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Mechanisms of Formation of Muscle Fiber Types

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Introduction

The formation of skeletal muscles as anatomic units is one of the earliest events in vertebrate development. While little is known about how muscles form, understanding of some the processes that lead to myogenesis, such as the early events in the formation of myogenic cells, has greatly increased in recent years (1-5). Many of the discoveries about myogenesis have focused on the formation of myogenic cells *per se*, with much less emphasis on the formation of myogenic cells of different functional types. For example, in a cross-section of the sartorius from a young or old bird there are fibers of at least three types: those that express just fast, those that express just slow, and those that express both fast and slow isoforms of myosin (6). Furthermore there is a pattern to the distribution of fibers and this pattern of fiber distribution is common to all birds within the species.

Individual fibers within a muscle can differ from one another in the members of the myosin heavy chain gene (MyHC gene) family that each expresses. These differences in genes expressed are reflected in the function, or rate of contraction of the fiber based on the enzymatic properties of the proteins they encode (7). The basis for how each of the adjacent fibers within a muscle initiates the expression of different members of this family is currently not known, nor is it known how the patterning, or precise distribution, of each fiber type within the sartorius or any other muscle is controlled. However, two hypothesis have emerged to explain these phenomena. One hypothesis suggests that the differences in fiber type and function emerge early in the commitment of mesenchymal cells to their myogenic fate, prior to the time a cell becomes a precursor to the muscle fiber, a myoblast. In the myoblast a state is established that influences which members of the myosin heavy chain gene family will be expressed when the myoblast differentiates into a muscle fiber, or myocyte. The other hypothesis suggests that mesenchymal cells become committed to a myogenic fate that is generic — they are restricted to a myogenic fate, but when they manifest that fate they have no predisposition to differentiate into a fast, fast and slow, or slow muscle fiber. In this hypothesis, extrinsic factors are the important determinates in commitment to different fiber types. While frequently proposed as competing hypotheses it is likely that each best explains one or more observations about myogenesis depending on the stage of development. Both hypotheses must be invoked to explain the early events of myogenesis in the vertebrate.

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The early origins of muscle fibers

In discussing the formation of muscles it is important to establish the developmental time frame of the organism in which a study is conducted. From studies on myogenesis in the limb it is widely accepted that the origins of all muscles are from at least three distinct populations of cells that appear in the development of the vertebrate embryo (2, 5, 8-10, 11). These populations all emerge from the myogenic regions of the somite in the early embryo, but each becomes active in anatomic muscle formation at differing, but precisely determined times in development. Distinct populations of myoblasts are active in embryonic development; the transition to fetal development: and near the end of fetal development in anticipation for post-natal life. While these three types of myoblasts, can be distinguished by several characteristics, the time at which they can be experimentally isolated from developing organisms has been used as a basis for naming them. Thus, embryonic myoblasts are those that can only be isolated in embryonic development, fetal myoblasts are those that can only be isolated during fetal development, and those that can be isolated late in fetal development as well as during adult life, are called adult myoblasts. They have also been named by the type of muscle fiber they form, or by the relationship they have to muscle fibers — primary myoblasts, secondary myoblasts, and satellite cells. Primary myoblasts can be isolated from that stage of muscle development when primary muscles fibers are forming (embryogenesis); while secondary myoblasts can be isolated when secondary fiber formation is occurring (fetal development); and satellite myoblasts can be isolated from there position beneath the basal lamina of adult muscle. Satellite cells are now known to first emerge not during adult or postnatal life, but rather in late fetal development as basal lamina formation is beginning. Adult myoblasts or satellite cells are the last population to emerge during the sequence of muscle formation (9, 12, 13, 14). It is widely recognized that these

three basic types of myoblasts do not each produce a single fiber type, either fast, slow, and fast plus slow. On the contrary these three myoblast types have within them cells capable of forming muscle fibers of more than one functional type. Thus identifying the cells that produce each succeeding wave of muscle fibers which results in the formation of anatomic muscles and in their growth, has not resolved the question of how the diversity among fibers emerges.

The earliest periods of muscle fiber formation

Only during the earliest periods of muscle formation in the embryo does myogenesis precede in an environment that is devoid of the effects of motor neuron function (15). Primary fibers first form in the vertebrate limb before axons from the motor neurons of the spinal cord reach them. Experimentally, formation of primary muscles fibers occurs in the embryonic limb in the absence of functional innervation (in the presence of curare) and in embryos in which the neural tube has been surgically removed prior to fiber formation (6, 16, 17). In each experimental case diverse primary fibers types form — expressing fast as well as slow isoforms of the myosin heavy chain in the precise patterns that are characteristic of each anatomic muscle of the limb. These observations indicate that the initiation of primary fiber subtypes (fast, slow, and fast/slow) and their distribution within a muscle can not be dependent upon innervation. Thus, other explanations should be sought for primary fiber types and patterns.

It was the observation that curare introduced into chick eggs prior to incubation, did not prevent the formation of the normal limb muscles, nor the diversity of fiber types, that lead to an analysis of the autonomous formation of muscle fiber types. When myoblasts are isolated from the limb bud of the 4–5 day chick embryo and incubated in cell culture, fast, slow, and fast/slow myosin heavy chain expressing fibers are seen. These fibers formed *in vitro* have a distinctive morphology initially described by Hauschka and colleagues (8). These fibers can be identified as primary, or embryonic fibers, because they contain few nuclei and have an irregular outline, rather than the straight-edged outline characteristic of muscle fibers formed *in vitro* when myoblasts from fetal stages of development are cultured. When clonal cultures of embryonic chick myoblasts isolated from the limb bud are grown *in vitro*, colonies form fibers derived from a single parental myoblasts. Three types of fiber colonies formed and each had only one fiber type, either fast, fast/slow, or slow (18). Even in mass culture, when thousands of embryonic myoblasts were cultured together, embryonic myoblasts autonomously form these three fiber types (18, 19, 20). When clones were picked and individual myoblasts from the picked clone were re-cloned, they continued to form colonies of the same type as the parental clone (18, 21). Thus, the progeny of each fast, or fast/slow myoblast "bred true". These findings are consistent with a process whereby myogenic cells (myoblasts) are committed to a specific myogenic fate, that is, to a subsetted fiber type.

It follows from these observations that if myoblasts are committed to particular fiber fates it should be possible to demonstrate this experimentally *in vivo*. If myoblasts that have an intrinsic commitment to either the slow or the fast type, are marked and grown *in vitro*, and then reintroduced into the early embryonic limb bud, the fiber type they become should be independent of surrounding fibers (22). For these experiments embryonic myoblasts were cloned and transfected with the *Drosophilia* alcohol dehydrogenase (ADH) gene and a selectable marker. Clones were isolated and using antibodies specific to fast and slow MyHC isoforms and to *Drosophilia* ADH, individual clones were characterized. Individual clones in which every fiber expressed only fast, or only fast and slow MyHCs, as well as *Drosophilia* ADH, were selected and expanded in cell culture. Ten thousand myoblasts of each clonal type were injected into the limb buds of 4 day embryos (prior to anatomic muscle formation) and the embryos were incubated *ex ovo* until day 10 of development. The limbs were sectioned and triple stained for the two classes of MyHC and for *Drosophilia* ADH. In each case ADH-marked fibers were of the same fiber type as the injected myoblasts would have formed in cell culture. The fiber type formed from injected myoblasts was independent of the muscle in which the myoblast came to occupy. The fast myoblasts formed only fast fibers even if they were found within muscles expressing slow MyHC isoforms. Likewise the fast/slow myoblasts formed fast /slow fibers even if they were located in an exclusively fast-fiber muscle. The results of these studies demonstrate that the myoblasts isolated from early limbs and expanded in cell culture retain a commitment to a particular fiber fate that can be demonstrated in cell culture as well as in *in vivo*.

Studies in animals other than birds

Attempts have been made to confirm the above observations on myoblast commitment to fiber fate in the early mammal. Until recently only a single type of myoblast has been cloned from the mouse or rat. Merrifield and colleagues (23) have demonstrated that embryonic rat myoblasts form fibers in cell cultures that express a slow isoform of MyHC while myoblasts from fetal stages express only fast isoforms. Similarly in the mouse, embryonic fibers formed in cell culture express slow MyHC whereas those myoblasts from the fetus express only fast isoforms (24). When embryonic rat myoblasts are re-introduced into the rat, in the absence of innervation these embryonic myoblasts form two types of

fibers — fast and fast/slow MyHC expressing fibers (25). They have also found that rat L6 cells form only a single type of fiber when injected into muscles of regenerating rat muscles (26). Hughes and colleagues also report that myoblasts can be cloned from the early mouse embryo that express differing amounts of slow myosin (Hughes, personal communication). Even at later stages of development of mammals as in birds, adult myoblasts are committed to form fibers that selectively express only certain MyHC genes (27).

The proposal that myoblasts are committed to different fiber fates is premised on use of regents that distinguish one fiber type from another when the progeny of a single myoblast differentiate into a fiber. If there are myoblasts of differing types in mammals, then one must have a regent that distinguishes a fast from a slow fiber in the proper time frame of muscle development. In the chicken and the quail the reason differences in fate were detected is that the there are several isoforms of myosin expressed at this early developmental time. In particular there is more than one gene encoding the slow, as well as the fast class of MyHCs. The patterning seen in the first muscles to form in the limb bud of birds is based on the expression of a slow MyHC that is not expressed at later stages of skeletal muscle development. The antibody used to distinguish fast from slow-fiber-forming myoblasts recognizes this embryonic slow form (slow myosin heavy chain 3) as well as the adult form (slow MyHC 2) (28). In mammal, until recently, it was believed that there is only a single isoform of slow myosin, β-cardiac MyHC, that is expressed in the embryo as well as the adult. While it may be true that the β-cardiac MyHC gene is expressed in the mammalian embryo and in the adult, recent evidence points to the existence of additional slow MyHC isoforms in mammals (17). In particular an embryonic slow MyHC isoform has been identified in the rat upon the basis of differential monoclonal antibody staining of primary muscle fibers in the embryo, suggesting that in mammals, as in birds, there is early commitment of myogenic precursors to a definitive slow fiber fate.

The importance of slow MyHC gene expression and the patterning of fiber types in vertebrates

The expression of slow isoforms of MyHC is fundamental to recognition of fiber patterning in birds, mammals, and fish. Recently it has been demonstrated that an embryonic form of slow MyHC is expressed in the primary fibers of the developing quail limb musculature (28). This myosin is expressed in muscle whenever there are slow fibers within that muscle. As the embryo enters the fetal stage of development, there is a down regulation of slow MyHC 3 and the emergence of the definitive slow isoform of MyHC, slow MyHC2, in parallel with the addition of slow secondary fibers within slow-fiber muscles. This suggests in birds that fibers of the slow type have origins that are different than those that are fast and that this can be recognized by the expression of an embryonic form of the slow MyHC not expressed in the fast fiber. Fast fibers at embryonic stages of the chick express a slow isoform of MyHC, slow MyHC 1, an isoform expressed subsequently in fibers of the embryonic limb, but only slow MyHC3 is expressed in those fibers and muscles destined to be slow in the adult.

Recently it has been demonstrated that the slow fibers of the fish are also set aside in the early embryo, forming from a group of myogenic cells separate from those that produce the fast fibers of the fish (29). These slow fibers not only have a separate origin from the fast fibers, but they express a MyHC that is not expressed in any other fibers within the early embryo. In elegant marking experiments Devoto and colleagues (29) demonstrated that the slow fibers form as a specific group of cells near the notochord of the embryo. These cells migrate to the lateral regions of the trunk of the embryo where they give rise to the slow muscles of the zebrafish. Thus patterning in the fish is also dependent on the setting aside of particular precursor myogenic cells. It is not know if this patterning in fish can occur in the absence of innervation as it can in birds.

Thus there is accumulating evidence in three classes of animals that slow-fiber types patterns is associated with the expression of an embryonic slow MyHC in the first fibers that form in a developing muscle (17, 18, 29). This evidence is based upon the expression of particular isoforms of slow MyHC (the gene of one of which has been cloned (28)), in a subset of the primary fibers which appear displayed in specific fiber patterns. The results of the work of these investigators suggests that fibers expressing these slow MyHCs are committed to form the definitive slow fibers of adult muscles. They also provide additional evidence that the mechanisms involved in the patterning of slow fiber expression in the embryo is by innervation independent mechanisms, because in both the avian and mammalian embryo blocking innervation does not prevent the formation of slow fiber appearance or patterning (6, 17).

The role of innervation in the expression of myosin heavy chain and fiber type

While the precursor cells of embryonic fish and bird fibers are set aside prior to the outgrowth of axons from the motor neurons of the spinal cord, this is not the case in the fetal vertebrate. By the time fetal or secondary fibers begin to form in birds, axons have reached the primary fibers in the limb, and innervation has occurred as evidenced by motor end plate formation and movements in the more proximal muscles of the limb. These events are important since the transition from embryo-

nic primary fiber formation to fetal secondary fiber formation requires innervation if muscle development is to continue (6, 30, 31). If curare, β-bungarotoxin, or d-tubocurarine is present during this developmental transition, the primary fibers persist, but few new fibers (secondary fibers) appear and the continued expression of slow MyHC declines (32, 33). Harris and colleagues (31, 34) have demonstrated the close relationship between innervation and the formation of the secondary fibers. Only in regions where innervation of primary fibers is initiated are secondary fibers laid down. Using β-bungarotoxin these investigators were also able to show, as in birds, that functional innervation was a requisite for secondary fiber formation in the mammal.

Both slow and fast fibers form during fetal development as secondary fibers are laid down. In fact, most of the fibers that will be slow in the organism are formed during this time. Thus it was surprising, when fetal chick myoblasts were cloned, that only a single type of fiber was observed. All muscle-fiber clones formed from myoblasts isolated from developing chicks of about 8 days until about 12 days of incubation express fast, but no slow myosin (20). The fibers in these clones, of course, had all formed in tissue culture in the absence of innervation. To determine if innervation has a central role in differentiation of myoblasts derived from the fetal stage of development, myoblasts were isolated from fetal muscles of two types, those muscles composed exclusively of fast fibers such as the pectoralis, and those composed of fibers all of which express slow myosin as in the medial adductor of the thigh (DiMario and Stockdale, unpublished observations). When permitted to differentiate in mass culture or in clonal culture, myoblasts from either source form fibers that exclusively express fast isoforms of MyHC. To determine if innervation could alter expression of MyHC and to determine whether myoblasts of either fast or slow fetal muscle origin would respond to innervation in the same fashion, mass cultures were prepared and small segments of neural tube from embryos were added to the cultures. The cultures were stained with antibodies specific to fast or slow MyHC, or RNA was extracted and subjected to Northern analyses. All cultures expressed fast isoforms of MyHC when co-cultured with segments of neural tube, but only the cultures of myoblasts derived from slow-muscle myoblasts expressed slow isoforms of MyHC when the neural tube was present. Staining of nerve-muscle co-cultures to visualize axons and motor end plates demonstrates an abundance of motor end plates on all fibers in contact with neurite outgrowths from the neural tube, regardless of whether the myoblasts were derived from fast pectoralis or slow medial adductor muscles. Furthermore, if the cultures were grown with tetrodotoxin, motor endplates formed, but the fibers formed from medial adductor myoblasts, like those formed from pectoral muscle myoblasts, expressed only fast MyHC isoforms. These experiments demonstrate that even at the fetal stages of development there are myoblasts committed to different fiber fates, but those differences in fate are only manifest when fibers become innervated. These results also demonstrate that differentiation of muscle beyond the embryonic period, must go hand-in-hand with development of the nervous system; that at fetal stages only through cell-cell interactions (innervation) do fiber types become apparent.

There are many experiments performed in the neonatal and adult period of muscle development that demonstrate the importance of the nervous system in fiber type formation at these later times in muscle development (35). Many of these are cross-reinnervation experiments, where fibers of one type are re-innervated by a nerve that normally innervate a muscle fiber of the opposite type. These experiments demonstrate that re-innervated fibers acquire the expression of MyHC genes expressed in fibers of the type that the nerve normally innervated. These experiments have been used to conclude that muscle fibers must all be of one type. The conclusion has also been drawn from these experiments that all fibers must be of the same type when they formed in the embryo. There are many reasons why such extrapolations are inappropriate. For example, most of the fibers analyzed in cross re-innervation were not present in the embryo, but were performed on fibers that formed after the embryonic period of development. On close analysis it is also apparent that these later fibers upon re-innervation are converted in an incomplete fashion. There is a range in the completeness of the reversal of fiber type in muscles following cross re-innervation, but they remain different in the genes expressed from the muscle fibers *normally* innervated by the nerve under study. Finally, it is sometimes forgotten that innervation can not explain embryonic fiber typing, because axons have not reached limb muscle until after fibers of particularly types have formed.

A unitary hypothesis on fiber type formation during the early period of muscle formation

Anyone who has looked at crossections at any level of the fore or hind limb recognizes that all the fiber types are present and in their precise distribution very early in morphogenesis. By late embryonic development all the anatomic muscles are clearly demarcated in the shape and the fiber distribution they will retain throughout the life of the organism. These are dependent upon two types of processes, those that commit mesodermal cells to a myogenic fate and those that lead to innervation of fibers once formed. These two processes are not mutually exclusive, but it is arguable that cellular commitment to fate is the primary process.

During the formation of the vertebrate body axis, the neural tube has a pivotal role in the initiation of myogenesis (36–41). The neural tube in the first few days of avian and mammalian development produces diffusible substances that in experimental systems act on the adjacent segmental plate mesoderm resulting in muscle fiber formation (15, 42). Fibers that express slow or fast myosin are formed within the paraxial mesoderm in these experimental systems (42). The anatomic locations which serves as the source of this factor(s), is controversial. Some groups report that the dorsal neural tube is the source of crucial myogenic signals (43, 44), while others indicate the ventral or ventral medial neural tube or notochord as an important signally source (39, 42, 45). While not completely resolved it appears that an important candidate for this factor is sonic hedgehog that is produced in the floor plate of the ventral neural tube in response to the underlying notochord. But there are a large number of other factors, including Wnt (43, 46), that have been shown to enhance a myogenic response in cultured segmental plate or somite tissue. In mice, the neural tube appears to activates myogenesis in the medial half of paraxial mesoderm through a myf-5-dependent pathway (41). The same authors report that the dorsal ectoderm activates myogenesis in lateral half of the paraxial mesoderm through a MyoD-dependent pathway (41). Additional evidence of the neural tube activating a myf-5 dependent pathway in the medial protion of murine somites comes from recent studies of the phenotypes associated with the sonic hedgehog knockout mouse (47). These animals which for the most part lack functional floor plate have significantly reduced levels of myf-5 expression while levels of MyoD expression are comparable with wildtype litter mates. While the activation of myogenesis is usually thought of a positive in character there are as well negative influences reported to emanate from the lateral plate (48, 49), ventral neural tube (45), and dorsal neural tube (42). These and surgical ablative studies (50) performed *in vivo* on developing chick embryos show that the neural tube is important in the initial events of myogenesis. These interactions between the neural tube and segmental plate mesoderm, clearly do not require innervation *per se*, or even axons, because axons have not appeared at these stages of development and because the initiation of myogenesis in either segmental plate or somites can occur across a filter that blocks cell-cell contact (innervation) (42). Thus all myogenesis begins with an interplay of neural and mesodermal tissues.

Those myogenic cells that will form the limb musculature migrate from the somites to populate the forming limb buds as an autonomous population of cells, in the sense that they will differentiate in the absence of innervation. The migratory cells are located, as one would expect, in the lateral position of the somites (51). Those that will form the slow fibers of the limb muscles migrate at a different time than those that will form the fast fibers. The precursors to the slow fibers migrate into the limb before those that will form the fast fibers of the limb (52). These latter observations by Lance-Jones and colleagues (52) are another demonstration that the early period of myogenesis in the limb, one launched in the somite by interaction with the neural tube and notochord, is autonomous with myogenic precursors of two or more cell kinds producing the differing fiber types seen in the limb muscle. These migratory precursors are those that form the embryonic myoblasts which in turn produce primary fibers. These migratory precursors are a complex population of cells as among them must be the precursors to the fetal and adult myoblasts a well, unless the assumption is made that these derive from one another or from embryonic myoblasts.

The embryonic myoblasts disappear from the developing chick limb at about day 7 or 8 of development, because beyond this time it is not possible to isolate cells from limb muscles that form fibers in culture with the distinctive morphology of an embryonic fiber or that express slow MyHC chain. The presumption is that these embryonic myoblasts have a limited proliferative life (2) and are consumed in the process of forming the first muscles and their primary fibers. Beginning at this time one can isolated increasing numbers of fetal myoblasts, or what Hauschka and colleagues (53) have call late myoblasts. These can be identified in the bird by the distinctive morphology of the fibers they form in mass and in clonal culture. They form fibers of great length — at times containing hundreds of nuclei clearly differing from the early fibers formed from embryonic myoblasts that contain and average of 2 to 3 nuclei. It is this new wave of myoblat that populate the limb which are dependent on the nervous system both for the initiation of fiber type and their subsequent maintenance. The fetal myoblasts of the fast and slow muscles differ from one another yet each posses the same requirement for interaction with motor neurons. Those that will form slow-MyHC-expressing fibers must be innervated to do so, but they as well as those that are of the fast-fiber forming type require innervation if they are to be maintained and continue to transcribe members of the MyHC gene family. Thus muscle development comes full circle — first dependent on the neural ectoderm to develop the autonomy in fiber formation and finally direct contact through a motor endplate to initiate new fiber formation on their surfaces and to sustain themselves.

Recent observations are provocative in suggesting that there may be yet an earlier period in the formation of cells destined to a myogenic fate. This is an even earlier period of myogenic development which is independent of the nervous system. George-Weinstein (54) has presented work that shows that myogenic cells exist

within the hypoblast of the blastodisc prior to formation of the neural tube and the mesoderm. She showed that cells isolated from pregastrulation stages of chick development will produce muscle fibers in cell culture. Such muscle forming cells appear to be abundant at this early stage of development. Because a number of investigators have demonstrated that once the segmental plate of mesoderm forms later during gastrulation, this tissue is not autonomous with regard to myogenesis in cell culture or in any other setting in isolation (37, 38, 39–41, 42), George-Weinstein's observations suggest that autonomy of the myogenic phenotype is primary, and that it must become masked at gastrulation. Only after gastrulation does interaction with the axial structures of the embryo (notochord and neural tube) unmask myogenic commitment in these cells. It is now well established that mesodermal cells within the segmental plate prior to somite formation express members of the myogenic regulatory factor family (MRF family), even though when placed in cell or organ culture early somite or segmental plate cells will not differentiate into muscle. Recent work by Emerson and colleagues (45) provides an explanation for this in that when removed beyond the influence of diffusible factor(s) from axial structures, the cells of the somite discontinue the expression of MRFs. Thus, in pregastrulation, during gastrulation, and immediately following gastrulation, and into morphogenesis there is constant interplay between the cells that will be, or are, myogenic and the cells of the nervous system. The nervous system over developmental time comes to wrest control of the system from the decreasingly autonomous myogenic cell precursors.

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