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Evidence for distinct fast and slow myogenic cell lineages in human foetal skeletal muscle

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Summary

To analyse the myogenic cell lineages in human foetal skeletal muscle, muscle cell cultures were prepared from different foetal stages of development. The *in vitro* muscle cell phenotype was defined by staining the myotubes with antibodies to fast and slow skeletal muscle type myosin heavy chains using immunoperoxidase or double immunofluorescence procedures. The antibodies to fast skeletal muscle myosin heavy chains stained nearly all myotubes dark in cell cultures prepared from quadriceps muscles at 10–18 weeks of gestation. The antibodies to slow skeletal muscle myosin heavy chains, in contrast, stained only 10–40% of the myotubes very dark. The remaining myotubes were further subdivided into two populations, one of which was unstained while the other stained with variable intensity for slow myosin heavy chain. The slow myosin heavy chain staining was not influenced by the nature of the substratum used to culture these cells, although the growth of muscle cell cultures was greatly improved on matrigel-coated dishes. The presence of both slow and fast myosin heavy chains was detected even when myotubes were grown on uncoated petri dishes. The myotube diversity was further investigated by analysing the clonal populations of human foetal skeletal muscle cells *in vitro*. When cultured at clonal densities, two types of myogenic clones were identified by their differential staining with antibodies to slow myosin heavy chain. As was the case with the high density muscle cell cultures, virtually all myotubes in both groups of clones stained with antibodies to fast myosin heavy chains. Antibodies to slow myosin heavy chains stained nearly all myotubes dark in one group of myogenic clones, but only a subset of the myotubes stained dark for slow myosin heavy chain in the second group of clones. The proportion of slow myosin heavy chain positive myotubes in this group varied in different clones. The myogenic diversity was thus apparent in both high density and clonal human muscle cell cultures, and myogenic cells retained their ability to modify their muscle cell phenotype.

Introduction

Most skeletal muscles in both mammalian and avian species are composed of two main groups of fibre types which can be further subdivided to different extents on the basis of their different mRNA or protein expression patterns in individual fibres (Pette & Staron, 1990; Schiaffino & Reggiani, 1996). The unique expression patterns of such molecules provide muscle fibres with contractile properties well suited to their differing functional demands. The emergence of different muscle fibre types is observed in foetal skeletal muscles, although the time at which these differences first become apparent varies in different species depending on the nature of molecular markers used to identify such differences. The muscle fibres in the embryo are formed by the fusion of myoblasts which may already be committed to form specific fibre types.

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It is still unclear, however, whether different muscle fibre types in all species are derived from a single or distinct myogenic cell lineage. The adult muscle fibre types have been shown to modify their biochemical and physiological properties in response to changes in innervation (Buller *et al*., 1960; Dhoot *et al*., 1981; Pette & Vrbova, 1985), hormones (Izumo *et al*., 1986) or stretch (Loughna *et al*., 1990), and it is possible that such factors contribute to the differentiation of different muscle fibre types during foetal development. Innervation, however, is unlikely to have a similar influence during embryonic development before neuromuscular junctions are fully developed. Neural deprivation during foetal development nevertheless has been shown to reduce muscle growth and the proportion of slow myotubes in both rodent and avian species, although a small number of slow myotubes persists in such muscles (Crow & Stockdale, 1986; Phillips *et al*., 1986; Condon *et al*., 1990; Dhoot, 1994b).

The possibility that muscle fibre diversity arises from the presence of distinct myogenic cell lineages is particularly supported by studies of avian species in which the existence of different myogenic cell lineages is well documented (Miller & Stockdale, 1986; Stockdale & Miller, 1987; Stockdale, 1992). A number of studies by Stockdale and colleagues have demonstrated the existence of distinct fast and slow myogenic cell lineages identified on the basis of differential myosin heavy chain expression. Unlike the avian species, fast and slow myogenic cell lineages using similar analyses were not observed in human foetal or adult skeletal muscle cell cultures (Cho *et al*., 1993; Edom *et al*., 1994). All myotubes *in vitro* were reported to co-express fast and slow myosin heavy chains. It is not clear whether such differences seen in human and chicken skeletal muscles reflect a species difference or the differing complexities of myosin heavy chains in these two species. We hypothesized that some of the observed species differences may result from different culture conditions or the differences in molecular markers used to identify fast and slow muscle fibre types. Both *in vivo* and *in vitro* studies of embryonic avian muscles have been helped by the easier identification and availability of a much greater number of different slow muscle cell type-specific antibodies that distinguish slow myosin heavy chains from the cardiac muscle type myosin heavy chains which are much more widely expressed during embryonic development. This differs from mammalian species in which usually a single slow myosin heavy chain is detected during early foetal development, although some additional slow myosin heavy chains have been reported during later development (Hughes *et al*., 1993). The slow myosin heavy chain expression in mammalian foetal skeletal muscles thus cannot be distinguished from the expression of ventricular myosin heavy chain, which unlike the avian species (Stewart *et al*., 1991) is encoded by the same gene in mammals (Lompre *et al*., 1984). It is highly probable that some further slow myosin heavy chain diversity also occurs in early foetal mammalian skeletal muscles (Dhoot, 1986, 1994a), but is not apparent from most slow myosin heavy chain antibodies used at present.

To determine if species-specific or general mechanisms for both human and avian species are employed to generate muscle fibre type diversity during early foetal development, we investigated human myogenic cell diversity *in vitro* by isolating the muscle precursors from foetal quadriceps muscles. The phenotype of such myotubes was analysed immunocytochemically using a set of myosin heavy chain antibodies prepared in our laboratory. As for the avian species, this study demonstrated the human myogenic diversity *in vitro*.

Materials and methods

Muscle cell cultures

Muscle cell cultures were prepared from human foetal quadriceps muscles at 10–18 weeks of gestation. Muscle tissues were dissected out in calcium/magnesium-free phosphate buffered saline (PBS) and transferred to Ham's nutrient medium F10 (Life Technologies). Muscles were then cut into small fragments and digested in 2% Trypsin (Sigma Chemical Co.) for 10 minutes at room temperature. Muscle suspension was titrated with a Pasteur pipette and the cell suspension collected in 15% foetal calf serum (FCS) in F10 medium. A single cell suspension was created by passing the suspension a few times through a 19 gauge needle and by filtering it through a single layer of 0.45 μm Nitex filter. Cells were plated on gelatin-coated petri dishes. In some experiments, petri dishes that were uncoated or coated with standard matrigel or growth-factor reduced matrigel preparations (Becton Dickinson Labware) were also used as described by Dusterhoft & Pette (1993).

For high-density muscle cell cultures, a 25 μl cell suspension containing approximately 1000 cells was spotted into a 4 cm petri dish. Petri dishes with 2–3 spots each were incubated for 1 hour at 37°C with 5% CO_2, and then flooded gently with 1.5 ml of DMEM or Ham's F10 nutrient medium containing 15–20% FCS, 0.5% chick embryo extract and 1% penicillin, streptomycin and fungizone. The cell cultures were grown in this growth medium for 3–15 days, after which it was replaced with differentiation medium for 3–4 days. The differentiation medium was either F10 medium with 3% FCS or DMEM medium with 10 μg ml^{-1} insulin and 100 μg ml^{-1} transferrin (Sigma Chemical Co.).

For clonal analysis, microfragments of muscle were plated on to gelatin-coated petri dishes and grown in growth medium for 2–3 days at 37°C with 5% CO_2. The cells from such PBS-washed cultures were removed by 0.025% trypsin and resuspended in growth medium. After washing with the growth medium and centrifugation, the resuspended pellet was filtered through 0.45 μm Nitex membrane. To obtain non-overlapping clones, cells were plated at a density of 3–6 cells per cm^2 in 1:1 fresh and filtered conditioned medium. Clonal cells were grown for 8–21 days with medium change every two days. The growth medium was then replaced with differentiation medium for a further growth of myotubes for 3–5 days.

Immunocytochemical procedure

For immunocytochemical staining, the muscle cell cultures growing on petri dishes were washed three times with PBS and fixed in either 70% methanol for 10 minutes or in 95% acetone for 15 seconds. The fixed cultures were either stained with antibodies or stored at −70° until required. Cell cultures were stained with different antibodies, diluted in 10% FCS in PBS Tween, using an immunoperoxidase or double immunofluorescence procedure. Antibody F2 was used to investigate the expression of fast myosin heavy chains. This antibody reacts with both adult and developmental isoforms belonging to the fast class of myosin heavy chain (Williams & Dhoot, 1992). Foetal myosin isoforms are usually present in all *in vitro* myotubes prepared from early

foetal muscles and *in vivo* embryonic muscles. Fast myosin heavy chain antibodies usually do not enable the distinction between myotube types in early developing muscle. We therefore used a single fast myosin heavy chain antibody which detects the whole class of fast myosin heavy chains. Two different antibodies, 96J and 9812, were used to investigate the expression of slow muscle type myosin heavy chains (Kilby & Dhoot, 1988). For double immunofluorescence, cell cultures were first stained with either slow or fast myosin heavy chain antibodies using either FITC- or TRITC fluorochrome-labelled secondary antibodies. After washing, the same cultures were then treated with the second primary antibody (fast if slow myosin heavy chain antibody was used in the first instance, but slow if fast myosin heavy chain was used first) which was then detected by the second fluorochrome-labelled antibody. After washing, treated sections were mounted in glycerol medium (Citifluor) and photographed using an Olympus microscope.

For immunocytochemical analysis of muscle tissues, 6 μm thick sections were cut from adult or foetal muscle blocks frozen in liquid nitrogen. Serial sections were then stained by fast and slow myosin heavy chain antibodies by the immunoperoxidase procedure.

Results

Distribution of fast and slow myosin heavy chains in adult and foetal human skeletal muscles

The expression of fast and slow myosin heavy chains in individual skeletal muscle fibres was analysed by staining of serial sections with antibodies to slow and fast classes of myosin heavy chains. Slow and fast myosin heavy chains in adult skeletal muscles were usually segregated into type I and type II fibres (Fig. 1). As expected, unlike the adult muscle, the fast class of myosin heavy chain in early foetal skeletal muscles is detected in all myotubes, whereas the expression of slow myosin heavy chain is generally restricted to a subpopulation of fibres (Fig. 2), most of which go on to form type I fibres (Dhoot, 1986).

Muscle cell phenotype of high-density human muscle cell cultures

Human muscle cell cultures were prepared from quadriceps muscles at different stages of foetal development, ranging from 10–18 weeks of gestation. The muscle cells at high densities (approx. 5×10^4 cells per cm^2) were routinely grown on gelatin-coated petri dishes, although their growth on other

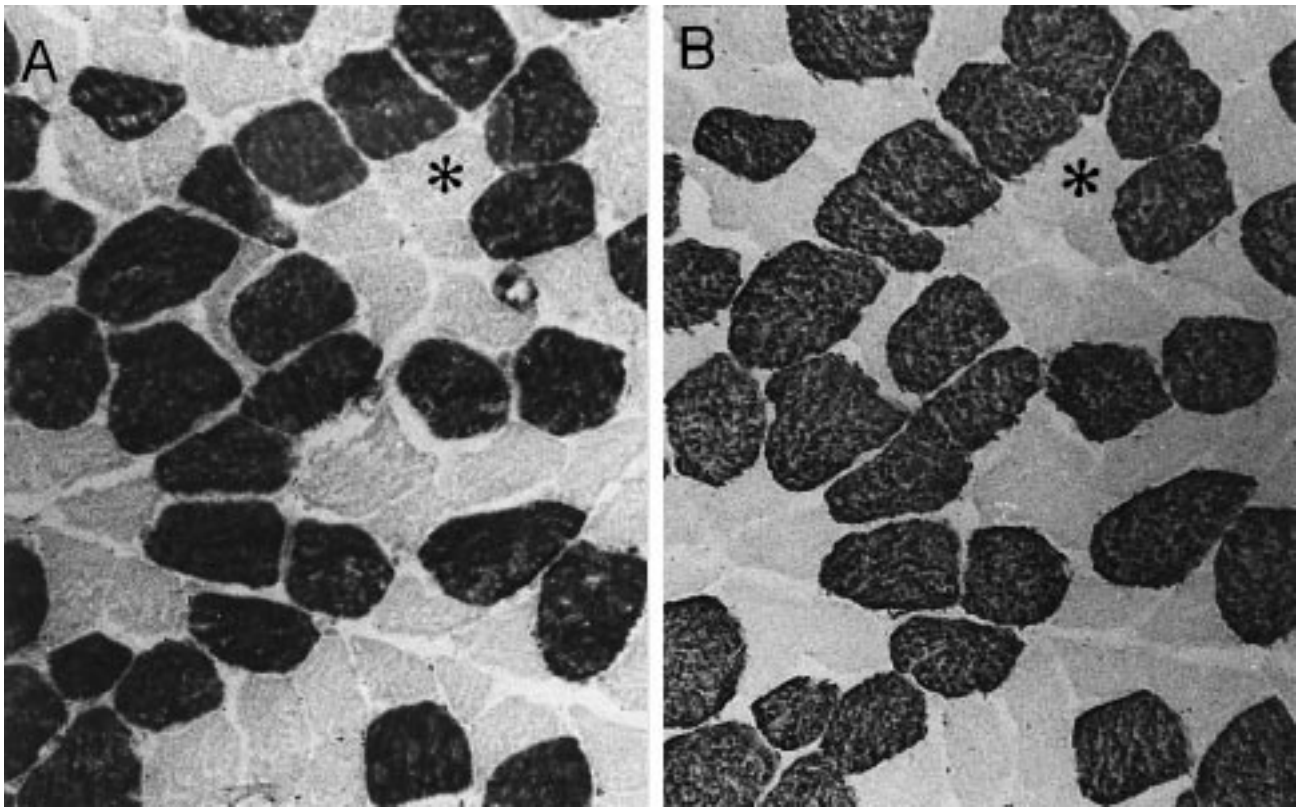


Fig. 1. Serial sections of adult human biceps muscle are stained with (B) an antibody (96J) to slow myosin heavy chain and (A) for histochemical myosin ATPase after acid preincubation at pH 4.3. Only type I fibres are dark after both staining procedures, while type II fibres remain unstained. Identical cells in A and B are labelled by asterisks.

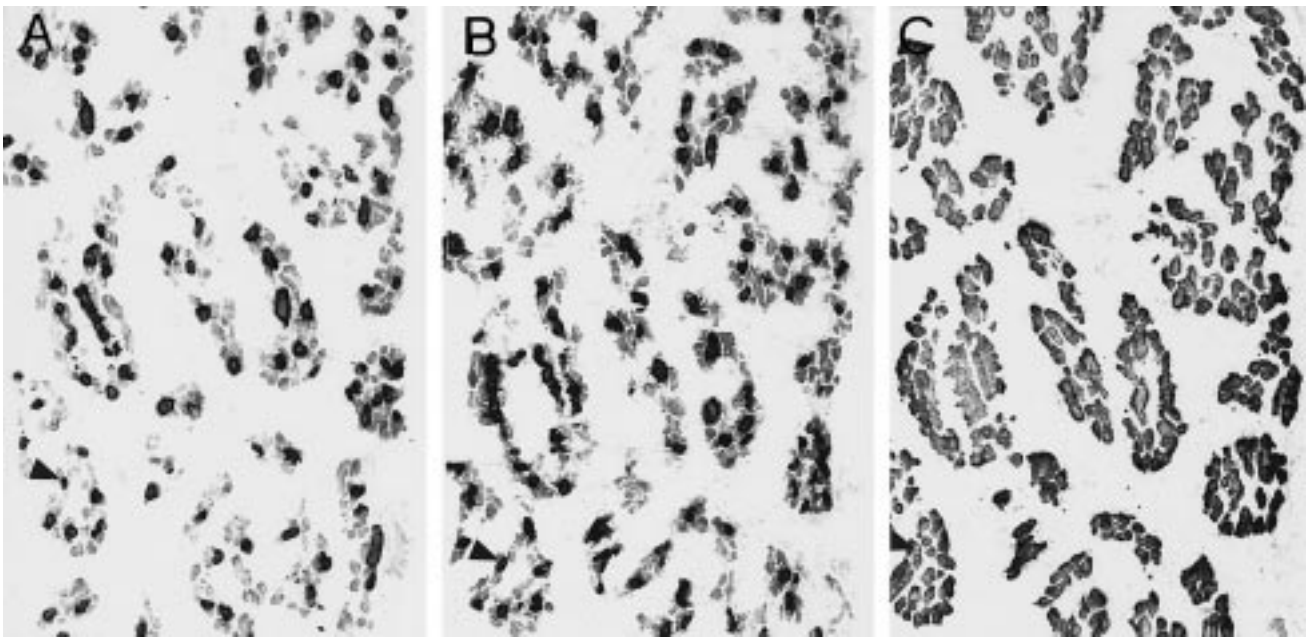


Fig. 2. Serial sections of human quadriceps muscle at 15 weeks gestation, stained with two different slow myosin heavy chain antibodies, 96J (A) and 9812 (B) and a fast myosin heavy chain antibody F2 (C) by the immunoperoxidase procedure. Antibody F2 stained all myotubes dark whereas the antibody to slow myosin heavy chain stained only some myotubes very dark. Identical cells are labelled by similar arrows.

substrata was also tested. The myogenic cell cultures were grown for 6–18 days before staining with antibodies to fast and slow isoforms of myosin heavy chain by either immunoperoxidase or double immunofluorescence procedures. Antibodies to fast myosin heavy chains in such cultures stained virtually all myotubes very dark. Unlike antibodies to fast myosin heavy chain, antibody 96J to slow myosin heavy chain stained only a small proportion of the myotubes present in these cultures (Fig. 3). The differential staining of only a subset of myotubes with antibody 96J was further confirmed by another antibody, 9812, to slow myosin heavy chain. Although it was clear that slow myosin heavy chain antibodies stained only a proportion of the myotubes, it was further established by double immunofluorescence procedure in which the presence of myotubes unstained by antibodies to slow myosin heavy chain could be seen directly by their positive staining with antibodies to fast myosin heavy chains in the same area (Fig. 3C, D). Size and morphology of slow myosin heavy chain positive and negative myotubes in high-density cultures did not appear to differ greatly.

Changes in the proportions of slow muscle cells occur during normal *in vivo* development of foetal skeletal muscles in most species (Dhoot, 1986; Cho *et al*., 1993). To determine if such a change could also be detected in the slow myotubes *in vitro*, the muscle cell cultures were prepared from different stages of human foetal development. The myotubes prepared from 10–18 weeks of gestation showed some variation in the proportion of myotubes staining for slow myosin heavy chain in different cultures, but there was no consistent change over the limited time period investigated in this study.

The effect of gelatin and matrigel coating of the petri dishes on growth and differentiation of muscle cells

To determine if the coating of petri dishes with different extracellular components affected either the growth or the muscle cell phenotype *in vitro*, muscle cells were grown on petri dishes either uncoated or coated with gelatin or matrigel or growth factor-reduced matrigel. Some muscle cell growth was observed even on uncoated petri dishes, although it was much poorer in comparison with both gelatin and matrigel-coated petri dishes (Fig. 4). The best muscle cell growth was observed on growth factor-containing matrigel-coated dishes. Although the growth of muscle cell cultures reflected the coating with different extracellular components, the presence of slow myotubes was observed under all conditions including the uncoated surfaces (Fig. 4). The proportion of slow myotubes did not appear to vary significantly with the nature of the surface coating used in this study.

Muscle cell phenotype of clonal myogenic cell cultures

To determine if the myotube diversity seen in high-density muscle cell cultures was reproduced in individual clonal populations, and how many different

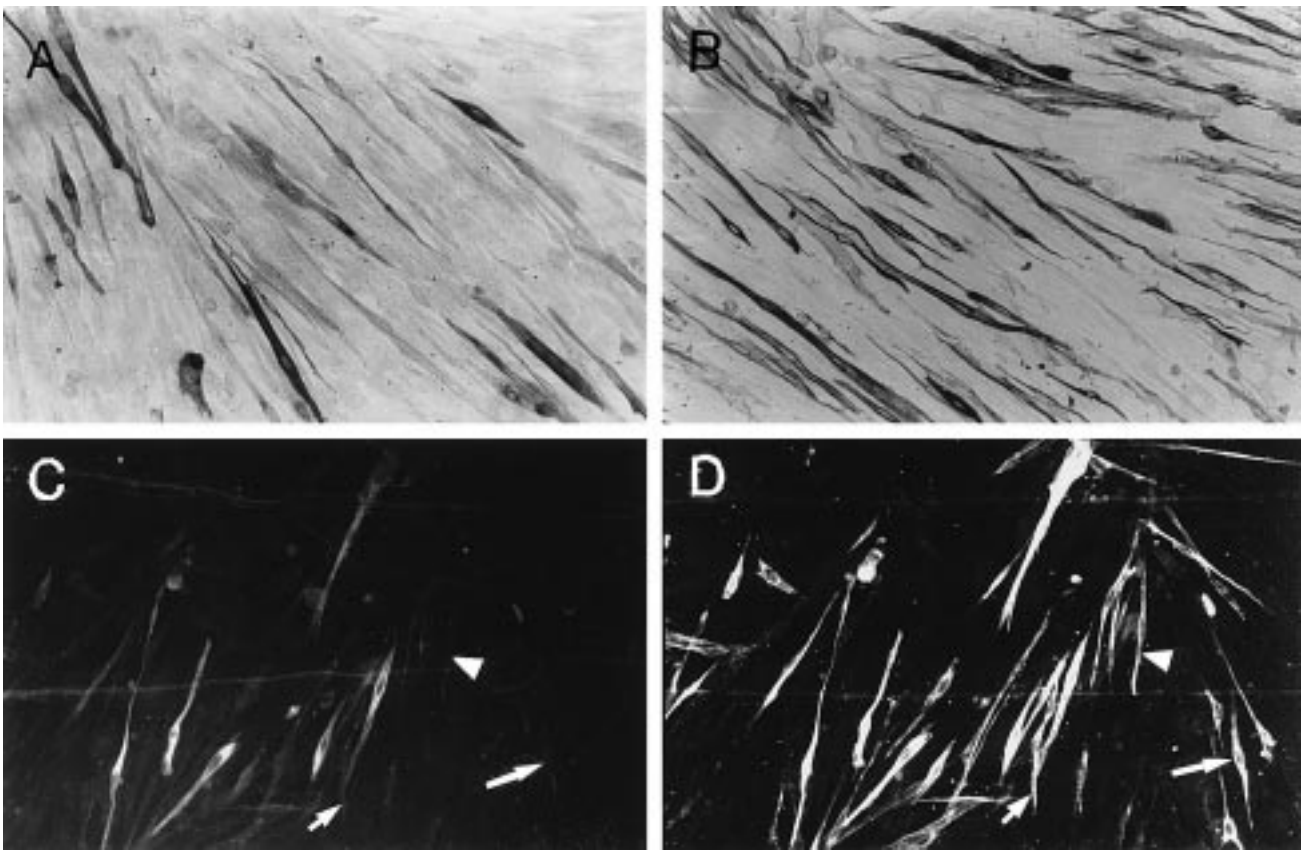
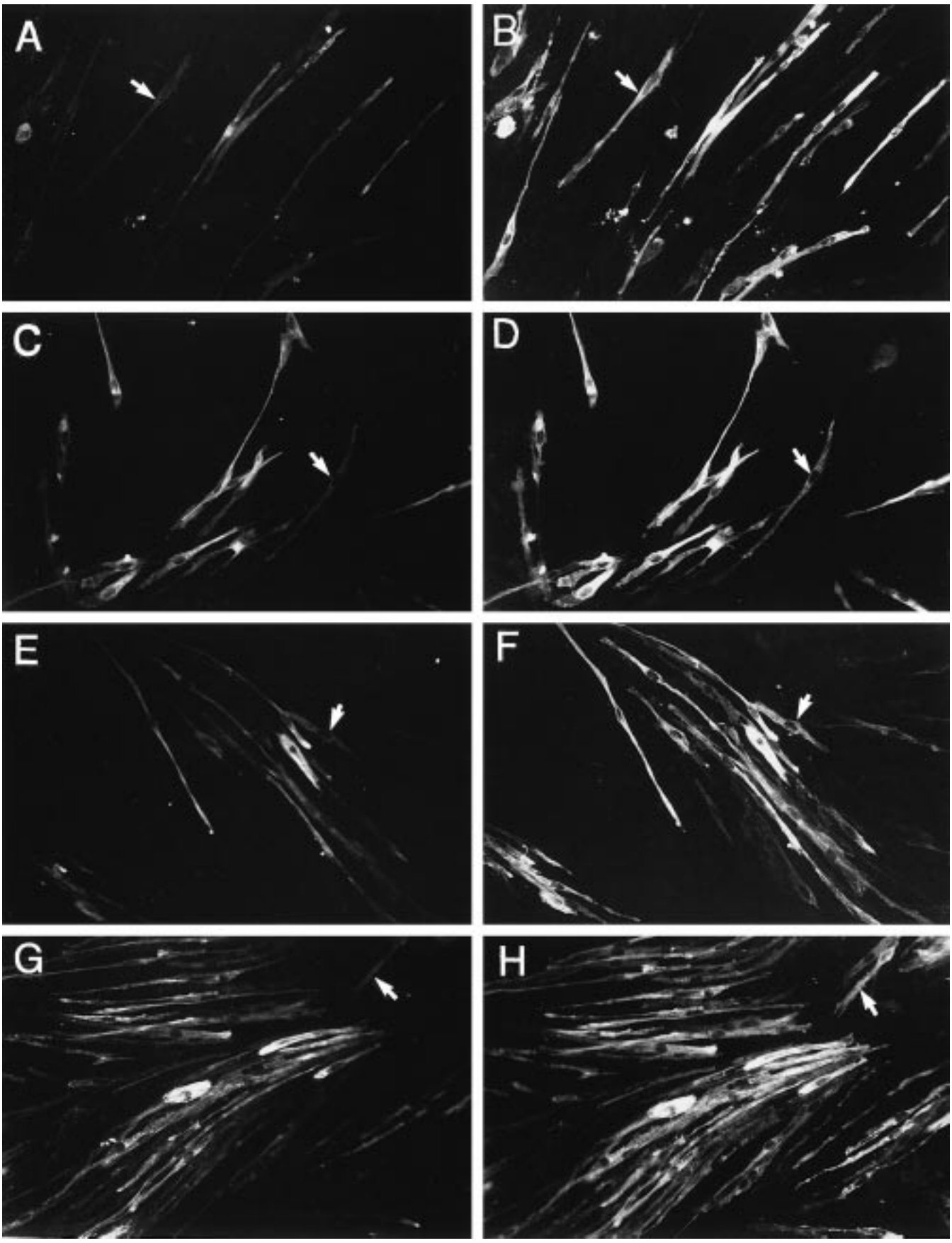


Fig. 3. Muscle cell cultures prepared from quadriceps muscle of 13-week human foetus, after six days of growth stained with slow myosin heavy chain antibodies (A with 96J, C with 9812) and fast (B, D) myosin heavy chain antibodies (F2), using immunoperoxidase (A, B) or double immunofluorescence (C, D) procedures. Identical myotubes in C and D are labelled by similar arrows.

types of myogenic cell lineages were identifiable *in vitro*, muscles were grown at clonal densities (3–6 cells per cm^2) for 8–21 days. The myogenic clones were stained with antibodies to fast and slow myosin heavy chains. As expected, and similar to high-density muscle cell cultures, nearly all myotubes in all clones stained positive with antibodies to fast myosin heavy chains, although the level of staining intensity was lower in a small number of myotubes or myocytes. The staining with antibodies to slow myosin heavy chains, in contrast, distinguished two types of clones. In 15% of the 42 myogenic clones examined, nearly all myotubes within a clone stained dark with antibodies to slow myosin heavy chain (Fig. 5). In a second group of clones, which represented 85% of the clones studied, only some myotubes stained dark with antibodies to slow myosin heavy chain, with the other myotubes remaining unstained (Fig. 6). Such a staining pattern for these clones was observed during both early (8 day) and later stages (21 days) of clonal growth. This heterogeneous staining pattern of clones with antibodies to slow myosin heavy chain did not appear to change over the 8–21 day period of growth examined in this study, although the proportion of myotubes staining for slow myosin heavy chain varied in different clones.

Discussion

Adult skeletal muscles in most species are composed of two main classes of fibre types, called type I and type II, or slow and fast. These two muscle fibre types in the adult usually express either slow or fast isoforms of a number of regulatory and contractile proteins, including myosin, which equip them with distinct specialized contractile or force-generating properties (Pette & Staron, 1990; Schiaffino & Reggiani, 1996). It is not clear, however, whether these two fibre types, characteristic of fully differentiated muscles, are derived from distinct fast and slow myogenic cell lineages, or if a single muscle cell lineage is modified gradually by extrinsic factors encountered during development. Evidence both for and against these two different possibilities has been presented based on *in vivo* and *in vitro* studies. For example, the rat satellite cells in regenerating soleus, a slow muscle, did not express slow myosin heavy chains in the absence of innervation (Whalen *et al*., 1990), indicating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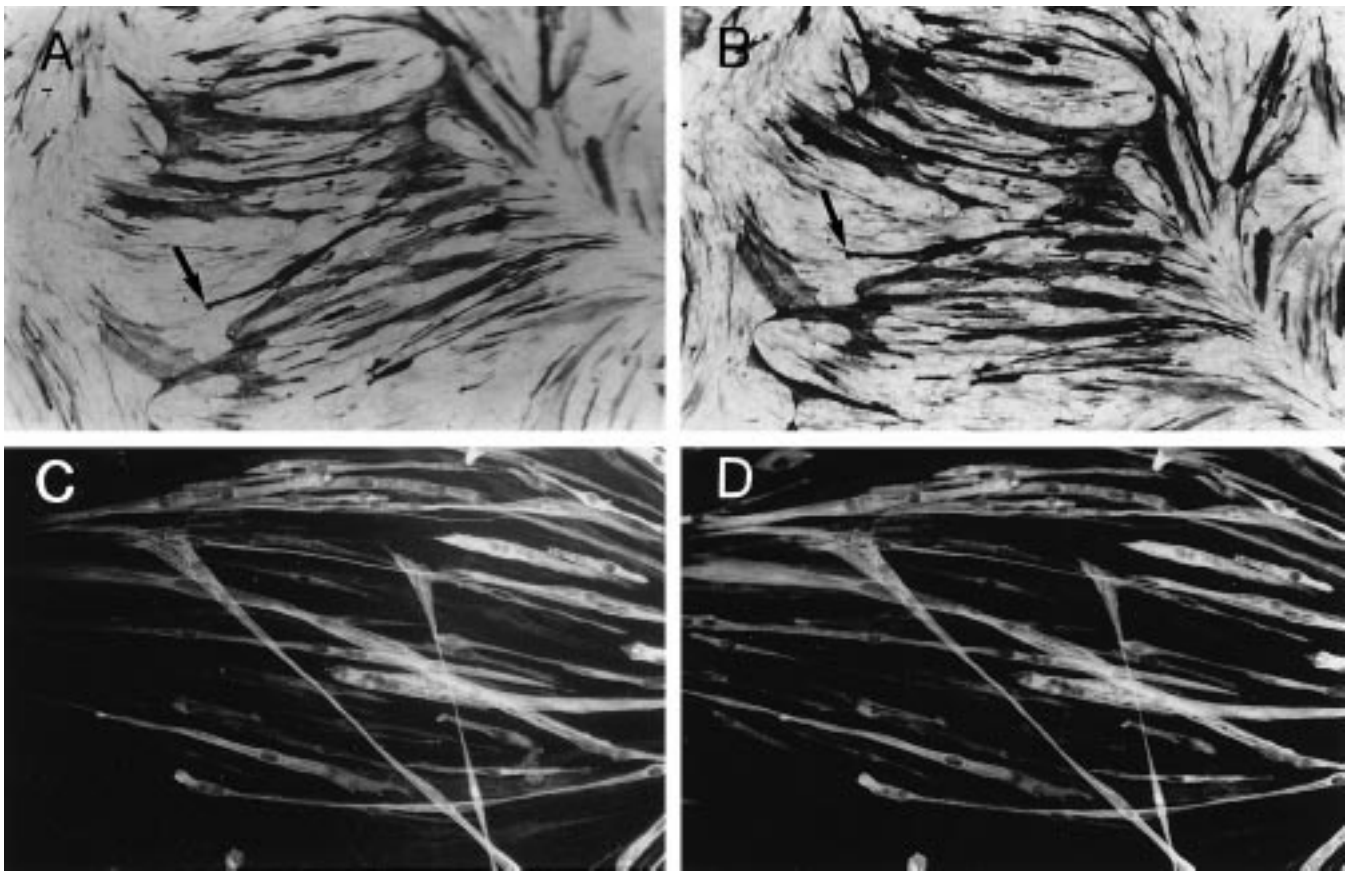

Fig. 5. Two different myogenic clones prepared from quadriceps muscle of 12-week (A, B) and 15-week-old foetuses (C, D), subcultured for 15 (A, B) and 10 (C, D) days after three days of primary culture, stained with 96J and F2 antibodies to slow (A, C) and fast (B, D) myosin heavy chains by double immunofluorescence procedure. Most myotubes stain positive for both slow and fast myosin heavy chains. Identical myotubes in A and B are labelled by similar arrows.

that the pattern of myosin heavy chain expression is not determined by satellite cells but rather the innervation pattern of the muscle. Muscle cells in such non-innervated regenerating soleus have been reported to express only fast muscle type myosin heavy chains and hence a single fibre type. Unlike myosin, the presence of slow troponin I positive and negative myotubes has been observed in non-innervated regenerating soleus and plantaris rat muscles (Dhoot & Perry, 1982). The studies of Hoh and Hughes (1988) have also demonstrated the expression of a specialized muscle specific myosin, called superfast, in some jaw muscle regenerates in both their own and inappropriate muscle beds in the absence of innervation.

The *in vitro* studies of avian muscles, mainly by Stockdale and colleagues (Miller & Stockdale, 1986; Stockdale & Miller, 1987; Schafer *et al*., 1987; Stockdale, 1992), have clearly demonstrated the importance of distinct embryonic fast and slow myogenic cell lineages in the establishment of fast and slow muscle fibre types during foetal development. Similar studies of mammalian muscles, however, have not been supportive of such a view that the fast and slow myogenic cell lineages contribute to muscle fibre type diversity seen in the adult, particularly in human muscles. For example, human muscles from all stages of development have been reported to generate a single myogenic cell type when isolated and grown *in vitro* as they all co-express fast and slow myosins (Cho *et al*., 1993; Edom *et al*., 1994). The *in vitro* myotubes prepared from embryonic mouse muscles have also been reported to express both fast and slow myosin heavy chains in all myotubes, but they differ from human muscles by producing these slow myosin heavy chain expressing cells from only

Fig. 4. Muscle cell cultures prepared from quadriceps muscle of 14-week human foetus grown on uncoated (A, B), gelatin-coated (C, D), growth factor-reduced matrigel (E, F) and standard matrigel (G, H) coated petri dishes for six days, stained with 96J and F2 antibodies to slow (A, C, E, G) and fast (B, D, F, H) myosin heavy chains. Identical myotubes in each set (A&B, C&D, E&F, G&H) are labelled by arrows.

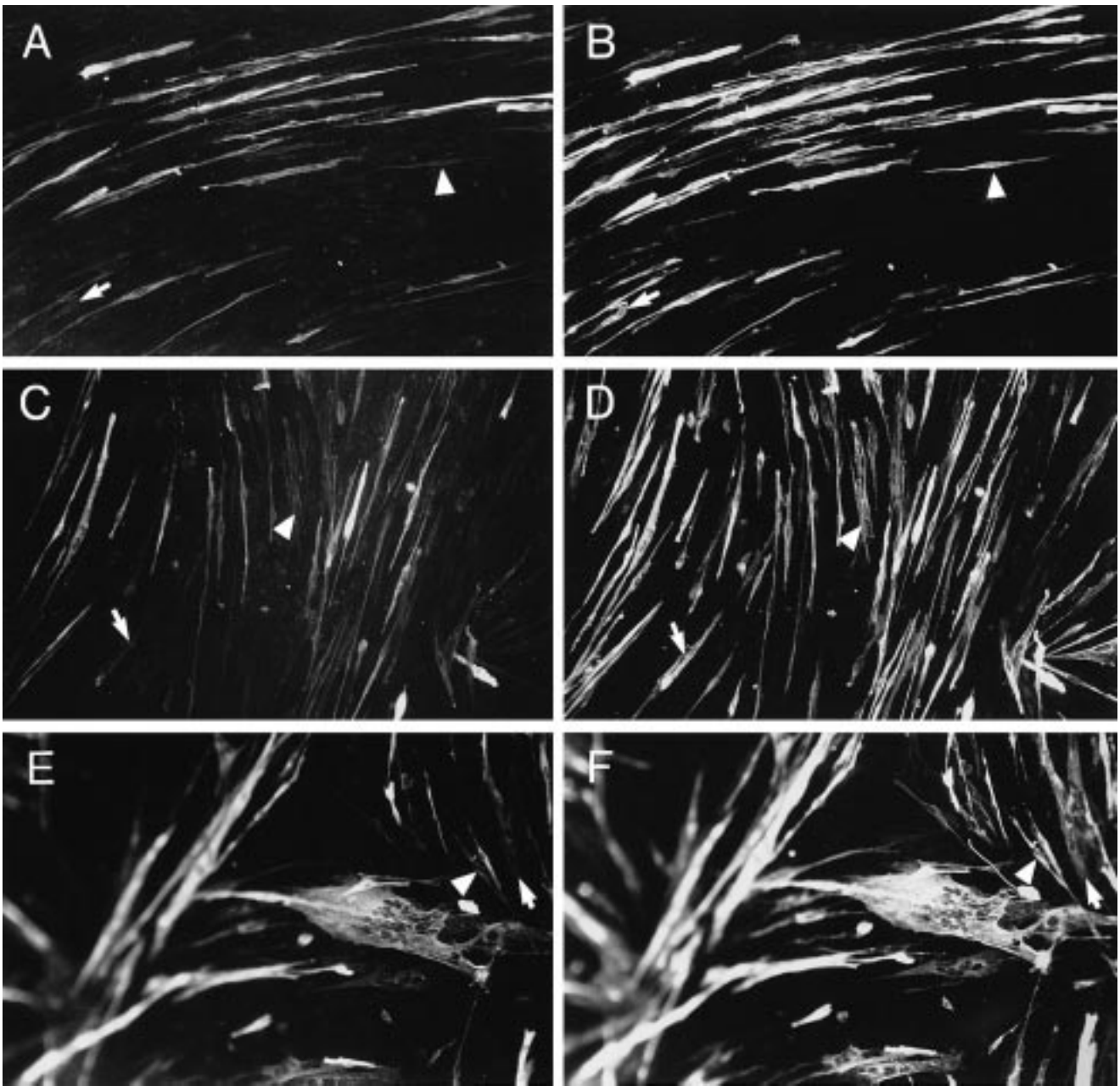

Fig. 6. Three different myogenic clones from quadriceps muscles of 13-week human foetuses subcultured for 16 days after three days of primary culture, stained with 96J and F2 antibodies to slow (A, C, E) and fast (B, D, F) myosin heavy chains by double immunofluorescence procedure. The antibody to fast myosin heavy chain stained all myotubes dark. The proportion of slow myosin heavy chain positive myotubes varies in different myotubes. Identical myotubes in each set (A&B, C&D, E&F) are labelled by similar arrows.

early embryonic muscles (Vivarelli *et al*., 1988). The muscle precursors isolated from later foetal mouse muscles grown *in vitro* express only fast myosin heavy chains.

Based on the expression pattern of slow skeletal muscle type myosin heavy chain, our *in vitro* study of human foetal skeletal muscles clearly demonstrates the presence of two distinct myotube types, one staining positive for slow myosin heavy chain while the other remaining unstained for this myosin heavy chain. Fast myosin heavy chain antibodies stained all myotubes dark because all myotubes express foetal and possibly neonatal muscle myosins during early development, which due to their greater homology with adult fast muscle type myosin heavy chains are often recognized by a large number of fast myosin heavy chain antibodies. It is assumed that the presumptive fast muscle cells express only fast muscle type myosin heavy chains, whereas presumptive slow myotubes express slow and fast muscle like foetal

myosins. This is similar to early *in vivo* developing foetal muscles which show similar staining patterns with slow and fast myosin heavy chain antibodies during early development. In rapidly developing *in vivo* muscles, the expression of foetal myosins is gradually suppressed with time, becoming more easily apparent in presumptive slow muscle cells, unlike presumptive fast muscle cells in which fast muscle-like foetal myosins are gradually replaced first by fast muscle-like neonatal and then by adult fast myosins. Our study thus differs from Cho and coworkers (1993) and Edom and coworkers (1994), which reported the expression of both fast and slow myosin heavy chains in all high-density myotubes prepared from foetal and postnatal muscles. The precise reason for this difference is not known, but may arise from differences in culture conditions or the differing extent of myosin heavy chain diversity identified by the different antibodies.

The slow myosin heavy chain expression in *in vitro* myotubes has been reported to be influenced by the extracellular matrices. For example, the presence of slow myosin heavy chain in some rat myotubes was detected only when the petri dishes on which the muscle cells grew had been coated with matrigel (Dusterhoft *et al*., 1990; Dusterhoft & Pette, 1993). We tested the effect of gelatin and matrigel coating on the expression of slow myosin heavy chain in human *in vitro* myotubes. While coating with various substrata improved the growth of myotubes, particularly with growth factor-containing matrigel, the expression of slow myosin heavy chain in human foetal muscle cell cultures did not appear to be dependent on the matrigel coating since the presence of slow myosin heavy chain in some myotubes was detected even on uncoated petri dishes. The proportion of slow myosin heavy chain expressing myotubes did not appear to change with matrigel coating. The differences in the expression patterns of slow myosin heavy chain in different human muscle cell culture studies may therefore not be entirely caused by interaction with extracellular components, since a subset of myotubes remained unstained in matrigel-coated dishes despite much improvement of growth.

In chicken, two different types of myotubes, fast and slow, are apparent *in vitro* only in cell cultures prepared from early embryonic muscles, less than 8–10 days *in ovo* (Miller & Stockdale, 1986). Most myotubes prepared from muscles of embryos older than 8–10 days express only fast myosin heavy chains. The presence of slow myotubes, however, can be detected in cultures prepared from the adult avian slow tonic muscle called anterior latissimus dorsi (Feldman & Stockdale, 1991). Similarly, studies of adult rat and mouse muscles have also demonstrated the presence of slow myosin heavy chain in a small proportion of the myotubes in cultures prepared from slow muscle (Dusterhoft & Pette, 1993; Rosenblatt *et al*., 1996). The presence of slow myotubes in our study was detected in all cultures prepared from human muscles at 10–18 weeks of gestation period. In our study of human foetal skeletal muscles, the proportion of myotubes staining for slow myosin heavy chain did not significantly alter over the 10–18 week gestation period examined. Since we did not test the later stages of foetal or postnatal development, we do not know whether dramatic changes similar to avian and murine species (Miller & Stockdale, 1986; Vivarelli *et al*., 1988) would have become apparent in such cell cultures. The dramatic reduction in the number of slow myotubes formed from muscle precursors isolated from later foetal or postnatal muscles in both avian and murine species may reflect the greatly reduced proportions of slow fibres, as a result of their dilution with secondary myotubes, in most *in vivo* muscles of these species during later development. This differs from the human in which most normal muscles go on to have fairly similar proportions of fast and slow muscle fibre types during both late foetal and postnatal stages.

To confirm the observations of human myotube diversity seen in high-density cell cultures, we further investigated the expression patterns of fast and slow myosin heavy chains in clonal myogenic colonies. Based on *in vivo* and our high-density *in vitro* studies, we expected to see two main types of myogenic cell colonies with the antibodies used in this study, one expressing only fast myosin heavy chains while the other expressing both fast and slow myosin heavy chains. We observed one group of myogenic clones in which most myotubes expressed both fast and slow myosin heavy chains as expected. The level of slow myosin heavy chain in a small number of myotubes or myocytes in some colonies was lower. We did not find a second group of myogenic clones expressing fast myosin heavy chain only. Instead, we found another group of myogenic clones in which nearly all myotubes expressed fast myosin heavy chains, but a subset of the myotubes expressed slow myosin heavy chain in addition. The clonal populations would normally be expected to be homogeneous, although cell diversification within the myogenic lineage has been reported to arise with time following further *in vitro* growth or subculturing in some avian species (Schafer *et al*., 1987). Using non-myosin markers to distinguish cell types, Baroffio and colleagues (1995) also demonstrated heterogeneity in the progeny of single human muscle satellite cells. Schafer and coworkers (1987) reported *in vitro* generation of two types of myoblasts from a single quail myogenic progenitor cell. They observed the conversion of a subset of homogeneously-staining fast myotubes to slow myosin heavy chain staining myotubes with subculturing or extended periods of growth, and hence an increase

in number of slow myosin heavy chain positive myotubes with time and some differences in myotube morphology. We observed the heterogeneity of some human myogenic clones within a short period of 8–10 days of growth, although all clonal cultures investigated in this study were subcultures which may have contributed to this heterogeneity.

In conclusion, antibodies to slow myosin heavy chains clearly identified distinct presumptive slow and fast human myotubes *in vitro*, and therefore highlight the role of different myogenic cell lineages in establishing the *in vivo* fibre type diversity not only in avian species but also in human muscle. Myogenic cells, however, retained the ability to modify their muscle cell phenotype further. Muscle cell diversity created by distinct fast and slow myogenic cell lineages during early stages of development must be modulated by extrinsic factors, such as innervation, stretch and hormonal changes, during later stages of *in vivo* development.

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