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Review

The generation of fiber diversity during myogenesis

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CONTENTS

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Introduction

Skeletal muscle is a tissue consisting of multinuclear fibers organized in parallel arrays. Each fiber is produced by the fusion of specialized mononuclear myogenic cells (called myoblasts). Muscle fibers are however not all alike and vary in their time of appearance, their metabolism and speed of contraction. This review discusses the mechanisms that may be involved in generating differences between fibers found in the limb muscles.

Formation of muscle fibers in the limb

The cells which form muscle fibers in the limb are derived from the somites adjacent to the developing limb bud. Cells detach from the lateral edge of the somite and migrate into the limb coming to rest in locations determined by the limb mesenchyme (Chevallier *et al.*, 1977; Christ *et al.*, 1977; Ordahl and LeDouarin, 1992). The formation of muscle fibers is described as biphasic, as the generation of new fibers falls into two periods. These are distinguishable by their temporal separation and the morphology of the fibers formed (Kelly and Zacks, 1969; Ashmore *et al.*, 1972; Ontell and Kozeka, 1984). The term primary fibers or primaries, is applied to the first wave of fibers. These only form during the early stages of myogenesis and are distributed throughout the muscle forming regions of the limb (Ontell *et al.*, 1988). Primary fibers rapidly increase in diameter and are distinguishable by their size and characteristic 'doughnut' shaped outline in section, from the smaller fibers which form subsequently (Wigmore and Stickland, 1983; Ross *et al.*, 1987). Primaries comprise only a minority of the final fiber number in a muscle, but play a significant role in the generation of later fibers.

After primaries have stopped forming, a new generation of small fibers is seen on the surface of each primary fiber. These new fibers, called secondaries, use the surface of primary fibers as a scaffold for the attachment and fusion of myogenic cells into the new fibers. After a short period, secondary fibers separate from the surface of the primary fiber so that, at any one time, an individual primary can have several secondary fibers still on its surface while being surrounded by a halo of secondaries which have detached (Fig. 1). In the rat, primary fibers form between embryonic day (E)14 and E16 while secondary fibers form between E17 and the neonatal period (birth being at E21). Both types of fiber continue to grow and increase their nuclear number by cell fusion well into the postnatal period (Harris *et al.*, 1989a; Condon *et al.*, 1990a; Wigmore *et al.*, 1992; Zhang and Mclennan, 1995). The number of secondary fibers which form on each

Abbreviations used in this paper: E, Embryonic day; MHC, myosin heavy chain; MRFs, myogenic regulatory factors; TGFβ, nansforming growth factor beta.

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primary fiber varies from between 5 and 9 in small mammals (mouse/rat; Ross *et al.*, 1987; Ontell *et al.*, 1988) to over 20 in larger species (human/ pig; Stickland, 1981; Wigmore and Stickland, 1983; Stickland and Handel, 1986). This correlation indicates that the requirement for increased muscle fiber number, as species increase in size, is met by an increases in the number of secondary fibers forming on each primary fiber rather than an increase in the numbers of primary fibers each supporting a small number of secondaries.

The end of myogenesis is marked by the cessation of fiber formation and a decrease in diameter of primary fibers (Wigmore and Stickland, 1983; Wigmore *et al.*, 1996). As a result of the growth of secondaries the size difference between these two generations of fibers disappears and they are no longer distinguishable by morphology.

The sequential formation of primary and secondary fibers raises the question of what determines their time of formation and the differences between them. Interest has focused on the myoblasts present in the limb when primaries and secondaries are forming. Evidence has accumulated that the cell populations present, during primary and secondary fiber formation, may be different and are responsible for producing these two generations of fibers.

The generation of primary and secondary fibers by different populations of myoblasts

A large body of work has established that myogenic cells isolated from the limb fall into three populations. These have been called embryonic, fetal and adult (Cossu *et al.*, 1988; Stockdale, 1992) and predominate at different stages of muscle development (see Fig. 2). Differences in the characteristics of these cells in culture can be used to distinguish these populations and indicate that they may have different roles during myogenesis.

Cells removed at the start of myogenesis require conditioned media to survive and in culture produce short fibers containing few nuclei (Bonner and Hauschka, 1974; Hauschka, 1974; White *et al.*, 1975; Rutz *et al.*, 1982; Seed and Hauschka, 1984). These cells have been called embryonic myoblasts and since they constitute the majority of cells present when primary fibers start forming, it has been assumed that they are responsible for the production of these fibers. Direct evidence for this has recently come by genetically marking cells *in vivo* using retroviral vectors (Wigmore and Dunglison, 1997). Cells marked at E15 in the rat, only produce primary fibers and do not persist into the later stages of myogenesis or contribute to secondary fibers. From these results embryonic myoblasts all fuse with primary fibers and their sole function is the initial formation of primary fibers. Fetal cells can be isolated from muscle during the period of secondary fibers formation. These cells have less stringent media requirements than embryonic cells and in culture produce long thin myotubes with numerous nuclei. Unlike embryonic cells they express the $\alpha 7$ integrin on their surface which may enable them to attach to the basal lamina of primary fibers (Song *et al.*, 1992; George-Weinstein *et al.*, 1993).

Retroviral marking of mammalian cells during the period of secondary fiber formation has shown that these cells are pluripotent in terms of fusing with both primary and secondary fibers. Clones of cells marked at E17 in either rat or mouse fetuses contribute to the formation of new secondary fibers and the continued growth of primary fibers (Evans *et al.*, 1994; Wigmore and Dunglison, 1997). It is unclear how individual fetal cells decide whether to fuse with a primary or secondary fiber. The initial fusion of cells to form new secondary fibers appears to be restricted to the vicinity of the motor end plate on the primary fiber (Duxson *et al.*, 1989). This would suggest that secondary fiber formation is under the control of the supporting primary fiber which may provide specific sites for new fiber production. This view is supported by the observation that localized adhesion molecules are found on the surface of primary fibers at the sites of secondary fiber attachment and that primary fiber diameter correlates with the numbers of secondary fibers present on its surface (Wigmore and Stickland 1983; Rosen *et al.*, 1992; Fredette *et al.*, 1993; Rose *et al.*, 1994; Cifuentes-Diaz *et al.*, 1995).

From the work described above, embryonic and fetal cells constitute the predominant cell type at different stages of prenatal myogenesis and are involved in different aspects of the initiation of primary and secondary fibers and their subsequent growth (Fig. 2 and 3). Prolonged culture of embryonic cells never causes them to convert into fetal cells and each population is therefore believed to be derived from different precursors. Small numbers of fetal cells have been isolated from early stages of myogenesis suggesting that the precursors of this population are already present during primary fiber formation but are not activated until secondary fiber formation starts (George-Weinstein *et al.*, 1993). Embryonic and fetal cells can readily fuse together in culture (DiMario and Stockdale, 1995; Pin and Merriefield, 1997) which raises the question of how they are kept separate *in vivo* . This has been at least partially answered by work on their response to the growth factor TGFβ (Cussella-De Angelis *et al.*, 1994; DiMario and Stockdale, 1995; Zapelli *et al.*, 1996). Embryonic cells can differentiate in the presence of this growth factor while fetal cell differentiation is inhibited. This response has been correlated with high levels of TGFβ in the limb during primary fiber formation which is sufficient to inhibit the differentiation of fetal cells.

During secondary fiber formation a third population of cells called adult cells has been identified. These are distinguishable from fetal cells by the expression of acetylcholine receptors, different myosin heavy chain (MHC) isoforms and their ability to differentiate in the presence of the tumor promoter TPA which prevents fetal cell differentiation (Cossu *et al.*, 1988; Hartley *et al.*, 1991; reviewed in Cossu and Molinaro, 1987; Yablonka-Reuveni, 1995). Adult cells form an increasing proportion of the cells which can be isolated from the limb during the later stages of myogenesis and persist into postnatal life when they are responsible for growth and regeneration (Feldman and Stockdale, 1992; Hartley *et al.*, 1992). Fetal and adult cells also differ in their sensitivity to surface features when cultured on grooved slides. Fetal cells change their orientation in response to fine (sub-micrometer) grooves which are ignored by adult cells (Wigmore *et al.*, 1995). This may reflect an ability of fetal cells to use extracellular matrix fibrils to bring cells into the correct position and orientation during secondary fiber formation.

Cells displaying adult characteristics have spontaneously arisen in clones of fetal cells (Hartley *et al.*, 1992). This suggests that while embryonic cells descend from progenitors which are different from those of later cells, adult cells are derived from the same precursors as fetal cells.

Differentiation of fast and slow fiber types

Primary and secondary fibers can be further classified into different fiber types characterized by differences in metabolism and speed of contraction (reviewed in Schiaffino and Reggiani, 1996). Slow twitch fibers are used to maintain posture while those with fast twitch characteristics are used in producing movement. Individual muscles have characteristic proportions of these fiber types which, in some muscles, are also distributed in a graded fashion with a higher proportion of fast fibers in more superficial regions (Condon *et al.*, 1990a). Many of the contractile proteins within a fiber have multiple isoforms which are specific for different fiber types and correlate with their speed of contraction. Myosin heavy chain (MHC) isoforms are the most commonly used for fiber typing. In adult muscle four types of fibers are normally distinguished by their metabolism and MHC isoforms. These are a single slow fiber (type I) and 3 fast fiber types (type IIA, IIB and IIX). During development, fibers are normally only classified as either fast or slow (Schiaffino and Reggiani, 1996). Fibers can be recognized as being fast or slow from the time of their formation by the expression of specific developmental MHC isoforms which predict their final fiber type (Schiaffino and Reggiani, 1996).

In mammalian muscle, a good correlation exists between primary and secondary fibers and their initial fast or slow fiber type (Fig. 4). All primaries are initially slow but a small proportion subsequently convert to being fast. Conversion of primary fibers occurs in the superficial parts of muscles, regions which are predominantly fast in postnatal muscle (Harris *et al.*, 1989b; Condon *et al.*, 1990a). Fiber type conversion may be under the influence of TGFβ derived from the surrounding connective tissue (McLennan, 1993). In contrast, secondary fibers are all initially fast but a proportion convert to being slow. Converting secondary fibers are generally found in predominantly slow muscles or slow regions of muscles (Condon *et al.*, 1990a). This conversion establishes the fast/slow gradient of fiber types distributed from the deep to superficial parts of some muscles.

The correspondence of primary and secondary fibers to fiber type in avian muscle is more complex as primary fibers can be one of three different fiber types from the time of their formation. Fast, fast/slow and slow primaries show characteristic proportions and distribution within each muscle. This establishes the pattern of fast and slow fiber types within individual muscles. Nearly all secondaries are fast (Crow and Stockdale, 1986; Fredette and Landmesser, 1991a; Robson, 1993). In both mammal and chick the origin of the fast and slow fiber types is independent of innervation. This is in contrast to adult muscle where neural activity plays a major role in determining the MHC isoform expression (reviewed in Pette and Vrbova, 1985; Schiaffino and Reggiani, 1996). During development nerves are present throughout myogenesis and make contact with the newly formed fibers. However, a variety of denervation experiments in both avian and mammalian embryos has shown that fast and slow fiber types still form and show the correct proportions and spatial distribution when nerves are absent or electrical activity is blocked (Crow and Stockdale, 1986; Condon *et al.*, 1990b; Fredette and Landmesser, 1991b). Absence of innervation or electrical activity does however reduce the total number of fibers which form and causes the subsequent degeneration of all fibers (Hughes and Ontell, 1992; Ashby *et al.*, 1993a,b; Wilson and Harris, 1993).

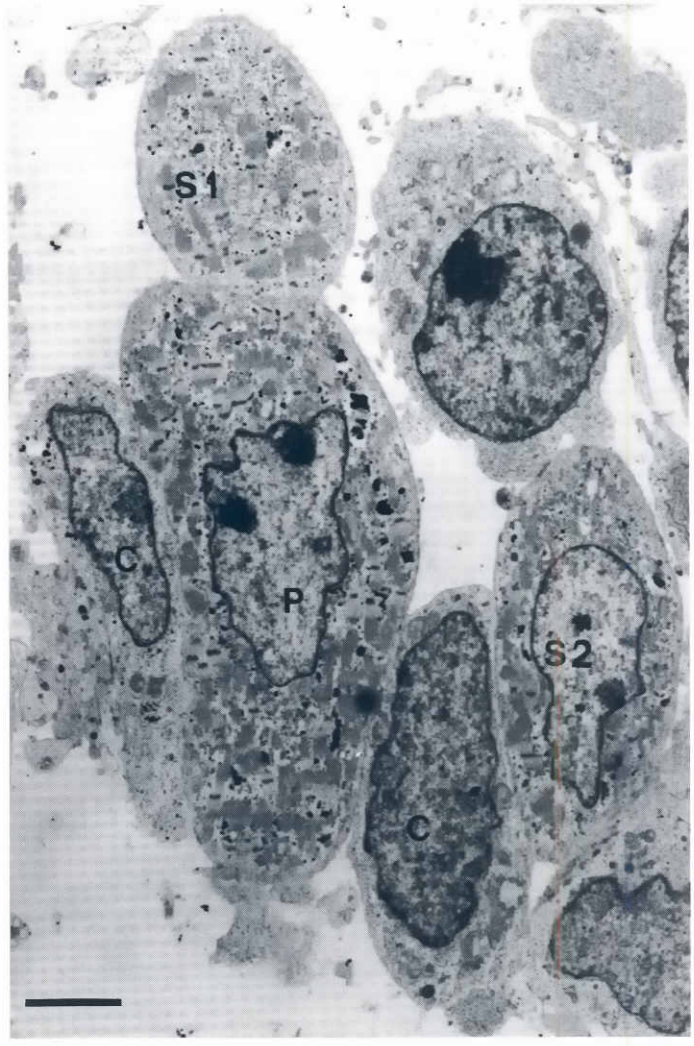

Fig. 1. Transmission electron micrograph showing primary and secondary fibers in an E18 rat extensor digitorum muscle. *A primary fiber (P) with a secondary fiber (S1) is on its surface while another secondary (S2) has detached. Two cells (C) are also on the surface of the primary fiber. Bar, 3 μm.*

Origin of fast and slow fibers

The origin of fast and slow fiber differences could be due to differences between the cells producing each fiber type. This has been demonstrated in avian muscle where clones of myoblasts, isolated *in vitro* , fuse to form a single fiber type. Each myogenic cell is therefore committed to forming either fast or slow fibers and this commitment is inherited by the daughter cells at division. When clones of cells are marked and transplanted back into the embryo they retain this commitment and continue to produce fibers of the same type as were formed *in vitro* (DiMario *et al.*, 1993). Clones of cells producing particular fiber types have now been isolated from embryonic, fetal and adult populations of avian cells (Miller and Stockdale, 1986; Schafer *et al.*, 1987; Feldman

and Stockdale, 1991; Stockdale, 1997). Commitment appears to be determined prior to the migration of embryonic cells into the limb as cells forming slow primary fibers enter the limb bud ahead of those forming fast primary fibers (Van Swearingen and Lance Jones, 1995). Coculture with nerves facilitates the expression of some MHC isoforms when fetal avian cells are cultured (Lefeuvre *et al.*, 1996; DiMario and Stockdale, 1997). These results showing cell commitment have led to the development of the lineage theory which suggests that fiber diversity is due to prior commitment by the cells forming different types of fiber (Stockdale, 1992).

Mammalian cells too can exhibit commitment to forming either fast or slow fiber types *in vitro*. Embryonic cells, which form primary fibers all of which are initially slow, also only produce slow fibers *in vitro* (Vivarelli *et al.*, 1988; Smith and Miller, 1992; Cho *et al.*, 1993; Pin and Merrifield, 1993).

Reports however vary on the commitment of mammalian fetal cells. Some authors have claimed that fetal cells only produce fast fibers *in vitro*, which would correlate with their role in the formation of fast secondary fibers (Pin and Merriefield, 1993). In contrast other authors have shown that single clones derived from fetal mouse or human cells can produce a mixture of fast and slow fibers in culture (Cho *et al.*, 1993; Robson and Hughes, 1997). The proportion of fast and slow fibers produced by individual clones in these studies, remained constant with repeated cell passaging indicating that this a heritable characteristic. From these results the role of cell lineage in determining mammalian fetal cells is less clear than that seen in avian muscle. *In vivo* retroviral marking of fetal cells in prenatal rats has shown that clones of fetal cells contribute both to slow primaries (which are still growing by incorporating cells) and the formation of new fast secondaries (Wigmore and Dunglison, 1997). The ability of clones of cells to contribute to both fast and slow fibers continues into the postnatal period when clones of adult cells fuse randomly with all surrounding fiber types (Hughes and Blau, 1992; Dunglison and Wigmore, 1996). Figure 3 is based on retroviral marking of mammalian myoblasts at different ages and shows which cells contribute to which types of fiber.

These results indicate that despite showing signs of commitment to particular fiber types *in vitro*, mammalian fetal and adult cells are pluripotent *in vivo* and able to fuse with all fiber types. At first sight there would seem little role for the commitment to producing either fast or slow fibers shown by mammalian cells *in vitro*. One clue has come from recent observations that adult cells derived from the surface of slow fibers tend to produce slow fibers in culture while those from fast fibers produce fast fibers (Rosenblatt *et al.*, 1996). This spatial aspect to cell commitment could be due to cells seeking out fibers of congruent type with which to associate or more likely, is caused by the fiber itself in some way dictating the expression of commitment by cells on its surface. In either event since cells on the surface of a fiber are likely to fuse with that fiber, it may be advantageous for cells to be preprogrammed to produce the MHC isoforms of the fiber type with which they are associated. This commitment can be overruled and does not prevent the cell fusing with other fiber types. Another possibility is that inherent cellular commitment gives a default path of differentiation which is used in the absence of any other cues. Results consistent with this have come from regenerating avian muscle where satellite cells from slow muscles, injected into regenerating fast muscles, do not form slow fibers. However, if the fast muscle is first X-irradiated to destroy endogenous satellite cells, slow fibers are formed by the grafted cells (Bourke *et al.*, 1995).

Myogenic regulatory factors

The four myogenic regulatory factors (MRFs) MyoD, Myf5, Myogenin and MRF4 were first identified by their ability to convert non-myogenic cells into myoblasts. MRFs are only expressed by presumptive myogenic cells once they have entered the limb. MRFs are then expressed in a sequential fashion in developing muscle (Sassoon, 1993). This pattern of expression reflects the maturation of the tissue and differential expression of these factors has not been found in embryonic, fetal or adult myoblasts populations *in vitro* (Smith *et al.*, 1993). Nor do developing fast and slow fiber types show differences in MRF expression (Grieshammer *et al.*, 1992). This contrasts with the situation found in adult muscle where fast and slow fibers differentially express MyoD and Myogenin respectively (Hughes *et al.*, 1993). One piece of evidence does however suggest that MRFs may play a role in distinguishing primary and secondary fibers. In the Myogenin knockout mouse only primary fibers form. Large numbers of myoblasts surround each primary fiber but fail to fuse into secondary fibers (Venuti *et al.*, 1995). Myogenin may therefore be required for the fusion of fetal and adult myoblasts but not for cells from the embryonic population.

MRFs dimerize with E proteins prior to binding to E boxes, a consensus sequence (CANNTG) found in the promoters and enhancers of many muscle specific genes (Firulli and Olson, 1997). As described earlier different fiber types are distinguished by the expression of different isoforms of contractile and enzymatic proteins. In the majority of cases these isoforms are coded by separate genes, the regulatory sequences of which must control fiber type specific expression. Use of reporter gene constructs coupled with fragments of promoters and enhancers from these genes has enabled the identification of sequences causing expression in particular fiber types. These sequences normally contain several binding motifs but in most cases include one or more E boxes indicating that MRFs may play a role in fiber type specific expression. Transgenic animals carrying reporter genes under the control of these sequences have now been made which faithfully reproduce the fiber type expression patterns of several

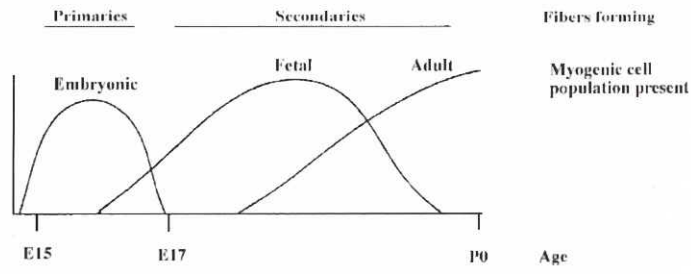

Fig. 2. Appearance of different populations of myogenic cells during prenatal myogenesis. *The ages are based upon myogenesis in the rat and the periods when new primary or secondary fibers are forming is also shown. Modified from Stockdale 1992.*

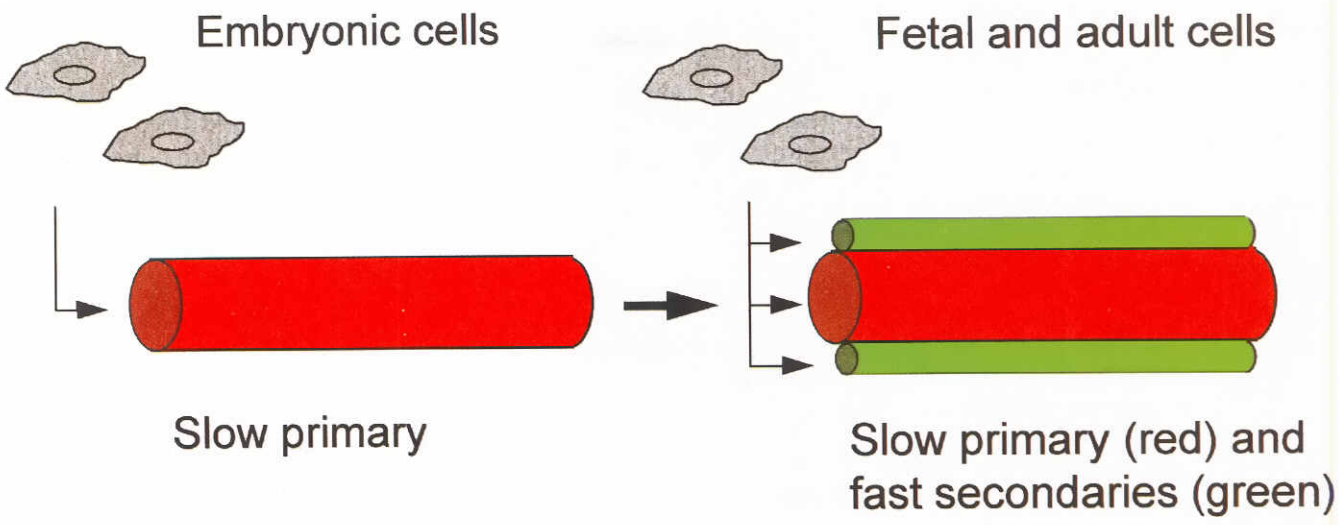

Fig. 3. Contribution of different populations of myogenic cells *in vivo* to primary and secondary fibers in mammals. *The contribution of each cell population is based upon following retrovirally marked clones of cells in vivo in rodents. Cells marked when only embryonic myogenic cells are present, only contribute to primary fibers and do not persist into the later stages of myogenesis. Cells marked when fetal or adult populations predominate contribute to both primary and secondary fibers.*

members of the troponin, myosin light chain and myosin heavy chain gene families in developing and adult animals (Corin *et al.*, 1995; Knotts *et al.*, 1996; Nakayama *et al.*, 1996; Wiedenman *et al.*, 1996).

The myosin light chain *1/3f* gene is an exception to the above in that differential splicing rather than activation of separate genes is responsible for the production of different isoforms. This gene is expressed only in fast fibers but codes for two isoforms which are expressed at different times during development. Dissection of the regulatory elements of this gene has shown 2 promoters and 2 enhancers, with the temporal expression differences due to sequences within one of the enhancers (Rao *et al.*, 1996; Kelly *et al.*, 1997). Reporter gene constructs containing regulatory elements from this gene surprisingly also show spatial differences in expression of the gene. Reporter gene expression is absent from rostral regions but shows progressively stronger expression in more caudal muscles (Donohue *et al.*, 1991). Although this pattern of expression is not found in the endogenous gene, it indicates that the regulatory sequences of muscle genes contain a variety of control elements conveying tissue, fiber type, temporal and spatial control to their expression.

The *ski* oncogene also shows differential expression in different fiber types with the highest expression being found in type IIB fibers (Sutrave and Hughes, 1991). Overexpression of this gene in transgenic animals causes selective hypertrophy of this fiber type. Unexpectedly however these fibers are weaker than those in normal animals (Leferovich *et al.*, 1995). *Ski* appears to be under the same control as other fiber type specific genes but its normal role in type IIB fibers remains unclear.

Concluding remarks

Myogenesis in both birds and mammals occurs as an unfolding sequence of different populations of both myoblasts and muscle fibers. It is worth noting that in fish birds and mammals, slow fibers are the first to differentiate (Condon *et al.*, 1990a; Van Swearingen and Lance-Jones, 1995; Devoto *et al.*, 1996). Cell commitment to produce these early slow fibers occurs before the cells have left the somite in the fish, while in birds, it is at least before the cells have entered the limb. Programming of cells before they migrate, may enable them to come under the influence of signaling molecules produced by midline axial structures. In the fish, the signaling protein, sonic hedgehog, derived from the notochord, induces the differentiation and migration of these early slow myoblasts (Blagden *et al.*, 1997; Devoto *et al.*, 1997). It is currently unknown at what point and how, avian and mammalian embryonic cells acquire their commitment but it is possible that they too receive instructions before leaving the somites.

Cell commitment through lineage is implicated in the production of fiber differences in both mammal and chick. In the mammal differences between embryonic and fetal cells appear to form the basis for the differences between primary and secondary fibers and the initial differentiation of muscle into slow (primary) and fast (secondary) fibers. The ability of fetal cells to produce secondary fibers which are all initially fast does not however prevent them from fusing with existing slow primary fibers. Further refinement of the pattern of fast and slow fibers occurs by fiber conversion which continues well into the postnatal period and is almost certainly not intrinsic to the muscle but dependent on neural or growth factor signals.

In avian muscle a similar sequence of myoblast populations occurs to produce primary and secondary fibers but differs from mammalian muscle in that three types of primary fiber (fast, fast/slow and slow) are produced. This is brought about by the commitment of individual clones of myoblasts, within the embryonic population, to produce each of these three fiber types. This mechanism of clonal commitment continues to be used in the

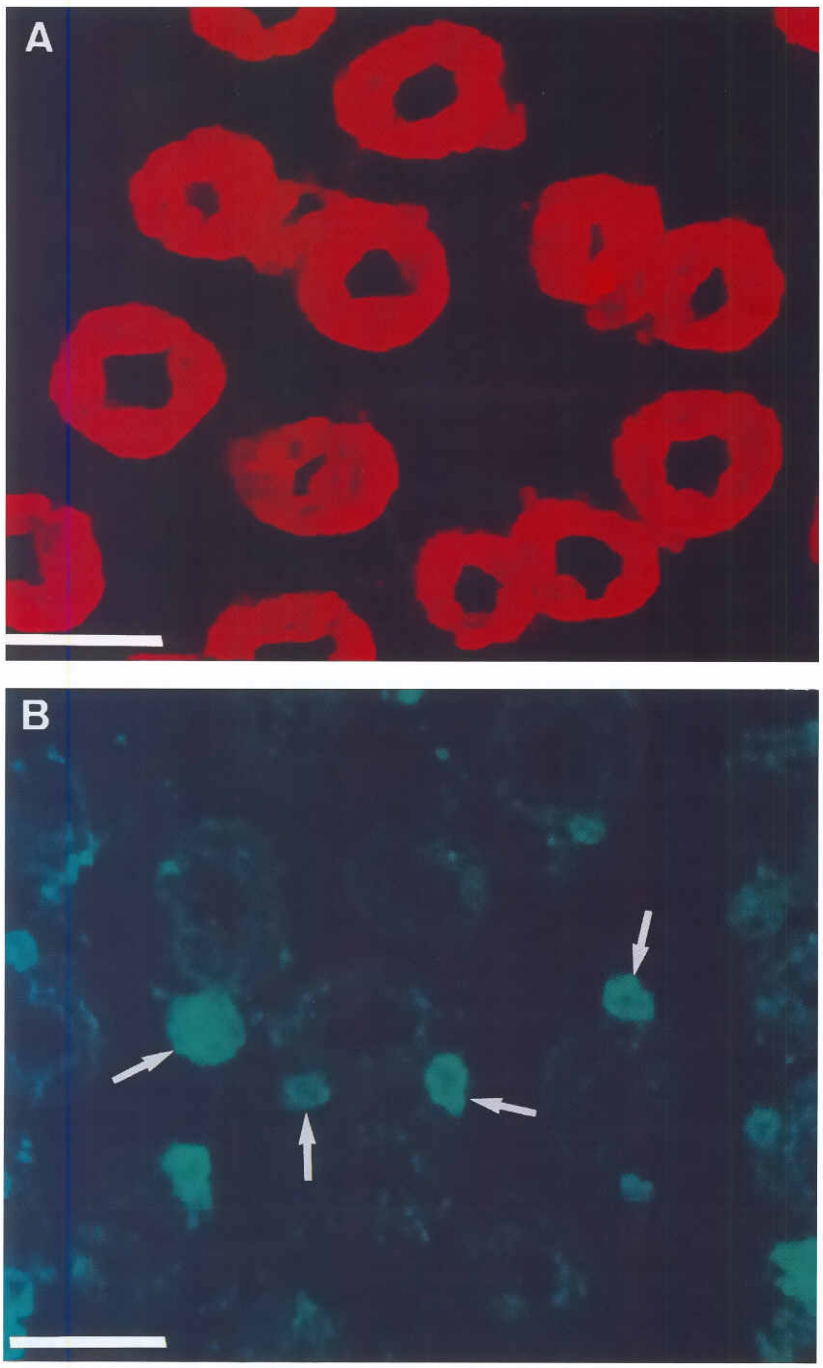

Fig. 4. Initial relationship between fast and slow fiber type and primary and secondary fibers in mammals. *Sections of E18 rat extensor digitorum muscle stained for either slow or fast MHC.* **(A)** *All primary fibers show positive staining for slow MHC.* **(B)** *Secondaries (arrowed) on the surface of primaries stain positively for fast MHC. Bar, 10 μm.*

subsequent stages of myogenesis and the resulting distribution of fiber types requires little fiber conversion to produce the adult pattern. The role of clonal commitment has been one of the bones of contention between results obtained from avian and mammalian myogenesis. During mammalian development only a single (slow) type of primary fiber is formed, making it unnecessary for mammalian embryonic myoblasts to be differentiated into forming more than one type of fiber. The secondary fibers formed by mammalian fetal cells are all initially fast but clones of fetal cells contribute randomly to both fast secondary and slow primary fibers indicating that clonal commitment does not determine the fate of these cells.

One difficulty in comparing avian and mammalian myogenesis is the different approaches used. Future work would usefully be directed to *in vivo* lineage tracing in avian embryos which would demonstrate the commitment of cells in this environment. Other gaps in our knowledge include what other factors in the limb environment are responsible for setting up and maintaining the pattern of fast and slow fibers during development. Nor is it understood how cells acquire and retain commitment during specific stages of myogenesis. Relatively few transcription factors have been identified whose expression correlates with the sequence of events during myogenesis, it remains to be seen whether existing myogenic factors will be found to have a role in these events or whether new factors will be discovered.

Summary

Adult muscle is composed of different fiber types distinguished by their speed of contraction and metabolism. The generation of these differences is related both to the sequence in which muscle fibers form and to differences between the myogenic cells involved. Fibers form in two successive waves (primary and secondary) whose time of appearance can be correlated with the existence of successive populations of myogenic cells (embryonic and fetal). The differences between fibers arise through an interplay between heritable cellular commitment, where cells are preprogrammed to produce particular types of fiber and influences from the limb environment. The techniques of genetically marking cells and clonal analysis *in vivo* and *in vitro* are starting to reveal the relationship between these different influences. Although the process of myogenesis is similar in birds and mammals it is likely that cell autonomous behaviour plays a more important role during avian development as compared to mammals. The identification of muscle specific transcription factors has provided some clues to the mechanisms by which development is controlled but the expression of relatively few of these has been correlated with the sequence of events seen in myogenesis.

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