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Molecular and cellular changes associated with the evolution of novel jaw muscles in parrots

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Vertebrates have achieved great evolutionary success due in large part to the anatomical diversification of their jaw complex, which allows them to inhabit almost every ecological niche. While many studies have focused on mechanisms that pattern the jaw skeleton, much remains to be understood about the origins of novelty and diversity in the closely associated musculature. To address this issue, we focused on parrots, which have acquired two anatomically unique jaw muscles: the ethmomandibular and the pseudomasseter. In parrot embryos, we observe distinct and highly derived expression patterns for *Scx*, *Bmp4*, *Tgf β 2* and *Six2* in neural crest-derived mesenchyme destined to form jaw muscle connective tissues. Furthermore, immunohistochemical analysis reveals that cell proliferation is more active in the cells within the jaw muscle than in surrounding connective tissue cells. This biased and differentially regulated mode of cell proliferation in cranial musculoskeletal tissues may allow these unusual jaw muscles to extend towards their new attachment sites. We conclude that the alteration of neural crest-derived connective tissue distribution during development may underlie the spatial changes in jaw musculoskeletal architecture found only in parrots. Thus, parrots provide valuable insights into molecular and cellular mechanisms that may generate evolutionary novelties with functionally adaptive significance.

1. Introduction

Within vertebrates, birds (class Aves) are the second largest group, consisting of over 10 000 species. Although birds display wide variations in body size, coloration and habitat, their body plan is highly uniform among almost all lineages, possibly owing to the biomechanical constraints of flight. Even the morphology of the jaw complex, which is highly variable in other groups of vertebrates, is less diverse in birds, being basically composed of toothless upper and lower beaks and a consistent number of jaw muscles (figure 1*a*, left). An exception to this is parrots (order Psittaciformes), which have acquired two novel jaw muscles that are anatomically peculiar and adaptive for feeding on hard-shelled nuts and seeds via strong adduction of the mandible [1–4]. The ethmomandibular (*Musculus ethmomandibularis*) originates high on the rostral part of the interorbital septum and inserts into the dorsomedial aspect of the mandible, passing the lateral surface of the palatine bone (figure 1*a*, right). The pseudomasseter (*Musculus pseudomasseter*) covers the jugal bone laterally, bearing a superficial resemblance to the mammalian masseter muscle. In no other avian taxon is the jugal bone covered with jaw muscle fibres. Therefore, parrots can serve as a useful model for understanding developmental mechanisms that establish evolutionary novelties not only in birds but in vertebrates in general.

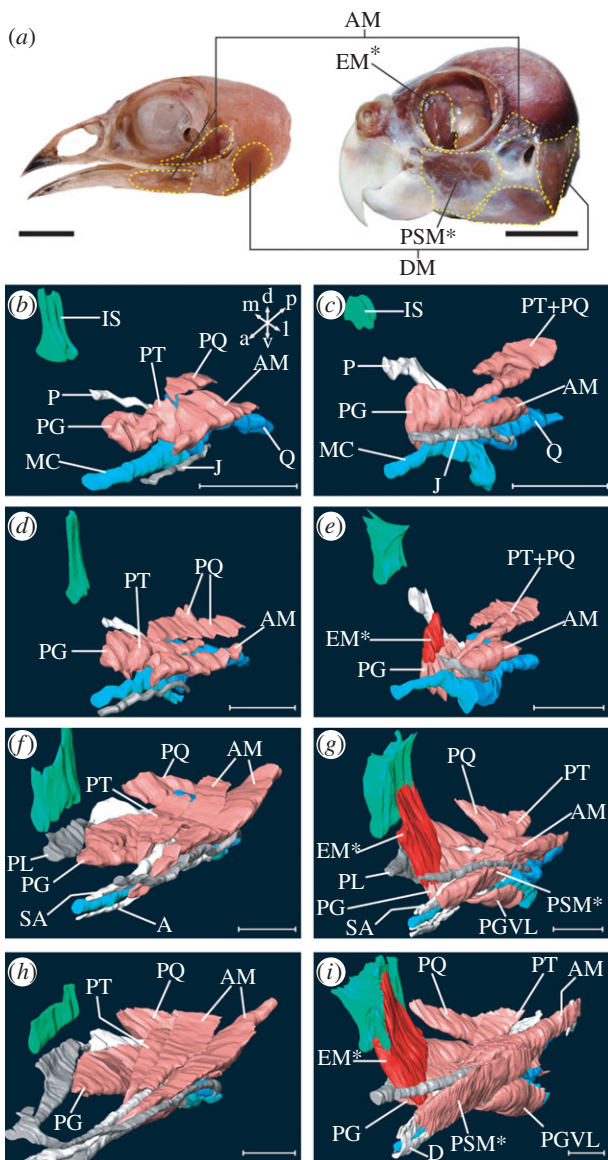


Figure 1. Morphogenesis of novel jaw muscles in parrots. (a) Quail (left) and parrot (right) heads with jaw muscles (yellow dashed lines). (b) Three-dimensional model of the jaw complex that is primarily composed of muscular, cartilaginous and intramembranous bone tissues, of quail at stage 30 reconstructed from a series of histological sections. Orientation of the model is indicated at right-upper corner: a, anterior; d, dorsal; l, lateral; m, medial; p, posterior; v, ventral. This orientation is applicable for other three-dimensional models. (c) Parrot at stage 30. (d) Quail at stage 32. (e) Parrot at stage 32. (f) Quail at stage 36. (g) Parrot at stage 36. (h) Quail at stage 38. (i) Parrot at stage 38. A, angular bone; AM, mandibular adductor muscle; D, dentary bone; DM, mandibular depressor muscle; EM, ethmomandibular muscle; IS, interorbital septum; J, jugal bone; MC, Meckel's cartilage; P, pterygoid bone; PG, pterygoideus muscle; PGVL, ventrolateral part of pterygoideus muscle; PL, palatine bone; PQ, quadrate protractor muscle; PSM, pseudomasseter muscle; PT, pseudotemporal muscle; Q, quadrate cartilage; SA, surangular bone. Asterisked muscles are specific to parrots. Scale bars, (a) 10 mm, (b–i) 1 mm.

Developmentally, jaw muscle fibres are derived from cranial paraxial mesoderm (reviewed by Noden & Francis-West [5]). By contrast, both the jaw skeleton and associated connective tissues, including ligaments, tendons and endomysia distributed in individual muscles, form from cranial neural crest mesenchyme (reviewed by Noden & Francis-West [5]). A series of studies, including mutant screens [6], tissue

ablation [7] and hetero-specific transplantation [8,9], have revealed that the cranial neural crest has a crucial role in the differentiation and shaping of mesoderm-derived cranial muscles. Based on these results, we hypothesized that the neural crest is elemental not only in maintaining the highly conserved patterns for individual jaw muscles but also in the evolution of novel jaw muscles.

To test this hypothesis, we have taken a comparative approach in this study. First, we described the pattern of jaw morphogenesis in both parrot and non-parrot birds, using computer-aided reconstructions of serial sections. Second, we analysed gene expression patterns in the cranial musculoskeletal tissues for two avian lineages. In this analysis, differentiated muscle fibres were specifically stained by immunohistochemistry with an anti-skeletal muscle myosin antibody. To label cranial mesoderm-derived muscle precursors as well as differentiated muscles, we performed *in situ* hybridization of *MyoD* [10]. We used *Runx2* as a marker for early osteoblasts [11], and *Scx*, *Six2* and *Tenascin-C* as markers for connective tissues [12–14]. We also examined expression pattern of genes involved in signalling pathways known to regulate connective tissue development in vertebrates: BMP, FGF and TGF- β [15–23]. In this study, we focused on *Bmp4*, *Fgf8* and *Tgf β 2*, which are expressed in tendinous connective tissues and affect tendon development. Third, we assayed for differences in the proliferation of cells distributed in a differentiated jaw muscle, and cells in surrounding non-muscle connective tissues of parrot and non-parrot bird embryos. This approach enabled us to assess the potential role of cell proliferation in the patterning of novel jaw muscles. Our analyses on the molecular and cellular levels reveal that parrot embryos exhibit an alteration of jaw connective tissue distribution. This spatial alteration may be linked to neural crest development, and ultimately may allow parrots to acquire novel jaw muscles that have been a key innovation for their highly successful granivorous lifestyle.

2. Results

(a) Morphogenesis of novel jaw muscles in parrots

Understanding how and when the novel jaw muscles of parrots develop is prerequisite information for identifying potential molecular and cellular mechanisms. Thus, we first described morphogenesis of differentiated jaw muscles and associated skeletons of parrot embryos, using three-dimensional reconstructions of serial sections. We compared parrot jaw muscle morphology with that of stage-matched quail (order Galliformes), which was used as a generalized and more basal representative of birds. At stage 30, the spatial pattern of jaw muscle precursors is quite similar in the two species (see figure 1b,c and electronic supplementary material, movie files). However, we observed a unique dorsal projection of the anterior part of the pterygoideus muscle precursor in parrot embryos. This corresponds to initiation of the ethmomandibular development. Subsequently (stage 32–34), this muscle projection further grew in a dorsal direction in parrots (figure 1e). Corresponding muscle projection was never seen in stage-matched quail (figure 1d). At stage 36, the ethmomandibular of parrots reached the cartilage of the interorbital septum, passing the lateral side of the palatine bone (figure 1g). In stage-matched quail embryos, the pterygoideus muscle attached to the

ventrolateral edge of the palatine bone and no muscle covered the lateral surface of the bone (figure 1*f*). Initiation of development of the pseudomasseter, another novel jaw muscle of parrots, was observed in embryos at stage 36. This muscle branched off from the parent muscle (i.e. the mandibular adductor), and grew in a dorsolateral direction, almost covering the lateral surface of the jugal bone (figure 1*g*). The architectural patterning of the jaw muscles was almost completed in both parrot and quail by stage 38 (figure 1*h,i*). In parrot embryos, the dorsal attachment site of the ethmomandibular further broadened over the interorbital septum (figure 1*i*). Although the pseudomasseter was not separated completely from the medially located mandibular adductor, the lateral surface of the jugal bone was completely covered by the muscle in the middle part of the head (figure 1*i*).

(b) Unique expression of *Scx*, *Bmp4* and *Tgf β 2* in connective tissues associated with the ethmomandibular muscle

In parrot embryogenesis, the ethmomandibular develops earlier than the pseudomasseter. Spatially, the ethmomandibular develops in a more medial location close to the palate, compared with the pseudomasseter, which forms at a more lateral region of the head (figure 1). In stage 32 parrot embryos, *MyoD*-positive ethmomandibular muscle precursor overlay medially located and *Runx2*-positive palatine bone anlagen from a lateral direction (figure 2*b,e*). Between the muscle and the bone, an *Scx*-positive elongated tendinous layer was detected (figure 2*f*, arrowheads). In stage-matched quail embryos, the pterygoideus muscle, a parent muscle of the ethmomandibular acquired in parrots, approached the palatine bone via an underdeveloped tendinous tissue (figure 2*a,c,d*). The lateral surface of their palatine bone was never overlaid by any muscular or tendinous tissues (figure 2*a,c,d*).

In younger parrot embryos (at stages 28–29), an *Scx*-positive tendon primordial layer uniquely formed between the dorsomedial edge of *MyoD*-positive jaw muscle and *Runx2*-positive palatine bone precursors (figure 3*f–h,o,p*). Because we detected this *Scx*-positive tendinous layer in most anterior portion of the jaw muscle precursor, it probably corresponds to future dorsomedial tendon of the ethmomandibular. In stage-matched galliform embryos, an *Scx*-positive tendinous layer was not detected between *MyoD*-positive jaw muscle and *Runx2*-positive palatine bone precursors (figure 3*a–c,k,l*). Instead, *Scx*-positive mesenchyme was diffusively distributed around the jaw muscle precursors. To screen candidate signaling pathways that govern the development of such spatially unique tendon in parrots, we examined expression patterns of *Bmp4*, *Fgf8* and *Tgf β 2*, which are reportedly expressed in tendinous tissues, and compared them in embryos of two avian lineages.

Although *Fgf8* was expressed only in the oral epithelium in early stage (stages 24–28) embryos of both parrot and galliform birds (see the electronic supplementary material, figure S1), *Bmp4* and *Tgf β 2* were expressed in neural crest-derived connective tissues associated with the jaw muscles. In galliform embryos at stages 28–29, expression of *Bmp4* was detected at the distal mesenchyme of the jaws and the mesenchyme within the eyelid primordium (figure 3*e,n*, arrows). By contrast, in stage-matched parrot embryos,

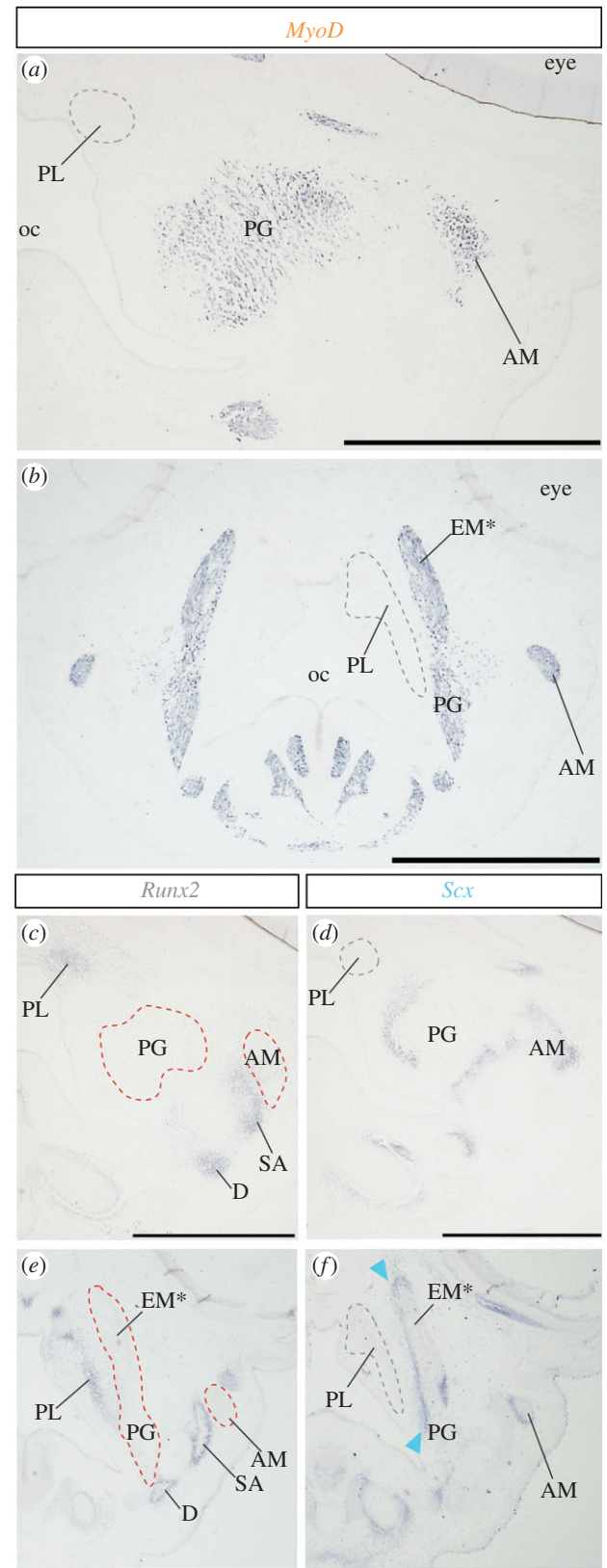


Figure 2. Substantial alteration of cranial musculoskeletal tissue arrangement in parrot embryos revealed by gene expression analysis. (*a,b*) *MyoD* expression in jaw muscles of quail and parrot embryos at stage 32, respectively. (*c*) *Runx2* and (*d*) *Scx* expression in adjacent sections of (*a*). (*e*) *Runx2* and (*f*) *Scx* expression in adjacent sections of (*b*). Note *Scx* expression in tendinous tissue that covers the palatine bone laterally in parrot embryo (arrowheads). oc, oral cavity. Other abbreviations as in figure 1. Scale bars, 1 mm.

unique expression of *Bmp4* was detected in the mesenchyme associated with the dorsal part of the ethmomandibular (figure 3*j,r*, arrowheads), as well as in the distal mesenchyme

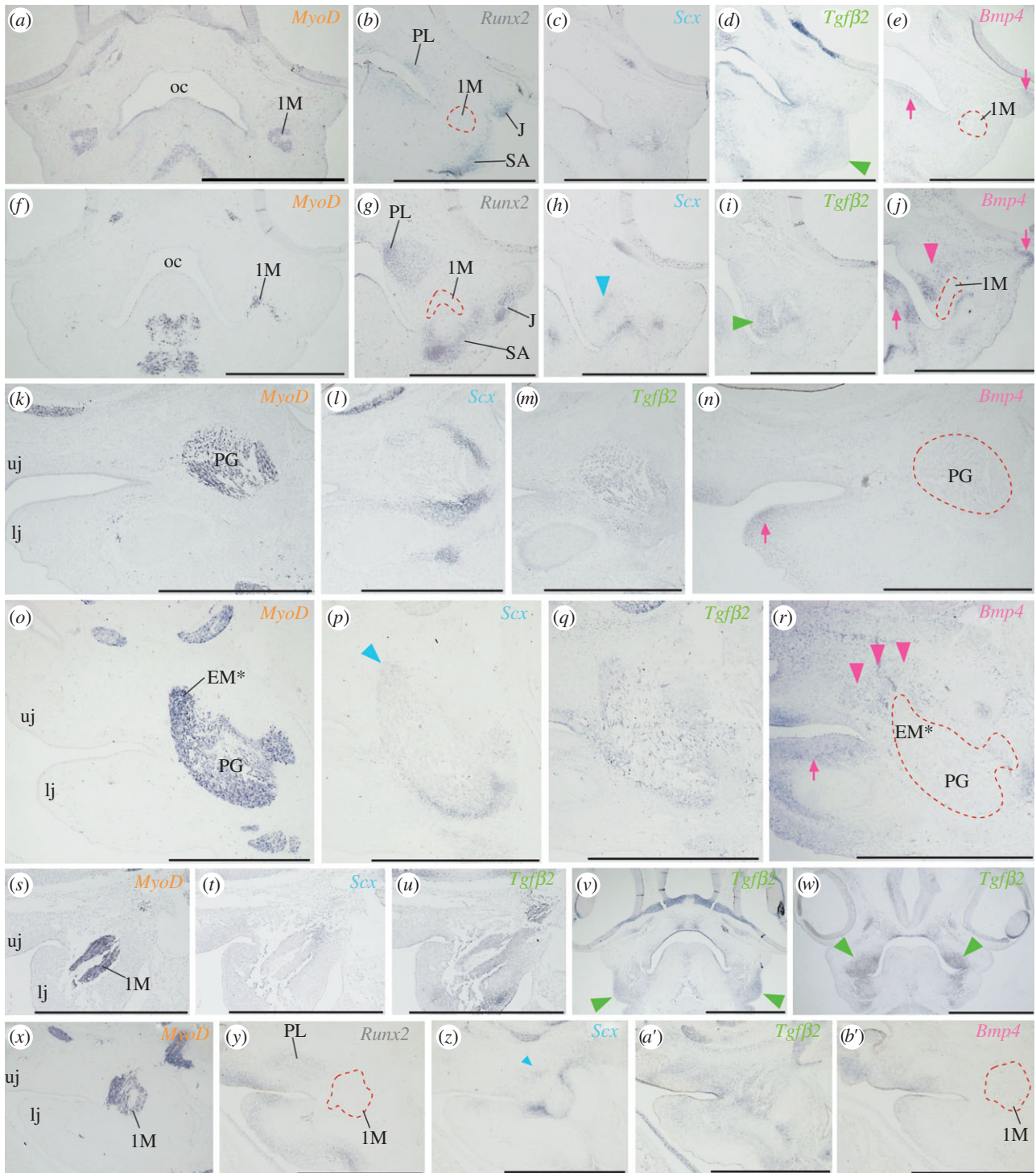


Figure 3. Unique expression of *Bmp4* and *Tgfβ2* in connective tissues associated with the ethmomandibular muscle. (a) *MyoD* expression in jaw muscle precursors (1M) of quail embryo at stage 28. (b) *Runx2*, (c) *Scx*, (d) *Tgfβ2* and (e) *Bmp4* expression in adjacent sections. (f) *MyoD* expression in jaw muscles of parrot embryo at stage 28. (g) *Runx2*, (h) *Scx*, (i) *Tgfβ2* and (j) *Bmp4* expression in adjacent sections. (k) Parasagittal section of chicken embryo at stage 29, where cranial muscles were labelled with *MyoD* probe. (l) *Scx*, (m) *Tgfβ2* and (n) *Bmp4* expression in adjacent sections. (o) Parasagittal section of parrot embryo at stage 29, where cranial muscular tissue was labelled with *MyoD* probe. (p) *Scx*, (q) *Tgfβ2* and (r) *Bmp4* expression in adjacent sections. (s) Parasagittal section of chicken embryo at stage 25, where cranial muscle precursors were labelled with *MyoD* probe. (t) *Scx* and (u) *Tgfβ2* expression in adjacent sections. (v) Frontal section of quail embryo and (w) of parrot embryo at stage 26, where expression domain of *Tgfβ2* was shown. (x) Parasagittal section of parrot embryo at stage 25, where cranial muscle precursors were labelled with *MyoD* probe. (y) *Runx2*, (z) *Scx*, (a') *Tgfβ2* and (b') *Bmp4* expression in adjacent sections. Arrowheads in (d), (h), (i), (j), (p), (r) (v), (w) and (z) indicate gene expression in neural crest-derived connective tissues associated with jaw muscles. Arrows in (e), (j), (n) and (r) indicate gene expression in neural crest-derived mesenchyme that is distant from jaw muscle tissues. lj, lower jaw; uj, upper jaw. Other abbreviations as in figure 1. Scale bars, 1 mm.

of the lower jaw and the mesenchyme within the eyelid primordium (figure 3j,r, arrows). In both parrot and galliform embryos at stages 28–29, expression of *Tgfβ2* was detected in the mesenchyme associated with the jaw muscle precursors (figure 3d,i,m,q). In galliform embryos at stages 24–26, *Tgfβ2* was

detected in the mesenchyme associated with jaw muscle precursors formed in the lower jaw primordium (figures 3s,u and 4v, arrowheads; electronic supplementary material, figure S2). In (doi:10.1007/s00442-004-1809-7) these embryos, an *Scx*-positive tendon precursor was diffusively distributed around

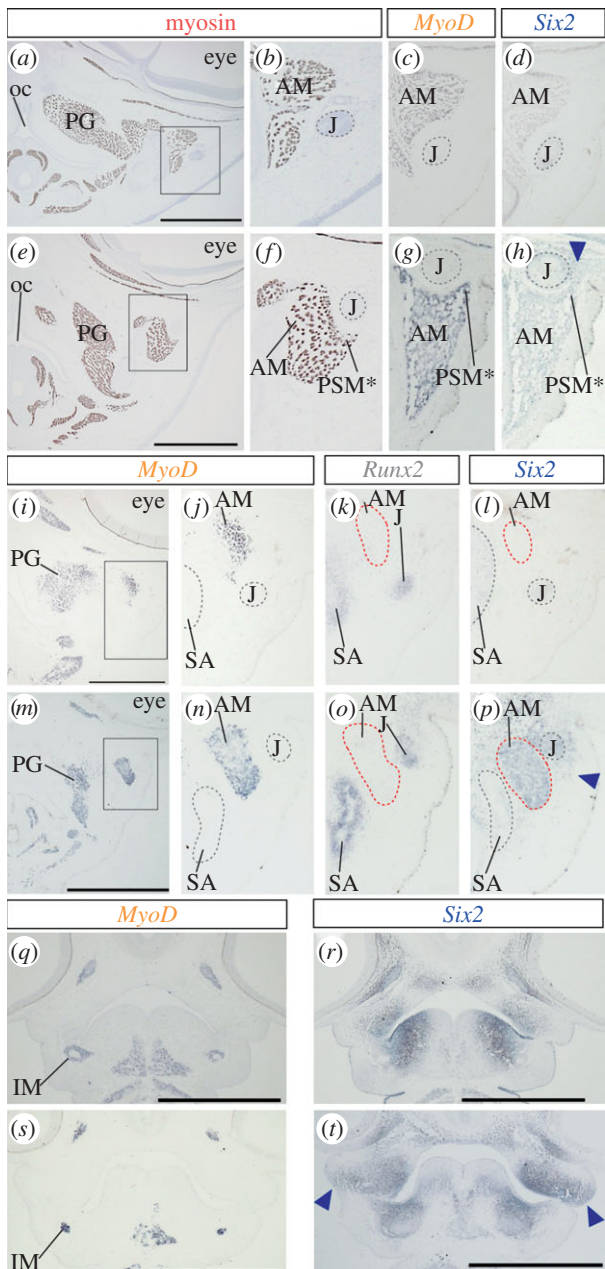


Figure 4. Unique expression of *Six2* in tendon of the pseudomasseter muscle. (a) Frontal section of a quail embryo at stage 36, where skeletal muscles were stained with myosin antibody. (b) Higher magnification of the rectangle in (a). (c) Frontal section of chicken embryo at stage 36, where *MyoD* expression in jaw muscle tissue was shown. (d) Expression of *Six2* in section adjacent to (c). (e) Frontal section of a parrot embryo at stage 36, where skeletal muscles were stained with myosin antibody. (f) Higher magnification of the rectangle in (e). (g) *MyoD* expression in the mandibular adductor muscle in parrot embryo at equivalent stage. (h) *Six2* expression in the tendon of the pseudomasseter muscle. (i) *MyoD* expression in cranial muscles of chicken embryo at stage 32. (j) Higher magnification of the rectangle in (i). (k) *Runx2* and (l) *Six2* expression in adjacent sections. (m) *MyoD* expression in cranial muscles of parrot embryo at stage 32. (n) Higher magnification of the rectangle (m). (o) *Runx2* and (p) *Six2* expression in adjacent sections. (q) *MyoD* and (r) *Six2* expression in frontal section of quail at stage 26. (s) *MyoD* and (t) *Six2* expression in parrot embryo at stage 26. Arrowheads in (h), (p) and (t) indicate unique expression of *Six2* in neural crest-derived connective tissue or mesenchyme in parrot embryos. Abbreviations as in figure 1. Scale bars, 1 mm.

the jaw muscle precursor (figure 3t). In stage-matched parrot embryos, *Tgfb2* was expressed in neural crest-derived mesenchyme distributed in both upper and lower jaw

primordia (figure 3w, arrowheads; figure 3a'; electronic supplementary material, figure S2). In the upper jaw primordium, *Tgfb2* was expressed in the mesenchyme distributed in proximity to both *Runx2*-positive palatine bone precursor and *MyoD*-positive jaw muscle precursor (figure 3w,a'). In these embryos, unique expression of *Scx* was just initiated in a population of mesenchyme located between jaw muscle and palatine bone precursors (figure 3y,z, arrowhead). We could not observe expression of *Bmp4* in the mesenchyme adjacent to the palatine bone and jaw muscle precursors of these embryos (figure 3b'). The results on expression domains for each gene analysed in parrot and galliform bird embryos are summarized in the electronic supplementary material, table S1.

(c) Unique expression of *Six2* in tendon of the pseudomasseter muscle

Of two novel jaw muscles of parrots, the pseudomasseter begins to develop at a relatively late embryonic stage (figure 1). The pseudomasseter was first recognized in parrot embryos at stage 36, as a small dorsolateral projection of mandibular adductor muscle (figure 4a–c,e–g). As described in a previous study [2], we found the condensation of connective tissue at the dorsal tip of the muscle (figure 4h, arrowhead). This connective tissue condensation occupied the domain lateral to the jugal bone and expressed connective tissue-marker genes: *Scx*, *Six2* and *Tenascin-C* (see figure 4h and electronic supplementary material, figure S3). This type of connective tissue condensation was absent in the corresponding domain of galliform quail and chicken embryos (figure 4d). In parrot embryos at stage 32, where the pseudomasseter has not yet formed (figure 4i–p), only *Six2* was expressed in the connective tissue cells distributed at the domain lateral to *Runx2*-positive jugal bone and *MyoD*-positive mandibular adductor muscle (figure 4p, arrowhead). In adjacent sections, *Scx* and *Tenascin-C* were not expressed in corresponding connective tissue cells (see the electronic supplementary material, figure S3). Expression of *Six2* was never detected at the corresponding domain in stage-matched galliform embryos (figure 4l). In younger embryos (at stages 26 and 24), no interspecific difference was observed in the spatial pattern of skeletal muscle precursors that express *MyoD* (figure 4q,s). By contrast, substantial differences were observed in the domain of *Six2* expression between parrot and galliform birds (figure 4r,t). The expression domain of *Six2* in neural crest-derived mesenchyme broadened in a lateral direction in the upper jaw primordium of parrot embryos (figure 4t, arrowheads; electronic supplementary material, figure S2).

(d) Cell proliferation activity in jaw muscle and surrounding connective tissue

Through the earlier-mentioned analyses, we found that both the ethmomandibular and pseudomasseter muscles of parrots acquired new tendons during embryogenesis. The formation of these muscles was initially achieved by expansion of the muscle into the new attachment sites where the new tendons were located. Based on those observations, we hypothesized that active cell proliferation in neural crest-derived endomysial connective tissues, which are distributed in the jaw muscle mass, drives this expansion. To test this hypothesis, we examined the level of cell proliferation activity in the cells composing a differentiated jaw muscle

Table 1. Comparison of the ratio of cell proliferation in jaw muscle and non-muscular tissues surrounding muscle.

species	specimen	tissue	total no. cells (=A)	no. PHH3-positive cells (=B)	B/A (%)
quail	1	muscle	1228	30 (myosin ⁺ : myosin ⁻ = 2:28)	2.44
		non-muscle	913	15	1.64
	2	muscle	987	27 (0 : 27)	2.74
		non-muscle	797	14	1.76
parrot	1	muscle	3892	52 (1 : 51)	1.34
		non-muscle	1003	8	0.80
	2	muscle	5974	166 (1 : 165)	2.78
		non-muscle	817	17	2.08
	3	muscle	4601	90 (3 : 87)	1.96
		non-muscle	1017	12	1.18

and the cells within surrounding connective tissue. We used an antibody specific for the mitotic marker phosphohistone H3 (PHH3; see electronic supplementary material, figure S4). For this experiment, we chose parrot embryos at stage 32 ($n = 3$) where a distinct growth of the ethmomandibular in an anterodorsal direction was observed (figure 1e). Then, we compared the numerical data on cell proliferation with that obtained from stage-matched quail embryos ($n = 2$) to find interspecific differences (table 1). As a result, although we did not see any remarkable interspecific differences in the mode of cell proliferation, cell proliferation was always more active (at least 50% more active on average) within jaw muscle compared with surrounding connective tissue in both species. Within jaw muscle, a majority of the cells undergoing proliferation (PHH3-positive) were not myosin-positive differentiated myocytes but myosin-negative cells (at least over 90% of the total number of cells counted).

3. Discussion

To date, much attention has been paid to the anatomical diversification of the bones and cartilages of the jaws [24–26], and especially the fundamental role of the cranial neural crest mesenchyme in this process (reviewed in recent studies [27–29]). By contrast, few studies have identified developmental mechanisms that account for the diversity of jaw muscle morphology. Previously, we conducted hetero-specific neural crest transplantations between quail and duck, to test whether quail neural crest-derived connective tissues could confer species-specific identity to duck mesoderm-derived jaw muscles [9]. In these chimaeras, duck host mesoderm-derived jaw muscles acquired quail-like shape and attachment sites owing to the presence of quail donor neural crest-derived connective tissues. Based on these results, we concluded that neural crest mesenchyme regulates the species-specific jaw muscle morphology [9].

Interestingly, the number and organization of jaw muscles have been extremely modified in a few vertebrate taxa. For example, pufferfish (order Tetraodontiformes) show substantial alteration of jaw muscle morphology, principally caused by duplication of mandibular adductor muscles [30]. Similarly, parrots have acquired two novel jaw muscles that are anatomically peculiar and not possessed

by any other avian lineages [1,2,4]. However, molecular and cellular mechanisms underlying the acquisition of such novel jaw muscles have not been investigated in these non-model vertebrates. Migration of neural crest mesenchyme from the neural tube into the first pharyngeal arch is relatively more advanced in parrots than in galliforms [31]. Such temporal shifts in neural crest cell migration may influence the organization of the jaw muscles [31]. To test our hypothesis that neural crest mesenchyme plays a crucial role not only in determining shape and attachment sites of individual jaw muscles but also in the evolution of architecturally unique new jaw muscles, we performed a series of comparative analyses using parrot embryos.

In this study, we observed unique expression patterns for several genes that are expressed in the neural crest-derived connective tissues of parrots. Regarding the ethmomandibular, we speculate that spatially unique expression of *Tgfβ2*, *Scx* and *Bmp4* in neural crest-derived connective tissue may be important in its development. TGF-β signalling is crucial for the development of cranial neural crest-derived tissues [25,32,33]. In this study, we focused on *Tgfβ2*, which is expressed in cranial neural crest-derived mesenchyme [15,34,35]. In stage 24 parrot embryos, we observed *Tgfβ2* expression in the neural crest-derived mesenchyme distributed in both the upper and lower jaw primordia. In subsequent stages (stages 25–26), the *Tgfβ2* expression domain was expanded into the mesenchyme that ultimately differentiates into the palatine bone and the mesenchyme associated with the jaw muscle precursors. At the periphery of the *Tgfβ2*-positive connective tissue, *Scx*-positive tendon precursor appeared. Corresponding tendon primordia did not form in the head of galliform embryos. This situation mimics the observation that TGF-β2-soaked beads transplanted into cranial or appendicular tissues can upregulate *Scx* expression [15,20,21,22]. Thus, we speculate that the spatial alteration of *Tgfβ2* expression in neural crest-derived mesenchyme may be crucial in enabling the formation of the new tendon necessary to attach the ethmomandibular in parrots.

BMP signalling also plays essential roles in the development of craniofacial tissues. In this study, we focused on *Bmp4*, which is expressed in neural crest-derived tissues, and regulates the development and morphology of the neural crest-derived skeleton [24,36–38]. In stage 28 parrot embryos, we observed unique expression of *Bmp4* in the

neural crest-derived mesenchyme distributed between the palatine bone and the ethmomandibular muscle precursors. In these embryos, *Scx*-positive tendon precursors have already formed at the dorsal tip of the ethmomandibular precursor. *Bmp4* is expressed in tendon, and regulates proliferation and differentiation of muscles through activation of BMP signalling [23]. Also, *Bmp4* expression in tendons is regulated by *Scx* [16], which is a downstream target of TGF- β signalling. Currently, much remains to be understood about how TGF- β signalling may affect the unique expression pattern of *Bmp4* in the neural crest-derived mesenchyme of parrot embryos. We speculate that spatial alteration of *Bmp4* expression in neural crest-derived mesenchyme may affect proliferation and differentiation of the ethmomandibular, and thus allow this muscle to expand into a new attachment site in the head of parrots.

FGF signalling is known to function in tendon development [19]. In this study, we focused on *Fgf8*, which upregulates *Scx* expression in trunk tendons of vertebrate embryos [17,18]. Although we detected *Fgf8* expression in tendinous and fascial tissues associated with jaw muscles in late-stage parrot embryos (at stage 36 or older), its expression was not observed in the neural crest-derived mesenchyme associated with the jaw muscle precursors in early-stage parrot embryos (younger than stage 36). These results may relate to a lesser contribution of FGF signalling in the evolution of the novel jaw muscles in birds.

Through comparative analysis of cell proliferation among tissues, we found that the cells within the jaw muscle proliferate more actively than the surrounding connective tissue cells in avian embryos. Of these actively proliferating cells within the jaw muscle, a majority were myosin-negative cells. Unfortunately, our method is unable to distinguish mesoderm-derived and undifferentiated muscle progenitor cells from neural crest-derived mesenchyme. However, we speculate that a majority of the cells inside the jaw muscle could be the latter because there is an increase in the number of neural crest-derived mesenchymal cells inside muscles after stage 27 [5,39], and muscle progenitor cells tend to be located at the periphery of the muscle [23]. Synthesizing the results above, we propose a working hypothesis about the developmental mechanism underlying the evolution of the ethmomandibular. This hypothesis will be tested in future studies (see the electronic supplementary material, figure S5).

Another novel jaw muscle of parrots, the pseudomasseter, develops at a relatively late embryonic stage (after stage 36) and at the lateral part of the head. We speculate that spatially unique expression of *Six2* in neural crest-derived mesenchyme may be crucial in pseudomasseter development. *Six2* is a transcription factor that regulates development of urogenital and digestive organs, and its transcripts are also expressed in connective tissues such as tendons and ligaments [12,13]. In the head, *Six2* is expressed in neural crest-derived mesenchyme [40] and regulates skull development [41,42]. In stage 32 parrot embryos where the pseudomasseter has not formed, *Six2*-positive neural crest-derived connective tissue was observed in the domain lateral to both the jugal bone and the mandibular adductor muscle precursors. In younger parrot embryos (stage 24–26), the *Six2* expression domain already broadened laterally in the upper jaw primordium, where future jugal bone develops. By contrast, expression of *Six2* was more biased into the medial portion of the upper jaw primordium

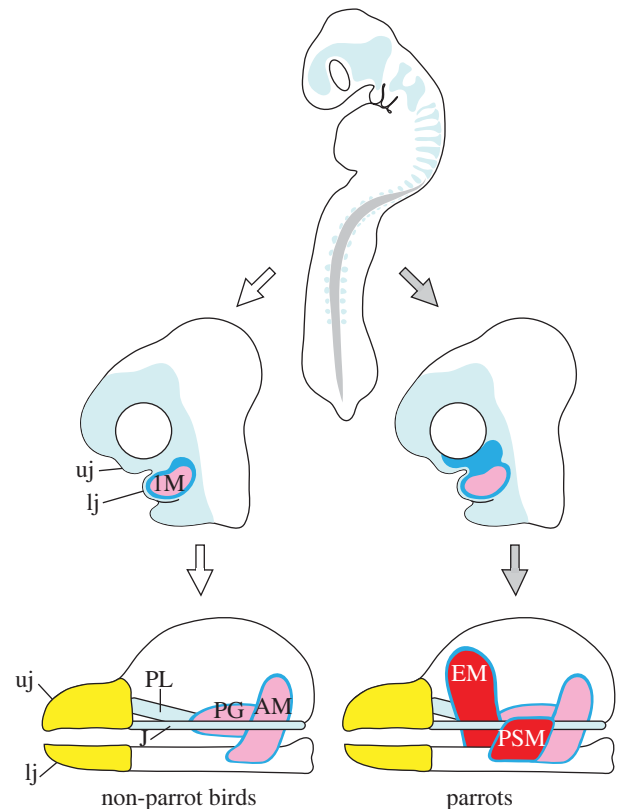


Figure 5. Schematic diagram depicting possible association of neural crest mesenchyme in the evolution of novel jaw muscles. Neural crest mesenchyme (pale blue) delaminates from the neural tube and migrates in a ventral direction in the head and trunk of early-stage embryos. In parrot embryos, development of populations of neural crest mesenchyme that later differentiates into jaw connective tissues (dark blue) is uniquely regulated, and this eventually leads to unique distribution of the mesenchyme (top black arrow), compared with the situation in non-parrot bird embryos (top white arrow). In parrots, spatially unique jaw muscle connective tissues that are derived from neural crest mesenchyme affect the organization of mesoderm-derived jaw muscles, and this eventually leads to the formation of novel jaw muscles with adaptive significance (bottom black arrow) that are absent in non-parrot birds (bottom white arrow). Abbreviations as in figure 1.

in galliform embryos. Expression of *Six2* in neural crest-derived mesenchyme distributed in pharyngeal arches is regulated by *Hoxa2* [43,44]. Alteration in *Hox* gene regulation may account for spatially unique expression of *Six2* that eventually forms the tendon of the pseudomasseter in parrots.

In conclusion, although the two novel jaw muscles of parrots form at different times and at different locations, they both appear to share a common developmental process, which is that their differentiation is preceded (and possibly even presaged) by the spatially unique populations of closely associated neural crest-derived connective tissues. We predict that this unique population of connective tissues can account for the evolution of novel jaw muscles (figure 5). As a next step, the nature of the interaction among genes that are uniquely expressed in neural crest-derived mesenchyme of parrots needs to be better defined, and the precise function of each of these genes should be tested in the context of novel jaw muscle development. Furthermore, understanding the interactions of adjacent cranial tissues (such as cranial nerves and blood vessels) with the jaw muscles will also be important for explaining how parrots and other vertebrate taxa can evolve novel features in their musculoskeletal systems.

4. Methods

A full description of methods, including sample collection, staging of embryos, cloning of genes, analysis of gene expression and tissue-marker protein localization, and comparative analysis of cell proliferation, can be found in the electronic supplementary material. All animal experiments were approved by the University of Tsukuba Committee for Animal Care.

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