

Cellular insertion of primary and secondary myotubes in embryonic rat muscles

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Summary

Mammalian muscles develop from two populations of myotubes; primary myotubes appear first and are few in number; secondary myotubes appear later and form most of the muscle fibres. We have made an ultrastructural study to investigate how primary and secondary myotubes in embryonic rat muscles transmit tension during the period of their development. Primary myotubes extend from end to end of the muscle from the earliest times, and attach directly to the tendon. In contrast, newly formed secondary myotubes are short cells which insert solely into the primary myotubes by a series of complex interdigitating folds along which adhering junctions occur. As the secondary myotubes lengthen and mature, their insertion is progressively

transferred from the primary myotube to the tendon proper. We suggest that this variable insertion of immature secondary myotubes, combined with complex patterns of innervation and electrical coupling in developing muscle, makes it difficult to predict the overall contribution of secondary myotubes to muscle tension development. This work extends other studies showing the unique relationship between a primary myotube and its associated secondary myotubes, indicating that these may constitute a developmental compartment.

Key words: primary myotubes, secondary myotubes, insertion, myotendinous junction, ultrastructure, rat embryo.

Introduction

In adult skeletal muscles, contractile force exerted by the myofilaments is transmitted to the dense collagenous connective tissue of the tendon (and thence to the skeleton) at the ends of the muscle fibres, at the muscle–tendon junction (Goss, 1944; Gelber *et al.* 1960; Trotter *et al.* 1983). The situation in embryonic and neonatal muscles is less straightforward. Skeletal muscle fibres develop asynchronously, the initial population of embryonic muscle fibres (primary myotubes) forming in a short period and subsequently acting as a scaffold on which the later developing secondary myotubes are progressively assembled during the foetal period of development (Kelly and Zacks, 1969). From the time of their first appearance, primary myotubes stretch from end to end of the muscle (Dennis *et al.* 1981) allowing them to insert directly onto the tendon. Secondary myotubes, however, originate as short, initially binucleate, cells in the midregion of the embryonic muscle (Ontell and Kozeka, 1984) and thus have no apparent relation to the tendon. How then are growing secondary myotubes maintained in their longitudinal orientation, and how do they transmit force?

The following work is a detailed ultrastructural study in which we describe the insertion of secondary myotubes at various stages of growth. For comparison, a brief study of the insertion of primary myotubes is included. Our results suggest that while primary myotubes form early attachments to the connective tissue, young secondary myotubes initially attach only to a single primary myotube via a complex system of inter-folding cellular ridges and primitive adhering junctions. Tension exerted by the secondary myotubes will therefore be transmitted through the elastic element of another myotube, rather than through the inelastic element of the collagenous connective tissue.

Materials and methods

All studies used white Wistar rat embryos. Pregnancies were dated by the appearance of a copulatory plug, 9 a.m. on the day the plug was found being designated E0.

Primary myotube insertion was examined in sternomastoid muscles taken from embryonic day 16 and 21 rats (E16 and E21) (3 animals at each date). Secondary myotube insertion was examined in a detailed serial section study of a single IVth

lumbrical muscle taken on E21, but observations in the text also draw on information from about 30 other IVth lumbrical muscles and 10 sternomastoid muscles at a range of developmental stages. E21 was chosen for the serial section study because, at this age, the IVth lumbrical muscle contains numerous secondary myotubes which range in maturity from newly formed binucleate cells to well-developed myotubes stretching the full length of the muscle. The entire length of the muscle is approximately 1 mm.

Electron microscopy

Foetuses were fixed by perfusion of 5 ml of warm fixative through the heart and the dissected muscles were then immersed in cold fixative for 2 hours. The fixative contained 1% paraformaldehyde, 1% glutaraldehyde, 120 mM-NaCl, 5 mM-CaCl₂ and 20 mM-Hepes buffer, at pH 7.2. Tissues were postfixed in 2% osmium tetroxide, block stained with 2% aqueous uranyl acetate and dehydrated through graded alcohols and propylene oxide before embedding in TAAB epoxy resin. Sections were collected on single-slot formvar-coated grids and viewed with a Philips 410 electron microscope.

The serial section study commenced at the midbelly of the muscle, near the point of nerve entry, and continued through to the tendon. The entire cross-section of the muscle was serially (section thickness 90 nm) or semiserially (section interval 2.7 µm) sectioned, for a total length of 400 µm. If the commencement point is designated as zero along the longitudinal axis of the muscle, then the sectioning proceeded as follows; 0–10 µm serial sections, 10–105 µm semiserial, 105–160 µm serial sections, 160–220 µm semiserial, 220–400 µm serial sections.

Analysis of the material proceeded at three levels. The most general information came from a series of 20 µm spaced photomontages which included approximately one-third of the muscle cross-section and covered the initial 200 µm of the sectioned length. Secondly, a group of four adjacent myotube 'clusters' (defined as a primary myotube plus related secondary myotubes and mononucleated cells sharing a common basal lamina) were selected for detailed study and photographed at intervals of 2.7 µm along the entire 400 µm length. Finally, one of the four clusters was used to make detailed serial three-dimensional reconstructions of selected features.

Three-dimensional reconstruction

Three-dimensional reconstruction was performed with computer graphics using a Macintosh Plus microcomputer (Apple), a digitizing tablet (EDT11 Hipad, Houston Instrument), a color printer (ImageWriter 11, Apple) for working documents, and a laserprinter (Apple) for presentation graphics. Two Pascal programs were developed for i) digitization and fitting of the serial sections and ii) graphical presentation of the reconstructed tissue.

Cell contours were traced from each micrograph in a series and stored on hard disc along with ancillary information such as cell type (primary myotube, secondary myotube, mononucleated cell). Contours of sequential sections were aligned by the operator, using an interactive process similar to that described by Street and Mize (1983). The reconstruction program used 3-D graphics (Newman and Sproull, 1978) to display cells at any possible angle of view and with various perspectives. Wire-frame representation, hidden face removal and a range of filling patterns were available and could be mixed to provide the most readable pictures. Nuclei were drawn as 'inlines' within the cell contours, and in the reconstructions are represented as holes within the cells.

Results

Insertion of primary myotubes

The insertion of primary myotubes was examined in E16 and E21 sternomastoid muscles and E21 IVth lumbrical muscle. Sternomastoid was preferred at the earlier date because it is well defined and easily dissected. The first filamented cells appear in sternomastoid on E15; at E16, the muscle is composed solely of primary myotubes, with the earliest secondary myotubes appearing on E17 (M. J. Duxson, personal observations).

Primary myotubes in E16 sternomastoid muscles usually terminated as single processes which could be either extremely attenuated (Fig. 1A) or bulbous (Fig. 1B) in shape. The region of the tendon contained many fibroblasts, but little organised extracellular material. The dense bundles of banded collagen fibrils which are prominent at adult myotendinous junctions were not observed. Extracellular material was generally of an amorphous character (Fig. 1C) with only occasional small bundles of longitudinally aligned collagen fibrils seen alongside the terminal processes of the myotubes. The myotubes made many sideways contacts with fibroblasts, and the extreme end of the myotube often terminated in a broad contact with a fibroblast. Specialised cell junctions were not apparent between myotubes and fibroblasts, even though both gap junctions and adhering junctions were common between neighbouring myotubes in the same material.

Myofilaments were sparse at the extreme ends of the primary myotubes, with organised myofibrils giving way to scattered thick and thin filaments over the last 15–20 µm of the cell (Fig. 1B). These disorganised terminal filaments were often seen to insert in a dense, subsarcolemmal region, but there was no clear pattern apparent in the termination (Fig. 1C, 1D). At adult myotendinous junctions, it is always the terminal actin band of a sarcomere that inserts into the dense subsarcolemmal zone ('internal lamina').

Primary myotube-tendon junctions in sternomastoid and lumbrical muscles from E21 embryos were highly organised, and in most respects similar to adult myotendinous junctions (Fig. 1E). Sternomastoid muscles were at a more advanced stage of organisation than IVth lumbrical muscles. The myotubes were divided into a number of finger-like processes at their ends, with well-defined myofibrils extending into the processes and terminating in an orderly way, the terminal actin band of the last sarcomere inserting in a dense region immediately underlying the sarcolemma. Fibroblast processes interdigitated with the terminal processes of the myotubes, and dense accumulations of banded collagen fibrils (arrows in Fig. 1E) attached to the ends of the myotubes in the region of the basal lamina. The general nature of the tendon was more cellular than in adult muscle, with proportionately more fibroblasts and less collagen present.

Insertion of secondary myotubes

The four myotube clusters analysed in detail from

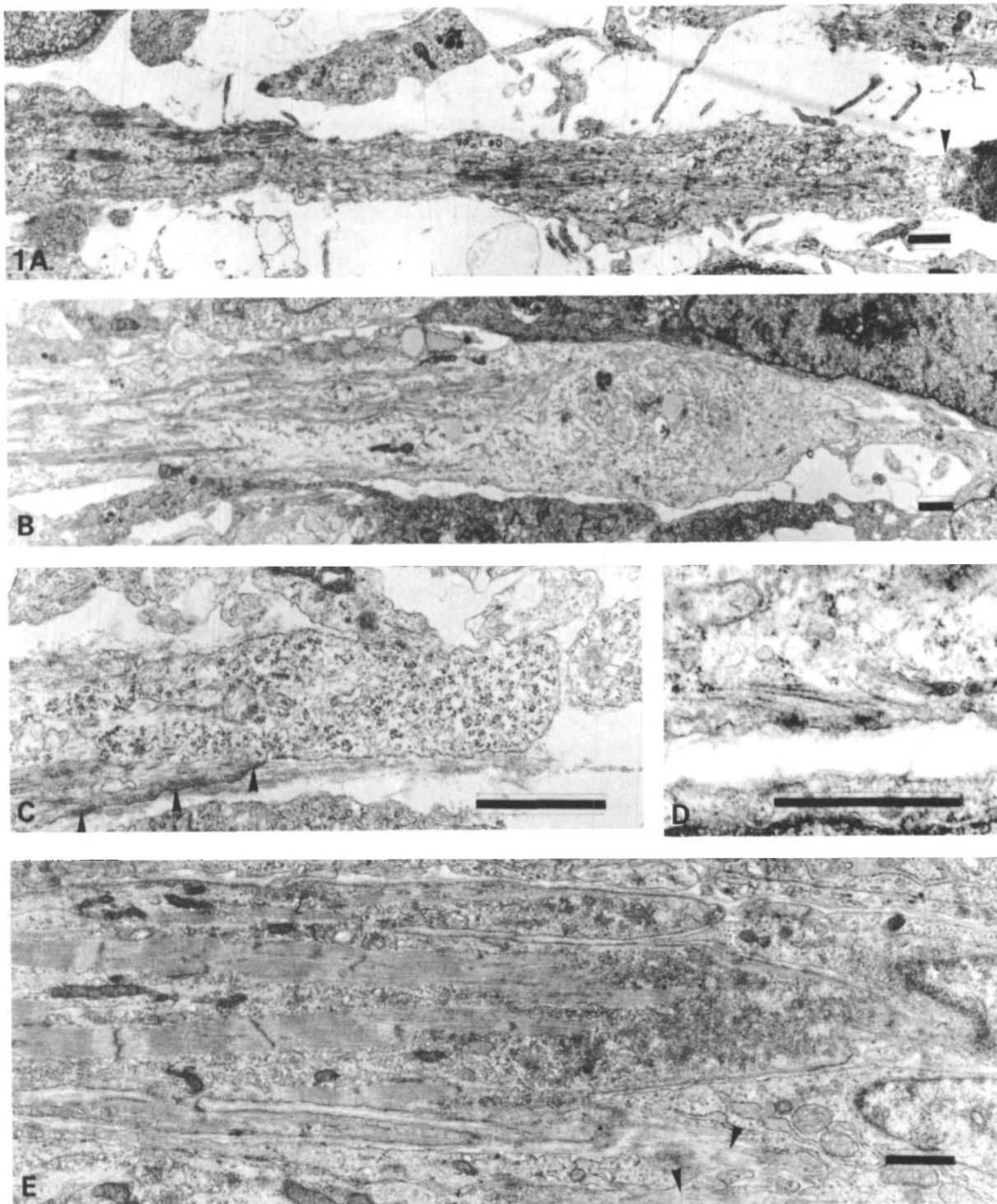


Fig. 1. Insertion of primary myotubes in developing sternomastoid muscles. (A) E16. A primary myotube ends in a single attenuated process which appears largely unattached. The process terminates (arrow) in contact with a fibroblast, at extreme right. There is a complete absence of the extracellular collagen normally found in the region of the tendon in more mature muscles. (B) E16. Another primary myotube ends in a more expanded process, which makes many sideways contacts with surrounding fibroblasts. (C) E16. The terminal region of this primary myotube shows myofilaments inserting in a sub-sarcolemmal dense zone (arrows). A small amount of amorphous extracellular material, probably collagen, is present adjacent to the sarcolemma. (D) E16. A closer view of myofilaments entering a dense region just underneath the sarcolemma. (E) E21. The terminal region of the primary myotube shows all the specializations characteristic of a mature myotendinous junction. The end of the myotube is divided into a number of villous processes which interdigitate with fibroblast processes. Dense bundles of collagen (arrows) are present in the extracellular spaces. Calibration bars = 1 μ m.

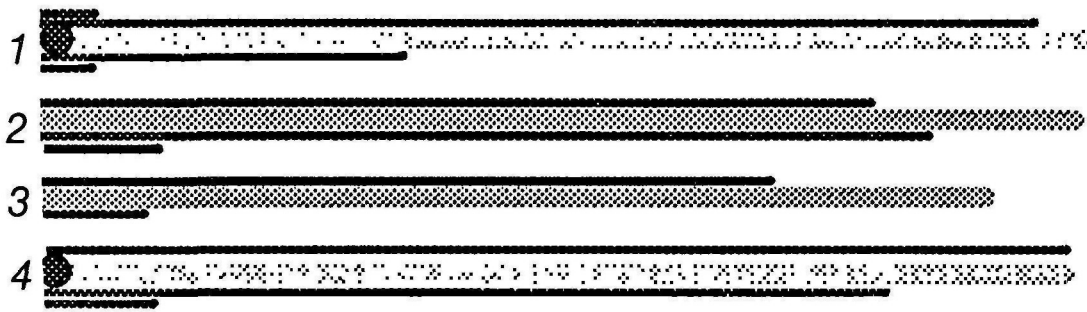


Fig. 2. Diagrammatic representation of the four myotube clusters analysed in detail showing primary myotubes (light shading), secondary myotubes (dark lines) and the positions of neuromuscular junctions (circles). The primary myotubes were about 400 μm long.

2.7 μm spaced electronmicrographs included 4 primary and 12 secondary myotubes. These clusters are represented in diagrammatic form in Fig. 2; a computer reconstruction of cluster 1 is shown in Fig. 5A. The secondary myotubes of the four clusters varied in half-length (i.e. endplate to termination of myotube) from <20 μm (one nucleus in the half-cell) to 390 μm (10 nuclei in the half-cell).

More general observations in this section come from the 20 μm spaced photomontages of the E21 IVth lumbrical muscle (which followed about 30 cell clusters) and from single section observations of many other muscles (see Methods).

Newly formed secondary myotubes

The youngest myotubes studied had one nucleus in the sectioned length and were probably binucleated cells. Such cells were never seen to form connections with the connective tissue framework of the muscle. On the other hand, they consistently showed a complex physical interlocking with the primary myotube. In their midregions, very young myotubes exhibited long outpushings which inserted deeply into the primary myotube (Fig. 3A). These varied in number along the longitudinal axis of the cell but tended to appear in relatively constant positions for each cell, suggesting the outpushings to be long ridges rather than fingers.

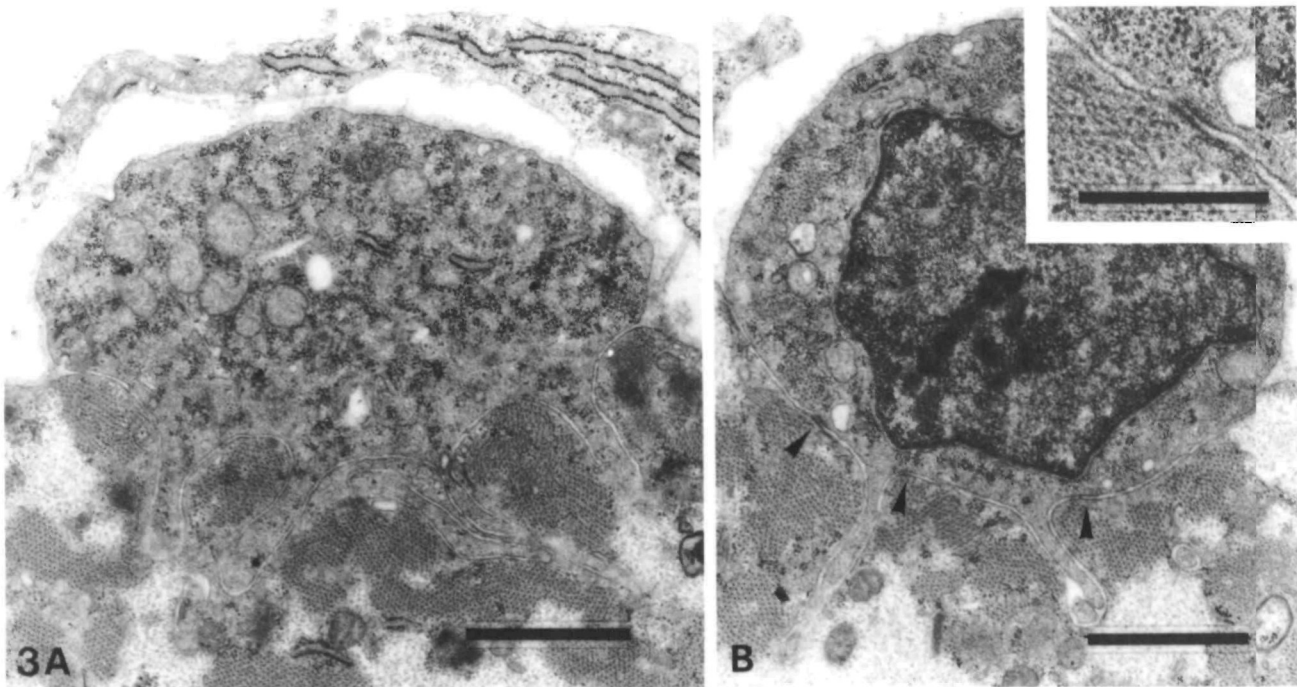


Fig. 3. Secondary myotube insertions into primary myotubes. (A) A binucleate secondary myotube (top) with very organised myofilament has long processes which insert deeply into the primary myotube (bottom). (B) The midregion secondary myotube of intermediate length (top), again displays complex interlocking with the primary myotube (bottom). At the cell interface, there are regions of parallel, electron-dense membranes (arrowheads) which are thought to be junctions. One of these is shown at higher magnification in the inset (top right). Calibration bars = 1 μm (main pictures), 0.5 μm (inset).

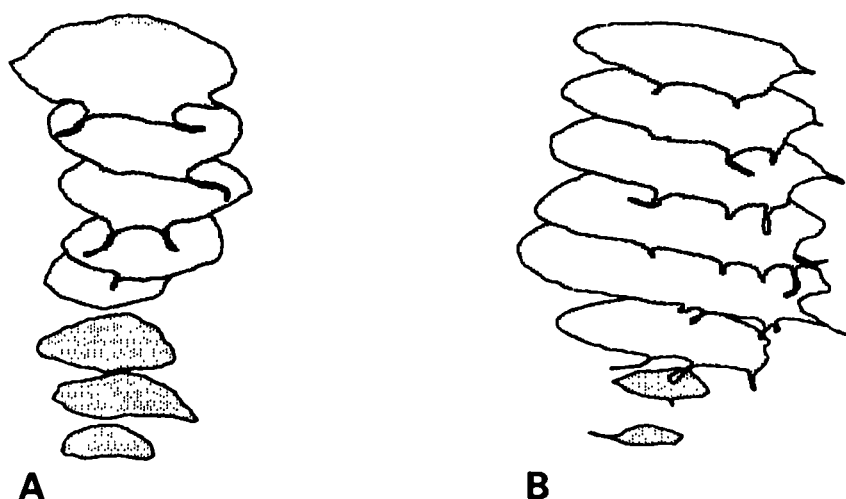


Fig. 4. Semiserial computer reconstructions of very young myotubes. These cells were $<20 \mu\text{m}$ long from endplate region (at top) to their termination, contained sparse myofilament predominantly at the midzone and one nucleus in the half-cell. (A) Cell 4 and (B) cell 3 of the cluster shown in detail in Fig. 5. Section interval = $2.7 \mu\text{m}$. The horizontal axis is expanded $\times 2$ with respect to the vertical.

The myotubes always ended in a smooth taper, with no interlocking ridges present over the most distal $5\text{--}10 \mu\text{m}$.

Figure 4 shows semiserial reconstructions of two very short secondary myotubes from cluster 1 (cells 3 and 4) as an example of cell form at this stage of development. The myotubes are shown from midpoint (endplate region) to termination, and oriented to reveal the cell face apposing the primary myotube. It was notable that, in single sections, young secondary myotubes were more reliably identified by their physical interlocking with the primary myotube than by the presence of myofilaments; mononucleated cells were never seen to form these complex junctions with the primary myotubes.

Zones of parallel, electron-dense membranes were common in all regions of the cell interface between primary and young secondary myotubes (Fig. 3B); these are thought to be adhering junctions, but lacked the subsarcolemmal dense bars and tonofilaments typical of desmosomes. The junctions appear identical to those described as *punctum adherens* by Fawcett (1981).

Secondary myotubes of intermediate length

Secondary myotubes of increasing nuclear number and length maintained a complex physical relationship with the primary myotube (Fig. 3B). Figure 5 shows reconstructions from serial electronmicrographs of the related faces of the primary myotube of a developing cluster and a secondary myotube of intermediate length (cell 5), at three different positions along the cluster. Cell 5 contained three nuclei in the half-cell (represented by apparent holes in the reconstruction of Fig. 6) and had a half-length of $\approx 140 \mu\text{m}$, about one-third the length of the primary myotube.

The reconstructions show that the outpushings of the secondary myotube are long, narrow cell ridges, rather than fingers, interlocking with deep channels in the complementary face of the primary myotube. At the midpoint (position i) of the secondary myotube only two substantial ridges are present, and large areas of smooth apposition occur between the myotubes. The most complex interfolding occurs in a region about two-

thirds of the way down the cell (position ii), where a total of six ridges, each extending up to $2 \mu\text{m}$, embrace and intrude into the primary myotube, in some cases pushing almost to the nucleus. Towards the end of the secondary myotube (position iii), the prominent ridges disappear and there is simple apposition of primary and secondary myotubes (see also Fig. 6C).

Analysis of 30 cell clusters from $20 \mu\text{m}$ spaced photomontages confirmed that, in general, secondary myotubes substantially shorter than the primary myotube were most intricately anchored to the primary in a region about two-thirds of the way between the endplate and the end of the secondary myotube. The myotubes always ended in a smooth taper, $15\text{--}20 \mu\text{m}$ long and lacking nuclei or prominent cell ridges.

The rotated reconstructions of Fig. 5D reveal prominent overhanging 'hooks' present at the distal ends of the interlocking ridges of cell 5. These extended up to $2 \mu\text{m}$ along the longitudinal axis of the cell and were regularly oriented with their free end directed towards the insertion. We suggest they might serve a function in anchoring the ridges within the primary myotube. Adhering junctions (arrowed in Fig. 3B) remained ubiquitous at the interface between these growing secondary myotubes and their supporting primary myotubes.

Secondary myotubes approaching the length of the primary myotube

Secondary myotubes which approached the length of the primary myotube began to lose their complex interlocking with the primary myotube, and eventually to separate from the parent cell, concurrently acquiring an independent basal lamina. These processes were first apparent in the midregion of the secondary myotube (e.g. cell 2 in Figs 5A, 6A–F). In the $2.7 \mu\text{m}$ spaced serial section study, six out of twelve secondary myotubes were of a length that approached ($>70\%$) or equalled that of their related primary myotube. Of these six, three cells were always contained under the common basal lamina of the cluster, but formed interlocking connections with the primary myotube only in regions well removed from the middle of the muscle.

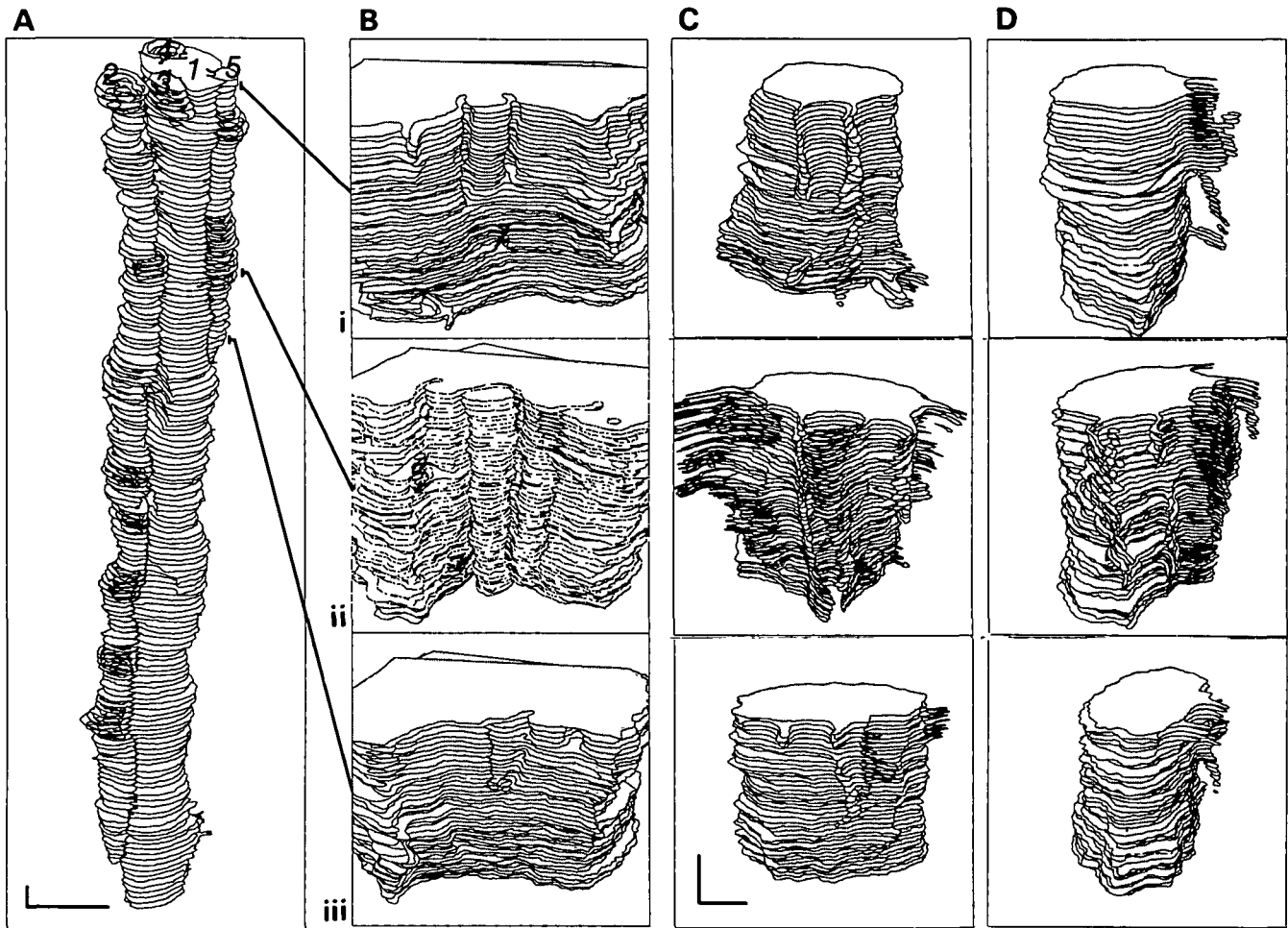


Fig. 5. (A) Semiserial computer reconstruction of cluster 1. The cluster includes one primary myotube (cell 1), and four associated secondary myotubes (cells 2–5). The reconstructed segment is 400 μm long, commences at the endplate region of the cluster (top) and continues almost to the end of the primary myotube (bottom). The bars identify short sectors where the cell interface between cells 1 and 5 has been serially reconstructed. Section interval = 2.7 μm . Bars = 10 μm . The horizontal axis is expanded four-fold with respect to the vertical axis. (B,C,D) Serial reconstructions of the related cell surfaces of the primary myotube (cell 1) and a secondary myotube of intermediate length (cell 5), shown at three points (i, ii and iii) along the length of the cluster. (B) Primary myotube. (C) Related face of the secondary myotube. (D) A rotated view of the secondary myotube interface to illustrate overhanging hooks on the ends of the interdigitating ridges. Section interval = 90 nm. Bars = 1 μm .

The other three cells appeared as independent myotubes in the midregion ('separate secondary myotubes') but lay under the common basal lamina at more distal points (compare cell 2 in Fig. 6A and 6B–F). The process of leaving and rejoining the cluster often occurred several times before the separate secondary myotube formed a more committed relationship with the primary myotube, interlocking with and embracing the parent cell. The physical coupling of large secondary myotubes with their supporting primary was never as complex as that formed by younger myotubes. Fewer interlocking ridges were present, and they intruded a lesser distance into the primary myotube. *Punctum adherens* remained common at all points where primary and secondary myotubes were closely apposed.

All secondary myotubes in this class extended into the region of insertion of the primary myotubes, and formed direct connections with the extracellular elements of the tendon. Regions of attachment of col-

lagen to the surface of secondary myotubes, similar to those seen on the primary myotubes, became increasingly common as the termination of the cell was approached (e.g. cell 2 in Fig. 6E and 6F). At their extremities, the largest secondary myotubes again separated from the primary myotube, and formed a final tendinous attachment indistinguishable from that of the primary. This means that these cells made dual insertions, partly to the primary myotube, and partly to the tendon proper. The final stage, not observed in this study, would presumably be for all attachment to the primary myotube to disappear and for the secondary myotube to insert solely onto the connective tissue framework of the tendon.

Observations on the association of fibroblasts with developing myotube clusters

Developing myotube clusters were often almost com-

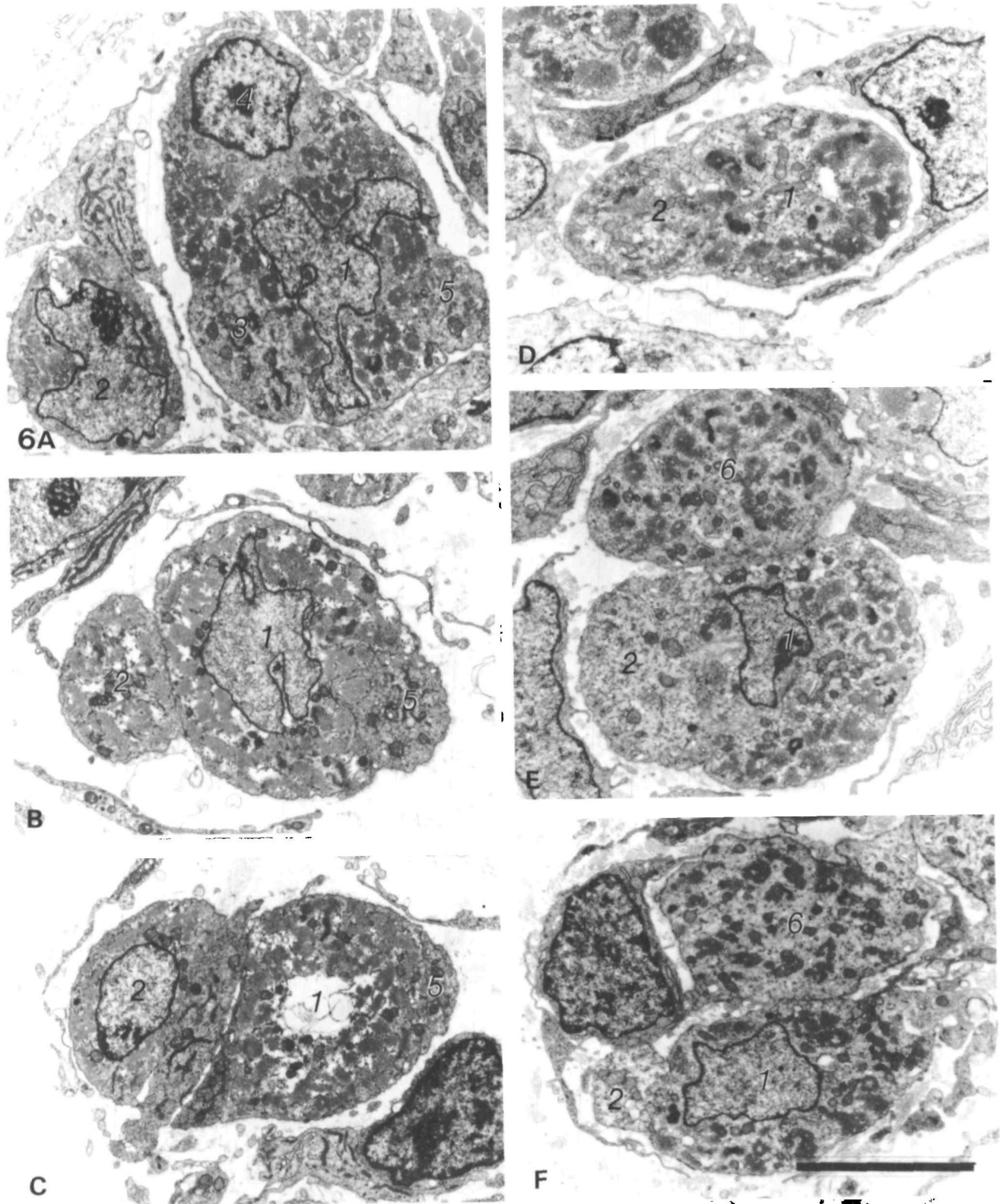


Fig. 6. Six electron micrographs showing the evolution of the reconstructed cluster 1. (A) Origin of sectioning in the innervation zone of the cluster. (B) 105 μm from origin. Secondary myotubes 3 and 4 have terminated and cell 2 has returned under the basal lamina of the cluster. (C) 135 μm . Secondary myotube 5 ends in a flattened process. (D) 280 μm . (E) 360 μm . The largest secondary myotube (cell 2) inserts processes deeply into the primary myotube in this region. A second primary myotube (cell 6) comes within the basal lamina of the original cluster. (F) 375 μm . Secondary myotube 2 and primary myotubes 1 and 6 all attach to extracellular collagen bundles in the region of the tendon. Calibration bar = 5 μm .

pletely encircled by fibroblasts and their attenuated processes (e.g. Fig. 6C and D). In the lumbrical muscle, the number of primary myotubes formed is 102 ± 4 (Ross *et al.* 1987); in the adult muscle, there are about 100 fascicles (M.J.D., unpublished observations). From this we surmise that each primary myotube and its associated secondary myotubes (i.e. each myotube cluster) will give rise to a single fascicle of the muscle. This conclusion is supported by the observation (Jones *et al.* 1987) that each fascicle of the adult lumbrical muscle contains a single slow-myosin-containing muscle fibre (derived from the primary myotube), associated with a number of fast fibres (secondary myotube derivatives). The encirclement of the developing myotube clusters by fibroblasts presumably represents an early stage of formation of the perimysium – the fibrous connective tissue layer that will finally completely separate adjoining fascicles.

Discussion

The development and exertion of muscle force is a complex process depending both on patterns of excitation within the muscle and on the way in which force is finally transmitted from the muscle cells to the tendon. Both these factors differ in foetal and embryonic muscles, as compared to adult muscles. Secondary myotubes are coupled to a primary myotube by both electrical and mechanical junctions, so that during development the cluster of myotubes ('muscle unit', Ontell and Dunn, 1978) may act as a single functional unit. Furthermore, a secondary myotube has a unique developmental relationship with a single 'parent' primary myotube, with implications for the regulation of muscle fibre type.

Cell insertion

Two essentially different populations of myotubes are found in developing muscles, and the work reported here shows clearly that they insert differently. A minority population, the primary myotubes, are a relatively uniform set of cells that stretch from end to end of the muscle, and attach to the skeleton via a myotendinous junction. At the earliest times examined (E16 sternomastoid muscle) this junction is tenuous, and probably serves an initial role in orienting myotubes and providing a small amount of tension to stimulate growth, rather than in transmitting movement from muscle to skeleton. However, the primary myotube-tendon junction rapidly matures so that by E21 (in sternomastoid and IVth lumbrical muscles) it is comparable to a mature myotendinous junction.

Secondary myotubes, by contrast, initially have no connections to the tendon, but insert directly onto the primary myotubes by a complex system of interlocking ridges associated with adhering junctions. Only in the final stages of their formation do secondary myotubes progressively make connections with the tendon and shed their mechanical coupling with the primary myotube. The unusual and varied nature of the insertion of

growing secondary myotubes means that the final tension they contribute during contraction of the muscle or of a single motor unit may not be simply related to their numbers.

Patterns of activation in developing muscle

A previous study from this laboratory (Ross *et al.* 1987) described the extensive and changing patterns of electrical coupling among developing myotubes in IVth lumbrical muscles until the end of gestation (E21), extending earlier reports of coupling in a range of other embryonic rat muscles (e.g. Kelly and Zacks, 1969; Dennis *et al.* 1981; Schmalbruch, 1982). Patterns of activation within developing muscles are further complicated by the occurrence of a rather variable polyneuronal innervation of myotubes. Although physiological studies report that virtually 100% of myotubes in rat muscles are polyinnervated in the late foetal and early neonatal periods (e.g. Dennis *et al.* 1981; Betz *et al.* 1979), we have recently reported evidence from ultrastructural studies (Duxson *et al.* 1986) that, while primary myotubes in E21 lumbrical muscles are always innervated by multiple axon terminals, young secondary myotubes are often contacted by only a single nerve terminal profile. The combination of complex and variable patterns of innervation with extensive electrical coupling leads to considerable complication in predicting activation patterns within developing muscles.

Implications for interpretation of physiological studies

The observed non-uniformities in primary and secondary myotube patterns of innervation and electrical coupling, and in their manner of transmission of contractile force to muscle tendons, make it impossible to use muscle tension measurements as a direct measure of motor unit size during the early stages of muscle development. In order to accurately calculate the number of cells in a motor unit from the tension it generates, it would be necessary to know the proportions of primary and secondary myotubes in the unit, the size dispersion of the secondary myotubes, and to make predictions as to the effects of the physical coupling between primary and secondary myotubes on the tension produced at the tendon. Further inaccuracies will be introduced by the existence of electrical coupling between myotubes, which would lead initially to overestimates of unit size followed by an apparent fall in the unit size as coupling is lost. Finally, the redundancy of innervation of primary but not secondary myotubes does not affect measures of the first unit recruited but would affect calculations of redundancy of innervation based on measures of contraction, and estimates of the number of motor units in a muscle.

Implications for developmental regulation of muscle fibre type

The complex attachments between secondary myotubes and their 'parent' primary myotube indicate that myotube clusters may constitute developmental compartments. In a subsequent paper (Duxson *et al.* 1989), we

discuss the distribution of myoblasts as well as myotubes within clusters, and suggest that the multiple nerve terminals that initially contact a primary myotube are involved in regulating secondary myotube formation within that cluster, by being transferred from the primary to a new-formed secondary myotube.

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