrium-like fibroblasts adjacent to the mucocartilage of the ventrolateral plate and ventrolateral longitudinal bar (Wright & Youson, 1982), and we looked to further these observations across all mucocartilages. We reasoned that all mucocartilage would have perichondrium encompassing the tissue and that these perichondrial fibroblasts would be largely similar to one another. As a reference, we used TB staining to first identify the perichondrium of cartilage in the trabecular and parachordal processes as well as the branchial arches. With TB staining, the perichondrium stains weakly purple and are stellate in shape, forming a small ring around the chondrocytes [Fig. 7C,D,G]. In the ventrolateral plate, we identify the band of cells previously reported to be the perichondrium (Wright & Youson, 1982), but these cells stained strongly blue using TB [Fig. 7E,F,G]. Upon further look into the literature, this patch of cells also corresponds to a band of pax3/7-positive cells that migrate ventrally from the lateral plate (Kusakabe & Kuratani, 2007; Onimaru et al., 2011), suggesting that these cells are likely part of the ventral body wall rather than perichondrium. We next looked at the vmlb and notice that its perichondrial are more cuboidal in shape than those of the ventrolateral plate [Fig. 7E,F,G]. Moving posteriorly, the reported perichondrial fibroblasts are connected to the epithelium of the endostilic hypobranchial grooves rather than the nearby mucocartilage. Considering that the vmlb itself is surrounded by thickened epithelium that joins posteriorly with the hypobranchial grooves of the endostyle, it is more likely that its “perichondrium” is either connective tissue associated with the pharyngeal epithelium or is itself epithelium. We next tested the mucocartilages that were not in the aforementioned work, and we find that none of them (oral hood, sub-otic mucocartilage, velum) had perichondrium-like tissues. In the case of the velum, the cells surrounding the mucocartilage are contiguous with the thickened epithelium that can be found at the medial most point of the velum, supporting that these are likely flattened epithelial cells rather than fibroblasts [Fig. 7B,C,D]. Without any unifying characteristics of the reported perichondrium across tissues and its absence in several others, our results together posit that mucocartilage is likely not surrounded by perichondrial tissue. Combined with its broader histological features, this lamprey cell type should be largely considered non-skeletogenic connective tissue.

Our results show that lamprey mucocartilage is more homogenous at the histological level than gene expression assays would predict, and this tissue consistently stains similar to mesenchyme and fibroblasts in gnathostomes. Toluidine blue is able to distinguish the vmlb and the medial flap from other mucocartilages, however, suggesting that there is still some heterogeneity in these lamprey skeletal tissues. We also found differences in lamprey hyaline types, with TB and RGB staining revealing the cartilage of the otic capsule to deviate from the features of traditional hyaline and, in the case of RGB
staining, reveal features similar to elastic cartilage. When taking a more holistic view of mucocartilage, we were unable to find any perichondrium surrounding these tissues. Our histochemical assays of larval lamprey cartilage are among the most comprehensive to date, and these comparative methods help give deeper insight into lamprey musculoskeletal anatomy. Paired with our gene expression analyses, these provide a powerful tool to assess the cellular identity of tissues.

Discussion

In this study, we compared lamprey mucocartilage and hyaline cartilage through more extensive means like comparative histochemistry, and we further looked at differences in gene expression between them. Morphologists have wondered about the homology and origin of lamprey mucocartilage for more than a century (Root, Gould, et al., 2021), and it has only been with renewed interest and an improved understanding of histology, genetics, and evolution that we have been able to address it. We have previously suggested that skeletal tissues exist along a spectrum of connective tissues with chondrocyte-like features (Root, Gould, et al., 2021), and our findings help further elaborate this schema. While mucocartilage is almost certainly not a skeletal tissue per se, these fibroblasts show a range of skeletal-like properties that indicate levels of chondrification. Paired with our results that show skeletal heterogeneity in lamprey hyaline-like cartilages, specifically the otic capsule, we posit that a combination of chondrocyte-fibroblast interactions helped generate the diversity of skeletal tissues we see in vertebrates, a critical aspect of vertebrate skeletal evolution and specifically the gnathostome jaw.

Lamprey mucocartilages are partially chondrified fibroblasts

The earliest reports of mucocartilage consistently described this tissue as fibroblast-like, yet still prescribed it cartilage-like features such as a strong responsivity to alcian blue staining and a ground substance rich in hyaluronic acid (Root, Gould, et al., 2021). The idea that mucocartilage was surrounded by perichondrial tissue further complicated our understanding of this cartilage-like cell type, supporting that it was a cartilage that significantly deviated from traditional chondrocyte development. Our results here suggest that mucocartilage does not have a perichondrium, supporting histological data that would categorize it as fibroblasts with cartilage properties rather than cartilage with fibroblast properties. Fibroblasts themselves are somewhat unclear in features that distinguish them from other connective tissues, so it will be important to identify these in order to determine what mucocartilage is and the extent that it has skeletal properties.

Despite decades of usage in stem cell biology, fibroblasts still do not have a fixed definition (Haniffa et al., 2009; Soundararajan & Kannan, 2018). The conflation of the word itself with other cell types like fibrocytes and mesenchymal stem cells (MSCs) further show that we still do not understand how fibroblasts differentiate and mature nor even if these cells are terminally differentiated. The extent that mucocartilage is differentiated has important implications of lamprey metamorphosis, a period in which this tissue reverts to a mesenchymal state before redifferentiating into traditional cartilage (Armstrong et al., 1987). Although there are similarities in histology and broad gene expression between fibroblasts and
MSCs, DNA methylation patterns may prove to be an effective marker to distinguish them, supporting the claim that these cells mostly represent a spectrum of multipotency states (Soundararajan & Kannan, 2018). Future work is needed to determine where mucocartilage exists along this continuum and how they are altered during metamorphosis.

The differences in gene expression between mucocartilage and normal fibroblasts is therefore of great interest, as mucocartilage does have alcian staining affinities closer to cartilage than non-skeletal connective tissues (Yao et al., 2011). Paired with differences in expression of key ECM genes involved with connective tissues like col1a2, col2a1, col3a1, and col9a1 as well as lecticans (Root et al., 2022; Root, Jandzik, et al., 2021), mucocartilage shares more ECM similarities with cartilage in comparison to other fibroblasts. The differences in expression of these genes between various mucocartilages need to also be considered in the broader context of development, as these differences may reflect subtypes of this tissue or differences in maturity and differentiation. Three key examples of this are the oral hood, the ventral pharynx, and the fin fold, which collectively show diminishing expression of col1a2a and col3a1a during later development, this expression being progressively confined to the ends respectively. This would suggest that Type I and III collagen, traditionally markers of fibroblasts and mesenchyme in gnathostomes, may be specific to the proliferative zone of these mucocartilage and thus help resolve some of the heterogeneity that we see across these tissues. In contrast to previous findings, we propose a more simplistic model for lamprey skeletal types, the main criteria distinguishing them being the presence or absence of lecticans, major and minor cartilage ECM collagens like type II and IX respectively, and core chondrogenic regulators like soxD/E homologs and gdf5/6/7 [Fig. 1B] [Fig. S1].

**Skeletal modularity is an ancestral feature for vertebrates**

Gnathostomes have evolved a diversity of skeletal tissues over the past five hundred million years, using distinct types of cartilage, bone, and a variety of cells with intermediate features between them (Eames et al., 2020). The fossil record also shows a range of mineralizing tissues across specimens, implying that skeletal diversity was present in ancient vertebrates as well. Because the phylogenetic position of cyclostomes amongst fossil and extant vertebrates is still unclear (Janvier, 2008; Miyashita et al., 2019), the skeletal repertoire seen in extinct groups like heterostracans and anaspids cannot tell us alone whether this diversity was present in the common ancestor of gnathostomes and cyclostomes. Our results here provide a more holistic view of lamprey mucocartilage from the perspective of vertebrate skeletal evolution, and our findings hint that non-skeletal cells like fibroblasts can display chondrocyte-like properties via regulation by gdf5/6/7 homologs. This would mean that core cartilage regulatory genes can act more broadly across connective tissues, an important step in the diversification of vertebrate skeletal tissues.

The manner in which gdf5/6/7 and soxD/E homologs drive chondrification across skeletal and non-skeletal cells in lamprey is still unclear. Previous studies which tested soxD/E expression in lamprey found that these genes are mostly restricted to hyaline cartilage during skeletogenesis, although soxE3 has also been detected in the external velum (Lakiza et al., 2011; Ohtani et al., 2008). With the exception
of the latter, these tissues also correspond to lecC-positive cells (Root, Jandzik, et al., 2021), suggesting that there is a connection between soxD, soxE1/2, lecC, and traditional hyaline cartilage. Conversely, we see gdf5/6/7 activity across both hyaline and mucocartilages. We see lecA as the dominant lectican across the majority of these mucocartilages, and this expression is even observed in the pre-chondrogenic mesenchyme of the branchial arches earlier in development (Root, Jandzik, et al., 2021). To explain the connection between sox-lecC and gdf5/6/7-lecA in skeletal development, we posit three scenarios. In the first, the gdf5/6/7-lecA module is specific to pre-chondrogenic mesenchyme, and the mucocartilage phenotype is due in part to the absence of soxD and soxE1/2 [Fig. 8A]. soxD and soxE1/2 would likely downregulate lecA and other aspects of gdf5/6/7 signaling, but soxE3 evolved new functions that change its interaction with this pathway, considering that the exterior velar bar is a pharyngeal arch mucocartilage yet expresses soxE3. In the second scenario, the gdf5/6/7-lecA module was ancestrally a cartilage module that was later co-opted into fibroblast tissues [Fig. 8B]. This scenario would allow us to explain the presence of type II and IX collagen in mucocartilage among others, as these cartilaginous ECM genes were likewise coopted. In the third scenario, the gdf5/6/7 module is more generalist in function in mesenchyme during development and only later acquired chondrocyte-like expression of genes like lecticans, fibrillar collagens, and type IX collagen [Fig. 8C]. The mechanism through which this happened is unclear, but this scenario allows us to reconcile the pleiotropy we see with gdf5/6/7 signaling as well as inconsistencies in gene expression across all cell types. Each of these scenarios has different implications for the evolution of skeletal diversity in lamprey. The first scenario would imply that mucocartilage is a pre-chondrogenic mesenchyme that semi-differentiates and commits to the fibroblast lineage later in development, the second would imply that mucocartilages are fibroblasts that directly co-opted cartilage modules, and the third would imply that mucocartilages are fibroblasts that indirectly acquired chondrocyte-like properties. While we are still uncertain about the specific changes which created mucocartilage, our results suggest that this tissue arose from regulatory interactions in mesenchyme between chondrocyte and fibroblast-associated pathways.

The question remains whether the repertoire of cartilage and cartilage-like tissues found in lamprey were present in the common ancestor of vertebrates. We have previously discussed the similarities between lamprey mucocartilage and hagfish pseudocartilage (Root, Gould, et al., 2021), and it is likely that it is a shared cell type amongst cyclostomes. There are no tissues in gnathostomes yet which have the same myriad of features as mucocartilage/pseudocartilage, implying that either jawed vertebrates lost the cell type or it is cyclostome-specific. Specifically to lamprey, this cell type has important functional roles in forming the oral cavity and providing hydrostatic support during filter feeding (Mallatt, 1981). It has been suggested that the common ancestor of vertebrates was a burrowing filter feeding in a way similar to that seen in the invertebrate amphioxus as well as larval lamprey (Satoh, 2009), meaning that much of the oropharyngeal anatomy at this stage may be similar. Although the tissue itself might have been structurally different, it is likely that chondrified fibroblasts were present in the common ancestor, being potentially lost in gnathostomes later as the oral cavity changed or developed different histologies from its previous form. Alongside these tissues, we must also consider whether the lamprey otic capsule cartilage is a homolog of gnathostome elastic cartilage. Our findings here posit that the otic capsule has
different histological properties than those seen in the hyaline of the branchial arches and trabecles, but these alone are not sufficient for homology. The differences between gnathostome elastic cartilage and hyaline seem minimal, as comparative studies between them only reveal a small set of different genes (Zhai et al., 2011) (Yao et al., 2022). Considering that lamprey hyaline is also distinguished by the presence of elastin-like genes like lamprin and pharymprin (McBurney et al., 1996a, 1996b; Yokoyama et al., 2019), it is likely that the staining differences between lamprey otic and hyaline cartilage are due to other differences in ECM structure than those between gnathostome elastic and hyaline cartilage. These two skeletal types are thus different hyaline types and we cannot posit whether this distinction was present in the common ancestor. Taken together, our findings support a complex skeleton at the base of vertebrates, although the specifics of this skeletal diversity and its deployment are still unclear.

Gnathostomes have been the dominant lineage of vertebrates for more than three hundred fifty million years in part to vast morphologies that have diversified land, air, and water. While there is no direct lamprey homolog for joint tissue, our results suggest that the ancestral vertebrate repertoire of cartilage and cartilage-like cells was highly diverse, an important step in the development and evolution of gnathostome morphologies. These different cells likely stem from changes to a core chondrogenic module, whether in the case of otic cartilage as smaller modifications to hyaline cartilage or in the case of mucocartilage as partially chondrified fibroblasts. This diversity of structural tissues was likely critical for the development of not only the gnathostome oral cavity but also that of cyclostomes and other jawless fishes. Based on our findings, we posit that skeletal modularity was pivotal for the evolution of gnathostomes, traces of which can be detected even in their distant ancestors.

**Methods**

**Isolation of lamprey homologs**

Lamprey collagen sequences were tiled from transcriptomic reads of Tahara st. 26.5 embryos and adult oral disc tissue that were previously gathered and submitted to GenBank (Wheeler et al., 2007). Sequences from these files were used for our phylogenetic and syntenic analyses. For *in situ* hybridizations for barxA, trps1, and gdf5/6/7b, primers were designed from lamprey genomic sequence to amplify conserved exon sequences, which were cloned into the pJet1.2 vector. For the remainder of the lamprey genes, 500-550bp regions from transcriptomic sequences were selected and ordered as fragments in pUC57-amp vector from Synbio Tech®. All sequences used in this study have been submitted to NCBI and can be found in Table S1.

**Embryo Collection and Staging.**

Embryos for *in situ* hybridization were obtained from adult spawning-phase sea lampreys (Petromyzon marinus) collected from Lake Huron, MI, and kept in chilled holding tanks as previously described (Nikitina et al., 2009). Embryos were staged according to the method of Tahara (Tahara, 1988), fixed in MEMFA (Mops buffer, EGTA, MgSO4, and formaldehyde), rinsed in Mops buffer, dehydrated into methanol, and stored at −20 °C.
In Situ Hybridization

Riboprobes were made for anti-sense fragments using SP6 RNA Polymerase. Sequences for probes and genes are available upon request. In our experience, full-length P. marinus riboprobes, or riboprobes generated against untranslated regions of P. marinus transcripts, give higher background than short riboprobes against coding sequences. We believe that this is because lamprey noncoding sequences, especially 3′ UTRs, often have an excessive GC-repeat content, causing corresponding riboprobes to hybridize nonspecifically to off-targets. To mitigate this, we made short 550-bp riboprobes against coding regions and used a high-stringency hybridization protocol (Cerny et al., 2010; Square et al., 2016). Key parameters of this protocol include post-hybridization washes at 70 °C and the use of a low-salt, low-pH hybridization buffer (50% formamide; 1.3× SSC, pH 5.0; 5 mM EDTA, pH 8.0; 50 μg/mL tRNA; 0.2% Tween-20; 0.5% CHAPS; and 100 μg/mL heparin).

Histology, Histochemistry, and Sectioning

After in situ hybridization, embryos were postfixed in 4% paraformaldehyde/PBS (4 °C, overnight), rinsed in PBS, cryo-protected with 15% sucrose/PBS, embedded in 15% sucrose, 20% gelatin/PBS (37 °C, overnight), and 20% gelatin/ PBS (37 °C overnight), frozen in liquid nitrogen, and mounted in OCT compound (Miles). Cryo-sections of 14 μm were collected on Super Frost Plus slides (Fisher Scientific), counterstained using Nuclear Fast Red (Vector Laboratories), and dehydrated and mounted in DPX (Fluka) (Jandzik et al., 2014). For Masson Trichrome and RGB staining, formaldehyde-fixed embryos were rinsed in PBS, dehydrated with alcohol and infiltrated with Histoclear II, and lastly embedded in Paraplast© overnight. Sections of 8-10 μm were collected on Super Frost Plus slides (Fisher Scientific). All slides were rehydrated and cleared with Histoclear II for 15 minutes before staining. For TB staining, embryos were progressively infiltrated in Infiltration Solution (JB4 Monomer A / benzoyl peroxidase) / EtOH for several hours before being left overnight in 100% Infiltration Solution. The following day, embryos were embedded in 25:1 Infiltration Solution and JB4 Monomer B. Sections of 4-6 μm were collected on Super Frost Plus slides (Fisher Scientific) using a glass knife.

Toluidine Blue, Masson Trichrome, and RGB staining were done on cleared slides with minor modifications to each. For Toluidine Blue, dehydrated slides were treated in 0.1% Toluidine Blue at 70°C for thirty seconds, washed in three series of distilled water for three minutes each, and progressively dehydrated, recleared, and mounted in DPX (Fluka). For Masson Trichrome, slides were treated in Bouin Solution at 56°C for 15 minutes, Weigert’s Solution (Sigma Aldrich) for 5 minutes, 1% Biebrich Scarlet / Acid Fuchsin (Sigma Aldrich) for 5 minutes, 5% PTA / PMA (Sigma Aldrich) for 10 minutes, 2.5% Aniline Blue (Sigma Aldrich) for 8 minutes, 1% acetic acid for 1 minute, and lastly progressively dehydrated, recleared, and mounted in DPX (Fluka). For RGB staining, slides were treated in 1% alcian blue pH 2.5 for 20 minutes, 1% Fast Green FCF for 20 minutes, 1% Picrosirius for 30 minutes, two washes in 1% acetic acid for 5 minutes each, and lastly progressively dehydrated, recleared, and mounted in DPX (Fluka).

Imaging
Whole-mount in situ hybridized *P. marinus* embryos and larvae were photographed using a Carl Zeiss Axiocam MRc5, Carl ZeissDiscovery V8 dissecting microscope, and Axiovision 4.9.1 software. Sections were photographed using a Carl Zeiss Imager A2 compound microscope.

**Declarations**

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**Ethics Approval**

The animal study was reviewed and approved by the IACUC Protocol 2392.

**Competing interests**

The authors declare no conflicts of interest regarding this study.

**Author contributions**

ZR, CA, CG, MB, and DJ did the experiments. ZR wrote the manuscript and created the figures. Edits were done by ZR. Final manuscript was approved by ZR, DJ, and DM.

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**Availability of data and materials**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in Table S1.

**References**


**Figures**
Figure 1

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Figure 3

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Figure 4

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Figure 5

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Figure 6

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Figure 7

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Figure 8

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