Patterning of fast and slow fibers within embryonic muscles is established independently of signals from the surrounding mesenchyme

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SUMMARY

During embryonic development, and before functional innervation, a highly stereotypic pattern of slow- and fast-contracting primary muscle fibers is established within individual muscles of the limbs, from distinct populations of myoblasts. A difference between the fiber-type pattern found within chicken and quail pectoral muscles was exploited to investigate the contributions of somite-derived myogenic precursors and lateral plate-derived mesenchymal stroma to the establishment of muscle fiber-type patterns. Chimeric chicken/quail embryos were constructed by reciprocal transplantation of somites or lateral plate mesoderm at stages prior to muscle formation. Muscle fibers derived from quail myogenic precursors that had migrated into chicken stroma showed a quail pattern of mixed fast- and slow-contracting muscle fibers. Conversely, chicken myogenic precursors that had migrated into quail stroma showed a chicken pattern of nearly exclusive fast muscle fiber formation. These results demonstrate in vivo an intrinsic commitment to fiber-type on the part of the myoblast, independent of extrinsic signals it receives from the mesenchymal stroma in which it differentiates.

Key words: Skeletal muscle, Chicken/quail chimeras, Muscle fiber-type, Hypaxial musculature, Limb development

INTRODUCTION

Skeletal muscle of the tongue, trunk and limbs (hypaxial muscle), including the pectoral muscles, is exclusively derived from myogenic precursors originating within the somites (Chevallier et al., 1977; Christ et al., 1977). These precursors of the hypaxial musculature originate within the ventrolateral edge of the somitic dermomyotome (Ordahl and Le Douarin, 1992) and subsequently migrate to peripheral sites where they ultimately form muscle fibers within a mesenchymal stroma derived from the lateral plate mesoderm (Jacob et al., 1978; Brand-Saberi and Christ, 1992). Once within the limbs, myogenic cell precursors, the myoblasts, undergo a proliferative expansion and begin the process of cell differentiation. At least two distinct myogenic precursor populations, embryonic and fetal myoblasts, migrate sequentially into the wing anlagen, giving rise to primary (embryonic) and secondary (fetal) muscle fibers, respectively, with distinct morphological and biochemical properties (Seed and Haushchka, 1984; Stockdale, 1992). Both primary and secondary muscle fibers can be broadly classified into two types: fast contracting and slow contracting, with the rate of contraction particularly dependent on the specific isoforms of the myosin heavy chain (MyHC) family each accumulates (Bárány, 1967; Reiser et al., 1988). A clonal analysis of myogenic precursors present in embryonic avian limbs identified distinct populations of myoblasts committed to form either slow MyHC- or fast MyHC-expressing primary fibers upon differentiation in vitro and in vivo (Miller and Stockdale, 1986; DiMario et al., 1993). These results suggest that, at least initially, slow and fast muscle fibers form within the limbs via a cell intrinsic mechanism (for review see Stockdale, 1997). Subsequently, the contractile speed and isoform content of muscle fibers can be modified by innervation, activity and hormonal influences (for review see Pette and Staron, 2000).

Individual muscles within an organism display highly stereotypical patterns of fast and slow muscle fiber distribution, established during the embryonic stage of development before and independent of innervation (Rubinstein and Kelly, 1981; Crow and Stockdale, 1986; Phillips et al., 1986; Condon et al., 1990), and largely maintained during subsequent growth. The reproducible pattern of fast and slow fibers, characteristic of each anatomic muscle within a species, has led to the view that muscle fiber-type is determined by extrinsic signals originating within the surrounding stroma (for review see Blagden and Hughes, 1999). This view derives in part from observations that muscle cell precursors within a somite of a specific axial level are not committed to migrate to specific regions of the limb, and thus are not committed to form specific anatomical muscles with their attendant slow and fast fiber-type pattern (Lance-Jones, 1988).

Heterochronic transplantation suggested that myoblast diversity could arise within the somite, prior to migration into the limb (Van Swearingen and Lance-Jones, 1995). In a series
of somite transplantation and fiber analysis experiments, these investigators demonstrated that slow muscle fibers derive from myogenic precursors that migrate from the somite earlier than those that form fast muscle fibers. This observation implies that fiber-type patterning signals present in the limb act not on naive myoblasts, but rather on fiber-type-committed myoblasts and/or their progeny.

To investigate the influence of the surrounding stroma on the specification of embryonic fiber-type as muscle cell precursors differentiate in situ, we have exploited a difference between the chicken and the quail in the pattern of slow and fast muscle fibers they form in the embryonic pectoralis muscle, prior to functional innervation. Whereas in the chicken the pectoral muscle is composed of nearly all fast-contracting primary fibers, at the same stage of development, in the quail pectoralis there is a nearly even mix of slow- and fast-contracting fibers. Chicken/quail chimeric embryos were constructed so that myoblasts of one species formed muscles in the stroma from the other. Results show that the fiber pattern that forms is dependent upon the origin of the myoblasts rather than the stroma, revealing a heretofore unidentified role for intrinsic differences between myogenic precursors in establishing fiber-type pattern within individual anatomic muscles.

MATERIALS AND METHODS

Embryo surgeries

Quail and chicken eggs were incubated at 37°C for approximately 2 days in a humidified chamber, at which time host and donor embryos were staged according to the number of somites that had formed. Donor embryos were removed from the egg and unspecified, epithelial somites (somites I-III according to the nomenclature of Christ and Ordahl, 1995), or the somatopleure of the lateral plate mesoderm adjacent to somites 16-23 (numbered from rostral to caudal), were removed using tungsten needles and transferred to a holding solution of 2% fetal calf serum in Tyrode’s solution (Sigma). Surgeries on host chicken and quail embryos were performed in ovo at 2 days of embryonic development (ED2). Holes were made in the shells of the egg, and embryos that had formed 16-23 somite pairs (stages 12-14 according to Hamburger and Hamilton, 1951) were used as transplant recipients.

Two types of transplantation were performed – transplantation of somites and transplantation of lateral plate mesoderm. For somite transplantation, between one and three epithelial somites were removed by aspiration from host embryos, and an equal number of stage-matched epithelial somites from donor embryos were transplanted into the space left following removal of the host somites. Previous work has demonstrated that somites located in the position of the transplants contribute myoblasts to pectoral muscles (Christ et al., 1974; Beresford, 1983). For reciprocal lateral plate mesoderm transplants, the somatopleure adjacent to somites 16-23 was removed, leaving the underlying splanchnopleure intact. All surgeries were performed on the host’s right side, with the consequence that normal pectoral muscle on the animal’s left side served as a control for the chimeric pectoral tissue that formed on the animal’s right side. Following surgery, the eggshell was sealed, eggs were returned to the incubator, and chicken/quail chimeric embryos were allowed to develop in ovo for an additional 8 days.

When chimeric or unoperated embryos had reached ED10, the entire pectoral muscle and intervening sternum were removed en bloc and embedded in a glycerol based tissue freezing medium (OCT) in such a way that subsequent cryostat sections passed through both right and left halves of the pectoral muscle. Frontal sections, from superficial to deep, were collected on gelatin-coated slides and immediately fixed in 70% ethanol. Left and right pectoral muscles from chimeric ED10 quail and chicks were embedded, sectioned and stained as a single unit to minimize potential differences arising from variations in the subsequent staining protocol.

Immunohistology

Fast and slow muscle fiber-types were identified in sections of pectoral muscle using previously characterized monoclonal antibodies F59 and S58, respectively (Crow and Stockdale, 1986). All muscle fibers express at least one isoform of fast MyHC which is recognized by mAb F59, allowing muscle fibers (staining blue) to be distinguished from non-muscle, stromal cells. Slow muscle fibers additionally express at least one slow MyHC isoform (staining green), which is recognized by mAb S58 and permits slow fibers (staining both green and blue) to be distinguished from fast fibers (staining only blue).

Lineage tracing in the avian system, pioneered by Le Douarin (Le Douarin, 1973), was modified by the use of a quail-specific monoclonal antibody, mAb QCPN, obtained from the Developmental Studies Hybridoma Bank. This antibody distinguishes both myogenic and non-myogenic quail cells from chicken cells based on the expression of a quail-specific nuclear antigen. Thus, quail cells were recognizable by a characteristic red nuclear fluorescence following immunostaining.

Following a blocking step with 2% horse serum and 2% bovine serum albumin in PBS, primary antibodies, S58 and QCPN (diluted 1:10 in blocking solution), were reacted with tissue sections in a humidified container. Secondary antibodies, FITC-conjugated goat α-mouse IgG (Zymed) and Texas Red-conjugated goat α-mouse IgG (gamma chain specific; Jackson ImmunoResearch), each diluted 1:100, were applied to sections for 1 hour to visualize S58 and QCPN epitopes, respectively. Subsequently, sections were reacted with biotinylated F59 (1:250) for one hour, followed by application of AMCA-conjugated avidin (1:100; Vector Labs) to visualize F59.

Microscopic fields representative of chimeric and contralateral control regions of ED10 pectoralis muscle were photographed under epifluorescent illumination. Each field was photographed three times using filter combinations that distinguish between the various fluorescently identified epitopes. Using Photoshop 4.0 software, composite photographs were generated to show the position of the quail QCPN marker relative to either the fast (F59) or slow (S58) MyHC marker.

Because there is a stereotypical distribution of slow and fast fibers in the unoperated pectoral muscle, care was taken to examine all areas of the breast muscle. Additionally, the regions depicted in photographs of control breast muscle from chimeric embryos were carefully selected to lie at the equivalent contralateral position (medial to the sternum, posterior to the clavicle, and deep to the surface) to regions depicted in photographs of chimeric breast muscles.

Analysis of data

From photographs of cross sections through control and transgenic muscle, counts of fast and slow embryonic muscle fibers were made. In chimeric muscles, fibers were counted in regions to which transplanted somites had made a contribution, or in the stroma provided by transplanted quail lateral plate. Because both chicken and quail show regional differences in the distribution of slow fibers within the pectoral muscle, and the region of transplant contribution varied in extent and position within the breast among individuals, the number of fibers that could be counted and the percentage of slow fibers varied between embryos. Three or more cross sections were counted in each embryo. To avoid counting the same fiber as it transversed multiple sections, counts were made from sections separated by at least 100 μm. The average number of slow fibers was calculated as a percentage of the total number of fibers counted for each chimeric and control breast muscle. These averages per individual embryo, in turn, were used to calculate the mean of slow
fibers resulting from each experimental transplantation paradigm as well as the standard error of the mean (s.e.m.) as displayed in Table 1.

RESULTS

The distribution of fast and slow muscle fiber-types within the chicken and quail pectoral muscles are quite different. At 10 days of development in ovo (ED10), nearly all the muscle fibers within quail and chicken pectoralis muscles have an embryonic fiber phenotype. In the quail pectoralis, there is a stereotypical distribution of slow fibers interspersed among fast muscle fibers, with more than half of the fibers throughout the muscle immunostaining with the slow MyHC-specific monoclonal antibody S58 (Fig. 1A,B and Table 1), with the exception that slow fibers were excluded from the lateral margins of the muscle. In contrast, nearly all embryonic muscle fibers in ED10 chicken pectoralis muscles express only fast-contracting MyHC isoforms. All chicken muscle fibers immunostained with the fast MyHC-specific monoclonal antibody F59 (Fig. 1C), while very few muscle fibers reacted with the slow MyHC-specific monoclonal antibody S58 (Fig. 1D; Table 1). Some slow fibers do occur adjacent to the clavicle in the ‘red stripe’ area (Matsuda et al., 1983), and adjacent to the sternum at the most rostral part of the chicken pectoral muscle. Otherwise, nearly all the fibers of the chicken pectoral muscle are of the fast type. At this stage of development, all fibers that express slow MyHC isoforms also express at least one fast MyHC isoform, whereas fast fibers exclusively express fast isoforms. In the context of this report, fibers expressing S58-reactive slow MyHC isoforms are designated as slow, or slow MyHC-expressing, fibers.

Using the QCNP quail-specific monoclonal antibody developed by Drs Bruce and Jean Carlson, the fate of pectoral muscle precursors was followed in chicken/quail chimeric embryos. Transplantation of somites or lateral plate mesoderm allowed us to separate the roles of the non-muscle mesenchymal stroma from that of the myoblasts themselves in establishing muscle fiber-type pattern. In the first set of experiments, between one and three epithelial somites (somites I-III according to the nomenclature of Christ and Ordahl, 1995) were removed in ovo from the right side of ED2 chicken embryos that had formed 16-23 somite pairs. An equal number of stage-matched epithelial somites was removed from donor quail embryos and transplanted into the right side of the donor quail embryos and transplanted into the right side of the embryos.
chicken host. Previous work has demonstrated that somites located in the position of the transplants contribute myoblasts to pectoral muscles (Christ et al., 1974; Beresford, 1983). When quail-into-chicken chimeric embryos had reached ED10, the right and left pectoral muscle and intervening sternum were removed en bloc and cryosectioned. Sections were triple immunostained with F59 and S58 to identify fast and slow muscle fibers, and with QCPN, a monoclonal antibody that specifically binds to a quail perinuclear epitope.

As expected, QCPN-positive quail cells within the pectoralis muscles were confined to the right half of quail-into-chicken chimeras (compare Fig. 2A with C). Many of the QCPN-positive nuclei were within differentiated muscle fibers (Fig. 2A). Within the region of quail-derived muscle, approximately 50% of the muscle fibers were of the slow type as demonstrated by the coincidence of QCPN and S58 immunostaining (Fig. 2B). At the same position on the contralateral control side of the pectoral muscle, there is no quail contribution to either muscle or stromal cells, and as expected, no slow fibers are found within this region. Scale bar is 100 μm.

To determine the effect of the quail pectoral stroma on chicken host. Previous work has demonstrated that somites located in the position of the transplants contribute myoblasts to pectoral muscles (Christ et al., 1974; Beresford, 1983). When quail-into-chicken chimeric embryos had reached ED10, the right and left pectoral muscle and intervening sternum were removed en bloc and cryosectioned. Sections were triple immunostained with F59 and S58 to identify fast and slow muscle fibers, and with QCPN, a monoclonal antibody that specifically binds to a quail perinuclear epitope.

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positive nuclei were observed in such close proximity to chicken muscle fibers that it was not possible to determine whether or not they were within chicken muscle fibers. What is clear is that if a limited number of quail nuclei are located within a multinucleated chicken muscle fiber, their influence is insufficient to establish a slow phenotype.

Of 25 embryos that survived somite transplantation and developed to ED10, 11 showed a unilateral donor contribution on the right side. In each case, donor fiber-type correlated with that normally found in the pectoralis of the donor species. The remaining embryos exhibited morphological defects in the body wall, explicable as a failure of the transplanted somite to heal into the host. Surgical manipulation of somites per se did not affect subsequent fiber-type formation, because morphologically normal ED10 embryos (4 cases) were obtained when chicken somites were transplanted back into a chicken host. In each case, as in the normal chicken breast, slow fibers were rarely found in the pectoralis of the transplanted side (data not shown).

To further demonstrate that the stroma does not by itself determine muscle fiber-type, chicken somatopleure – the dorsal portion of the lateral plate mesoderm into which muscle precursors migrate – adjacent to somites that contribute to the pectoral muscles, was removed and replaced with the equivalent quail tissue. At the developmental stages at which the transplants were made, muscle precursors have not yet entered the somatopleure (Christ et al., 1977), and muscle fibers forming in chimeric embryos are from the host, while the stroma is of donor origin. Sections of breast muscle from chimeric embryos that had continued their development in ovo to ED10 were examined immunohistologically for fiber-type and species of origin. Again, chimeric embryos were easily identified by the expression of QCPN in cells of the right breast. The muscle fibers that formed in the chimeric right pectoral muscle were almost exclusively of the fast fiber-type, expressing the F59 epitope, but not the S58 epitope (Fig. 4A,B and Table 1). Close inspection showed that none of the QCPN-positive stained nuclei was associated with differentiated muscle fibers. In the contralateral control side of the pectoralis, the nearly pure fast fiber-type typical of the chicken was observed (Fig. 4C,D). As expected, the stroma on the control side of the embryo arose from chicken lateral plate, as evidenced by the complete absence of QCPN staining.

**DISCUSSION**

The cells that form the hypaxial musculature originate within the somites and migrate into a mesenchymal stroma, derived from the lateral plate, in which the myogenic cell population expands and begins to differentiate. Individual muscles form...
committed myoblast populations are expanded. In the chicken, while in the quail, both fast- and slow-fiber-myoblasts committed to form fast fibers are preferentially amplified population. In the case of the pectoralis muscle shown here, muscles is through expansion of the appropriate myoblast to establish the size and fiber-type pattern of individual anatomic muscles is through expansion of the appropriate myoblast population. In the case of the pectoralis muscle shown here, myoblasts committed to form fast fibers are preferentially amplified in the chicken, while in the quail, both fast- and slow-fiber-committed myoblast populations are expanded.

in specific locations, and the fiber-type composition and pattern within each is established as the first (embryonic) muscle fibers form. Experiments in which somites from non-brachial levels were shown to give rise to anatomically appropriate muscles when transplanted to brachial levels (Christ et al., 1978; Butler et al., 1982) demonstrated the plasticity of somitic muscle precursors and suggested that positional cues within the limbs control which anatomic muscle forms.

Work in this and other laboratories has demonstrated that myoblasts isolated from embryonic limb bud have an intrinsic predisposition to form either fast or slow fibers when allowed to differentiate in culture or in the embryo (Miller and Stockdale, 1986; DiMario and Stockdale, 1995; Cossu and Molinari, 1987; Pin and Merrifield, 1997), demonstrating that fiber-type committed myoblasts are present in the limb bud prior to the appearance of differentiated muscle fibers. Van Swearingen and Lance-Jones (Van Swearingen and Lance-Jones, 1995) elegantly showed that myoblasts migrating early from the somite preferentially contribute to slow muscle fibers while myoblasts leaving the somite later are more likely to be found within fast muscle fibers. Their results suggest that either distinct populations of slow and fast fiber-forming myoblasts arise within the somites and home to appropriate locations within the extremities, or that different instructive signals within the stroma are received by early and late arriving myoblasts to specify fast and slow muscle fiber phenotypes.

To distinguish between these alternatives, we have taken advantage of a species difference in the pattern of fast and slow embryonic muscle fibers that form in the pectoral muscles of the chicken and the quail. While the chicken pectoralis is composed nearly exclusively of fast fibers, the quail counterpart is a mixed fast and slow fiber muscle (Fig. 1). When chicken myogenic precursors migrated into a stroma composed of quail cells, nearly all of the primary muscle fibers that formed were of the fast fiber-type, recapitulating the chicken phenotype (Figs 3, 4). Conversely, when quail myogenic precursors migrated into a stroma composed of chicken cells, the quail phenotype of a mixed population of half fast and half slow muscle fibers formed (Fig. 2). Thus, our work presents evidence that embryonic myoblasts derived from somites are intrinsically programmed to form slow or fast fibers regardless of signals originating in the stroma.

The hypothesis that fiber-type patterning is determined by the myoblasts is supported by experiments in which avian embryonic myoblasts formed fast or slow fibers independent of the fiber-type composition of muscle in which they came to lie following injection into avian limb buds (DiMario et al., 1993). In contrast, when myoblasts from fetal mice were cultured and then transplanted into embryonic chicken limb buds, at least some introduced myogenic cells appeared to adopt a fiber-type fate as a result of local signals (Robson and Hughes, 1999). Thus, fetal myoblasts differ from embryonic myoblasts in that they are not committed to particular fiber types before transplantation between classes of animals. This confirmed earlier observations on myoblasts isolated from fetal stages that showed that the fiber-type formed by later differentiating avian myoblasts can be altered by environmental factors (DiMario and Stockdale, 1997).

While innervation is clearly one mechanism modulating fiber-type in fetal muscle, numerous studies have found no evidence that the initial formation of individual muscles, or the pattern of slow and fast fibers within them is affected by innervation (Laing and Lamb, 1983; Phillips and Bennett, 1984; Vogel and Landmesser, 1987; Crow and Stockdale, 1986; Condon et al., 1990). Furthermore, experiments in which embryonic chicken hind limb muscles were innervated by inappropriate motor neurons suggest that myofibers initially differentiate into specific fiber-types according to an intrinsic program based on their position within the limb (Laing and Lamb, 1983; Vogel and Landmesser, 1987).

Sonic hedgehog (Shh) has been implicated in myogenesis in the somite (Münsterberg et al., 1995) and in muscle patterning in the limb (Duprez et al., 1999). Previously we have shown that addition of exogenous Shh to somites in explant cultures, at the embryonic stages used in the experiments reported here, leads to a marked expansion of the slow fiber population (Cann et al., 1999), consistent with a role for Shh in commitment to fiber-type within the avian somite. While it is not clear that Shh plays an instructive role in establishing fiber-type, in zebrafish an instructive role in formation of slow muscle fibers in myotome-derived muscle has been suggested for signaling molecules of the hedgehog family (Blagden et al., 1997; Currie and Ingham, 1996; Du et al., 1997). The demonstration that Sonic hedgehog and Indian hedgehog are expressed in the limb (Riddle et al., 1993; Blagden and Hughes, 1999) provides a basis for either having a role in selectively increasing and/or maintaining slow fiber-type precursors by increasing cell survival and proliferation (Teillet et al., 1998; Cann et al., 1999; Krüger et al., 2001).

Combining previous reports with results presented here suggests a hypothesis of primary fiber-type patterning in hypaxial musculature, such as the pectoralis (Fig. 5). It is well
established in both birds and mammals that cells that form the hypaxial muscles form from a population that migrates from the dermomyotome of adjacent somites (Christ et al., 1974; Christ et al., 1977; Chevallier et al., 1977; Ordahl and Le Douarin, 1992). The work of Van Swaeringen and Lance-Jones (Van Swaeringen and Lance-Jones, 1995) showed that slow and fast muscle fibers form in the limb bud from myogenic precursors that migrate from the somites at early and late times, respectively. While early and late arriving myoblasts to the limb could be specified to a unique fiber-type by signals in the limb, the somite transplantation experiments presented here gives support to the hypothesis that commitment to fiber-type occurs within the somite before migration into the limb bud. This hypothesis requires amplification of migratory myogenic precursors because only small numbers of cells migrate and there is no proliferation of nuclei once myogenic differentiation begins, thus there must be amplification of myoblasts in the limb bud. Selective amplification of fast- or slow-fiber-committed myoblasts, in response to proliferative signals that originate in the limb stroma, is one mechanism by which the size, position and fiber-type pattern of individual anatomic muscles could be established. The results presented here suggest that any extrinsic signal originating in the stroma or overlying ectoderm acts on fiber-type-committed cells. While one might propose that among species of birds migratory muscle precursors differ in their response to stroma signals and thus there is no commitment imposed in the somite, the hypothesis in Fig. 5 is more parsimonious in its treatment of the data presented here and from other laboratories.

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