ARTICLES

Neural crest origins of the neck and shoulder

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The neck and shoulder region of vertebrates has undergone a complex evolutionary history. To identify its underlying mechanisms we map the destinations of embryonic neural crest and mesodermal stem cells using Cre-recombinase-mediated transgenesis. The single-cell resolution of this genetic labelling reveals cryptic cell boundaries traversing the seemingly homogeneous skeleton of the neck and shoulders. Within this assembly of bones and muscles we discern a precise code of connectivity that mesenchymal stem cells of both neural crest and mesodermal origin obey as they form muscle scaffolds. The neural crest anchors the head onto the anterior lining of the shoulder girdle, while a *Hox*-gene-controlled mesoderm links trunk muscles to the posterior neck and shoulder skeleton. The skeleton that we identify as neural crest-derived is specifically affected in human Klippel–Feil syndrome, Sprengel's deformity and Arnold–Chiari I/II malformation, providing insights into their likely aetiology. We identify genes involved in the cellular modularity of the neck and shoulder skeleton and propose a new method for determining skeletal homologies that is based on muscle attachments. This has allowed us to trace the whereabouts of the cleithrum, the major shoulder bone of extinct land vertebrate ancestors, which seems to survive as the scapular spine in living mammals.

The vertebrate neck has undergone a drastic evolutionary transformation from an immobile bony bridge between head and shoulder in early vertebrates with paired fins[1] to a mobile system of muscle scaffolds interconnecting the head and shoulders in early jaw-bearing fish such as placoderms[2]. These scaffolds have retained a structure and function that has been remarkably conserved in jaw opening and head mobility ever since[3]. The fundamental changes in the skeleton of the neck and shoulders reflect evolving embryonic differentiation processes of mesenchymal stem cells: from bone to muscle connective tissues and cartilage. These have defied mechanistic analysis because the detection of fate changes in homologous cell populations requires experimental long-term lineage labelling which has so far only been possible in the chick[4], a species with a highly modified neck architecture[5]. Gills and most of the head skeleton are derived from the embryonic cranial neural crest[4], whereas the limb skeleton is derived from trunk mesoderm[6]. The neural crest and mesoderm do not provide obvious landmarks for their respective boundaries in the intervening neck transition zone: the cranial neural crest is not segmentally deployed in this post-otic region (behind rhombomere 5)[7] and limb lateral plate mesoderm does not seem to pattern the shoulder girdle proper[8]. With post-otic neural crest (PONC) and paraxial (somitic) mesoderm as candidate components, the neck between the ear (otic) capsule of the head and the trunk forelimbs has remained an uncharted embryonic territory.

Bone formation versus muscle scaffolds

Neural-crest and mesodermal cells seem to differ in the way in which they form bones: neural crest forms dermal and endochondral bones in the head whereas mesoderm forms endochondral skeleton in the trunk. So far no evidence for mesoderm-derived dermal bones has been produced. The shoulder girdle and neck between head and limbs contains both dermal and endochondral bones. All previous investigations into the evolution of this region have therefore assumed this dermal–endochondral distinction to be a safe indicator for bone origins and homologies: Accordingly, all dermal bones in the post-otic region are considered to be derived exclusively from the neural crest, whereas all endochondral bones are considered mesodermal[9,10]. The validity of this widely held 'ossification model' has remained untested in the neck of any living vertebrate. Indeed, in apparent contradiction of it, a current view holds the posterior boundary of neural-crest-derived skeleton to be the parietal (or frontal) bone of the skull[11,12]: no neural-crest-derived skeleton behind the ear capsule has yet been identified.

Comparative neck anatomy in living jawed vertebrates challenges the likelihood of the prevailing 'ossification model 1'. We note that the pattern of neck muscles (red in Fig. 1) is far more conserved than the ossification modes of the shoulder bones to which these muscles are attached (Fig. 1; pale grey regions are dermal, dark grey regions are endochondral bones). This poses a serious problem for muscle homologies: in all cranial and trunk regions of the vertebrate body so far examined the embryonic cellular origin of muscle connective tissues and their respective skeletal attachment regions are identical[13,14]. This implies that if attachment regions change in their cellular origins and ossification type, their coordinated muscle connective tissues also change in their composition. This would force us to reject the homology of all neck musculature in jawed vertebrates, although it has a highly similar and complex connectivity pattern (red in Fig. 1, ref. 5). A similar problem is posed by the composition of the clavicular bone itself (box 3 in Fig. 1). It is dermal in fish and amphibians, and of mixed dermal plus endochondral composition in mammals[15,16]. If the 'ossification model' holds, we would have to refute the homology of a bone that has changed its histogenesis but

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not its position inside the head–neck assembly over more than 400 Myr. In this light a competing 'muscle scaffold model 2' can be considered, according to which bones 'morph' around a highly constrained muscle attachment scaffold[13]. Cell population boundaries remain stable and are the structural basis for conserved muscle scaffolds, but the differentiation of PONC and mesodermal cells into bone, cartilage or muscle connective tissues varies because of changes in signalling pathways. This implies that cell population boundaries are cryptic; they do not coincide with bone sutures but with muscle attachment sites.

By using a recombinase-mediated genetic lineage labelling strategy in transgenic mice we can now discriminate between these two models. We map neck neural crest and mesoderm with single-cell resolution onto muscular (connective tissue) attachment points and skeletal structures of a given (dermal versus endochondral) ossification type (Fig. 2). The two models make mutually exclusive predictions for shoulder girdle origins: if model 1 is correct, the anterior scapular spine would be mesodermal because it is endochondral (left part of box 1 in Fig. 1); if model 2 is correct, the same skeletal region (box 1 in Fig. 1) would be neural crest because it serves as the attachment region for the trapezius muscle (T in Fig. 1) with expected neural-crest connective tissues.

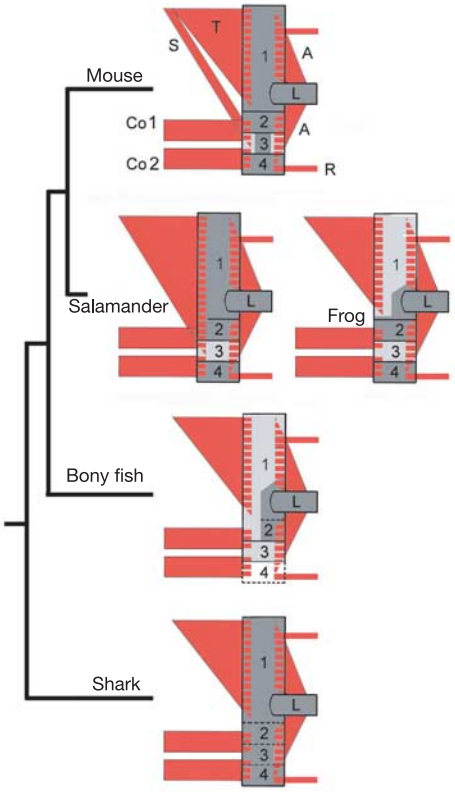

Figure 1 | Conservation of muscle scaffolds and diversity of shoulder ossification patterns. Highly conserved neck muscle scaffolds (red) attach (hatched areas) on a shoulder skeleton (boxes 1–4) that displays variable dermal (light grey) and endochondral (dark grey) ossification type. Attachment regions (hatching) of the gnathostome trapezius muscle (T)[5,50] are endochondral in sharks, salamanders[5] and all amniotes but are dermal in fish and frogs. A, limb muscles; Co1 and Co2, coraco-branchialis and coraco-hyoideus; L, limb skeleton; R, trunk muscles; S, sterno-cleido-mastoid. In the shoulder skeleton, box 1 is the dorsal cleithrum (dermal) in bony fish (*Polyodon*[5] or *Amia*[35]) and frog (*Rana*[36]) and the scapular region (endochondral) in salamander[5], mouse and living amniotes; box 2 is the acromio-coracoid (endochondral); box 3 is the clavicular region (dermal/dermal + endochondral), although in sharks[5] bone is absent and its space is taken by part of the scapulo-coracoid (stippled); and box 4 is the sternal region, comprising the sternum (endochondral) or connective tissue (bony fish).

Here we reveal a cryptic neural crest–mesoderm boundary inside the neck and shoulder girdle skeleton, which ignores traditional skeletal landmarks or (endochondral versus dermal) ossification types and thus invalidates the traditional 'ossification model'. Instead, cellular distributions of neural crest and mesoderm correspond precisely to muscle attachment scaffolds to the shoulder girdle, corroborating the non-intuitive 'scaffold model'. This finding illuminates the aetiology of various hitherto poorly understood congenital diseases in humans that are co-extensive with neural-crest-derived shoulder structures. By using the 'scaffold model' as a new arbiter of bone homologies, palaeontology can date fate changes of common precursor populations in fossils. This reveals an unexpected evolutionary directionality in underlying fate decisions of mesenchymal stem cells that originate from mesoderm and neural crest.

Cryptic neural crest in neck and shoulders

The key problem we wish to address is the full distribution of skeletal PONC. By using *Wnt-1* (ref. 17) and *Sox-10*–Cre-recombinase-mediated fate mapping (Fig. 2a) we ask three questions. First, can we find evidence that PONC forms endochondral bones? This determines whether either the 'ossification' or the 'scaffold model' are applicable to the neck region (Fig. 2b). Second, is the entire dermal skeleton behind the otic capsule derived from the neural crest, or is some of it mesodermal (Fig. 2a, b)? This will test the validity of the 'ossification model' in the only species that is currently accessible to high-resolution lineage mapping: the mouse. Third, does the distribution of neural crest and mesoderm correlate with muscle attachment points or with ossification types in the neck and shoulder skeleton? This will distinguish the explanatory value of the 'ossification model' from that of the 'scaffold model' because each model makes non-overlapping predictions about anterior shoulder girdle origins.

Neural crest proves to have an unexpectedly pervasive role in the mouse neck region, forming bone, cartilage and muscle connective tissue within two domains: first, an external, essentially tubular domain dominated by pharyngeal arch muscles that extends from the head to the entire ancestral shoulder girdle and incorporates its anterior part; second, a ventral internal domain comprising internal pharynx and larynx constrictors, tongue muscles, thyroid, cricoid and arytenoid cartilages and their respective muscle attachments at the oesophageal entry (Fig. 2b). Ensheathed between these two tubular domains lies a mesodermal domain centred on the somite-derived vertebral column, which reaches forward to the occipital region of the head (oc in Fig. 3).

The largest component of the external crest domain is the trapezius muscle and its attachment regions (tra in Fig. 3a–c). This is a branchial muscle, innervated by the accessory nerve X/XI with a position that has remained remarkably conserved in all jawed vertebrates[5]. The cranial neural crest connectivity code revealed by our previous chick–quail work[13] led us to predict that the connective tissue of the trapezius and all its post-otic attachments should be formed from PONC—even if they are endochondral. This indeed proves to be so. At the anterodorsal end, a patch of $LacZ^{+}/GFP^{+}$ PONC endoskeleton forms the occipital protuberance inside otherwise mesodermal (occipital) territory; that is, the trapezius attachment point in the skull (Fig. 3a). The labelling extends to the attached nuchal ligament (nl in Fig. 3a), the trapezius muscle connective tissues (Fig. 3b) as well as their respective endoskeletal attachment region on the entire anterior margin and inside the scapular spine (pc, Fig. 4a, b), the coracoid, acromion (Fig. 4c, box 2 in Fig. 1) and also the periosteal muscle attachment caps on spinous processes of all cervical vertebrae (sp of C_4 in Fig. 3c). Thus, the posterior neural crest boundary is found inside the shoulder girdle endoskeleton, where it forms an anterior attachment region of branchial muscles.

PONC thus generates extensive areas of endochondral ossification in the shoulder girdle and cervical vertebral column, contradicting the traditional notion that these regions are wholly mesodermal[4,11]. However, the crest contributions are morphologically cryptic: their only visible anatomical landmarks are the branchial muscle attachments. Tracing the posterior PONC boundary more ventrally we investigate the sterno-cleido-mastoid muscle (N. XI; scm in Fig. 5a, b, c, e). This originates on the post-otic mastoid process of the skull (m in Fig. 5a, b) and attaches onto the endoskeletal anterior sternum (st in Fig. 5a, e) as well as along the anterior margin of the dermal clavicle (cl in Fig. 5a, c, d). We find green LacZ$^+$/GFP$^+$ PONC cells inside all these attachment sites. Our genetic labelling provides first detailed insights into cellular architecture and origins of the clavicle (cl, black box in Fig. 5). The clavicle bone forms from an anterior (buccal) dermal ossification centre (Fig. 5d) and a posterior dermal ossification centre (cld, Fig. 5g), which later fuse and surround a cartilaginous core in mammals (Fig. 5c, g)[15,16]. The anterior dermal ossification is derived purely from PONC (green box, Fig. 5d). More medially, attachment regions of the (sterno-cleido-mastoid (scm) and the fascial sling of omohyoid (ohs) branchial muscles as well as most of the cartilaginous core of the clavicle are also derived from neural crest (Fig. 5c). Thus, as inside the scapula, PONC inside the clavicle gives rise to endochondral bone.

The ventral shoulder girdle carries a series of muscles that connect it to ventral branchial elements (Box 4, Co1 and Co2 in Figs 1 and 7): M. omohyoideus (connecting the anterior scapula next to the coracoid, and the internal clavicle, with the hyoid; oh in Figs 4c and 5a), M. sternohyoideus (connecting the manubrium sterni and clavicle to the hyoid; sh in Fig. 4d) and M. sternothyroideus (sth in Fig. 4d), connecting the manubrium sterni (mst) with the thyroid cartilage (t in Fig. 5a). These are homologues of the coracobranchial muscles (Co1 and Co2), which are present in all jawed vertebrates and effect rapid jaw opening and retraction of the branchial skeleton[3,5]. As these are in origin branchial muscles (innervated by cranial nerves[5]) we proposed that their connective tissue would be crest derived; this is indeed so. Swallowing in all jawed vertebrates requires internal pharynx and larynx constrictors (const. phar. in Fig. 6g), which constitute the internal tubular crest domain outlined above. These are connected to the mesodermal ventral neck vertebrae through the pre-vertebral ligaments (Fig. 6e) as well as to the mesodermal cranial base (the so-called clivus) anterior to the foramen magnum through the pharyngobasilar fascia (Fig. 6g). Their branchial innervation by N.IX./X. suggests that their attachment regions area also PONC-derived in all vertebrates. Despite being at odds with the commonly held notion that 'chordal' cranial base is entirely mesodermal[4,11], our PONC-labelled mice show constrictor muscle attachment points of neural crest origin (attp in Fig. 6g) focally inserted into the otherwise mesodermal endochondral cranial

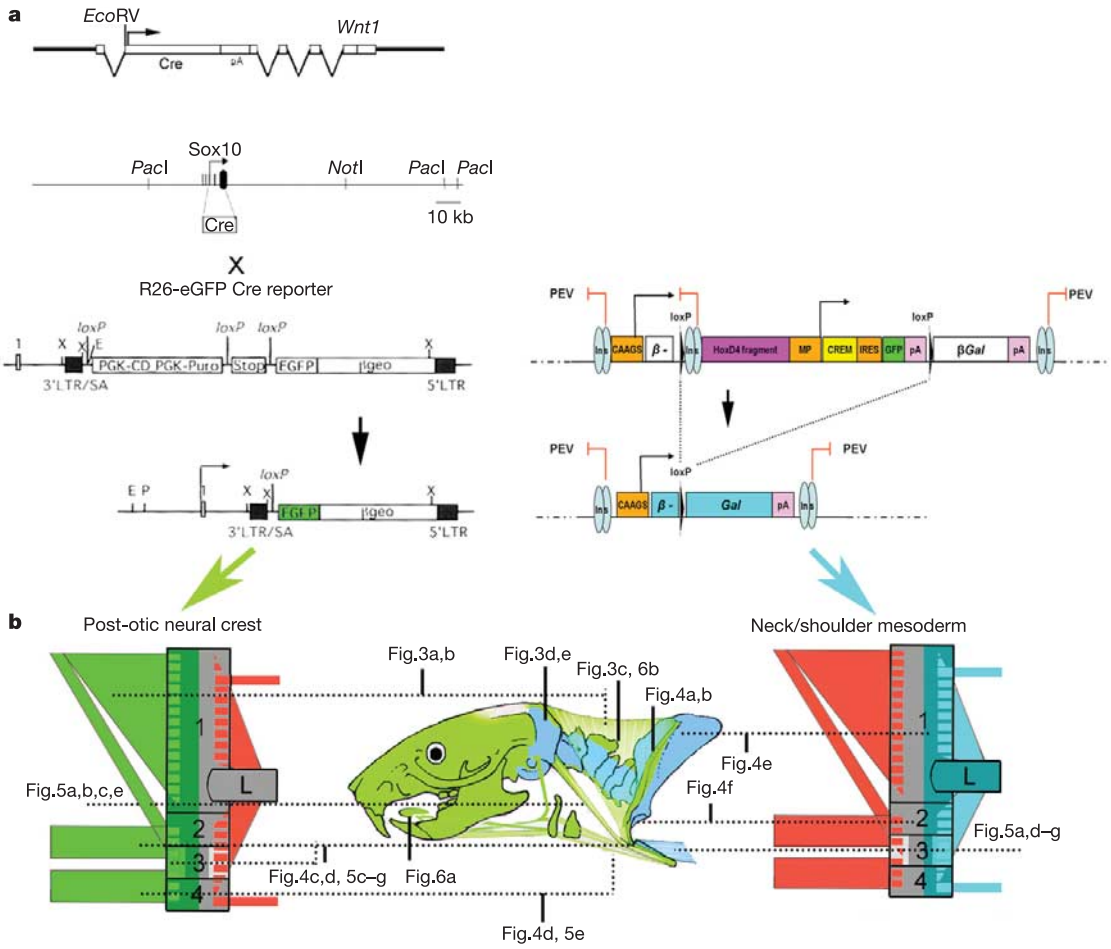

Figure 2 | Genetic lineage labelling of PONC and somitic mesoderm in the neck. a, Transgenic mice carrying a *Wnt*–Cre construct (ref. 17) (top left) or a 170-kilobase *Sox10*–Cre construct (bottom left) were crossed into R26-LacZ [45] and R26-eGFP (ref. 46) reporters, to permanently label all pre- and post-migratory post-otic/neck neural crest (green). Right: a self-excising Cre recombinase leads to the enhancer-dependent reconstitution of a β-Gal reading frame (blue) in the complementary (HoxD4+)mesodermal stem cell population of somite 5 and posterior (blue). Technical details are given in Methods and Supplementary Methods S1. **b**, Anatomical guide through mouse neck and shoulder skeleton, with keys to diagrams and micrographs.

base (mes in Fig. 6g). More posteriorly, thyroid, cricoid and arytenoid cartilages and their respective muscle attachments at the oesophageal entry are also derived from neural crest, with tracheal cartilages demarcating the anterior mesoderm boundary (data not shown). The entire intrinsic tongue musculature that is attached to crest-derived branchial skeleton has crest-derived connective tissue, despite being innervated by N.XII and cervical spinal nerves (Fig. 6a). This demonstrates that motor innervation alone cannot serve as a reliable indicator of connective-tissue origins of embryonic muscle, but skeleton-muscular connectivity can. These genetic PONC-labelling experiments show that PONC in mouse behaves in the same way as the pre-otic crest studied in our previous experiments[13]. Crest cells form not only the connective tissue of the muscle but all its attachment points, irrespective of how these attachment points ossify, whether endochondrally or dermally or whether—as inside the tongue (Fig. 6a)—they do not ossify. This unveils a pervasive but anatomically cryptic 'muscle scaffold system' that is sharply defined at the single-cell level.

Rules of engagement in the mesodermal neck

In addition to branchial muscles at its anterior margin, the shoulder girdle in all jawed vertebrates serves as an attachment for mesodermal trunk and limb muscles (with spinal motor neuron innervation and connective tissues derived from mesoderm) at its posterior margin[5]. Mesodermal trunk muscles also attach to the occipital head (oc in Fig. 3)[11,14]. Although the mesodermal (somitic) origins of vertebrae and occiput have long been established, the rules of connectivity between muscles and bony elements have remained unclear. This prompted us to test whether skeletal attachment sites of muscles with mesodermal connective tissues are also mesodermal and of the same (*Hox* gene) axial identity. As axial *Hox* gene expression boundaries sometimes do not coincide with somitic boundaries[18], a genetic approach is required (Fig. 2a, right side and Supplementary Methods S1).

An analysis of HoxD4–CREM–LacZ transgenic mice shows that that posterior margin of the scapular spine (right part of Box 1 in Figs 1 and 7; blue in Figs 2b and 4e, f) and also that the entire scapular blade is of somitic ($LacZ^+$) origin at places where mesodermal

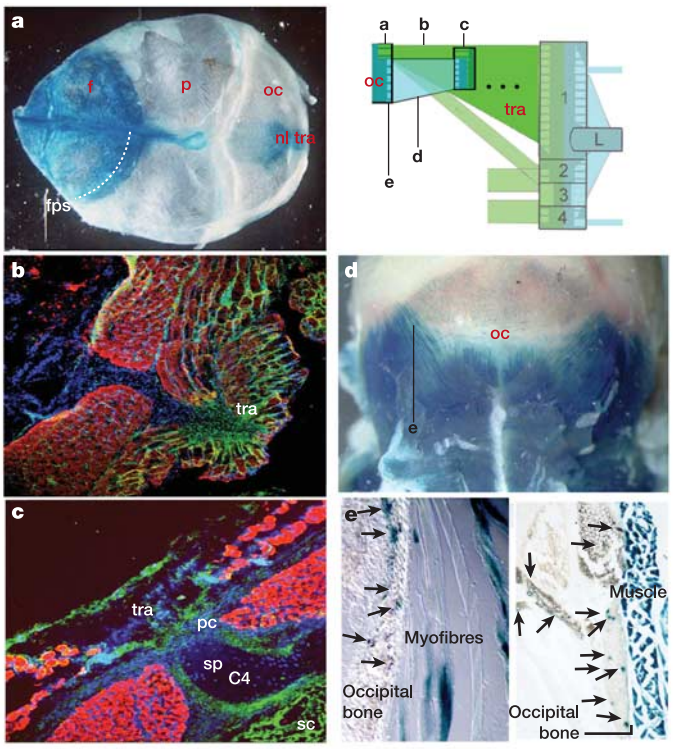

Figure 3 | Neural crest and somitic mesodermal origins of the neck. Green areas in the diagram are neural-crest-derived connective tissues; blue areas are mesodermal (somitic) connective tissues and muscle fibres. **a**–**c**, Neural-crest-derived (nl, nuchal ligament) attachment regions of the trapezius (tra) onto the occipital skull (oc in **a**) and perichondral (pc) sheaths around spinous processes (sp) of cervical vertebrae (C_4) are $LacZ^+$ in Wnt-1 transgenic (**a**) and GFP^+ (green) in Sox10 transgenic (**b**, **c**) mice. As an internal control the dorsal spinal cord (sc) is GFP^+ in Sox10-Cre-reporter crosses. Here and in all following figures, nuclear 4,6-diamidino-2-phenylindole (DAPI) stain is blue, myosin heavy-chain immunoreactivity of muscle fibres is red, and GFP^+ neural crest cells are green. The fronto-parietal suture (dotted line in **a**, fps) between frontal (f) and parietal (p) bones does not correspond to any cellular boundary of $LacZ^+$ neural crest (compare with Supplementary Methods S1). **d**, **e**, The mesodermal connectivity system. HoxD4–$LacZ^+$ mesodermal occipital bone (oc in diagram and **d**, arrows in **e**) is connected to neck vertebrae (box in diagram; dotted lines represent other vertebrae omitted) directly through striated myofibres (**e**) of the same genetic (HoxD4–$LacZ^+$) axial identity.

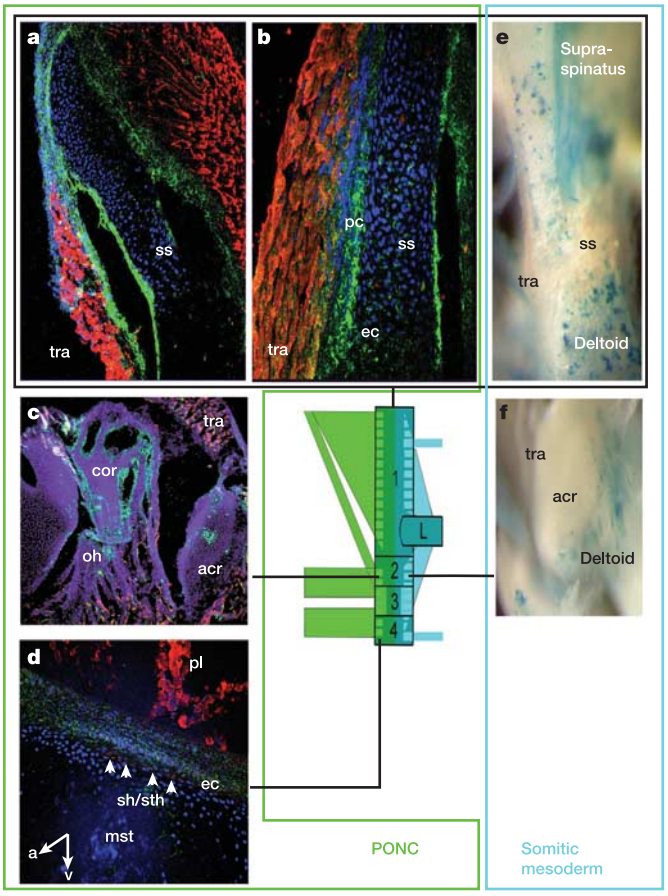

Figure 4 | Dual neural crest and mesodermal origins of the endochondral shoulder girdle. Green box, PONC GFP^+ green components (**a**, **b**); blue box, mesodermal $LacZ^+$ blue components (**e**) of the scapular spine (box 1 in diagram; **a**, **b**, **e**), the acromio-coracoid (box 2; **c**, **f**) and sternum (box 4; **d**). Inside the scapular spine (ss in **a**, **b**) GFP^+ neural crest cells are found to form endochondral skeleton (ec) and perichondrium (pc) exactly at the places where the trapezius (tra) carrying crest connective tissue is attached. Conversely, blue mesodermal muscle fibres are attached to punctate areas of the posterior scapular spine (**e**) and acromion (**f**). Note that areas of trapezius attachment in mice with mesodermal labelling are white (that is, unlabelled), showing that neural crest and mesodermal muscle attachment systems do not mix with each other. Coraco-branchial sterno-hyoideus/-thyroideus (sh/sth) muscle fibres in **d** are inserted by the endochondral PONC component (white arrowheads) onto the mesodermal sternum (mst). a, anterior; acr, acromion; oh, omo-hyoideus muscle; pl, pleural muscles; v, ventral.

muscles (supraspinatus and deltoid; Fig. 4e, f) are attached. Similar to the scapular spine, the more ventral coracoid and acromion (Box 2 in Figs 1 and 7; Fig. 4c, f) are also of dual neural-crest–mesoderm origin with attachment regions corresponding to muscle connective tissue origins. More ventrally, the clavicle reveals an as yet unrecognized potential of somitic mesoderm to differentiate into dermal bone (Box 3 in Figs 1 and 7; cld in Fig. 5g). According to the commonly held 'ossification model', all dermal clavicular parts (that is, both the anterior and the posterior parts) are expected to be derived from neural crest[9,10]. We find this to be an incorrect prediction for the clavicle. The posterior margin of the clavicle in HoxD4–LacZ transgenic mice is $LacZ^+$ at all dermal (cld in Fig. 5g) and endochondral (cle in Fig. 5g) attachment sites of $LacZ^+$—that is, mesodermal pectoral and deltoid—muscle fibres (pe and de in Fig. 5f, g).

This shows not only that the clavicle itself is a neural-crest–mesodermal interface but also that postcranial mesoderm gives rise to dermal skeletal elements. It was well known that the posterior dermal clavicle ossifies independently from the anterior dermal ossification centre, but its separate (mesodermal) origin was unknown[15,16]. This is the first experimental precedent for interpreting other trunk dermal armour plates among fossil and extant vertebrates as mesodermal. On the basis of our findings it is conceivable that the posterior dermal clavicle is the last remnant of a more widespread body armour of mesodermal origin. More ventrally, the sternum (Box 4 in Figs 1 and 7) is of mesodermal origin at its core and posterior margin, in line with the connectivities described above (Fig. 5e, unlabelled st area, and data not shown), and its anterior margin (manubrium sterni, mst in Fig. 4d and st in Fig. 5a, e) harbours crest-derived endochondral attachment points for coracobranchial musculature (Co2, box4 in Fig. 1, 4, 7, arrowheads in Fig. 4d).

For the occipital head region (Fig. 3d, e), examination of *HoxD4* transgenic mice shows that blue (HoxD4–$LacZ^+$) muscle fibres are focally and directly attached to $HoxD4^+$ skeletal regions (arrows in Fig. 3e). This shows by genetic means that somitic mesoderm strictly

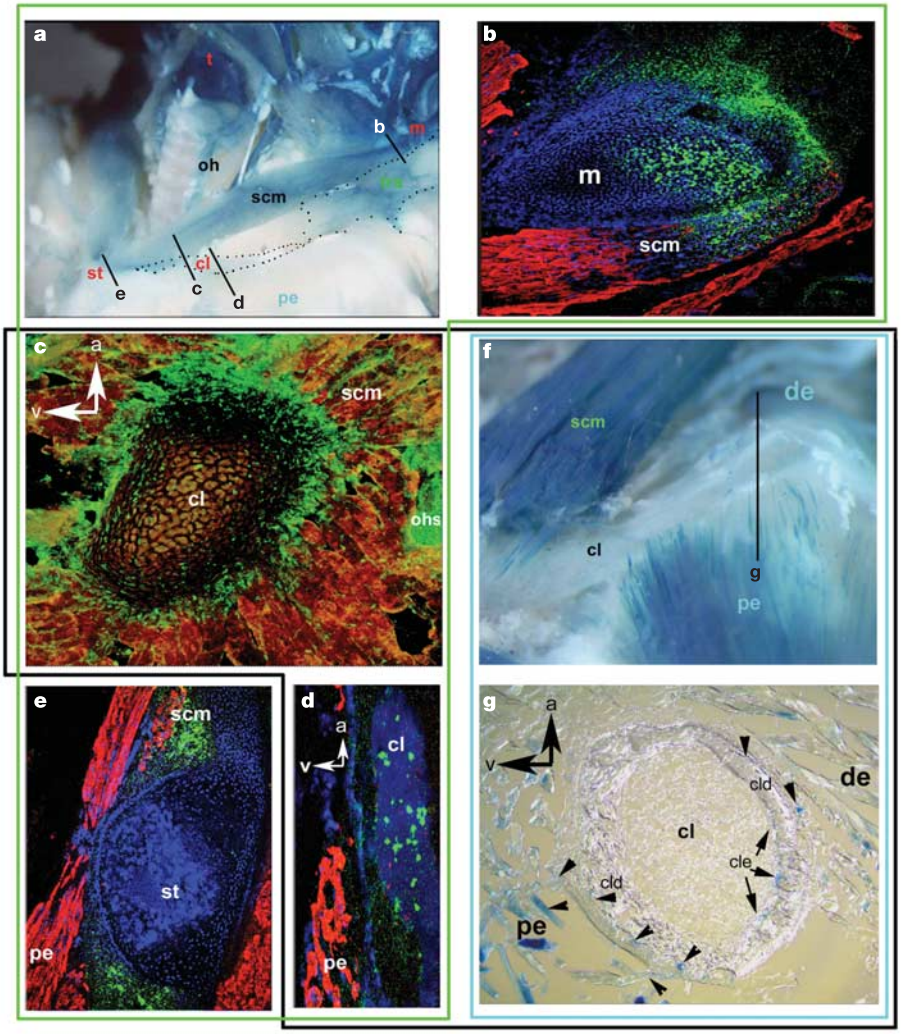

Figure 5 | The dual architecture of the clavicle: cell population boundaries coincide with muscle attachments and not with ossification modes. The black box surrounds data for the clavicle as a bone. **a**–**e**, PONC-derived parts (surrounded by green line); **f**, **g**, mesodermal parts (surrounded by blue line). **a**, Anatomical key to other panels. **b**–**e**, PONC connective tissue of the sternocleidomastoid (scm) attaches onto the mastoid process (m) of the post-otic skull (**b**) and reaches down to the clavicle (cl), which is endochondral (in **c**) and dermal (in **d**) anteriorly, and onto an endoskeletal PONC patch of the sternum (st in **e**). Contiguous DAPI staining of bone matrix in **d** shows that no other unlabelled (that is, mesodermal) cells are present inside the anterior clavicle. **f**, **g**, Complementary to this, $LacZ^+$ mesodermal myocytes of the pectoral (pe) and deltoid (de) muscles are attached exclusively onto the posterior dermal (cld) and endochondral (cle) clavicle margin (arrowheads in **g**). The anterior clavicle is unlabelled in the transgenic mouse with mesodermal labelling (white in **f**): $LacZ^+$ mesodermal cells are confined to mesodermal attachment points. Ohs, fascial sling of the M. omo-hyoideus (oh) onto the clavicle; a, anterior; v, ventral.

obeys a *Hox*-mediated genetic connectivity code directly comparable to that of the neural crest. Although the trapezius and sternocleidomastoid muscle also receives all its muscle fibres from HoxD4^{+} somitic mesoderm (Fig. 3d; Fig. 5f, blue scm) these fibres are connected to skeletal elements only through PONC-derived connective tissues (Figs 3b, c and 5a–c, e). In contrast, HoxD4^{+} mesodermal muscle fibres of occipital, pectoral or deltoid muscles connect directly to mesodermal skeletal structures, without mediation through mesodermal connective tissues (Figs 3e, 4e, f, 5g). Postotic somitic mesoderm is similarly capable of forming dermal and endoskeletal bone as well as connective tissue, a fact that invalidates the universal use of the 'ossification model' in comparative morphology or palaeontology. Instead, we find that the muscular 'scaffold model' more appropriately describes the dual composition and complex connectivity pattern of the neck and shoulder girdle.

Neural crest and human neck pathology

The flexibility of shoulder ossification types inside a highly constrained (trapezius) muscle scaffold as observed in comparative neck anatomy (T in Fig. 1) might reflect an innate flexibility of PONC cells to respond to osteogenic stimuli that, in turn, might render them prone to pathological malformation. The dual origin of neck and shoulder structures would also make it likely to find modular—that is, PONC-specific versus mesoderm-specific—pathological phenotypes. We therefore searched for human congenital syndromes with a tissue distribution that precisely corresponds to the PONC-population territory but displays pathological differentiation of PONC cells only: crest connective tissues would adopt new cartilaginous or osseous fates and vice versa (Fig. 6). The complex distribution of PONC-derived structures and the strict muscular connectivity rules yield safe criteria for discriminating patterning from differentiation defects. Several hitherto poorly understood human syndromes precisely match the profile of a PONC syndrome and permit first insights into their common cellular aetiology. Klippel–Feil disease (Fig. 6d, f)[19], Sprengel's deformity (Fig. 6d)[20], cleidocranial dysplasia (Fig. 6c)[21], Arnold–Chiari I/II malformation (Fig. 6h)[22] and 'cri-du-chat' syndrome (data not shown)[23] are all characterized by co-occurrence of pharyngeal/laryngeal, (sub-)occipital, cervical and shoulder dysmorphologies and swallowing problems. They also share a spina bifida occulta (Fig. 6c, d) that is unusually confined to the cervical region normally occupied by the trapezius muscle (tra in Figs 3c and 6b). In Sprengel's deformity a large fibrous, sometimes endochondral, so-called omo-vertebral bone replaces all dorsal neural-crest-derived endochondral elements of occipital region, cervical spinous processes, spina scapulae and trapezius[20] inside the PONC trapezius territory (Fig. 6d). On this basis we identify Sprengel's deformity, which is one of the phenotypic facets of Klippel–Feil syndrome, as primarily affecting PONC fate choices and not cervical segmentation as is currently thought[19]. Moreover, the cervical hypomobility of Klippel–Feil patients can also be understood as caused by defects in PONC fate choices: ectopic

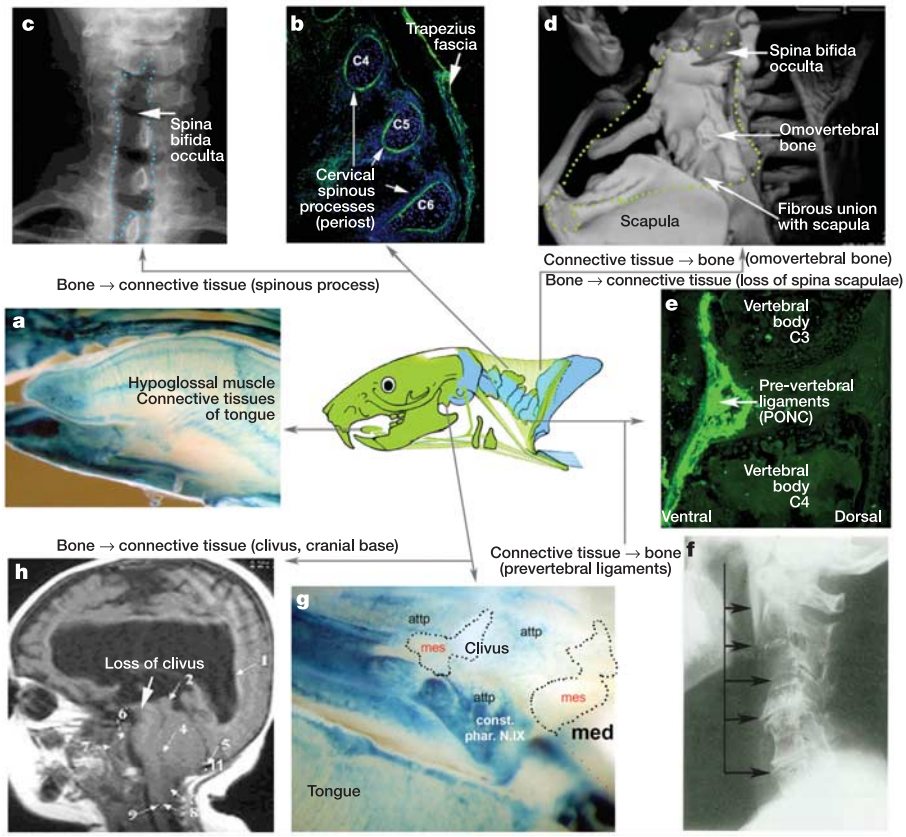

Figure 6 | Pathological flexibility of PONC differentiation. All tongue-muscle connective tissues are entirely derived from neural crest (blue areas in **a**), explaining enlarged tongues (in patients with trisomies) as neurocristopathic. Left: changes in neural crest fate specification from bone periosteum (**b**) into connective tissue can explain localized cervical defects in cleidocranial dysplasia (**c**) and Arnold-Chiari I + II syndrome (**h**). In the latter, the PONC-derived clivus (blue in **g**) of the otherwise mesodermal (mes) cranial base, which serves as the attachment point (attp) for pharynx constrictor muscles (constr. phar. N.IX/X.) in front of the medulla (med), fails to form and is replaced by fragile connective tissue. Right: conversely, PONC-derived connective tissues of pharynx constrictor muscles (const. phar. N.IX/X in **g**) that are connected to cervical vertebrae (**e**)can ectopically become bone (**f**), leading to neck immobility in Klippel–Feil syndrome (**f**) or ectopic, 'omovertebral' bones inside trapezius territory (stippled line in **d**) in patients with Sprengel's deformity, a frequent facet of Klippel–Feil syndrome[19,20]. Note also the concomitant loss of the PONC-derived (but not mesodermal) scapular spine in patients with Sprengel's deformity (**d**).

ossifications of PONC (trapezius) connective tissues around the somitic mesodermal neck vertebrae and an ectopic ossification of the PONC pre-vertebral ligaments of pharyngeal muscles (Fig. 6e, f). Similarly, loss or dysplasia of PONC-derived basicranial (clivus) bone attachments for the internal pharynx and larynx constrictors (Fig. 6g, attp of constr. phar. N.IX) and ensuing widening of the foramen magnum are the primary mechanical cause of the Arnold–Chiari I and II malformation, a serious human congenital malformation associated with swallowing problems and sudden infant (cot) death syndrome (SIDS) (Fig. 6h)[22]. In this case PONC re-specification from endochondral attachment bone to connective tissue is the likely cause of cryptic basicranial instability and early death. Detailed aetiologies of these congenital syndromes will be discussed elsewhere (T.M. and G.K., unpublished observations). Given the cellular identification of these defects and corresponding anatomical phenotypes of particular transcription factor mutants in mice (Supplementary Fig. S1) the underlying genetic causes can now be investigated in a more focused manner: impaired transcription factor networks acting inside PONC cell populations during development.

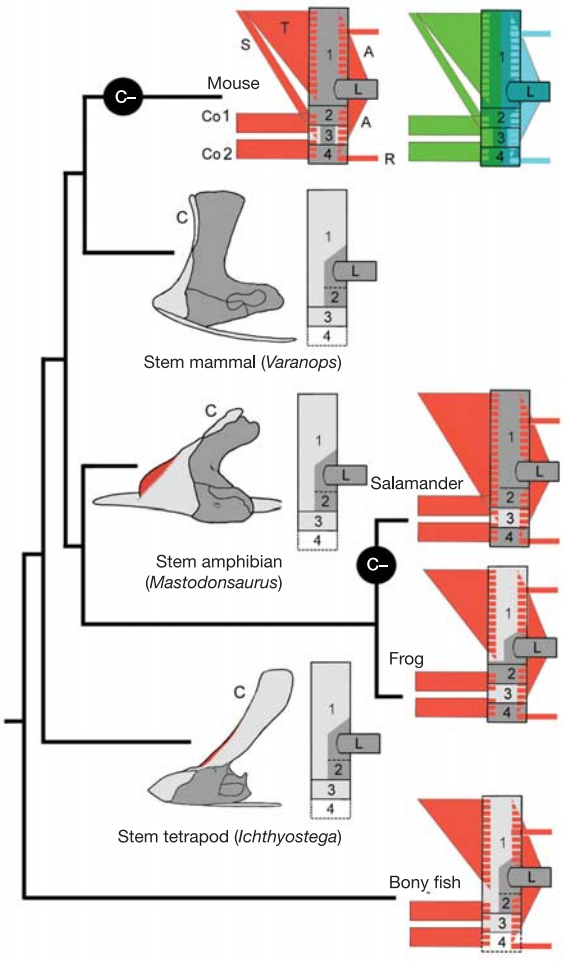

Figure 7 | Muscle scaffolds in fossils. The cleithrum (light grey box 1) has been lost (C−) several times independently in evolution and 'morphs' into scapular spines. In all species with a cleithrum—be they fossil mammalian (*Varanops*[38]), amphibian (*Mastodonsaurus*[37]) or tetrapod (*Ichthyostega*[35]; P.E.A., unpublished observation) stem-group representatives or extant frogs[36]—it is identical in position to the scapular spine of extant tetrapods with respect to the dual trapezius (T) and mesodermal (A) muscle connectivity and its dorsoventral position. Red zones on fossil forms indicate observed trapezius (T) complex attachment regions. Additional independent cleithrum losses among amniotes have been omitted for clarity (see the text). For abbreviations see Fig. 1.

Discussion

Scaffold model, homologies and mechanisms. We have identified the neck and shoulder region as the interface of the neural crest and mesodermal cell populations. We show that boundaries of embryonic cell populations precisely correspond to muscle attachment regions but not to ossification modes. The evolutionary conservation of muscle patterns (red in Figs 1, 7) is therefore likely to be a reflection of conserved cell population boundaries. The latter seem to be far more stable than the signalling pathways that determine their dermal–endochondral ossification as attachment points (grey and white areas in Fig. 1). An alternative hypothesis would have to find multiple independent developmental explanations for such highly constrained muscle patterns. Verification of cell boundary stability and the validity of the 'scaffold model' will have to await further genetic-fate mapping in a wider phylogenetic range of species when this becomes possible. However, our present high-resolution data set for the mouse allows us to refute the widely held competing 'ossification model'[9,10]. Dermal versus endochondral ossification modes are not safe criteria for identifying cellular origins and homologies of neck and shoulder structures. The rather counter-intuitive 'scaffold model' perceives muscle connectivities as the basic units (because they precisely correspond to cell populations) but considers the bones that everyone can see as mere epiphenomena and subjects of change. This prompts a new heuristic strategy for experimentally establishing neck homologies on the basis of attachment criteria, details of which can be found in Supplementary Methods S2.

The connectivity patterns that we observe with single-cell resolution in the mesodermal occipital and shoulder girdle are stricter than expected[14,24]. Muscles are directly connected to skeleton of the same axial *HoxD4*$^{+}$ gene identity, without mediation through connective tissue (blue dots in Figs 3e and 4e, f; arrowheads in Fig. 5g). Our findings corroborate the emerging notion[25,26] that in vertebrates, as previously found in *Drosophila*[27], myoblast (muscle) precursors still harbour positional identity inherited from their somitic mesenchymal stem cell precursors, and are not as naive as commonly perceived. Neural crest and mesoderm independently acquire *Hox* gene expression during evolution[28]. *Hox* genes have been proposed to be responsible for population contiguity in neural crest[13,29,30]. We note that mouse mutants of *Runx2*$^{+/-}$ (ref. 21), as well as those of interactors or downstream targets of autonomously acting *Hox* genes such as *Pbx1* (ref. 31), *Pax1* (ref. 32) and *Pax 9* (ref. 33), display modular neck/shoulder defects only of PONC but not mesodermal bones and replicate the human syndromes mentioned above (Supplementary Fig. S1 and data not shown). This supports the notion that cell-type-specific modularity and connectivity are controlled by *Hox* genes. In a complementary manner, *Emx2* mouse mutants lack the mesodermal scapular blade but not PONC shoulder derivatives[34]. Whereas HoxD4^{+} somites also provide muscle cells to the trapezius and other branchial neck muscles, these myoblasts seem to be subjugated to neural-crest-derived muscle connective tissue: they do not attach directly onto skeletal regions as mesodermal muscles do. The challenge will be to disentangle the molecular causes for such patterning dominance of neural crest over mesoderm in areas of spatial overlap, where neural crest and somitic *Hox* codes 'collide'.

Fossil fates: chasing the cleithrum's ghost. The conservation of the neck muscle scaffold among jawed vertebrates and its precise correspondence to cell population boundaries provides refined single-cell criteria for tracing skeletal fate changes of a more fundamental nature. This permits us to determine the whereabouts of elements such as the elusive cleithrum, the centralmost shoulder bone of all bony fish (osteichthyan) ancestors, which is absent from all extant land living vertebrates (tetrapods)[35] except frogs[36]. The cleithrum is uniquely defined by its position and connectivity. In extant bony fish and frogs it serves as the sole attachment region for the trapezius/cucullaris muscles anteriorly[5,36] and for fin, limb

and trunk muscles at its posterior margin (light grey in box 1 in Figs 1 and 7)[35,36]. Two main historical hypotheses have been proposed to explain its absence from most living tetrapods: first, the cleithrum was lost in common tetrapod (stem-group) ancestors and re-acquired only in frogs; second, it was independently lost from the lineages leading to living salamanders, diapsids, turtles and mammals. These two hypotheses have divergent implications for understanding macro-evolutionary trends in neck skeletogenesis. Comparative anatomy of extant species cannot distinguish between these two hypotheses (Figs 1 and 7; compare salamander and mammal). Palaeontology, in contrast, has unearthed a rich data set of fossil stem-group shoulder morphologies that can resolve the timing and polarity of these changes. Using these attachment criteria as a guide we re-examine representative examples of stem tetrapods (*Ichthyostega*)[35], stem amphibians (*Mastodonsaurus*)[37], stem amniotes (*Seymouria* and *Diadectes*, not shown) and stem-group mammals (*Varanops*)[38] (Fig. 7). All stem-group tetrapods discovered so far possess a cleithrum (C in Fig. 7) covering the entire antero-dorsal margin of the shoulder girdle. Indeed, trapezius/cucullaris muscle scars can be found on the cleithra of *Jacubsonia*[39], *Pederpes* (J. A. Clack and S. M. Finney, personal communication)[40] and *Ichthyostega* (P.E.A., unpublished observation) (red areas on Fig. 7), which demonstrates a cleithrum muscle connectivity pattern primitively retained from the fish condition. We also find a cleithrum in stem reptiles such as *Araeoscelis*[41] and stem turtles *Proganochelys*[42] and *Kayentachelys*[43]. Thus, the cleithrum has been lost at least four times independently: in salamanders, mammals, turtles and diapsids (C− in Fig. 7, and data not shown). Meanwhile, the muscle connectivities embracing the cleithrum have remained unchanged from the fish condition: exactly like the ancestral cleithrum, the mammalian scapular spine is positioned between the conserved trapezius and limb/trunk muscle attachment systems as a common abutment of the two. Based on identical cell-population-mediated connectivity and position, our genetic labelling proposes to identify the endochondral scapular spine as the 'cell population ghost' of the cleithrum. It remains to illuminate the molecular causes for this unidirectional trend to dismantle the dermal shoulder girdle, replace it by endochondral skeleton or lose it altogether, which seems to continue in mammals and amphibians and also extends to other bones such as the clavicle[16,36]. In the framework of the highly constrained neck muscle scaffolds we find no evidence for histogenetic reversals; that is, that endochondral bones of ancestors turned into dermal bones of descendants during the course of evolution. We speculate that a common, as yet unknown, genomic *cis*-regulatory architecture governing neck ossifications in tetrapod ancestors might have predisposed different descending tetrapod lineages to similar parallel trends.

The present study identifies the embryonic cell populations involved in neck patterning: PONC and somitic mesoderm. These mesenchymal stem cell populations are subject to considerable muscle patterning constraints while retaining pathological and evolutionary flexibility in their osteogenic differentiation. The molecular basis of such constraints and flexibilities and their integration into single cells remains to be discovered. Ultimately, this 'protean' flexibility of mesenchymal stem cells to 'morph' into cartilage, bone and connective tissue will have to be explained in the language of evolving gene-regulatory circuitry. This genetic circuitry will have to be placed into future reconstructions of phylogenetic trees because it was causative for the diverse neck morphologies that we observe. We expect that traces of other major evolutionary transformations and novelties will become detectable on a single-cell level once comparative genetic lineage mapping becomes possible.

METHODS

We generated two independent transgenic mouse lines in which all PONC cells are permanently labelled by means of recombinase-activated marker cassettes. *Wnt1* is expressed in all pre-migratory PONC cell precursors[17] and *Sox10* is expressed strongly in the entire post-migratory PONC population during early embryonic development and not at all in mesoderm[44]. We therefore labelled pre-migratory PONC with a *Wnt-1–Cre* transgene[17] and post-migratory PONC with a *Sox-10–Cre* bacterial artificial chromosome transgene, introduced into founders by pronuclear injection. Transgenic founders were crossed to Cre-conditional R26-LacZ and R26-eGFP reporter lines[45,46] (Fig. 2a, left). This allowed us to reveal for the first time the entire $LacZ^+$ and GFP^+ PONC population of the head, neck and shoulder region with single-cell resolution (green in Fig. 2b). Complementary to these lines, we generated a third transgenic line in which all post-occipital paraxial (neck and trunk) mesoderm was permanently labelled, carrying a novel *HoxD4–CREM–LacZ* construct (Fig. 2a, right). Landmark studies on the correspondence between *Hox* gene expression and axial somitic identity had predicted Hox-4 paralogues to define the non-occipital/occipital boundary[18,47]. These genetic and embryological studies[24] had shown that the occipital plane in amniotes runs exactly through somite 5, which expresses *HoxD4*. *HoxD4* is not expressed in neural crest and its mesodermal expression is regulated by a well-characterized genomic fragment[48]. We cloned this fragment into a novel lineage labelling construct for $HoxD4^+$ somitic mesenchymal stem cells and all of their progeny from somite 5 backwards (blue in Fig. 2b). In brief, LacZ marker activation was made conditional on a *HoxD4*-enhancer-controlled self-excision of Cre recombinase. This new strategy obviates traditional problems of differential position-effect variegation (PEV in Fig. 2a, right) of transgenic Cre drivers and reporters. It also avoids deleterious effects of high Cre-recombinase levels in tissues[49]. Technical details about constructs, controls and analysis are provided in Supplementary Methods S1.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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