Appendix S3: New methodology for determining shoulder girdle homologies

Comparative scope

Detection of conserved muscle pattern constraints in the neck region requires high-resolution long-term lineage mapping in a species with a neck musculature sufficiently generalized for gnathostomes. This is difficult to do. The chick as the only vertebrate in which long-term lineage labeling (by chick-quail chimeras) was feasible so far [2]is not informative: the ancestral jawed vertebrate neck musculature was dismantled during the evolution of the avian flying apparatus and a re-configured neck region with unique muscle innervation patterns bars detailed comparisons beyond archosaurs[5,14]. The mammalian neck and shoulder region is far more conservative. This determines our choice for the mouse as a model system to study the mechanisms behind the plesiomorphic muscle pattern shared among all living gnathostomes.

Fortunately, recent advances in recombinase-mediated lineage tracing now permit experimental verification of lineage descendants for the very first time.
Following this approach 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 conservation of muscle patterns (Fig. 1) is therefore likely to be a reflection of conserved cell population boundaries. The latter appear to be far more stable than the signaling pathways that determine their (dermal-endochondral) ossification as attachment points (Fig.1). An alternative hypothesis would have to find multiple independent developmental explanations for such highly constrained muscle patterns (Fig.1). 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 reject the widely held competing 'ossification model'[9,7]: dermal-endochondral ossification modes are not safe criteria for identifying cellular origins and homologies of neck and shoulder structures. Notably, muscle attachment patterns in the neck of mice follow the same connectivity rules that have already been observed in the cranial (hindbrain) neural crest of birds[13] and amphibians [31] and would therefore be at least a shared tetrapod character. The unity of skeletal attachment region and connective tissue origin has been demonstrated for mesoderm in the trunk and scapular blade of birds and turtles [24], is therefore at least a common amniote feature. The highly conserved nature of the crest-mesoderm neck muscle scaffold across jawed vertebrates might suggest that these connectivity rules are in fact universal for gnathostomes.

Methodology

The rather counter-intuitive 'scaffold model' supported by our single cell labeling perceives muscle connectivities as the basic units (as they precisely correspond to cell populations) and the skeletal structures everyone can see as subjects of change. This prompts a new heuristic strategy for establishing neck homologies in an experimentally falsifiable manner.
We first determine the connective tissue origins of the attached muscles on a given skeletal element and then infer the cellular origin of the skeletal attachment site. Muscles are either branchial (with neural crest connective tissue) or trunk/mesodermal (with somite-derived connective tissues). A muscle is branchial if it is either 1. motor-innervated by a branchial (hindbrain) nerve or 2.connected to branchial skeleton (connective tissue) or 3.connected to anterior shoulder girdle skeleton. These rules take into account the unusual structure of the hypoglossus (tongue muscle connectivity) system (rule2, Fig.6a) as well as coraco-branchial system (rule 3, Fig.4c,5a,c). Conversely, a muscle is to be considered mesodermal if 1. it is innervated by spinal nerves only or 2. attached onto the posterior margin of the shoulder girdle. Once muscle origins are determined, their respective skeletal attachment regions can be safely attributed to neural crest or mesoderm and the bone can be subdivided according to these criteria. Our 'scaffold model' makes precise predictions that will become testable once other informative species (such as *Amia* and sturgeon [5]) become accessible to genetic long-term lineage tracing. Zebrafish would not be particularly useful in this regard, as highly derived cyprinids have independently lost their cucullaris/trapezius musculature[11].
Depending on the phylogenetic distribution of connectivity mechanisms(which might be older than the emergence of skeleton), these criteria will permit us to disentangle strange fossil morphologies deep in the gnathostome tree that have hitherto defied analysis. These will be discussed elsewhere (TM, GK and PEA, manuscript in preparation).

Appendix S1-S3 references

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