Normal and aberrant craniofacial myogenesis by grafted trunk somitic and segmental plate mesoderm

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Summary

Our research assesses the ability of three trunk mesodermal populations – medial and lateral halves of newly formed somites, and presomitic (segmental plate) mesenchyme – to participate in the differentiation and morphogenesis of craniofacial muscles. Grafts from quail donor embryos were placed in mesodermal pockets adjacent to the midbrain-hindbrain boundary, prior to the onset of neural crest migration, in chick host embryos. This encompasses the site where the lateral rectus and the proximal first branchial arch muscle primordia arise. The distribution and differentiation of graft-derived cells were assayed using QCPN and QH1 antibodies to identify all quail cells and quail endothelial cells, respectively. Chimeric embryos were assayed for expression of *myf5*, *myod*, *paraxis* and *lbx1*, and the synthesis of myosin heavy chain (MyHC), between 1 and 6 days later (stages 14-30). Heterotopic and control (orthotopic) transplants consistently produced invasive angioblasts, and contributed to the lateral rectus and proximal first branchial arch muscles; many also contributed to the dorsal oblique muscle. The spatiotemporal patterns of transcription factor and MyHC expression by these trunk cells mimicked those of normal head muscles. Heterotopic grafts also gave rise to many ectopic muscles. These were observed in somite-like condensations at the implant site, in dense mesenchymal aggregates adjacent to the midbrain-hindbrain boundary, and in numerous small condensations scattered deep to the dorsal margin of the eye. Cells in ectopic condensations expressed trunk transcription factors and differentiated rapidly, mimicking the trunk myogenic timetable. A novel discovery was the formation by grafted trunk mesoderm of many mononucleated myocytes and irregularly oriented myotubes deep to the eye. These results establish that the head environment is able to support the progressive differentiation of several distinct trunk myogenic progenitor populations, over-riding whatever biases were present at the time of grafting. The spatial and temporal control of head muscle differentiation and morphogenesis are very site specific, and head mesoderm outside of these sites is normally refractory to, or inhibited by, the signals that initiate ectopic myogenesis by grafted trunk mesoderm cells.

Key words: Myogenesis, Quail-chick chimera, Craniofacial muscles, Extra-ocular muscles, Myotome, *myf5*, *myod*, *paraxis*, *lbx1*, Myosin heavy chain

Introduction

Vertebrate skeletal myogenesis begins in paraxial mesoderm with a commitment of cells to the muscle lineage, as evidenced by the activation of the bHLH transcription factors *myf5* and *myod*. This is accompanied or followed by dramatic changes in cell-to-cell relations among myogenic precursors, marked by the formation of epithelial dermamyotomes in the trunk or aggregation of mesenchymal cells in the head. (Trunk muscles are all muscles originating in somites, including those that subsequently move into craniofacial regions to form tongue and laryngeal muscles. Head muscles refer to those derived from unsegmented, non-epithelial paraxial mesoderm flanking the hindbrain and midbrain regions; all branchial and extra-ocular muscles are included in this category.) Subsequent muscle morphogenesis involves movements of individual or aggregated myoblasts to their sites of terminal differentiation, followed by the segregation and alignment of myotubes. Superimposed on these events is the establishment of fiber-type diversity within early primary and later-forming secondary myotubes (reviewed by Pownall et al., 2002).

Many of the signals and transcription factors necessary for myogenesis in murine and avian somites have been identified and their roles characterized experimentally (Borycki and Emerson, 2000; Sabourin and Rudnicki, 2000; Tajbakhsh and Buckingham, 2000). However, few studies have investigated the interactions controlling myogenesis in the head. The objective of this research was to characterize the cephalic myogenic environment by assaying its ability to direct the development of three distinct trunk mesodermal populations: medial half somite, lateral half somite and segmental plate (presomitic) mesoderm.

Differentiation and compartmentalization in somites

Trunk myogenesis requires a progressive series of tissue interactions that begin in the segmental plate region shortly after gastrulation (Arnold and Braun, 2000; Buckingham, 2001; Stockdale et al., 2000). Although most myogenic

interactions are similar throughout the trunk, peripheral signals unique to the occipital, brachial and lumbosacral levels elicit the formation of migratory laryngoglossal and appendicular myoblasts from the lateral myotome in chick embryos (Alvares et al., 2003; Brand-Saberi et al., 1996; Hayashi and Ozawa, 1995).

The onset of commitment to the muscle lineage is difficult to ascertain precisely and shows considerable interspecies variation (Buckingham et al., 2003). Classical explant experiments indicate that commitment coincides with the formation of somites, and autonomous competence to differentiate is acquired shortly thereafter (Christ and Ordahl, 1995; Ordahl and Le Douarin, 1992). This conclusion is consistent with segmental plate cell culture experiments (Buffinger and Stockdale, 1994; Gamel et al., 1995; Stern and Hauschka, 1995). However, PCR assays have found *myf5* or *myod* transcripts in cranial segmental plate mesodermal cells in the chick (Kiefer and Hauschka, 2001), suggestive of an earlier bias towards myogenesis.

Orchestrating myogenic initiation, differentiation, movements, proliferation and survival among trunk muscle precursors requires a consortium of extrinsic signals from adjacent tissues (Alves et al., 2003; Borycki and Emerson, 2000; Christ and Brand-Saberi, 2002; Pownall et al., 2002), as well as intra-somitic signals. Some signals are distinct for medial and lateral myogenic zones of the dermamyotome, and maintaining the ratio among signals is essential for proper spatio-temporal coordination of myogenesis (e.g. Wagner et al., 2000). In addition, the disparate origins of medial and lateral trunk paraxial mesoderm may predispose these two sets of cells to respond differently to extrinsic signals (Sporle, 2001). At present it is difficult to distinguish these historical biases from those acquired because of subsequent differences in their position in relation to neighboring tissues.

In avian embryos, the medial somite compartment gives rise to at least two, temporally distinct myoblast populations (Buckingham, 2003; Denetclaw and Ordahl, 2000; Summerbell et al., 2000). These form all epaxial muscles and those hypaxial muscles that remain close to developing vertebrae and proximal ribs (Burke and Nowicki, 2003). Thus, muscles derived from the medial half somite always remain within the paraxial mesoderm community. By contrast, lateral myotome-derived myogenic cells at restricted sites along the body axis migrate into neural crest or lateral mesoderm (Nowicki et al., 2003), forming laryngoglossal and appendicular muscles, and some body wall muscles close to the ventral midline. These cells are *pax3*-dependent and during their migrations express the transcription factors *paraxis*, *lbx1* and *six1*, and the receptor c-met (Delfini and Duprez, 2000; Gross et al., 2000; Laclef et al., 2003; Mennerich et al., 1998; Uchiyama et al., 2000; Williams and Ordahl, 2000). *Paraxis* expression occurs throughout the dermamyotome of all somites, and is initiated in cells within the cranial border of the segmental plate. By contrast, expression of *lbx1* is restricted to the lateral margins of somites, and is transient at all axial levels, except those adjacent to the limb buds and the occipital region (somites 1-5), from which appendicular and laryngoglossal precursors arise, respectively (Brohmann et al., 2000; Gross et al., 2000; Schäfer and Braun, 1999).

All skeletal myoblasts express *myf5* and *myod*. In both avian and murine embryos, tongue and laryngeal muscle precursors maintain *myf5* expression throughout their ventral movements; appendicular myogenic cells do not (Bladt et al., 1995; Dalrymple et al., 2000; Mackenzie et al., 1998; Noden et al., 1999).

Craniofacial myogenesis

Developing branchial and extra-ocular muscles originate in paraxial mesoderm that does not undergo epithelialization or form separate segmental units. Myogenic condensations occur in discrete and separate foci within the head paraxial mesoderm (Couly et al., 1992; Hacker and Guthrie, 1998; Noden, 1983b; Noden et al., 1999). Each primordium initiates the formation of multinucleated myotubes while simultaneously changing its position and relation to neighboring tissues. Initially surrounded by nonmyogenic paraxial mesoderm, head muscle primordia move peripherally to become surrounded by, and later, infused with, neural crest-derived connective tissues. The directions and routes of these morphogenetic movements are unique for each extra-ocular and branchial muscle. These movements separate primordia that are initially neighboring [e.g. lateral rectus (LR) from dorsal oblique (DO), palpebral depressor from other proximal first branchial arch (BA1) muscles], or may bring initially divergent primordia into close proximity [e.g. the dorsal rectus (DR), medial rectus (MR) and LR all converge near the ciliary ganglion].

The LR is the first head muscle to exhibit mesenchymal condensation and to initiate *myf5* transcription (Noden et al., 1999). The LR primordium moves rostrally towards the ciliary ganglion, then myotubes at the leading edge turn laterally and expand towards the equatorial zone of the eye (Wahl et al., 1994). The dorsal oblique (DO) primordium arises rostral to the LR, lateral to the mesencephalon, and then moves rostrally along an arc that parallels the dorsal margin of the eye. Expression of *myf5* and myosin follows shortly after the LR. The movements of LR and DO primordia begin during day 3 of incubation (stages 16-18) and are not complete until day 5 (stages 24-25) (Noden et al., 1999). Myoblasts of the first branchial arch (BA1) originate at the same axial level as the LR, but these precursors are dorsolateral to, and separate from, the LR progenitors (Couly et al., 1992; Noden, 1991b). They are initially in close contact with the overlying surface ectoderm, but become separated from this epithelium by migrating neural crest cells.

Muscle differentiation begins before the onset of these movements. The chick LR, for example, initiates *myf5* expression immediately ventrolateral to rhombomeres 1 and 2, close to the notochord and trigeminal ganglion. *Myf5* and *myod* expression are detectable in the LR at stages 13.5 and 14.5, but myosin heavy chain (MyHC) synthesis does not occur until stage 21.5 (Noden et al., 1999). This two-day delay in initiating MyHC production is characteristic of head muscles, and contrasts with the rapid progression to MyHC synthesis in most medial myotome-derived cells. Lateral somite-derived cells that form the hypoglossal cord also have a delayed onset of MyHC production.

Experimental comparisons of trunk and head mesoderm

Accounts of heterotopic transplants of paraxial mesoderm have been available since the pioneering work of Adelmann (Adelmann, 1938), but it was not until methods of identifying

and characterizing the progeny of transplanted cells became available that the totality of outcomes could be assessed. In an extensive series of quail-to-chick transplants of whole somites, or segmental plate mesoderm plus overlying ectoderm, Noden found trunk mesoderm cells capable of contributing to normal extra-ocular and branchial arch muscles while also forming large, irregular ectopic muscles (Noden, 1986).

More recently, Hacker and Guthrie, and Mootoosamy and Dietrich, implanted newly formed somites beside the hindbrain and assayed chimeric embryos for expression patterns of trunk and head muscle markers (Hacker and Guthrie, 1998; Mootoosamy and Dietrich, 2002). Grafted somites formed ectopic condensations that expressed *myf5* and *myod* plus several somite markers, including *paraxis*, *pax1* and *pax3*, but failed to express *lbx1*, suggesting that the lateral myotome program is not activated in the head environment. Both groups also transplanted segmental plate mesoderm, but the results were inconsistent. Mootoosamy and Dietrich found no differences between segmental plate and somite grafts (Mootoosamy and Dietrich, 2002), whereas Hacker and Guthrie found very limited dispersal or myogenesis by transplanted segmental plate cells (Hacker and Guthrie, 1998). Thus, the ability of the head environment to promote somitogenesis, muscle lineage activation and myoblast migration in these somite precursors remains controversial.

Experiments reported here were designed to resolve inconsistencies in these previous studies, and, in particlur, to examine both early differentiation and later morphogenesis of cell populations derived from medial and lateral half somites, or from presomitic mesoderm (segmental plate) grafted in place of cephalic paraxial mesoderm. We find that grafts of all trunk paraxial mesoderm populations produce both highly mobile and stationary cells, and are able to contribute to both normal and ectopic head muscles. Ectopic muscle condensations typically express trunk muscle markers (e.g. *paraxis*, *lbx1*) and differentiate rapidly, mimicking the trunk timetable. Not seen previously is a population of cells that emerge from grafted trunk mesoderm and form scattered individual myocytes and myotubes. In these cells, lineage commitment and differentiation have become uncoupled from the normal process of muscle morphogenesis.

Materials and methods

Surgeries

Quail and chicken embryos were staged using the Hamilton and Hamburger stage series (Hamilton and Hamburger, 1951). Three different tissues were excised from quail donor embryos at stage 11-12 (12-14 somites): medial or lateral halves of the most recently formed somite [somite I, as defined by Ordahl and LeDouarin (Ordahl and LeDouarin, 1992)], or segmental plate mesoderm located at –S2 (Fig. 1). These mesodermal tissues are adjacent cranially to sites from which wing myoblasts normally arise (somites 16-21). Donor embryos were explanted onto sterile Sylgard-lined dishes. Trunk surface ectoderm overlying segmental plate and newly formed somites was cut and removed following brief incubation in 1% dispase. After rinsing, somite I was bisected longitudinally. Half somites or pieces segmental plate were immediately transferred to stage 8.5 to 9+ (4-8 somites) host embryos. Orthotopic transplants of head mesoderm dissected from beside the isthmus of stage 8-9 donor embryos served as controls. Segmental plate and head mesoderm grafts were comparable in rostrocaudal and mediolateral dimensions with a newly formed somite, but were smaller in the dorsoventral axis and had a lower cell density.

Immediately prior to use, chick host embryos were cooled to room temperature, windowed and stained with 0.2% Neutral Red. After opening the vitelline membrane of the host, a shallow transverse incision was made in the surface ectoderm lateral to the caudal metencephalon (Fig. 1), and a pocket extending rostrally to the level of the mesencephalon was dissected within paraxial mesoderm. Underlying pharyngeal endoderm and overlying surface ectoderm were not lesioned. Grafts were placed with random orientation inside the pockets. Host eggs were then resealed using transparent book tape and returned to the incubator for 2-6 days.

In situ hybridization

Embryos were fixed at stages 14-22 by immersion in 4% buffered formaldehyde at 4°C, pH 7.4, then washed, dehydrated and stored in 100% methanol at –20°C. Whole embryo visualization of mRNA was as described previously (Wilkinson, 1998; Noden et al., 1999). In most cases, the roof of the hind- and midbrain regions was opened longitudinally to enhance antibody access to deeper tissues. Probes used were digoxigenin-conjugated *myod* and *myf5* (Kiefer and Hauschka, 2001), chick *paraxis* (Barnes et al., 1997), and *lbx1*. *lbx1* was made by RT-PCR of stage 22 limb tissue using primers of bases 758-777 and 1056-1074 from the murine *lbx1* sequence published by Jagla et al. (Jagla et al., 1995), ligated into Promega pGEM-T Easy vector. After clearing and examination in 100% glycerol, some embryos were rehydrated, embedded in 4% low-melting point agarose and sectioned at 60 μm on a vibratome. Embryos were visualized using darkfield illumination on a Wild M400 Macroscope. Digital images (QImaging) were processed in Photoshop.

Immunohistochemistry

Embryos were fixed at stages 18-30 in Serra's fixative at 4°C for 2-3 hours, then either embedded in Paraplast and sectioned (6 μm), or rehydrated and cleared for whole embryo antibody visualization. Primary antibodies included QCPN (diluted 1:3, Developmental Studies Hybridoma Bank) to identify all quail cells, F59 (1:10, a gift from Frank Stockdale) to identify cells producing skeletal (and embryonic cardiac) myosin heavy chain, and QH1 (1:200, Developmental Studies Hybridoma Bank) to identify quail endothelial cells. Whole-mount immunohistochemistry for QCPN was performed on stage 18-24 embryos using methods previously described (Noden, et al., 1999); many of these embryos were subsequently paraffin embedded and sectioned. Immunoassays were performed sequentially or on adjacent sections. Secondary antibodies including biotinylated anti-mouse (Jackson labs), streptavidin-HRP, streptavidin-AP, anti-mouse-HRP and anti-mouse-AP (DAKO) were used according to manufacturer's recommendations. With any sequential analysis, immunohistochemistry for QCPN was performed first, using the streptavidin-HRP. Slides were then incubated in protein block (3% BSA in TBS) overnight prior to application of additional antibodies. DAKO double-stain kit was used for sequential immunohistochemistry with QCPN, QH1 and F59. Slides were counterstained using eosin or thionin.

Results

Only embryos with no compromising craniofacial dysmorphology were included in this study. Table 1 summarizes the number of cases in each transplant category, and for each type of assay.

Distribution and cell:cell relations

The distribution of transplant-derived cells 2 days after surgery (stages 17-19) was analyzed using the anti-quail QCPN antibody (Fig. 2). Heterotopic transplants consistently generate

Table 1. Summary of transplants by category and assay

Transplant category	Stages 14-23* Number	Immunocytochemistry** QCPN	QH1	F59	In situs *myf5*	*paraxis*	*Ibx1*	Stages 24-30 QCPN + QH1 + F59	Total number of cases
Segmental plate	27	11	8	7	4	4	3	5	32
Lateral somite	15	6	1	2	8	3	3	5	20
Medial somite	18	12	1	3	5	3	2	4	22
Orthotopic	5	5	1	0	2	1	1	8	13
Totals	65	30	11	12	19	11	9	22	87

*Stage at fixation.
**Many embryos were double stained; thus, these numbers exceed the number of transplants.
QCPN, quail cell marker; QH1, quail endothelial cell marker; F59, myosin heavy chain marker.

more cells than orthotopic transplants, which is a reflection of the greater cell density in trunk mesoderm at the time of surgery. Medial, lateral, and segmental plate transplants generate qualitatively identical results. All produce large and multiple small ectopic cell condensations surrounded by a cloud of widely dispersed QCPN-positive cells. No comparable large or small ectopic condensations are present following orthotopic transplantation. Typically, in trunk-into-head recipients a dense, epithelial-like mass of quail cells forms at the implantation site, ventrolateral to the isthmus region. Dispersed around this are multiple smaller condensations in supraorbital or dorsal isthmic regions, locations where no cell aggregates are normally present. Graft-derived cells populate the proximal first branchial arch (BA1), but their numbers diminish distally.

Previous studies revealed that many grafted somite cells form angioblasts in the head environment (Noden, 1989; Noden, 1991a). To determine which cells among the dispersed mesenchymal population are committed to the endothelial lineage, QCPN-treated embryos were sectioned and assayed with the anti-quail endothelial antibody QH1. These data reveal that the majority of scattered quail mesenchymal cells are angioblasts that have moved invasively and omnidirectionally from the graft site (Fig. 3), with some already participating in the formation of primitive arterial and venous channels. Segmental plate transplants consistently give rise to fewer angioblasts than half somite transplants. QH1-negative quail cells are interspersed among these endothelial precursors. Some may be angiogenic precursors that have not yet expressed QH1, but others may later contribute to connective tissue and myogenic lineages. Angioblasts are rarely found within normal muscle condensations at these stages (Ruberte et al., 2003), although they are present abundantly in the surrounding periocular and proximal first branchial arch regions.

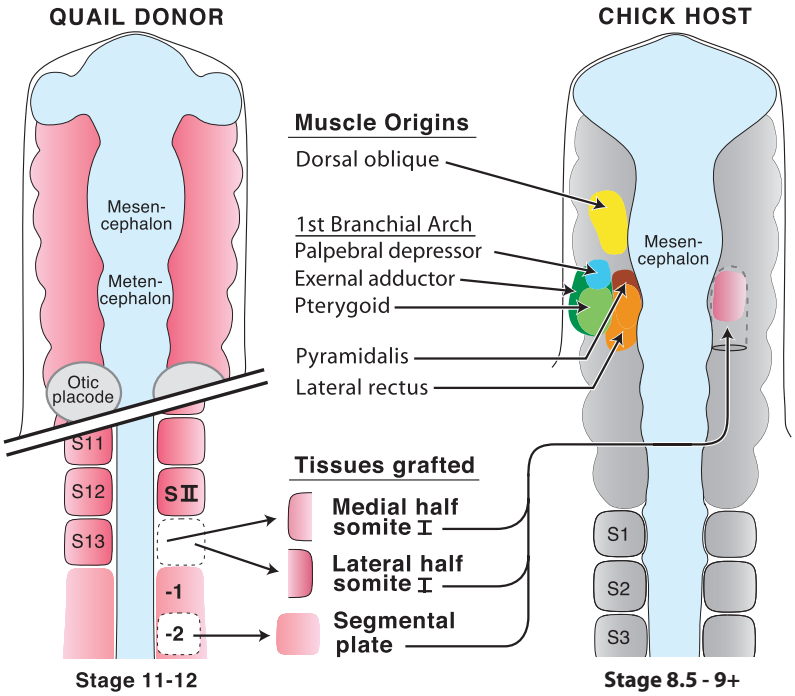

Fig. 1. Transplantation design for this study. Medial or lateral halves of newly-formed somites or pieces of segmental plate mesoderm from quail embryos were grafted into pockets cut into chick head mesoderm. Also shown are the sites of origin within head paraxial mesoderm of extraocular and jaw muscles discussed in this paper. These foci have been identified by orthotopic transplantations (Noden, 1986; Couly et al., 1992) and retroviral injections (Noden, 2002), and were confirmed in this study. The lateral rectus and pyramidalis primordia arise medial and ventral to the first branchial arch progenitors.

Early muscle gene expression

The early pattern of gene expression by transplant-derived cells was assessed using markers for dermamyotome (*paraxis*), lateral myotome (*lbx1*) and myogenic lineage (*myf5, myod*) commitment. In the head, *paraxis* is normally expressed in the LR, with expression beginning coincident with *myf5* expression at stage 13.5, but it is not expressed in other extra-ocular or branchial arch muscles. Trunk graft-derived cells in both large and small, dispersed ectopic aggregations, as well as in the LR primordium, express *paraxis* (Fig. 4). Expression is seen in all trunk-into-head transplantations. The area encompassed by the dispersed *paraxis*-positive cells is substantially smaller than that populated by transplant-derived angioblasts.

Lbx1 is normally expressed by lateral myotome cells prior to and during their migrations into the limb buds and hypoglossal cord. In the head, only the LR and DO muscle primordia express *lbx1*; transcripts are evident by stages 14.5 and 15,

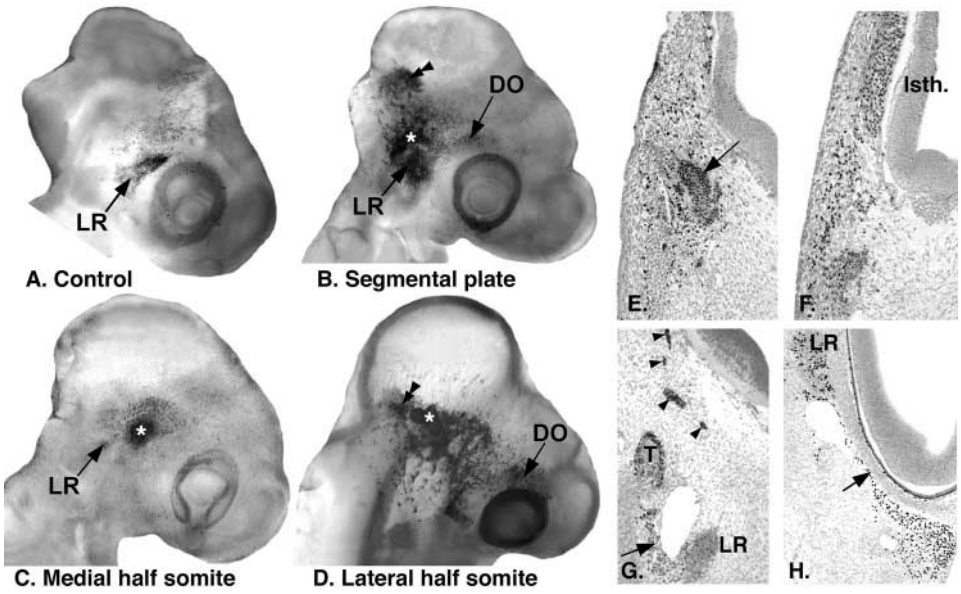

Fig. 2. The distribution of grafted quail mesoderm cells 1.5 to 2.5-days after surgery (stages 19-22) is revealed by the application of anti-quail (QCPN) antibody. (A-D) Whole embryos. Asterisks indicate ectopic somite-like condensations; double arrowheads indicate a mesenchymal condensation adjacent to the isthmus. DO (dorsal oblique) and LR (lateral rectus) are sites where eye muscle primordia condense. (E,F) Sections from segmental plate grafted hosts fixed at stage 22. These show an abundance of graft-derived cells within and scattered around an ectopic somite-like condensation (arrow, E), and a mesenchymal aggregate adjacent to the isthmus (Isth). (G,H) Segmental plate hosts fixed at stages 22 (G) and 26 (H). These reveal the presence of mesenchymal bridges (arrows) extending to the LR muscle during and after the translocation of the LR to its definitive position. Note the presence of quail cells circumscribing and within the trigeminal ganglion (T). Arrowheads indicate dense cords of graft-derived cells that extend ventrally from the isthmus region.

respectively. In all heterotopic transplant categories, *lbx1* was expressed in the LR and DO primordial, but was never detected in proximal first arch muscle primordia. *Lbx1* transcripts were present in large and small ectopic aggregates, but not in surrounding, scattered mesenchymal cells derived from the grafts. This labeling was identical in all trunk-into-head categories, even though medial half somites would not normally express this transcription factor.

Expression of myogenic transcription factors *myf5* and *myod* in host embryos occurs in both normal head muscle primordia and ectopic condensations. In ectopic quail cells, it is most robust in cell aggregations, but loose mesenchymal tissues surrounding these aggregates are also positive, often presenting a multipunctate appearance in whole embryos.

To determine the timeline of myogenesis in ectopic condensations, embryos were examined for *myf5* at stages 13-14, and for MyHC synthesis at stages 19-22, well in advance of *myf5* and MyHC expression in normal head muscles. Transplanted trunk mesoderm cells located in ectopic condensations but not those in normal head muscles are already expressing *myf5* within 24 hours of transplantation (Fig. 5A-D). This is evident following segmental plate and half somite transplants. MyHC is present in both large and small ectopic, QCPN-positive condensations by stage 20 (Fig. 5E,F), nearly a day before its appearance in normal head muscles. Again, no differences between any of the three classes of heterotopic transplants could be detected. The scattered QCPN-positive, QH1-negative quail mesenchymal cells were not immunopositive for MyHC at these stages (18-22).

No precocious expression of *myf5* or synthesis of MyHC was detected in any normal head muscles formed by grafted trunk cells. Nor did the proximal first arch produce detectable levels of mRNA for *paraxis* or *lbx1*. Thus, the times of muscle lineage-specific gene activation by trunk mesoderm cells differ markedly between ectopic condensations and normal head muscles. Moreover, the particular origin of transplanted mesoderm cells neither predisposes nor restricts their differentiation in the head environment. We saw no differences in the onset of gene expression among the three trunk graft categories, but did not assay for possible variations any time between 0 and 24 hours after transplantation.

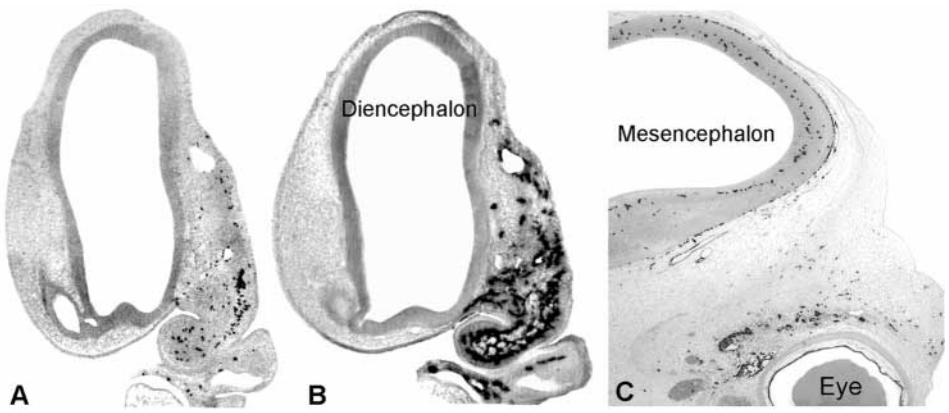

Fig. 3. (A,B) Adjacent sections from a stage 19 segmental plate recipient embryo stained with QCPN (A), to reveal the locations of all quail cells, and QH1 (B), to identify quail endothelial cells. These show that most of the cells that migrated from transplanted trunk mesoderm are angioblasts. (C) A QH1-stained section from a stage 28 lateral somite recipient, showing the substantial contributions of trunk-derived angioblasts to endothelial cells within and surrounding the brain.

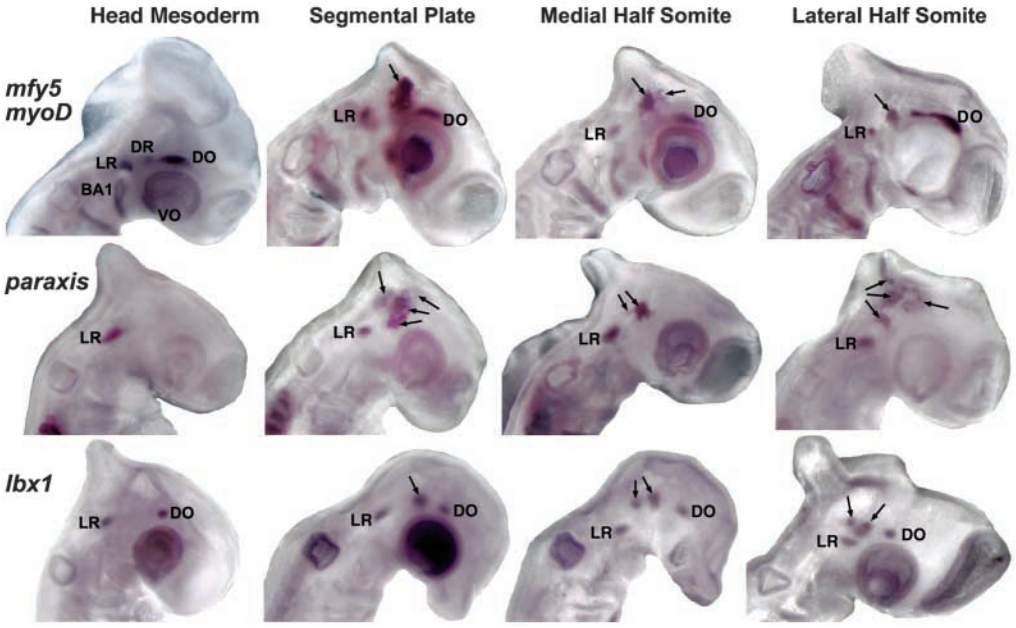

Fig. 4. Pattern of gene expression following head-into-head or trunk-into-head mesoderm transplantation. Column headings indicate the donor tissue; row headings are the gene products assayed. Sites of ectopic expression (black arrows) are evident in all heterotopic categories. *myf5/myod* and *paraxis* are expressed in aggregates, and also in cells dispersed around the site of implantation. *lbx1* is evident in ectopic aggregates but not in dispersed cells. Neither *paraxis* nor *lbx1* mRNA was detected in the first branchial arch (BA1). DR, dorsal rectus; VO, ventral oblique.

Differentiation and morphogenesis

To analyze the later distribution and phenotypes of transplant-derived cells, assays using the preceding antibodies were supplemented by application of the anti-MyHC antibody F59 to sections of embryos fixed 3.5-6 days after surgery (stages 23-30). Reconstructions made from these sections show the spatial distribution of graft-derived cells (Fig. 6). Endothelial cells (green dashes in Fig. 6) derived from grafted trunk mesoderm are consistently present in the proximal mandibular prominence, throughout the maxillary prominence, beside the temporal and dorsal quadrants of the eye, and adjacent to the isthmus, midbrain and diencephalon. Beyond these areas, variable and diminishing numbers of quail endothelial cells are present in the distal first arch, and in the frontonasal, telencephalic and myelencephalic regions. All trunk-into-head categories were qualitatively similar, but orthotopic transplants generated fewer cells.

The overall perimeter and distribution of graft-derived endothelial cells are asymmetric, reflecting the proximo-to-distal asymmetries in growth of the mandibular, maxillary and frontonasal prominences, and the caudo-to-rostral asymmetries of periocular and brain growth. The majority of cells do not cross the midline; however, angioblasts are an exception, and quail endothelial cells are often present on the contralateral side. Angioblasts are always the most widely dispersed and distantly located cells arising from mesoderm transplants. Quail endothelial cells are interspersed with host endothelial cells in the walls of normal blood vessels, including the medial and lateral branches of the cranial cardinal veins, the dorsal aorta, aortic arches and their derivatives, and the basilar artery, in addition to forming numerous diffuse capillaries. This distribution is similar to that seen after transplantation of whole somites and limb mesoderm (Noden, 1986; Feinberg and Noden, 1991). There was widespread contribution by grafted

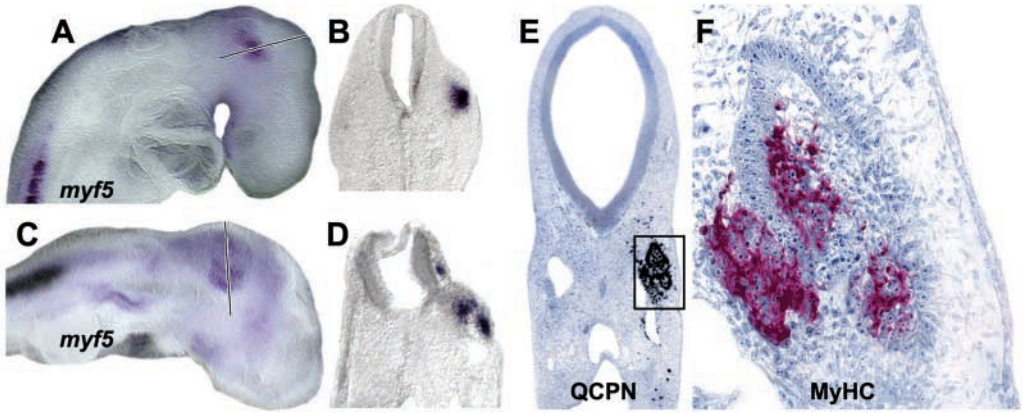

Fig. 5. (A,C) Segmental plate recipients fixed 24 hours after surgery and processed for *myf5* expression. (B,D) Vibratome sections from the embryos shown in A and C at the planes indicated. *Myf5* is activated on a normal trunk timetable, which is earlier than is appropriate for head muscles. (E,F) Adjacent sections from a stage 20 segmental plate recipient. Graft-derived cells within a somite-like condensation synthesize MyHC, nearly a day earlier than it would be found in developing head muscle cells.

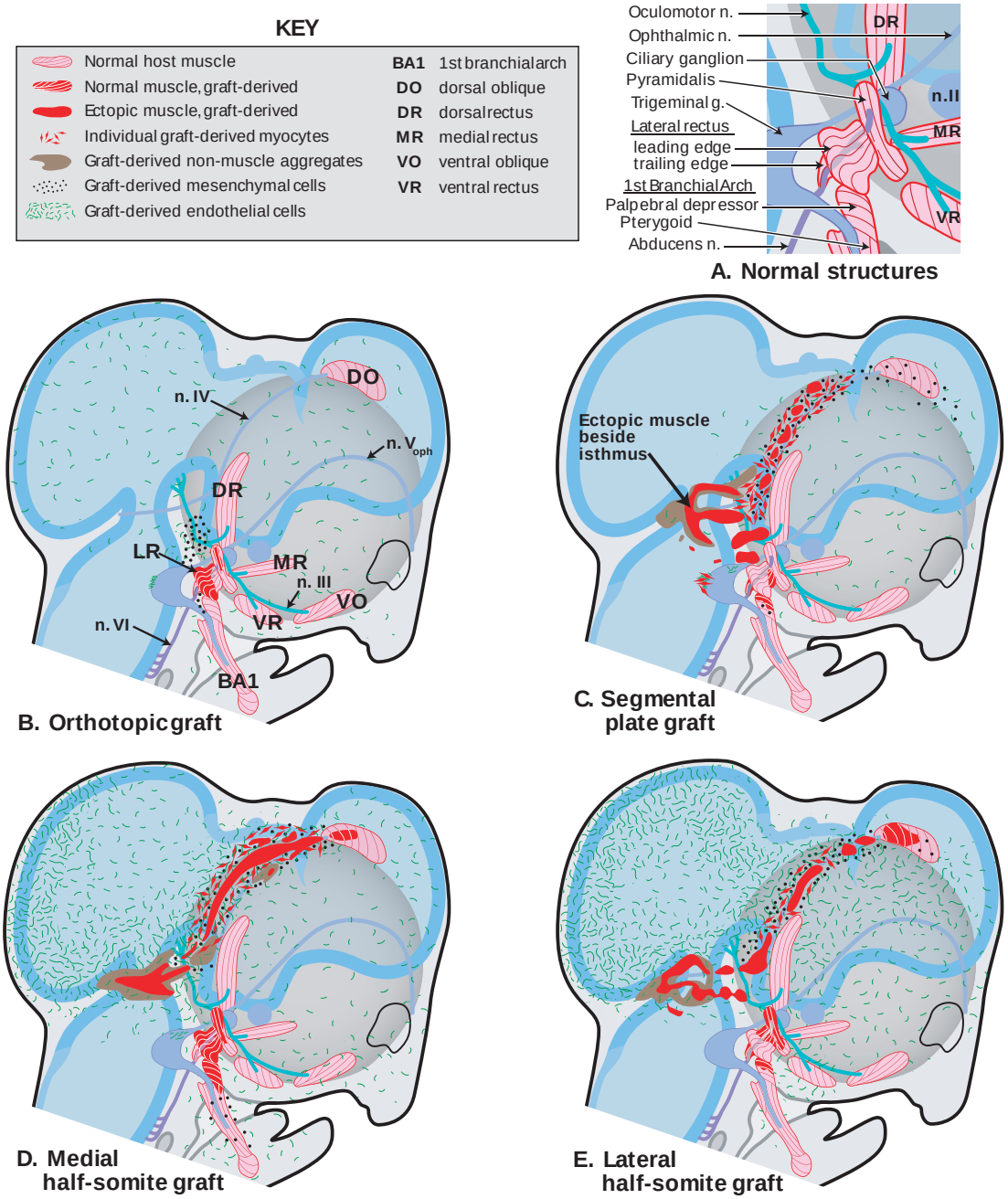

Fig. 6. Reconstructions of stage 29-30 (6-6.5 day) embryos showing the distribution and differentiation of cells derived from mesoderm transplants. A shows an enlargement of the LR and adjacent structures. All grafts contribute to the LR and the pyramidalis muscles, and usually to proximal first arch muscles, especially the palpebral depressor primordium. Many embryos also exhibited labeling in the DO. In contrast to control grafts (A), all trunk-into-head grafts (B-E) produce a large number of ectopic muscles. These are not randomly distributed, but form in large irregular aggregates beside the isthmus, and as small clusters and single myotobes dispersed in a supra-orbital band deep to and paralleling the dorsal margin of the eye.

mesoderm cells to the vascularization of the developing central nervous system, including both meningeal and intraneural vessels (Fig. 3C).

All heterotopic transplants produce cells that contribute to the lateral rectus muscle (Figs 7, 8). Contributions to the LR and pyramidalis range from total to a localized blaze of myotube nuclei. In chimeric LR muscles, quail and chick cells tend to remain segregated, with the host-derived myotobes usually found primarily at the leading (originally rostral) or trailing margin. This probably reflects slight differences in the site of graft implantation.

Graft-derived cells consistently contribute to proximal first branchial arch muscles, especially the precursor of the palpebral depressor muscle (Fig. 7E), which normally originates lateral and slightly rostral to the LR. Contribution to the DO (Fig. 7D) is more variable. In some cases the quail

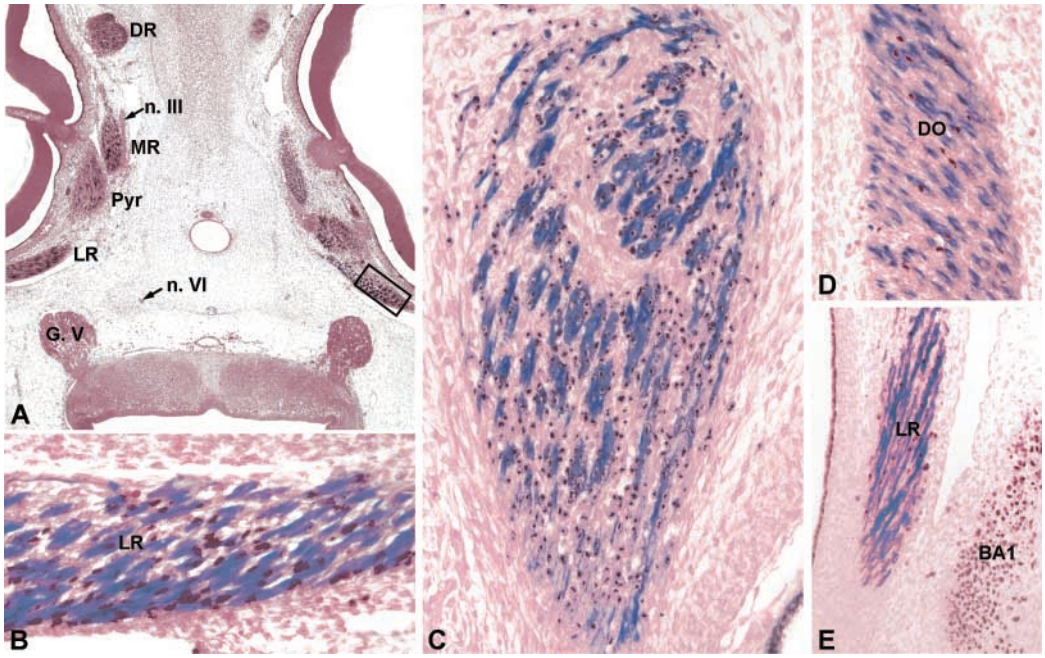

Fig. 7. Control (A,B, orthotopic) and trunk-into-head (C,E, lateral half somite; D, medial half somite) transplantations. Embryos were fixed at stage 28-30 and double stained with F59, to show myosin heavy chain (purple), and QCPN, to show quail nuclei (brown). (A) The labels are on the unoperated side. (B) Graft-derived cells contributing to the lateral rectus (box in A). (C) A lateral rectus muscle in which nearly all cells are derived from the transplant. Blazes of unlabeled intramuscular cells are connective tissues derived from neural crest cells. (D) Dorsal oblique muscle, and (E) lateral rectus and proximal first branchial arch (BA1) muscles, formed by grafted trunk mesoderm. Proximal BA1 muscles normally initiate MyHC synthesis at a later stage, and graft-derived myocytes follow this head timetable.

marker is present in myotubes scattered throughout the entire muscle, but, more commonly, graft contributions are found only in the caudal (proximal) part of this muscle. This is consistent with data that map the DO progenitors to a site beside the mid-mesencephalon, which is close to the cranial boundary of the implant site.

After the initial condensation of myoblasts, there is no movement of myogenic cells between muscle primordia. This is equally true for normal muscles, such as the LR and DR (dorsal rectus), which move into close proximity near the ciliary ganglion (Fig. 8), and for ectopic muscles (Fig. 9E).

As is evident from the reconstructions (Fig. 6C-E), the distribution of small ectopic muscles is not random; most are present within a broad supra-orbital band extending from the distal ophthalmic lobe of the trigeminal ganglion towards the DO muscle, passing tangential to the middle and distal parts of the DR and trochlear nerve (Fig. 9C,D). Less frequently, a second band extends from the isthmus to the proximal first branchial arch. The organization of muscle cells within and around these supra-orbital clusters is highly variable, ranging from tightly aggregated to dispersed.

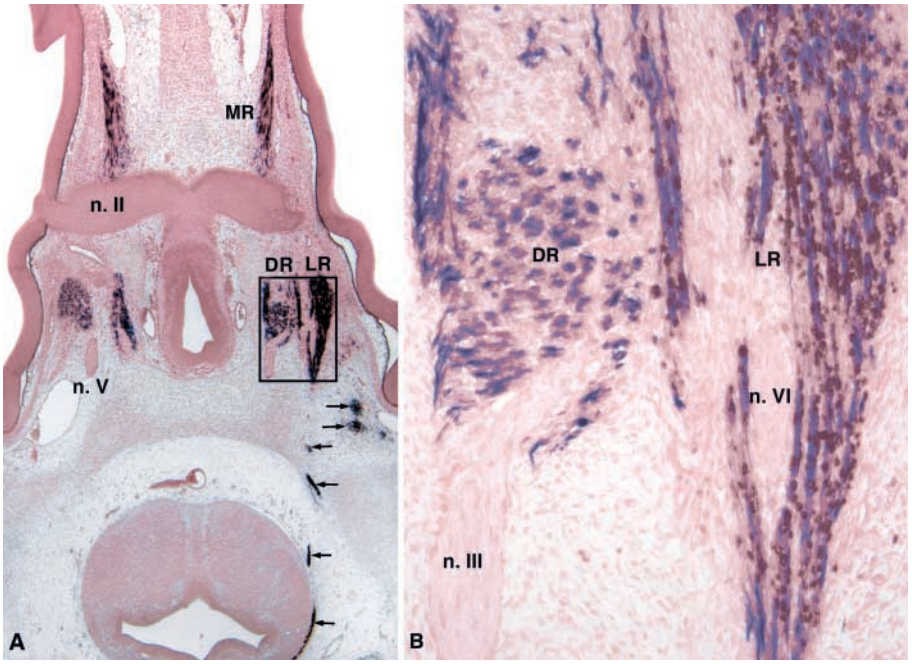

Fig. 8. Low (A) and high (B) magnification images of a stage 29 embryo, showing the ability of grafted medial somitic mesoderm to form the lateral rectus muscle. Note that grafted cells do not contribute to the adjacent dorsal rectus muscle. Purple, myosin heavy chain; brown, quail nuclear marker. Arrows indicate ectopic muscles.

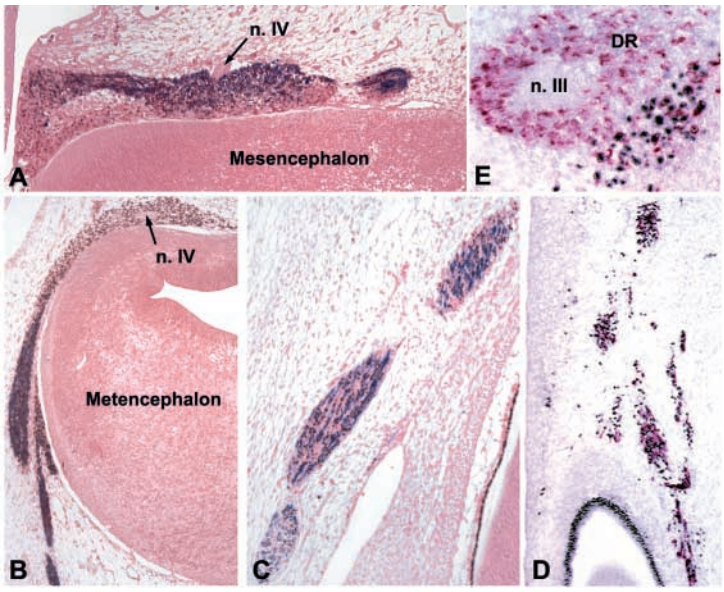

Fig. 9. Ectopic muscles formed by grafted trunk mesoderm. (A) A large mesenchymal aggregate dorsal to the isthmus is shown; note that myosin is synthesized primarily in abaxial cells (stage 29 embryo, segmental plate graft). This ectopic condensation has not disrupted the emergence of trochlear nerve fibers (n. IV) from the roof of the isthmus. (B) Multiple finger-like projections of ectopic muscle extend ventrally from the isthmus (stage 29 embryo, medial half somite graft). (C,D) Multiple small, ectopic muscle clusters are dispersed along a band deep to and paralleling the dorsal margin of the eye; the edge of the pigmented retina is visible. (E) An ectopic muscle adjacent to the dorsal rectus muscle; there is no mixing between them. Labels: MyHC is purple in A-C and red in D,E; quail nuclei are brown in A-C and black in D,E.

A large population of scattered mononucleated myocytes and short multinucleated myotubes was present in all trunk-into-head host embryos (Fig. 10). These cells exhibit typical immature myotube morphology and synthesize MyHC proteins, but do not express endothelial antigens or smooth muscle actin (data not shown). Nothing comparable to these cells was present in control embryos.

Transplanted trunk mesoderm cells often circumscribe one or both lobes of the trigeminal ganglion (Fig. 2G). This mimics the behavior of trunk paraxial mesoderm cells relative to spinal ganglia, but contrasts with the normal situation in the head, where neural crest-derived cells contribute to this epineural layer. Penetration of the ganglion by graft-derived invasive angioblasts occurs in all mesoderm transplant categories, including controls. An atypical behavior, however, is the presence of quail cells synthesizing MyHC within the trigeminal root, and occasionally within the ganglion and the adjacent brainstem (Fig. 11). This inculcation of MyHC-expressing cells into the trigeminal root is unique to segmental plate transplants, and is the only outcome restricted to one set of transplants.

Discussion

All trunk paraxial mesoderm grafts form normal and ectopic muscles

Three spatiotemporally distinct trunk tissues – segmental plate, medial half somite, and lateral half somite – rapidly generate substantial mesenchymal populations containing cells that activate skeletal muscle-specific genes and later form both normal and ectopic muscles in the embryonic head. Mechanisms underlying the rapid generation of large populations of mesenchymal cells by grafted somites or half-somites are not known. Some undoubtedly arise from somitocoel cells (Wong et al., 1993; Huang et al., 2000), and possibly from mesenchymal cells that underlie somites and

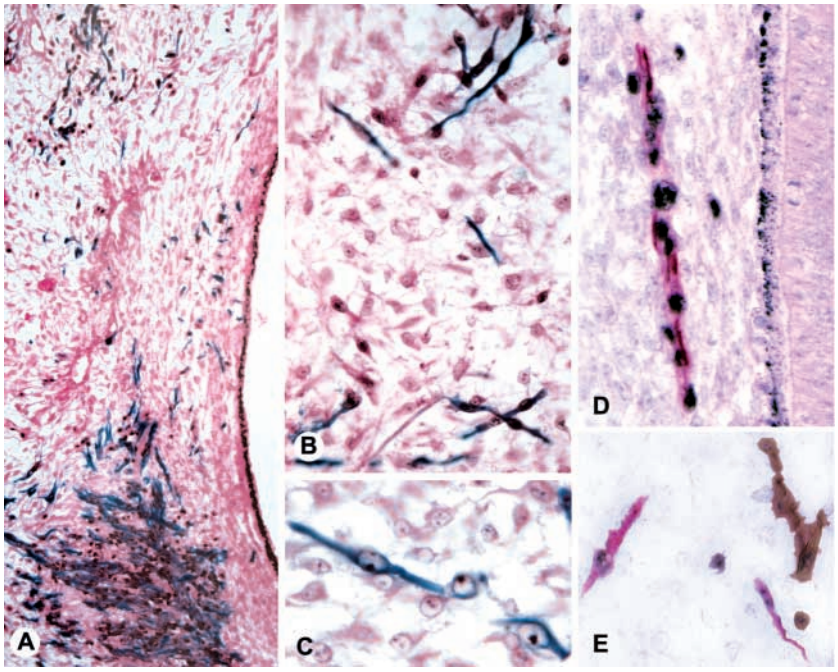

Fig. 10. Transplanted trunk mesoderm forms scattered myocytes and myotubes, as shown in sections from stage 28-29 embryos. (A) An ectopic aggregate (bottom of figure) containing non-aligned myotubes, identified by their shape and synthesis of MyHC (F59-positive, blue), with individual MyHC-positive cells dispersed deep to the dorsal quadrant of the eye. Some MyHC-positive cells are adjacent to the pigmented epithelium of the eye (right side), which normally is populated by connective tissues (e.g. sclera) formed by periocular neural crest cells. (B,C) Scattered myoytubes in the supra-orbital band; both cases shown are from segmental plate grafts. (D) A multinucleated myotube (MyHC, magenta) adjacent to the eye; the pigmented epithelium is visible on the right. (E) A triple-stained section from a lateral half somite recipient, showing graft-derived endothelial cells (QH1, brown), myocytes (MyHC, magenta) and quail nuclei (QCPN, black). Although invasive movements of grafted angioblasts have been reported, comparable behavior by myoblasts is novel. No double-labeled myoendothelial cells were found.

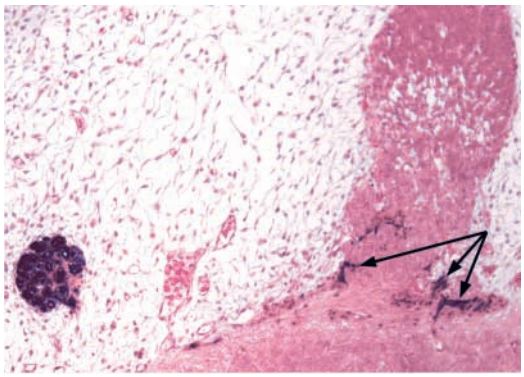

Fig. 11. MyHC-positive quail cells (arrows) are present in the root of the trigeminal ganglion. Epineural connective tissues and endothelial cells within the ganglion and brainstem were found in all transplant categories, but only segmental plate grafts generated myotubes within the ganglion. The other quail cells evident within the trigeminal root are endothelial cells. An ectopic muscle is visible to the left. A stage 29 embryo is shown.

segmental plate (Reiss and Noden, 1989). Exaggerated epithelial-mesenchymal transformation due to physical disruption of epithelial integrity is also likely.

The implantation site selected for this study includes the LR and proximal BA1 primordia, and is adjacent to the DO precursor. Transplanted trunk mesoderm cells that occupy these exact sites will aggregate and express muscle-specific transcription factors and MyHC on the normal head timetable. Moreover, they coordinate their differentiation and morphogenetic movements with surrounding host-derived cells to form normal extra-ocular and jaw muscles, thereby exhibiting a community effect (Buckingham, 2003). These results indicate that some members of the signal consortium necessary to direct head myogenesis are highly localized, and are able to initiate and sustain myogenesis in nearby paraxial mesoderm cells taken from any part of the body axis. The requirement for early spatial integration may explain why some previous trunk-into-head grafts failed to populate the LR, whose primordium is deep within head paraxial mesoderm ventral to rhombomere 2.

Why, then, do many grafted trunk mesoderm cells also initiate myogenesis at ectopic sites, where they express the transcription factors *paraxis*, *lbx1* and *myf5*, and synthesize myosin rapidly, mimicking their ancestral trunk timetable? Possibly because of a combination of the more widespread availability of some myogenesis-promoting signals (e.g. sonic hedgehog) and the reduced levels of inhibitors (e.g. Bmp4) (Pourquie et al., 1996). In addition, the transient exposure of some grafted cells to migrating neural crest cells, which produce myogenesis-modulating signals such as frzb1 (Ladher et al., 2000), noggin and gremlin (Tzahor et al., 2003), may potentiate their myogenic differentiation.

The presence of *myf5*- and MyHC-positive cells in somite-like epithelial condensations is not surprising, as populations within segmental plate acquire the ability to form epithelial tissues prior to the onset of somitogenesis (Zheng, 1993). By contrast, myogenesis in large mesenchymal aggregates adjacent to the isthmus, and within long, finger-like projections extending from them, is unexpected, because this is not normally a site of muscle formation. However, head mesoderm cells in this location will express a *lacZ*-reporter construct driven by one of the *myf5* enhancer sequences (Teboul et al., 2002), and will also transiently express *tbx1*, which is found later in some branchial arch muscles (D.N.N., unpublished) (Garg et al., 2001). Thus, this isthmic environment may be subthreshold for head mesoderm but sufficient to activate myogenic genes in trunk mesoderm cells.

Previous studies reported a more limited ability of transplanted segmental plate to contribute to normal head muscle development, either owing to a failure to populate muscle primordia (Hacker and Guthrie, 1998), or to an inability to recognize and respond to head myogenic stimuli (Mootoosamy and Dietrich, 2002). Both these studies limited their analyses to younger stages, so detection was based on the presence of fewer graft-derived cells. Hacker and Guthrie placed grafts beside rhombomere 4 (Hacker and Guthrie, 1998), the normal site of origin of second branchial arch (BA2) muscles. This presents a special challenge to migrating trunk myoblasts, many of which may be committed to the slow fiber type lineage (Noden et al., 1999). Indeed, we find that somites grafted into this area fail to participate in the formation of the large, fast fiber-dominant mandibular depressor muscle in BA2, but do contribute to smaller muscles (e.g. serpihyoid, stylohyoid), whose primary myotubes express the slow S3 MyHC isoform (R. Marcucio and D.M.N., unpublished). The possibility that the second arch environment may differ in the spatiotemporal distribution of other myogenic factors, e.g. sonic hedgehog (Bren-Mattison and Olwin, 2002) or noggin and gremlin, from migrating crest cells (Tzahor et al., 2003) cannot be excluded.

The identical behavior of medial and lateral half somites, including the expression of *lbx1* by both, suggests that this mediolateral polarity is lost following transplantation. Indeed, somite rotation experiments have shown that medial and lateral domains of newly-formed somites are labile (Aoyama and Asamoto, 1988; Dockter and Ordahl, 2000). It is possible that grafted half somites undergo regulation, restoring the entire mediolateral complement of myogenic lineages (Gamel et al., 1995). However, this alone would not account for the widespread expression of *lbx1*. Because both the LR and DO normally express *lbx1* and originate on either side of the midbrain-hindbrain boundary, it is possible that signals emanating from this center may participate in *lbx1* activation. Assaying for markers of trunk migratory myoblasts (e.g. *pax3*, *six1*, *c-met*), and for known activators of *lbx1* (e.g. Fgf4) (Alvares et al., 2003), might help resolve this uncertainty.

The differentiation of other lineages

All trunk grafts give rise to multiple cell types. Angioblasts contribute to the formation of all types of peripheral vessels, and to meningeal and brain vessels. Half-somite transplants consistently generate larger numbers of angioblasts than segmental plate grafts, and all generate more than orthotopic mesoderm grafts. This correlates with differences in the density of these mesenchymal populations at the time of transplantation (Feinberg and Noden, 1991). Previous studies have shown that somites contain angioblasts (Ambler et al., 2001; Noden, 1989; Noden, 1990; Wilting et al., 1995) and will generate a greater number of migratory angioblasts when grafted into the head than they normally do in situ (Spence and

Poole, 1994). This exaggerated angiogenic competence may be correlated to the large number of mesenchymal cells generated by somites in the head environment. How this epithelial-to-mesenchymal transformation is linked to the induction of angioblasts is not known.

Results from in vitro analyses of avian segmental plate and epiblast cells have led to the suggestion that myogenic and angiogenic differentiation may be non-specific default pathways (George-Weinstein et al., 1996; Grim et al., 1994). Although the results of our segmental plate transplants are consistent with this hypothesis, the diversity of lineages that form and the heterogeneity, especially among myogenic populations, favors an active role for local myogenic signals.

Muscle compartmentalization

The time at which each individual muscle becomes a distinct and separate entity is unclear, especially in the limb and head regions where many myogenic mesenchymal progenitors are contiguous. These transplantation results indicate that extra-ocular muscles become closed compartments, analogous to embryonic cartilage, soon after the initial aggregation stage. Muscle growth and differentiation continue without recruitment of additional myogenic cells from surrounding mesenchyme.

Axons, and later, angioblasts and connective tissues, penetrate each muscle condensation (McClearn and Noden, 1988; Ruberte et al., 2003). Also, progenitors of satellite cells or other stem cell-like populations might subsequently become incorporated in these embryonic muscles (Asakura et al., 2002). Although closed to myogenic cell recruitment, embryonic eye muscles do interact with adjacent structures. This is evidenced by the ability of a denervated/hypoinnervated LR primordium to attract inappropriate oculomotor axons from the nearby ciliary ganglion (Wahl and Noden, 2001), as also occurs in Duane syndrome (Gutowski, 2000).

Muscle differentiation uncoupled from morphogenesis

A diffuse population of quail mesenchymal cells expressing *paraxis* and *myf5*, but not *lbx1*, was present surrounding the graft site 1-2 days after transplantation. However, in contrast to myocytes within ectopic condensations, these scattered cells did not synthesize MyHC before stage 24, which corresponds closely with the normal time for appendicular, as well as head muscle, differentiation. A few scattered myocytes are occasionally present in normal embryos close to the trailing edge of muscle primordia, such as the DO, en route to their sites of terminal differentiation, or at sites where individual muscles separate from a common precursor aggregate (e.g. MR from VR). Scattered myocytes are also normally present around subcutaneous muscles such as the cranial cucullaris, which originates from occipital somites, but not around deeper head and neck muscles.

Unlike angioblasts, these graft-derived muscle cells do not move omnidirectionally, but are largely restricted to a supra-orbital band that is coincident with the normal DO morphogenetic pathway. These dispersed myocytes and myotubes do not show any preferential orientation, as is normally a hallmark of head (McClearn and Noden, 1988) and appendicular (Lance-Jones, 1979; Kardon, 1998) immature myotubes. Whether this reflects their non-responsiveness to extrinsic signals or rather is due to the absence of muscle-aligning cues (Kardon et al., 2004) in this part of the periocular mesenchyme is not known.

Normally, myoblast aggregation and fusion, which involve integrin and cadherin-mediated interactions (Mulieri et al., 2002; Kang et al., 2003), are prerequisites to sustained differentiation of embryonic muscle cells. However, among some grafted trunk cells, the ability to differentiate is not dependent upon maintenance of close cell-to-cell contacts; this population does not respect the requirement for a community effect (Buckingham, 2003; Standley et al., 2002). These single cells may be analogous to *lbx1*-positive myoblasts that normally leave the lateral myotome and move into appendicular tissues; assaying for c-met would clarify their identity. The c-met ligand, HGF, is present in branchial arch 1 and is associated with several extra-ocular muscles, including the DO (Caton et al., 2000) (D.M.N., unpublished), but whether it plays a role in myogenesis as well as being chemoattractive for efferent axons is not known.

The presence of segmental plate-derived myocytes within the trigeminal ganglion and root, where no known myogenic signals are present, was unexpected and suggests that these cells may be a unique population of either already committed or default pathway progenitors. The default pathway hypothesis suggests that trunk mesoderm cells, some of which transiently express *myf5* but do not normally commit to the myogenic lineage in situ, maintain this expression in the head environment and progress to myotube differentiation. This is consistent with our suggestion that some members of the myogenic signal consortium are present throughout much of the cephalic paraxial region. Recent studies (Kardon et al., 2002; Tamaki et al., 2002) have defined a unique lineage of bipotential endothelial-myogenic precursors that move into the limb. Possibly some of the scattered myocytes/myotubes, particularly those co-localized with angioblasts in the trigeminal root, represent cells that initially emigrated as myoangioblasts then diverged to the muscle lineage, albeit in inappropriate locations. Only by performing clonal analyses on transplanted tissue could the possibility of bi- (or multi-) potential lineages be confirmed. The signals directing the divergence of these bipotential cells are not known.

Muscle morphogenesis: active migration versus passive displacement

Embedded in unsegmented cephalic paraxial mesoderm are restricted sites where progenitors of extra-ocular and branchial muscle primordia arise, and graft-derived myogenic cells placed outside of these sites do not participate in head myogenesis. Surrounding these sites are progenitors of angioblasts, and a variety of hard and soft connective tissues, each of which exhibits a distinct set of morphogenetic movements. Grafted trunk mesoderm can mimic angiogenic, but not chondrogenic/osteogenic, morphogenetic events in the head (Noden, 1986), similar to the results reported for grafts of somites to different trunk levels (Alvares et al., 2003). The distribution of graft-derived myotubes in normal (dorsal oblique) and ectopic clusters around the dorsal margin of the eye provides clues as to how head muscle morphogenesis is orchestrated.

It has been proposed that for each extra-ocular muscle there is a portal at the neural crest-mesoderm interface that defines

subsequent routes of movement available to aggregated myoblasts (Noden, 1991b). A muscle primordium situated beside such a portal would migrate as a unit from its site of origin towards and then across the interface, whereas nearby non-myogenic mesodermal cells are excluded. The flaw in this model is that it implies an active migration of large aggregates of differentiating myotubes and myoblasts, a process for which there is no precedent. Indeed, many events classically described as involving embryonic cell migration are not accomplished by individual cell motility, but rather by continually elongating and remodeling cords of cells in which only the leading edge cells execute displacement behavior. This is equally true for neural crest cells (Conner et al., 2003; Kulesa and Fraser, 2000; Tosney et al., 1994) and some appendicular myoblasts (Jacob et al., 1979), and for some but not all angioblasts (Noden, 1990).

A second model, proposed by Mootoosamy and Dietrich, is that some head muscle primordia remain stationary while the neural crest population expands and surrounds them, pushing non-myogenic mesenchyme aside (Mootoosamy and Dietrich, 2002). However, mapping studies have shown that extra-ocular and branchial muscle progenitors do indeed change their positions relative to fixed objects such as the brain, cranial ganglia and the eye (Noden, 1983a; Wahl et al., 1994), and that, certainly for the LR and DO, crest cells do not encroach upon the sites where myogenesis is initiated.

Recent analyses of the interface between paraxial and lateral mesoderm populations at limb levels (Nowicki et al., 2003) offer an alternative model that applies well to the head. We propose that the neural crest:mesoderm interface is in fact not crossed by aggregated muscle primordia or by crest-derived connective tissues. Instead, the interface is a deformable plane. Localized sites of differential growth or changes in adhesive properties within populations on either side of the interface cause the formation of finger-like projections that appear to penetrate the interface but in fact only deform it. This model closely resembles ‘convergent extension’ (Wallingford et al., 2002), the process by which cell reorganization within embryonic epithelial sheets occurs. Structures such as the DO do not move across the interface, but rather are embedded within tips of expanding mesodermal projections. Later, crest cells encircle these mesodermal projections, isolating extra-ocular or branchial muscles from the more proximal parts of the mesodermal projections.

The supra-orbital mesodermal projection develops in our chimeric embryos, but is atypically populated by graft-derived myoblasts that remain dispersed along its length. In most cases, crest cells circumscribe the distal part of this projection, thereby isolating the DO. However, they are unable to displace the ectopic population of trunk-derived myocytes and connective tissues. A similar disruption of normal neural crest movements occurs when grafted trunk mesoderm populates the maxillary prominence (Noden, 1986).

The molecular signals and extracellular matrix components guiding the deformation of this interface are not known. Indirect evidence suggests that eph-ephrin mediated interactions (De Bellard et al., 2002; Santiago and Erickson, 2002), twist activity (Soo et al., 2002) and HGF expression (Caton et al., 2000) (D.M.N., unpublished) may be involved. Also, although we use the term growth, this process may involve physicochemical interactions (Newman and Comper, 1990) instead of, or in addition to, proliferative pressures. With the availability of animals in which the neural crest side of the interface can be labeled genetically, e.g. *wnt1-cre* mice (Chai et al., 2000; Jiang et al., 2002), by the transplantation of labeled cells (e.g. quail-chick chimeras), or by applied labels, e.g., DiI in zebrafish (Whitlock et al., 2003), it should be possible to define the molecular basis for maintaining and deforming the mesoderm:neural crest interface. Beyond immediate interest to myogenesis, slight changes in the location of this interface are likely to underlie evolutionary changes in vertebrate craniofacial musculoskeletal structure (Helms and Schneider, 2003).

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