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# The occipital lateral plate mesoderm is a novel source for vertebrate neck musculature

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## SUMMARY

In vertebrates, body musculature originates from somites, whereas head muscles originate from the cranial mesoderm. Neck muscles are located in the transition between these regions. We show that the chick occipital lateral plate mesoderm has myogenic capacity and gives rise to large muscles located in the neck and thorax. We present molecular and genetic evidence to show that these muscles not only have a unique origin, but additionally display a distinct temporal development, forming later than any other muscle group described to date. We further report that these muscles, found in the body of the animal, develop like head musculature rather than deploying the programme used by the trunk muscles. Using mouse genetics we reveal that these muscles are formed in trunk muscle mutants but are absent in head muscle mutants. In concordance with this conclusion, their connective tissue is neural crest in origin. Finally, we provide evidence that the mechanism by which these neck muscles develop is conserved in vertebrates.

**KEY WORDS:** Muscle development, Myogenesis, Skeletal, Somite, Vertebrate, Chick, Quail

## INTRODUCTION

The innovation that permitted free movement of the head independently of the body is considered to be a landmark adaptation in vertebrates, facilitating predatory avoidance and prey capture. Free movement of the head was achieved by the development of the neck. For this milestone to be reached, the body plan underwent two major modifications: firstly, anterior vertebrae reduced or lost their ability to form ribs; and secondly, new skeletal muscles developed that enabled a turning and bending motion of the head. The muscle that evolved to exert these movements is highly conserved in jawed vertebrates and is called the cucullaris muscle (Kuratani, 2008). The mammalian homologue of the cucullaris muscle is divided dorsoventrally into the larger dorsally positioned trapezius and a comparatively smaller portion, the sternocleidomastoid muscle (Williams, 1995).

The origin of the cucullaris muscle has been the subject of considerable debate (Krammer et al., 1987). Origins as diverse as the branchial arches, somites and the lateral plate mesoderm have all been proposed, evidenced often by innervation (Addens, 1933; Allis, 1897; Couly et al., 1993; Derjugin, 1908; Edgeworth, 1911; Edgeworth, 1926; Favaro, 1903; Fuerbringer, 1897; Gegenbaur, 1898; Harrison, 1918; Huang et al., 1997; Huang et al., 2000; Lewis, 1910; Luther and Lubosch, 1938; Noden, 1983; Noden and Francis-West, 2006; Piekarski and Olsson, 2007).

The head versus trunk origin of the cucullaris muscle will have considerable bearing on the molecular mechanism used during its development. The origins of myogenic cells that form head and

trunk muscles differ greatly, as do the source of their connective tissues. Head muscles develop from unsegmented head mesoderm or the splanchnic mesoderm (Noden and Francis-West, 2006; Nathan et al., 2008). Neural crest cells form the connective tissue of these muscles (Noden and Trainor, 2005; Matsuoka et al., 2005; Noden and Francis-West, 2006). By contrast, trunk muscles are derived from the somites and their connective tissue forms locally or from lateral plate mesoderm (Christ et al., 1974; Christ et al., 1982).

Following the advent of robust lineage-tracing techniques, it is believed that trunk muscles, including those of the neck and tongue, are derived from the paraxial mesoderm (somites), whereas head muscles have multiple origins, such as the prechordal, paraxial and splanchnic mesoderm regions, which are all located anterior to the somites (Harel et al., 2009). Apart from differences of head and trunk muscle development based on origin, recent publications document that they form according to distinct molecular programmes (Tzahor et al., 2003; Nathan et al., 2008; Harel et al., 2009; Mootoosamy and Dietrich, 2002; Sambasivan et al., 2009). Although the molecular end points of both myogenic programmes are the same, culminating in the expression of muscle structural proteins, they differ significantly during their early phases of development. In the trunk, *Pax3* is expressed in proliferating progenitor cells and is downregulated as *MyoD* is upregulated (Amthor et al., 1999). By contrast, *Pax3* is not expressed in head myogenic precursors, a role enacted by a suite of transcription factors, including *Tbx1*, *MyoR*, *Pitx2*, *Isl1* and *Capsulin* (Hacker and Guthrie, 1998; Mootoosamy and Dietrich, 2002; Harel et al., 2009; Sambasivan et al., 2009). The head and trunk myogenic programmes also display distinct outcomes in response to individual signalling molecules. For example, trunk myogenesis is promoted by the action of Wnt and Shh. By contrast, Wnt and Shh have been shown to inhibit head myogenesis. In the head, the repression mediated by Wnt molecules (and other molecules) needs to be lifted in order for differentiation to proceed, a process facilitated by the secretion of signalling molecule

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antagonists produced by neural crest cells (Tzahor et al., 2003). In addition, these cells provide the support tissue for the muscle. The timing of muscle differentiation in the head is also distinct, taking place later than in the trunk (Noden et al., 1999). Therefore profiling of genes and identifying the origin of the connective tissues are likely to yield telling clues as to the character of a particular myogenic programme.

In this study we report that the cucullaris muscle of birds and its mammalian homologues develop by deploying the head myogenic programme. This conclusion is reached from our studies on the cucullaris muscle showing that: (1) the majority of its myofibres develop from a non-somitic source; (2) its molecular profile is similar to that of other head muscles; (3) its connective tissue is derived from neural crest. Most significantly, our work experimentally demonstrates a new source of skeletal muscle – the occipital lateral plate mesoderm – as the major source of the cucullaris muscle.

## MATERIALS AND METHODS

### Preparation of embryos

All experiments were performed on *Gallus gallus domesticus* chicken embryos unless stated otherwise. Lineage-tracing experiments were performed with either quail (*Coturnix coturnix japonica*) or transgenic chick embryos expressing cytoplasmic GFP under control of the beta-actin promoter (Dr H. Sang, Roslin Institute, UK). Eggs were incubated at 38°C and 80% humidity.

### Grafting procedure

In order to avoid cross-contamination of somitic and lateral plate mesoderm tissues during grafting, an alternative to our previous methods (Huang et al., 1997) was used in this study. Here we firstly removed donor material using the Chapman easy culture method to isolate embryos from the eggshell (Chapman et al., 2001). Somites were then prepared firstly by making a precise incision with a sharpened tungsten needle between the somites and the medial edge of the lateral plate mesoderm. Subsequently, an incision was made between the somites and neural tube. Finally, the dissected tissue was briefly incubated in Dispase I (Sigma, 1 U/ml) to isolate whole somites free of mesenchymal contamination. A minimum of five procedures for each individual somite were performed. For lateral plate mesoderm transplantation, a strip of tissue 1.5 somites wide from quail or GFP<sup>+</sup> embryos was transplanted homotopically into the wild-type hosts ( $n > 20$ ). Grafting into the host was performed according to a modified protocol of Huang et al. (Huang et al., 1997), but with the omission of sub-epidermal ink injection. A minimum of five procedures was performed for homotopic regionalised lateral plate mesoderm grafts. Heterotopic transplantations were performed either by replacing chick lateral plate mesoderm adjacent to somites 1-3 of a Hamburger-Hamilton stage 9 (HH 9) embryo with quail lateral plate mesoderm originating adjacent to somites 10-12 from a HH 11 embryo (caudal to cranial graft,  $n = 10$ ). Alternatively, chick lateral plate mesoderm adjacent to somites 21-23 of a HH 14 embryo was replaced with quail lateral plate mesoderm originating adjacent to somites 1-3 from a HH 9 embryo (cranial to caudal graft,  $n = 7$ ).

### Immunohistochemistry

Quail/chick chimeras were fixed in 4% PFA/PBS and processed for paraffin sectioning (10 µm). Grafted material was detected with QCPN and MF20 (Developmental Studies Hybridoma Bank, DSHB) and subtype-specific (IgG1 or IgG2b) goat-anti-mouse DyLight antibodies (Jackson ImmunoResearch). Alternatively, QCPN was detected using goat-anti-mouse AP conjugated antibody. Paraffin sections (10 µm) from GFP/WT chimeras, fixed for 2 hours in 4% PFA/PBS, were subjected to immunohistochemistry using MF20 and the anti-GFP rabbit polyclonal antibodies (Torrey Pines Biolabs) and detected using goat-anti-mouse IgG 594 and goat-anti-rabbit 488 antibodies (Jackson ImmunoResearch). Paraffin embedded tissue for myosin heavy chain (MyHC) expression (antibody MF20), was subjected to antigen retrieval by boiling in citrate buffer (0.1 M, pH 6) for 10 minutes. Non-specific binding was blocked in 10% fetal calf serum (FCS).

*Pax3<sup>Cre</sup>:Rosa<sup>STOP/NFP</sup>* embryos were harvested at 15.5 days post-coitum (dpc), fixed in 4% PFA/PBS for 2 hours and processed for paraffin sectioning (7 µm). Immunohistochemistry was performed using MF20 and biotinylated anti-GFP antibodies (Abcam, UK). Secondary detection was performed using goat-anti-mouse IgG 488 and streptavidin 594 antibodies (Jackson ImmunoResearch).

*Pax3<sup>Sp/Sp</sup>:Myf5<sup>nlacZ/nlacZ</sup>* and *Tbx1<sup>-/-</sup>* embryos were harvested at 13.5, 13.75 or 14.5 dpc, fixed for 1 hour in 4% PFA/PBS and stored in 1% PFA/PBS until processing for paraffin sectioning (7 µm). MF20 antibody was used for primary detection. Secondary antibody conjugated with horseradish peroxidase (1:200, Sigma) was applied for 1.5 hours at room temperature. The antibody localisation was determined using DAB staining. Sections were counterstained with haematoxylin.

*Wnt1<sup>Cre</sup>/R26RlacZ* embryos were harvested 13.5 dpc and processed as described by Valasek et al. (Valasek et al., 2010). Turtle (*Trachemys scripta*) embryos were fixed in 4% PFA/PBS, processed for paraffin sectioning (10 µm) and subjected to MyHC immunohistochemistry using MF20 and goat-anti-mouse IgG 594 (Jackson ImmunoResearch). Images were captured using a Zeiss fluorescence microscope.

### Whole-mount in situ hybridisation

Whole-mount in situ hybridisation was performed according to Nieto et al. (Nieto et al., 1996). Sections of whole mounts after in situ hybridisation were generated after embedding samples in 4% agarose for vibratome sectioning (55 µm).

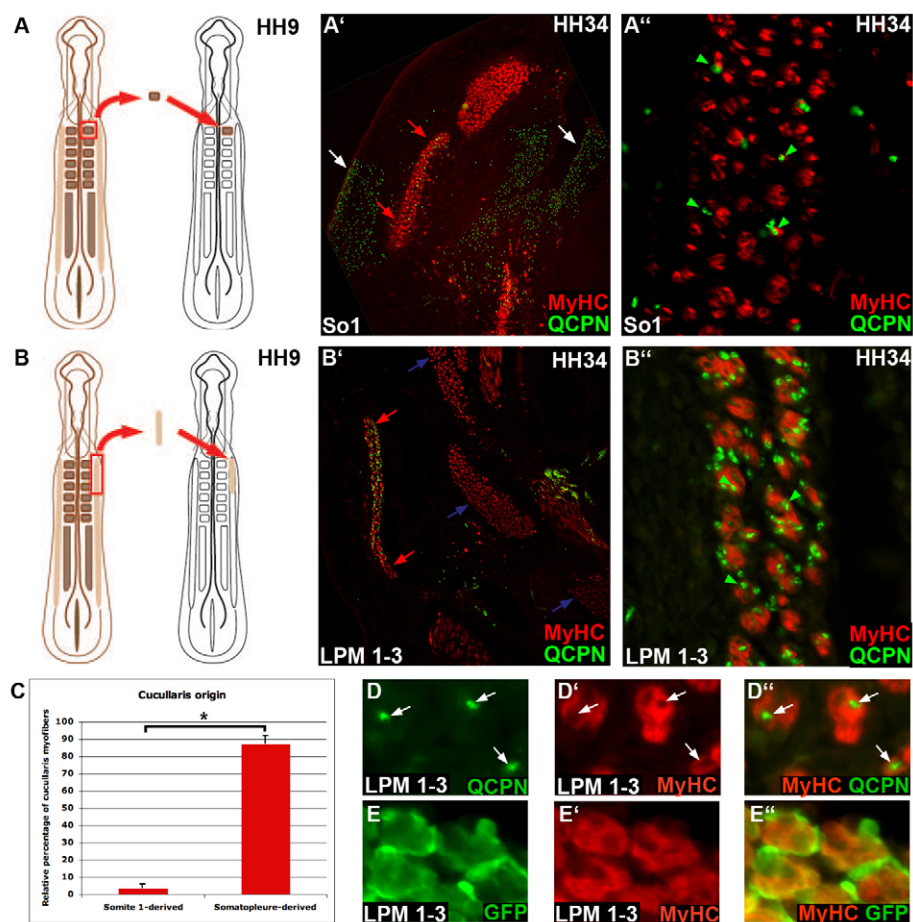
## RESULTS

### The majority of the cucullaris muscle is derived from the lateral plate mesoderm and not from somites

The cucullaris muscle in birds has been previously reported to derive from the somites (Couly et al., 1993; Huang et al., 1997; Huang et al., 2000; Noden, 1983). More specifically, Huang et al. (Huang et al., 1997; Huang et al., 2000) found a contribution from the first three somites, whereas Noden (Noden, 1983) reported contributions from somites 1-4. In this study, we homotopically transplanted, individually, the first seven somites from HH 9 quail embryos (before neural crest migration) free of contaminating tissues (facilitated through the use of Dispase I) into chick hosts and found that somitic contribution to the cucullaris muscle was relatively minor (Fig. 1A-A'). Quantification of the contribution from somite 1 to the cucullaris revealed that approximately 3.6% of fibres contained graft-derived cells (Fig. 1C). A similar number of fibres was found to develop from somites 2 and 3. The third, fourth and fifth somites contributed to the hypoglossus anlagen and did not contribute to the cucullaris muscle (data not shown). Furthermore, somites 6-7 did not contribute to the cucullaris muscle (data not shown) in agreement with Huang et al. (Huang et al., 2000).

We hypothesised that results from previous studies reporting a somitic origin for this muscle (Huang et al., 1997; Huang et al., 2000) could have arisen as a result of contamination by lateral tissue. To this end we transplanted quail lateral plate mesoderm adjacent to the occipital somites 1-3 into a chick host. In contrast to transplantation of somites, we found very high densities of tissue originating from quail in the cucullaris muscle (Fig. 1B-B'). Quantification of the contribution from the lateral plate mesoderm revealed that approximately 90% of the fibres were quail in origin (Fig. 1C).

Next, we determined whether the lateral plate mesoderm actually formed skeletal muscle in the cucullaris, as opposed to other tissues. This was addressed using two approaches. Firstly we identified numerous lateral-plate-grafted quail nuclei located within MyHC-expressing fibres (Fig. 1D-D'). In addition we used the newly developed ubiquitously expressing cytoplasmic GFP chick line as a source of donor tissue and found that homotopic lateral



**Fig. 1. Cucullaris muscle develops predominantly from the occipital lateral plate mesoderm.** (A) Schematic representation of homotopic somite graft at HH 9. (A', A'') Homotopic transplantation of quail somite 1 into chick host at HH 9 and processed at HH 34 (day 8). (A') Low-magnification image showing relative distribution of quail cells in the cucullaris muscle area. Quail cells were found predominantly in the skin and more centrally in cartilage of the skull (QCPN<sup>+</sup> cells, white arrows; cucullaris muscle, red arrows). (A'') High magnification of the cucullaris muscle after somite 1 transplantation showing sparse QCPN<sup>+</sup> cells (green arrowheads) in the cucullaris muscle but distinct from the MyHC<sup>+</sup> territory. (B) Schematic representation of homotopic lateral plate mesoderm graft at HH 9. (B') Low-magnification image of homotopic transplantation of lateral plate mesoderm at somite level 1-3 into chick host at HH 9 and processed at day 8, showing high density of QCPN<sup>+</sup> cells in the cucullaris (red arrows) and not in axial muscles (blue arrows). (B'') High-magnification image showing QCPN<sup>+</sup> cells tightly associated with MyHC<sup>+</sup> fibres within the cucullaris muscle (green arrowheads), but absent in the surrounding connective tissue. (C) Quantification of somitic and lateral plate origin contributions to the cucullaris muscle. Four somite 1 transplants were analysed and found to give an average of 3.60±2.57% somite-derived cells in the cucullaris muscle that express MyHC. By contrast, 87.22±4.70% of fibres in the cucullaris muscle were derived from the lateral plate mesoderm ( $n=4$ ,  $P<0.001$  by  $t$ -test). (D-E'') High-magnification images of cucullaris myofibres after lateral plate mesoderm transplantation adjacent to somite level 1-3 generated using the quail/chick procedure (D-D'') or GFP-WT chick transplants (E-E''). (D-D'') QCPN<sup>+</sup> quail-derived nuclei are present in the MyHC<sup>+</sup> cucullaris myofibres. (E-E'') Lateral plate mesoderm-derived GFP expression overlapped with MyHC.

plate mesoderm grafts at level of somites 1-3 gave rise to fibres that co-expressed GFP and MyHC (Fig. 1E-E''). Importantly, the somatopleure transplants did not give rise to any of the tissues that we showed to develop from the somites – for example, deep neck muscles or the vertebrae. These findings allowed us to conclude that transplanted somatopleure tissue was free of somitic contamination. These results show that the lateral plate mesoderm has myogenic capacity and is a major contributor to the formation of the cucullaris muscle.

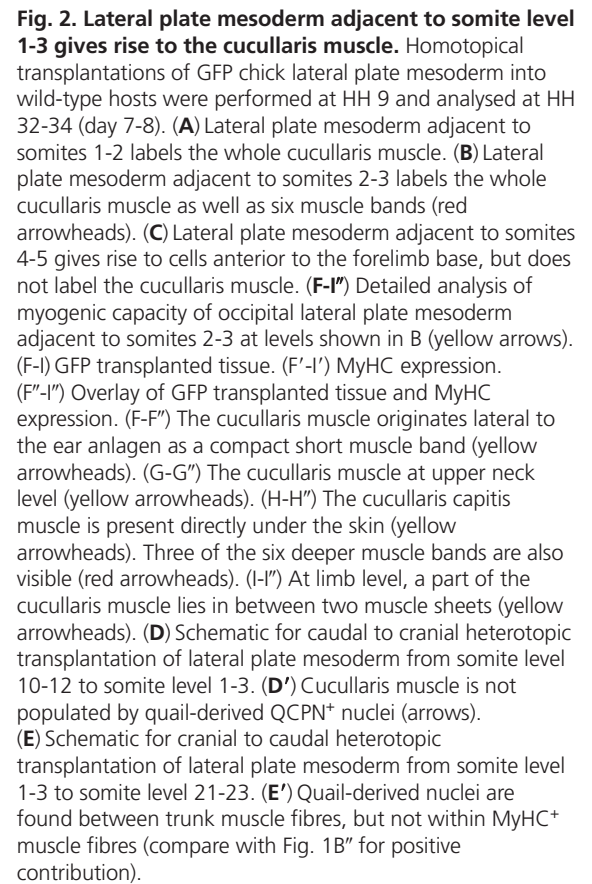
### Mapping of the myogenic territory in the occipital lateral plate mesoderm

Our work suggests that a relatively small population of cells adjacent to somites 1-3 give rise to the large cucullaris muscle. We made use of the superficial localisation of the muscle and lineage-

tracing properties afforded by the ubiquitously expressing GFP chick line that allows visualisation of the transplanted cells in whole mount to study the development of this anlagen.

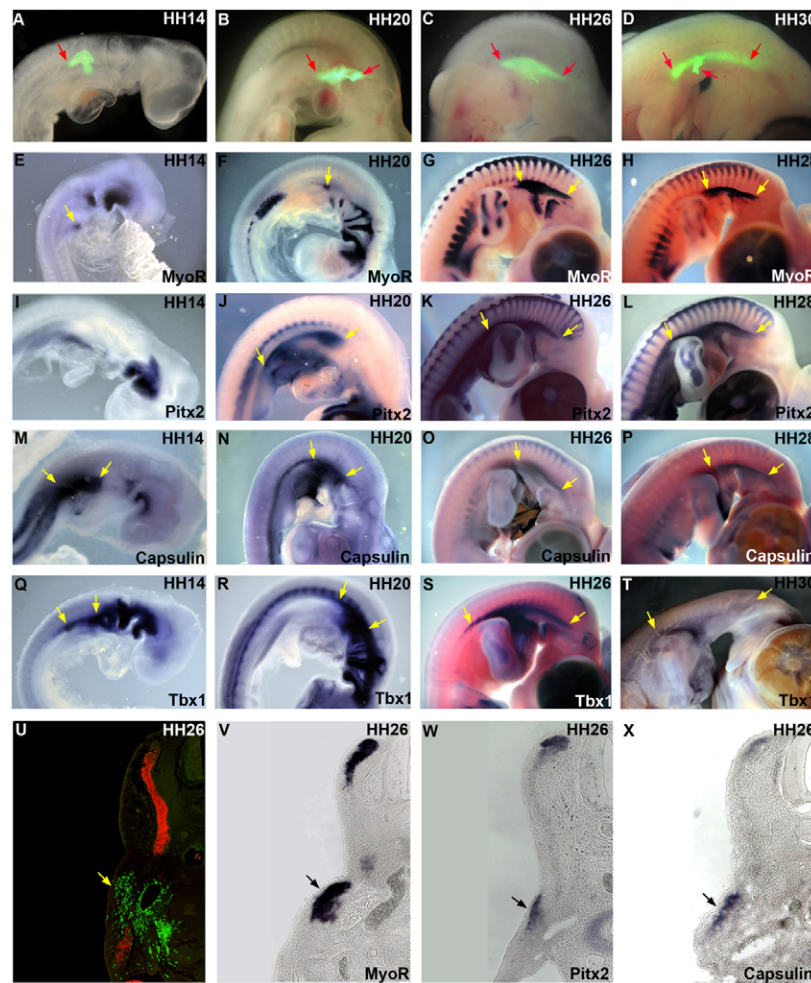
Firstly, a detailed contribution of specific regions of the lateral plate mesoderm to the cucullaris muscle was determined by transplanting tissue adjacent to somites 1-2, 2-3 and 4-5. Transplants of lateral plate mesoderm lateral to somites 1-2 as well as 2-3 gave rise to all parts of the cucullaris muscle identified through the expression of MyHC (Fig. 2A,B,F-I''). In addition we detected a contribution to six deeper muscle bands, to date not described in the literature (Fig. 2B,H-H''). A labelled deeper band of muscle possibly representing part of the cucullaris cervicis muscle was also labelled (Fig. 2I-I''). By contrast, lateral plate mesoderm adjacent to somites 4-5 failed to give rise to cells of the cucullaris muscle but instead formed tissue anterior to the wing (Fig. 2C).





Next we heterotopically transplanted lateral plate adjacent to somites 1-3 to the level of 21-23 of a HH 15 embryo. After incubation until day 9 we determined whether the caudally

Next we determined the temporal expansion of the lateral plate mesoderm lateral to somites 1-3. Transplantations were performed at HH 9. Extension of the transplanted region was detectable by HH 14 (Fig. 3A). By HH 20, the transplanted tissue had doubled its size to six somites in length and had reached the base of the heart anlagen (Fig. 3B). The transplanted GFP population lost its compact character and reached, as a band, the anterior limb base by HH 26 (Fig. 3C). A morphological form resembling the adult muscle was not realised until HH 30, when transplanted tissue divided into a ventral and dorsal part just anterior to the wing base (Fig. 3D).



**Fig. 3. Temporal development of the cucullaris muscle and its association with head muscle gene expression.** (A-D) Homotopic transplantation of chick GFP lateral plate mesoderm adjacent to somites 1-3 into a wild-type host at HH 9. Embryos were analysed at stages indicated. Red arrows mark the cucullaris muscle. (A) By HH 14 the transplant shows a caudally directed extension. (B) At HH 20 the cucullaris lateral plate mesoderm transplant extends to the heart. (C) By HH 26 the transplant loses its compact character and reaches the base of the wing. (D) By HH 30 the GFP lateral plate mesoderm resembles the adult muscle and splits into a dorsal and ventral part anterior to the wing base. (E-X) Temporal expression of genes expressed during early head myogenesis: *MyoR* (E-H,V), *Pitx2* (I-L,W), *Capsulin* (M-P,X) and *Tbx1* (Q-T). Yellow arrows indicate expression within the occipital lateral plate, which expands slightly caudally by HH 20. (G) At HH 26 *MyoR* expression is distinctly in the cucullaris muscle and resembles the GFP transplant profile in C. (H) *MyoR* expression extends to the base of the wing of a HH 28 embryo. (I,J) *Pitx2* was expressed in the lateral plate mesoderm at HH 14 and as a fine strip in the cucullaris muscle anlagen between the somites and the branchial arches at HH 20. (K,L) *Pitx2* was detected in the cucullaris muscle at HH 26 and 28. (M,N) *Capsulin* expression corresponds to the cucullaris muscle territory at HH 14 and HH 20 and is expressed in the muscle during the following stages to HH 28 (O,P). (Q) *Tbx1* is expressed in a continuous domain from branchial arches to the occipital lateral plate mesoderm at HH 14. (R) At HH 20 the *Tbx1*-expressing territory expands and includes the area of cucullaris development. (S,T) Between HH 26 and HH 30, *Tbx1* expression is distinct and mirrors the cucullaris muscle anlagen. (U-X) GFP-transplanted cells derived from the lateral plate mesoderm overlap with the expression of head muscle genes. Sections of HH 26 embryos shown in C, G, K and O. (U) Localisation of GFP lateral plate mesodermal cells (green; yellow arrow) and MyHC expression (red). (V-X) The expression of *MyoR*, *Pitx2* and *Capsulin* (black arrows) overlaps with the domains of GFP-expressing lateral plate mesodermal cells.

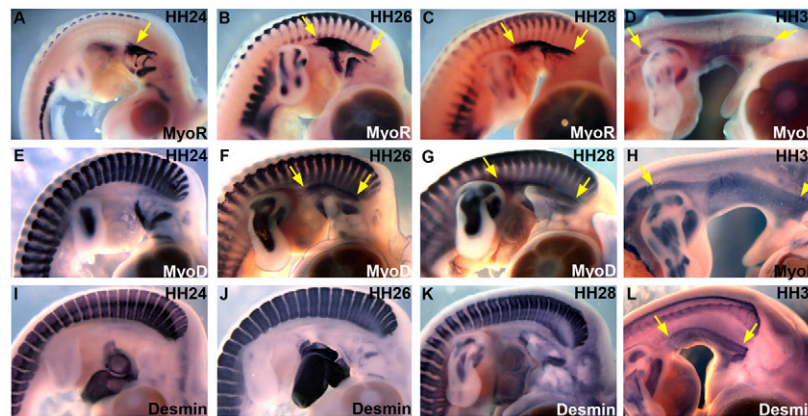
### Late onset of muscle differentiation in the cucullaris muscle

Having established the temporal development of the cucullaris muscle, we next determined the timing of its differentiation. We started by investigating the appearance of desmin, a marker for terminal muscle differentiation, in the area of cucullaris muscle development. This protein is robustly expressed in the somites and the heart anlagen at HH 24 (Fig. 4I). At HH 26, expression was also detected in the second and third branchial arch, but not in the cucullaris muscle anlagen (Fig. 4J). At HH 30, muscle

differentiation was detected in a distinct muscle band representing the cranio-ventral portion of the cucullaris muscle (Fig. 4L). Expression of MyHC was also detected in the cucullaris muscle only after HH 30, in contrast to its prior expression in axial, limb and head muscle (data not shown).

We next investigated *MyoD* expression as a marker for committed precursors and found that it too was initiated late in the cucullaris muscle. Robust *MyoD* expression was detected in the somites, the branchial arches and the limb by HH 24 (Fig. 4E). The first faint expression of *MyoD* in the cucullaris muscle was detected





**Fig. 4. Late myogenic differentiation characteristics of the cucullaris muscle.** Yellow arrows mark the cucullaris muscle anlagen. (A–D) *MyoR* expression is used as a reference for the cucullaris muscle. (A) *MyoR* is expressed in the branchial arches and the cranial area of cucullaris muscle development at HH 24. (B) Distinct expression in the cucullaris muscle anlagen extends caudally towards the base of the wing at HH 26. (C) *MyoR* expression has reached the wing base at HH 28. (D) *MyoR* is expressed along the entire length of the cucullaris muscle from just caudal to the ear anlagen beyond the limb at HH 30. (E) *MyoD* is expressed in the branchial arches, the somites and the limbs, but is not detected in the cucullaris muscle area at HH 24. (F) *MyoD* is expressed in the cucullaris muscle anlagen and reaches the forelimb at HH 26. (G) *MyoD* expression in the cucullaris muscle is upregulated at HH 28. (H) Expression of *MyoD* is present throughout the muscle and covers the lateral neck at HH 30. (I, J) Desmin is expressed in the somites and the heart anlagen at HH 24 and 26 and in the branchial arches at HH 26, but is not present in the cucullaris muscle anlagen. (K) Faint expression in the cucullaris muscle anlagen is observed at HH 28 in the ventral portion of the muscle. (L) Robust expression is detected in the cranial portion of the muscle at HH 30.

at HH 26 (Fig. 4F). By HH 30 the *MyoD* expression in this domain had elongated along the full extent of the neck, and broadened to cover its entire lateral side (Fig. 4H). In summary, the onset of cucullaris muscle differentiation is initiated later than in the somites, branchial arches and limbs.

### The trunk myogenic programme is not employed in the formation of the cucullaris muscle

As the cucullaris muscle develops adjacent to the somites and reaches far into the body, we investigated the expression of *Pax3*, *Pax7* and *Myf5* as evidence for the involvement of the trunk myogenic programme during its formation. *Pax3* was readily detectable in the dermomyotomes (HH 14), limb muscle precursors (HH 20 onwards) and the hypoglossal cord at HH 20 (see Fig. S1E,F in the supplementary material). Importantly, we never detected the expression of *Pax3* in the cucullaris muscle at HH 28 (see Fig. S1H in the supplementary material), a stage when *MyoD* is robustly expressed in this tissue (Fig. 4G). Furthermore more we were not able to detect clear (non-background) expression of *Pax3* in the cucullaris at later stages (HH 30–32, data not shown). Likewise, *Pax7* was not found during early cucullaris development but was faintly expressed at HH 28 (see Fig. S1I–L in the supplementary material). Similarly, *Myf5* expression was initiated late in the cucullaris muscle (HH 26) compared with somites, branchial arches and the limbs (see Fig. S1C in the supplementary material). The lack of the early specification markers *Pax3*, *Pax7* and *Myf5* indicates that the cucullaris muscle develops out of phase with the trunk myogenic programme.

### Genes regulating head muscle formation are expressed in the cucullaris muscle

The late expression of differentiation markers and the absence of genes involved in trunk myogenesis raised the possibility that the cucullaris muscle develops according to a head muscle programme. *MyoR*, *Pitx2*, *Capsulin* and *Tbx1* were used to mark early head muscle precursors. In contrast to the lack of early trunk muscle

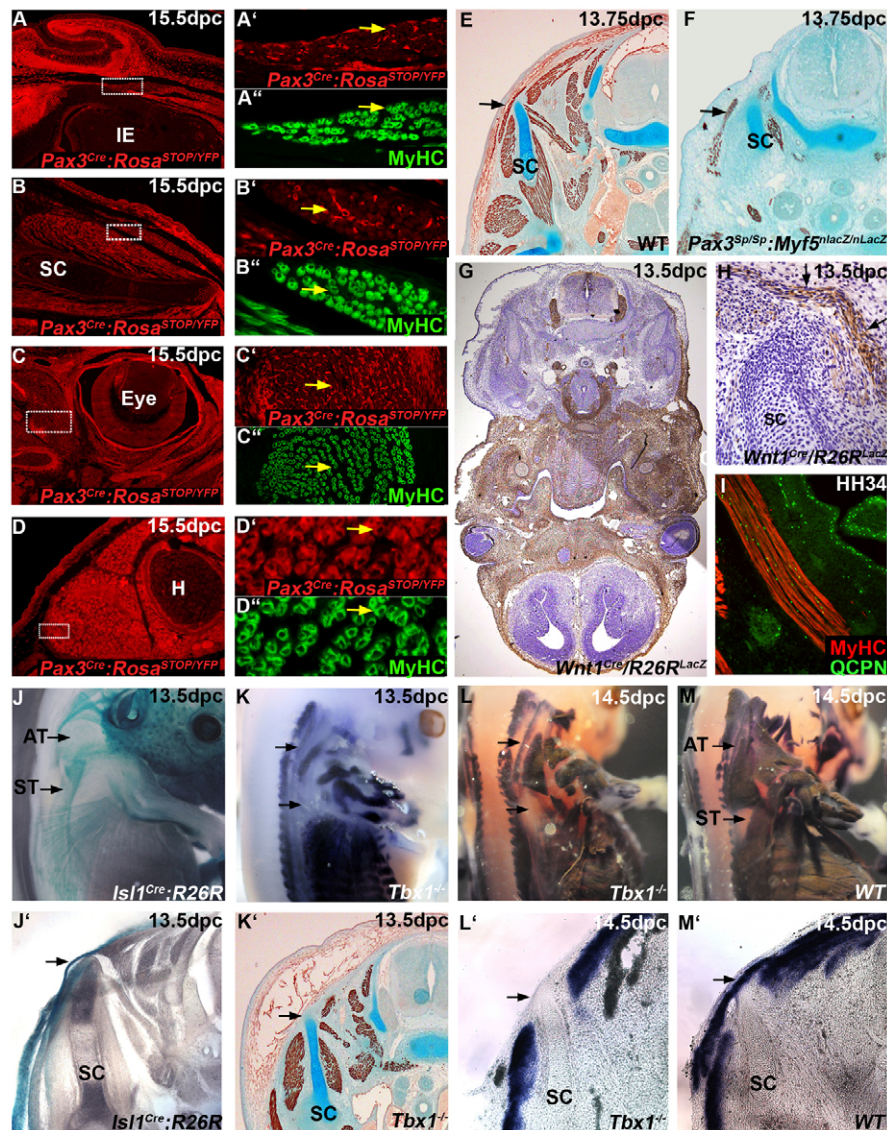
markers, *MyoR* was expressed in a small spot-like area of the cranial cucullaris muscle anlagen at HH 14 (Fig. 3E). By HH 26 robust *MyoR* expression in the cucullaris muscle anlagen was clearly evident (Fig. 3G,V) and overlapped with the lineage-tracing profiles of GFP lateral plate mesoderm homotopically transplanted adjacent to somites 1–3 at HH 9 (Fig. 3C,U). At HH 30 *MyoR* expression in the cucullaris muscle covered the entire lateral neck and reached into the back beyond the base of the limb (Fig. 3H). *Pitx2* was detected symmetrically in a very fine stripe in the cucullaris muscle anlagen between the hypaxial domain of the somites and the branchial arches at HH 20 (Fig. 3J). The transcription factor was distinctly expressed in the cucullaris muscle anlagen at HH 26 and HH 28 (Fig. 3K,L,W). *Capsulin* was expressed in the area of cucullaris muscle development at HH 14 (Fig. 3M). At HH 26 the gene was distinctly expressed in the cucullaris muscle (Fig. 3O,X). The expression of *Tbx1* was found to mark the cucullaris muscle from the time of its early anlagen to the fully formed muscle; initiated at HH 14 in a territory both of the muscle cells and in a prospective caudal region (Fig. 3Q). By HH 20 the expression of *Tbx1* had increased (Fig. 3R). At HH 26 the *Tbx1* expression mirrored the cucullaris muscle anlagen (Fig. 3S compared with 3C).

In summary, head-muscle-related genes, such as *MyoR*, *Pitx2*, *Capsulin* and *Tbx1*, are expressed in the cucullaris muscle territory from its earliest time of development.

### Genetic evidence for the deployment of the head developmental programme during cucullaris muscle development

In order to substantiate our findings and to determine whether the mechanisms identified in the chick were conserved in mammals, we turned to the power afforded by mouse genetics. Given our findings that the avian homologues of the trapezius and sternocleidomastoid muscles develop late compared with other muscle groups we first established when these muscles were clearly identifiable in the mouse. Our in situ and immunohistological





**Fig. 5. Mammalian trapezius and sternocleidomastoid muscle development is Pax3 independent and their connective tissue is derived from neural crest.** (A-D') YFP expression controlled by *Pax3<sup>Cre</sup>* was used to identify *Pax3* lineage cells (red) and compared to MyHC expression (green). Yellow arrows point at the corresponding muscle fibres in the *Pax3<sup>Cre</sup>:Rosa<sup>STOP/YFP</sup>* image and the MyHC image. (A) The sternocleidomastoid muscle at inner ear level shows muscle fibres predominantly negative for the reporter gene. (A,A') High-magnification images show YFP expression between the myofibres but is seldom found in the muscle (yellow arrow). (B,B') The trapezius muscle at scapula level displays a predominantly *Pax3*-negative developmental history (yellow arrow). By contrast, connective tissue between the myofibres is positive. (C,C') The eye muscle is predominantly negative for a *Pax3* history (yellow arrow) in the myofibres but connective tissue is positive for the transcription factor. (D,D') Limb level muscle fibres are positive for a developmental history of *Pax3* (yellow arrows), whereas the connective tissue is negative. (E,F) Comparison of MyHC expression in wild type and *Pax3<sup>Sp/Sp</sup>:Myf5<sup>nlacZ/nlacZ</sup>* at 13.75 dpc. (E) Wild type shows a trapezius (arrow) at the level of the scapula. (F) Trapezius is present in *Pax3<sup>Sp/Sp</sup>:Myf5<sup>nlacZ/nlacZ</sup>* (arrow) at the level of the scapula. (G,H) *Wnt1<sup>Cre</sup>/R26R<sup>lacZ</sup>* mice were analysed for a contribution of neural crest to the cucullaris muscle homologues at 13.5 dpc. (G) Robust Cre activity was detected throughout head tissues. (H) Strong Cre activity is present in the trapezius muscle (black arrows) at scapula level. (I) Chick/quail chimera of neural tube transplant at somite level 1-3. Cells of quail origin are located throughout the cucullaris connective tissue. (J-M') The involvement of head muscle associated genes in the development of the trapezius. (J) Both the cranial and the caudal part of the trapezius muscle are labelled in a 13.5 dpc *Isl1<sup>Cre</sup>;R26R* mouse embryo. Arrows indicate acromio-trapezius or spino-trapezius. (J') Sections of a 13.5 dpc *Isl1<sup>Cre</sup>;R26R* mouse embryo in which the trapezius muscle overlies the scapula, showing beta-galactosidase activity (arrow). (K,L) The entire trapezius muscle is absent in 13.5 and 14.5 dpc *Tbx1<sup>-/-</sup>* embryos (arrow). The surrounding trunk musculature is unaffected. (K',L') Sections showing absence of the trapezius muscle overlying the scapula in the *Tbx1<sup>-/-</sup>* animals (arrow). (M,M') The position of the acromio-trapezius and spino-trapezius is shown in a wild-type littermate of L. AT, acromio-trapezius; H, humerus; IE, inner ear; SC, scapula; SP, spino-trapezius.

analysis revealed that both muscles are formed before 11.0 dpc in the mouse (data not shown). Therefore in this study we analysed embryos at stages considerably after this point to avoid the issue of delayed development.

We have so far established that the cucullaris muscle is late to differentiate, reminiscent to head rather than trunk musculature. Furthermore key genes (*Pax3/7*), which are normally expressed during trunk muscle development, were not expressed during

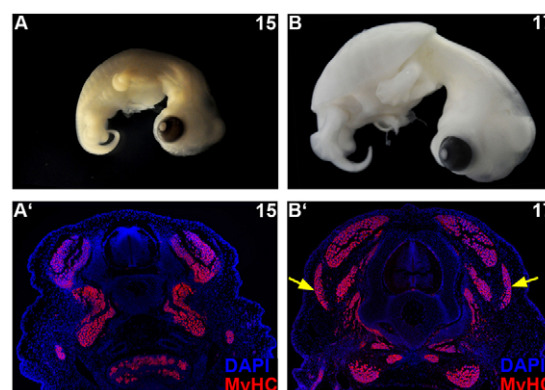
myogenesis of the cucullaris muscle. To firmly establish the lack of the involvement of *Pax3* in cucullaris muscle development and to homologue between birds and mammals, we examined *Pax3<sup>Cre</sup>;Rosa<sup>STOP/YFP</sup>* mice. This line expresses Cre-recombinase under the endogenous *Pax3* promoter, which ultimately initiates YFP expression from a floxed Rosa allele. YFP fluorescence marks all cells with a past or present history of *Pax3* expression. Skeletal muscle in these mice was detected using a MyHC antibody. MyHC<sup>+</sup> sternocleidomastoid myofibres, as well as trapezius myofibres, were negative for YFP, whereas expression was detected between the myofibres in 15.5 dpc embryos (Fig. 5A,B). Most trunk muscles showed YFP activity throughout the tissue (Fig. 5D), whereas its activity was absent from myofibres in head muscles (Fig. 5C). These results confirm the expression profiling data by providing genetic evidence that the cucullaris muscle and its homologues develop in a *Pax3*-independent manner. The independence of the cucullaris muscle group from the trunk myogenic programme was established by examining myogenesis in the *Pax3<sup>Sp/Sp</sup>;Myf5<sup>nlacZ/nlacZ</sup>*. This double knockout lacks all trunk and limb muscles (Tajbakhsh et al., 1997). Remarkably, both the trapezius (Fig. 5E,F) and sternocleidomastoid (data not shown) were present in the double mutant.

Our expression data suggest that the mechanism regulating the cucullaris homologues resembles that of head musculature. Recent work has shown that a subset of head muscles develop through a programme requiring the transcription factor *Isl1* (Nathan et al., 2008). We investigated whether the trapezius and the sternocleidomastoid muscles had a developmental history for the expression of this gene. We were able to show that both the trapezius and the sternocleidomastoid muscles expressed this transcription factor using the *Isl1<sup>cre</sup>/R26R* line (Fig. 5J,J'). Furthermore we examined *Tbx1<sup>-/-</sup>* mice that have previously been shown to be severely deficient in head muscles (Kelly et al., 2004). Examination of the trunk of these animals at 13.5 dpc revealed the absence of both the trapezius and sternocleidomastoid muscles (Fig. 5K,K'). To exclude the possibility that the absence of these muscles was due to late development we also examined the mutants at a later stage (14.5 dpc). Even at this advanced stage of development, we found that both the trapezius and sternocleidomastoid muscles were absent in the *Tbx1<sup>-/-</sup>* (Fig. 5L-M'), confirming and extending previous results of Kelly et al. (Kelly et al., 2004).

In conclusion, these results suggest that the cucullaris muscle develops according to the head and not the trunk myogenic programme.

### Neural crest origin of the cucullaris muscle connective tissue

The cucullaris muscle did not express *Pax3* during its development, and its mammalian homologues were shown to be largely negative for a history of *Pax3* expression. However, we found *Pax3*-lineage-derived expression in between the myofibres, indicating a somitic or neural-crest-derived connective tissue. Connective tissue of head muscles is known to be derived from neural crest. By contrast, the connective tissue of trunk muscles is derived from local mesoderm (Noden and Trainor, 2005; Noden and Francis-West, 2006; Matsuoka et al., 2005). To determine whether neural crest contributes to connective tissue, including the endomyosium of cucullaris muscle homologues in mammals, we investigated *Wnt1<sup>Cre</sup>/R26RlacZ* mice. This reporter line marks neural crest cells, but the reporter protein is not expressed in somite-derived



**Fig. 6. Conserved mechanism of cucullaris development in the turtle.** MyHC expression in the turtle at stages 15 and 17. Yellow arrows identify the cucullaris muscle. (A,B) Whole embryo morphology. (A',B') Sections showing MyHC expression (red) and the nuclear stain DAPI (blue). (A') At stage 15, differentiated axial muscles are present but the cucullaris is not detected. (B') The cucullaris muscle expresses MyHC at stage 17.

cells (Jiang et al., 2000). Sections of 13.5 dpc mice showed that the endomysial connective tissue of the trapezius muscle is derived from the *Wnt1* lineage, indicating that it is neural crest in origin (Fig. 5G,H).

We established the origin of the connective tissue for the cucullaris muscle by homotopically transplanting quail dorsal neural tube at the level of somites 1-3 into chick hosts. Immunohistochemistry of day 8 chimeras showed that cells expressing the nuclear antigen QCPN were found between MyHC<sup>+</sup> myofibres (Fig. 5I).

In conclusion, we confirm and extend the findings of Matsuoka et al. (Matsuoka et al., 2005) that neural crest cells contribute to the endomysial connective tissue of the cucullaris muscle of birds and its mammalian homologues.

### Conserved mechanism for cucullaris muscle development

The cucullaris muscle is a highly conserved muscle in higher vertebrates. We have shown that it not only has a unique origin but also develops late compared with all other muscle groups. To determine whether the latter feature is conserved in vertebrates, we explored the temporal development of the cucullaris muscle in the turtle. At stage 15 we found differentiated muscle in the head, trunk and limb. Significantly at this stage, the cucullaris muscle did not express MyHC (Fig. 6A,A'). By contrast, the cucullaris muscle was clearly evident at stage 17 (Fig. 6B,B'). We conclude that the differentiation of the turtle cucullaris muscle takes place later than head, trunk and limb muscles – a situation similar to that in birds.

## DISCUSSION

### The lateral plate mesoderm has myogenic properties

In this study we have shown that the lateral plate mesoderm has myogenic properties and is the major source of the cucullaris muscle. We show that the occipital lateral plate mesoderm, in contrast to other lateral plate regions, possesses the unique property of forming skeletal muscle. The cucullaris muscle and its mammalian homologues extend from the neck, reaching far into the trunk. In birds, this muscle fails to express genes associated



with the trunk myogenic programme, but does express markers linked to the head myogenic programme, differentiates late during embryogenesis, and has connective tissue originating from the neural crest. These findings led us to propose that cucullaris muscle development is regulated according to the head myogenic programme.

The origin of the cucullaris muscle and its mammalian homologues – the trapezius and the sternocleidomastoid muscle – have been the subject of much debate over the past 100 years. A diverse range of tissues has been postulated to give rise to these evolutionarily conserved muscles. Classical histology examination in salmon and flying fish by Harrison (Harrison, 1895) and discussed in 1918 (Harrison, 1918) and 1908 (Derjugin, 1908) led to the notion that skeletal muscle associated with the pectoral fin originated from the dorsal lateral plate mesoderm. However, contemporary experiment-based studies underpinned by robust lineage-tracing techniques showed that the cucullaris develops from the occipital somites (Couly et al., 1993; Noden, 1983; Huang et al., 1997; Huang et al., 2000). Our study shows that the majority of the cucullaris muscle develops from the lateral plate mesoderm.

At this point it is worth comparing the outcomes of this study with those from previous investigations seeking to establish the origin of the cucullaris. In this study we show, through quantification analysis coupled with lineage-tracing techniques, that the majority (approximately 90%) of cucullaris myofibres originate from the lateral plate mesoderm, with only a minor contribution (3.6%) from each of somites 1-3. In previous studies the origin of this muscle was reported to be from the somites, but it was noted that some successful somite grafts gave a very minor contribution (similar to those seen here), whereas others showed greater contribution (Huang et al., 1997; Huang et al., 2000). We believe that findings from previous studies can be reconciled with the present data simply by acknowledging that in our previous studies (Huang et al., 1997; Huang et al., 2000) and that of Noden (Noden, 1983) lateral plate mesoderm tissue was present in the somitic grafts. Examination of our previous grafting protocols in light of our present studies shows that efforts aimed at extracting entire somites would have included small amounts of lateral plate mesoderm in the quail donor tissue – a tissue believed not to have myogenic properties. Indeed, Noden magnanimously states that in his study, lateral plate mesoderm was more than likely to be present in the somite grafts (Noden, 1983). Our conclusion that the majority of the cucullaris muscle is non-somatic in origin, reached using an alternative tissue preparation approach to minimise lateral plate mesoderm contamination, is reinforced by two findings reported herein: (1) the extremely low level of *Pax3/7* expression during chick cucullaris embryogenesis; (2) the near absence of muscle cells with a present or past history of *Pax3* expression in the mouse trapezius.

The caudal myogenic boundary within this tissue lies at the level of somite 3, as transplantation of lateral plate mesoderm adjacent to somites 4-5 failed to give rise to the muscle. Transplantation of a smaller portion of the contributing lateral plate mesoderm gave rise to myogenic tissue along the entire length of the cucullaris muscle, suggesting that there is little, if any, anteroposterior compartmentalisation.

Our results provide experimental evidence that the occipital lateral plate mesoderm is a source of skeletal muscle and confirms suggestions made to this end by Harrison (Harrison, 1895; Harrison, 1918) and Derjugin (Derjugin, 1908) in their histological examination of the somatopleura development of fish. As intriguingly, our work shows also that the lateral plate mesoderm

in this region of the embryo forms predominantly skeletal muscle. This finding raises the issue of how the cucullaris lateral plate mesoderm gained myogenic capacity. The paraxial mesoderm of the trunk is initially segmented as epithelial somites, and contrasts the mesenchymal organisation of both the lateral plate and head mesoderm (Christ and Ordahl, 1995). Indeed the head mesoderm and the lateral plate are continuous, and this observation could be incorporated into one of two simple models to explain the myogenic origin of the cucullaris muscle. One possibility is that cells of the head mesoderm spread caudally into the area lateral to the first three somites. Alternatively, the occipital lateral plate mesoderm could adopt the molecular myogenic programme due to a caudal shift of the head myogenic programme. Boundary shifts resulting in the acquisition of novel tissue characteristics are common within the animal kingdom. For instance, in snakes a cranial shift of the trunk molecular boundary has been proposed to result in the loss of forelimbs and a development of ribs in the cervical region (Cohn and Tickle, 1999).

Connective tissue is either derived locally from the mesoderm (for trunk muscles) or from the lateral plate mesoderm (for limb muscles) (Christ et al., 1974; Chevallier et al., 1977; Christ et al., 1982). By contrast, all head muscle connective tissue originates from the neural crest (Noden and Trainor, 2005; Noden and Francis-West, 2006; Matsuoka et al., 2005). In this study we found that the connective tissue of the cucullaris muscle group of both birds and mammals originates from the neuroectoderm. These results confirm (in mammals) and extend (in birds) the findings of Matsuoka et al. (Matsuoka et al., 2005). Summarising the data regarding the origin of the myogenic and connective tissue components of the cucullaris muscle we show that the head muscle programme is far more extensively deployed than previously thought.

### Cucullaris progenitor proliferation is not controlled by *Pax3/7*, but by genes expressed during head progenitor proliferation

Our lineage-tracing studies in chicks showed that the lateral plate mesoderm movement starts at HH 14, but that myogenic differentiation is not initiated until HH 26. We considered that the intervening period may be required to generate the number of cells needed to form a large muscle like the cucullaris. In the trunk, proliferation is mainly driven by *Pax3* and *Pax7*, a process that simultaneously suppresses differentiation (Amthor et al., 1999; Amthor et al., 1998). However, neither of these genes was expressed in the developing cucullaris muscle. Our molecular analysis was confirmed using a genetic approach in mice through the deployment of the *Pax3<sup>Cre</sup>:Rosa<sup>STOP/YFP</sup>* and the *Pax3<sup>Sp/Sp</sup>:Myf5<sup>nlacZ/nlacZ</sup>* lines (Engleka et al., 2005). We found that myoblasts of the trapezius and sternocleidomastoid muscles were negative for a recent or past history for *Pax3* expression. These results are in keeping with our lineage-tracing experiments involving somite transplantations. Indeed, these results offer a mechanistic explanation of the presence of the large muscles found in the forelimb region of the *Pax3<sup>Sp/Sp</sup>:Myf5<sup>nlacZ/nlacZ</sup>* [see Fig. 2H in Tajbakhsh et al. (Tajbakhsh et al., 1997)]. We show that the trapezius and sternocleidomastoid muscles (which develop through a head myogenic programme) are present in the *Pax3<sup>Sp/Sp</sup>:Myf5<sup>nlacZ/nlacZ</sup>*, which lacks all trunk and limb muscles. This is concordant with our findings that connective tissue between the myofibres was positive and derived from the *Pax3* neural lineage, confirming our previous result of the neural crest lineage tracing in the chick.



In contrast to finding that the trunk precursor cell markers are not expressed in the cucullaris muscle at the time of proliferation, we found that genes thought to carry out a similar role in the head such as *MyoR*, *Tbx1* and *Capsulin* were robustly expressed throughout the development of the muscle (Bothe and Dietrich, 2006). Furthermore, the expression of the head precursor markers matches the movement of the cucullaris anlagen as shown in the temporal lineage-tracing studies. The role of the head muscle programme in the development of neck muscles was confirmed by examining the trunk of *Tbx1*<sup>-/-</sup> mice. These animals failed to form either the trapezius or the sternocleidomastoid muscle (see Fig. 5), confirming and extending the results of Kelly et al. (Kelly et al., 2004). In addition, we found that the trapezius also displayed a molecular history for the expression of *Isl1*, a gene recently implicated in the ontogeny of specific head muscles (see Fig. 5) (Nathan et al., 2008). We conclude that the cucullaris muscle and its mammalian homologues use the same myogenic programme as that employed in the formation of head musculature.

### Maintaining an extended phase of progenitor proliferation may be necessary for the extension of the cucullaris muscle into the trunk

An intriguing feature of the cucullaris muscle and its mammalian homologues is that it is derived from a small area lateral to the first three somites, yet eventually it extends over 14 segments, a process that is not completed until relatively late during embryogenesis (day 8 in chick). By this stage all other major muscle groups (axial, branchial arch and limb muscles) are at an advanced stage of differentiation. This late differentiation was found to be evolutionarily conserved, as we show that the cucullaris of turtles also differentiates after axial, head and limb muscles.

During cucullaris muscle development, an extended period of time for proliferation may be required to generate an adequate number of cells to form the muscle. This phase of proliferation may be especially important for cucullaris muscle development, as the muscle starts off from a small population of mesenchymal cells that reside under the occipital ectoderm.

In this study we found that the cucullaris muscle displays the exceptional property of employing the head myogenic programme, despite the fact that a considerable part of the muscle lies within the trunk territory. The development of the cucullaris muscle at the transition of the head-trunk territory has an interesting consequence for its differentiation. The development of muscles in the trunk and head differ in that they show very different responses to the same environmental cues. For example, muscle development is supported in the trunk by either Wnt or Shh. However, the same signalling molecule suppresses head muscle development (Tzahor et al., 2003). Release of the myogenic suppression in the head has been shown to be a result of the production of signalling protein antagonists secreted by the cranial neural crest. Assimilating the results generated in the present study in consideration of the differences apparent in the regulation of head versus trunk myogenic programmes, we propose the following scenario for the development of the cucullaris muscle. Wnts from the occipital neural tube and ectoderm suppress myogenesis of cells residing in the occipital lateral plate mesoderm. Under this influence they proliferate and express *Tbx1*, *MyoR* and *Capsulin*. As the pool of cucullaris progenitors expands, it extends caudally into the trunk. Here the cucullaris precursors are in close proximity with progenitor cells of trunk musculature. However, these two myogenic populations respond differentially when exposed to

influential signalling cues. In the presence of Wnts, the trunk precursors initiate differentiation, whereas the cucullaris precursors respond by proliferating. Again similar to the situation in the head, we suggest that the key to initiating differentiation is to antagonise the action of the environmental signalling molecules and that this action could be executed by the neural crest.

### Evolution of the cucullaris muscle

An evolutionary explanation of how the cucullaris muscle was transformed from a relatively short muscle (in sharks) into an entity stretching into the trunk still remains unknown. The short cucullaris muscle in the shark moves the gill arches, but also attaches to the pectoral girdle. The sharks' ancestral crossopterygian relatives, such as the Eusthenopteron and Sauripterus, possessed a neck girdle, which linked the pectoral girdle to the base of the skull. The neck girdle was dermal in origin and consisted of four membranous bones (post-temporal, supracleithrum, cleithrum and clavicle) (DePalma, 2008). During evolution, the size of the neck girdle components was reduced or lost and the skull became free of its attachments to the pectoral girdle. The advantage for animals with this modification was increased mobility of the head; however, at the cost of a destabilized head. We suggest that in the absence of the neck girdle, the cucullaris muscle expanded caudally – a feature seen during its development in birds – to provide both stability for the head (similar to that played by postural muscles) while permitting mobility. The unique developmental origin of this muscle will be valuable in order to identify its precise homologues in lower vertebrates, a prerequisite towards gaining a fuller understanding of the evolution of the neck (Kuratani, 2008).

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### Competing interests statement

The authors declare no competing financial interests.

### Supplementary material

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