ULTRASTRUCTURAL FEATURES OF SKELETAL MUSCLE DIFFERENTIATION AND DEVELOPMENT\textsuperscript{1,2}

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\textbf{Summary}

It is obvious from even this cursory review that ultrastructural studies have provided the basis for much of our existing knowledge about differentiation and development of muscle. It is also evident that a great deal is known about differentiation of muscle cells. Because the major myofibrillar proteins of muscle are well characterized and possess unique features that permit their isolation and identification from very small amounts of tissue, muscle tissue may well constitute a very favorable system for the study of cell differentiation in general. It seems probable that future progress in the study of muscle differentiation will rely on an integrated application of biochemical, immunological, and ultrastructural techniques. Information from such efforts should eventually lead to the ability to control the rate of synthesis of myofibrillar proteins, and the rates and times at which myoblasts fuse to form muscle cells. Possession of this ability will make it possible to achieve extraordinary increases in feed efficiency and muscling in our domestic animals, and indeed, may usher in an entirely new era in animal science.

\textbf{Introduction}

Differentiation and development of muscle has intrigued and perplexed biologists since before the beginning of this century. Until the past 10 to 15 years, however, virtually no information was available on the chemical events accompanying cell differentiation, and the light microscope was the most powerful tool available to early biologists for study of muscle development. Consequently, before 1950, light microscopy was used extensively, and in some instances almost exclusively, in studies of muscle differentiation and development. Although many of these early light microscope studies were of excellent quality and exhibited extraordinary biological insight (Weed, 1936), the inherent limitations in resolving power of the light microscope caused considerable disagreement about the exact nature and sequence of structural events during myogenesis. These disagreements were fueled by a lack of understanding of the structure and action of the myofibril and by the almost complete absence of information on the mechanism of protein synthesis. After 1950, electron microscopes became more widely available, and the greater resolving power of these instruments together with substantial progress in our understanding of the myofibril and the mechanism of protein synthesis has led to resolution of many of the earlier controversies about myogenesis. As will become evident in reading this review,
Characteristics of Mature Muscle Cells

Before beginning a discussion of skeletal myogenesis, it may be helpful to briefly review the endpoint of such myogenesis, i.e., the structure of the mature muscle cell. Figure 1 shows a schematic of skeletal muscle structure at several different levels of organization ranging from that visible with the naked eye to that seen only at very high magnifications in the electron microscope. Mature skeletal muscle cells have all the features associated with other animal cells; they contain a cell membrane, lipid inclusions, mitochondria and ribosomes, although the latter may be observed infrequently in mature muscle. However, mature skeletal muscle cells differ from other animal cells in at least five important ways: 1) mature skeletal muscle cells are much more elongated than ordinary animal cells, and may average 1 to 2 cm in length; 2) mature skeletal muscle cells are multinucleated; each cell may contain from 100 to 200 nuclei, and in normal, healthy cells, these nuclei all lie immediately under the sarcolemma or outer cell membrane (figure 1B and 1C); 3) mature skeletal muscle cells contain elongated protein threads whose long axes are oriented parallel with the long axis of the cell; these protein threads are called myofibrils and are the contractile units of the cell (figure 1C and 1D); 4) the endoplasmic reticulum membranes normally observed in animal cells assume a very specialized structure in muscle cells and are associated with another set of membranous tubules that are oriented perpendicular to the long axis of the cell; in recognition of its specialized structure, the endoplasmic reticulum in muscle cells is termed the sarcoplasmic reticulum, and the membranous tubules associated with the sarcoplasmic reticulum are called T-tubules (these structures will be described in more detail later); 5) lysosomes, the spherical, membrane-enclosed particles that contain hydrolytic enzymes, are not seen in healthy, mature skeletal muscle cells. The first four of these five unique features of skeletal muscle cells are important in understanding a discussion of the structural features of muscle development.

The Myofibril. Because myofibrils constitute over 50% of the total protein in a mature skeletal muscle cell, and because myofibrils exhibit a very characteristic and unique structure, most studies on differentiation and development of skeletal muscle have focused on the development of myofibrils in presumptive muscle cells. Consequently, it is important when discussing muscle development to be completely familiar with the structure of myofibrils in mature skeletal muscle. Figure 2 shows that, when viewed in the electron microscope, fully developed myofibrils consist of a series of alternating light and dark bands. The light or I-bands are bisected by a dark line called the Z-disk, and the dark or A-bands contain a lighter zone, the H-zone, in their center. The light H-zone in turn is bisected by a dark line called the M-line. As shown schematically in figure 1, the alternating light and dark bands of the myofibril originate directly from a unique arrangement of two sets of interdigitating filaments. The larger filaments (diameter = 14-16nm) are found only in the A-band and are called thick filaments. The other filaments, the thin filaments (diameter = 6-8 nm), are attached at one end to the Z-disk, extend from the Z-disk through the I-band, and end in the A-band at the edge of the H-zone (figure 1E). Interdigitating thick and thin filaments can be readily observed in thin sections of skeletal muscle (figure 3). It is now known that thick filaments are composed principally of the myo-
Figure 1. Schematic showing the structure of a mature skeletal muscle at different levels from the intact muscle (A) to the molecular architecture of the myofibril (E). A. Intact skeletal muscle such as the semitendinosus or the biceps femoris showing tendon attachment to bone. B. Portions of three muscle cells or fibers showing striations and peripherally located nuclei; this structure can be seen at the light microscope level. Small lines scattered through these cells are meant to depict mitochondria. C. Part of a single muscle cell showing a mitochondrion and nucleus, and illustrating how a muscle cell is literally packed with myofibrils. This structure is seen at high light microscope magnifications. D. A single myofibril illustrating the cross-striated banding pattern observed in skeletal muscle myofibrils and indicating a sarcomere. Very high light microscope level. E. Arrangement of the interdigitating thick and thin filaments that constitute the myofibril, and an illustration of how actin and myosin molecules may be arranged to form the thin and thick filaments, respectively. From Novikoff and Holtzman (1970) and reproduced by permission of Holt, Rinehart and Winston, Inc. and the authors.
fibrillar protein, myosin, whereas thin filaments are composed mainly of the protein, actin, but also contain several proteins in addition to actin. The protein composition of the Z-disk is unknown, although recent evidence suggests that it is composed at least partly of α-actinin (Goll et al., 1969; Robson et al., 1970; Stromer et al., 1969).

The Sarcotubular System. The endoplasmic reticulum of animal cells is a labyrinth of membrane-enclosed tubules that open to the extracellular space at one end and that wind a tortuous course to the nuclear membrane in the interior of the cell. The endoplasmic reticulum has a variety of physiological functions; many enzymes, including those involved in phospholipid and lipid synthesis as well as some of those involved in carbohydrate metabolism (such as glucose 6-phosphatase), ostensibly are bound to the inner surface of endoplasmic reticular membranes. In cells that synthesize protein for export (pancreas cells, for example), the inner surface of endoplasmic reticular membranes contains numerous ribosomes. The presence of ribosomes adhering to endoplasmic reticular membranes produces a structure that has been called “rough endoplasmic reticulum” because of its appearance in electron micrographs. The lumen of endoplasmic reticular tubules opens directly to extracellular space, and it has been proposed that proteins synthesized for export outside a cell are made on the inner surface of endoplasmic reticulum membranes and then transported extracellularly by passing through the membrane into the lumen. Hence, the endoplasmic reticulum may also have a transport function in animal cells.

In muscle cells, the endoplasmic reticulum
exists as two separate sets of membrane-enclosed tubules that lie closely apposed over part of their surfaces but do not open directly into each other. One of these two sets of membranous tubules is called the transverse or T-system and consists of a series of invaginations of the outer cell membrane. These invaginations extend perpendicularly into the cell at regular intervals along the length of the striated muscle cell. In some muscles, T-tubules occur at the level of each Z-disk, whereas in other, faster-acting muscles, they are found at the level of each junction of the A and I bands. Because T-tubules are invaginations of the outer cell membrane, the lumen of the T-tubule is extracellular. Hence, T-tubules resemble the endoplasmic reticulum of other animal cells, but they are distributed much more periodical-ly through the cell and are generally oriented more perpendicular to the long axis of the cell than ordinary endoplasmic reticular membranes are.

The second set of membranous tubules in striated muscle cells is called the sarcoplasmic reticulum or SR-system. These tubules are generally oriented parallel with the long axis of the cell and extend longitudinally in both directions from each T-tubule to meet and anastomose with membranes extending from adjacent T-tubules at a point halfway between adjacent T-tubules (figure 4). At the point where the SR membranes meet the T-tubules, they are expanded to form large sacs called lateral cisternae. Longitudinal sections of striated muscle cells cut T-tubules in cross-section (because T-tubules run perpendicular to the long axis of the cell), and the cross-section of the T-tubule flanked on both sides by lateral cisternae produces a structure called the triad (figure 4). Triads are seen at the level of each A-I junction (figure 4) or each Z-disk, depending on the location of the T-tubules. Because the T-system and the SR-system have distinct anatomical structures and different physiological functions, the term, sarcoplasmic reticulum, is inadequate to describe both these sets of membranous tubules in muscle. Therefore, the term, sarcotubular system, is often used to refer to the T-system and the SR system, collectively. It should be stressed that the sarcotubular system forms a very regular and extensive three-dimensional network around each myofibril in a muscle cell. The complexity of this three-dimensional network is often not appreci-ated by examination of single sections in the electron microscope (such as that shown in
Figure 4. A longitudinal section of a fish muscle (toadfish swim-bladder muscle) capable of very rapid contraction. This muscle has a very regular and highly developed sarcoplasmic reticulum and T-system. T-tubules (T) are seen in cross-section approximately at the junction of the A- and I-bands and are bounded on each side by lateral cisternae of the sarcoplasmic reticulum system. Two lateral cisternae and one T-tubule constitute a triad. Membranes extending from opposing lateral cisternae meet and anastomose in the center of each A-band. From Fawcett and Revel (1961) and reproduced by permission of the Rockefeller University Press and the authors. X22,700.

Figure 4); figure 5 shows an attempt to represent the three-dimensional nature of the sarcotubular system schematically.

Extensive study of the sarcotubular system of skeletal muscle cells has shown that the T-system is involved in transmission of the nerve impulse into all interior portions of the cell and that the SR-system contains a Ca\textsuperscript{2+}-stimulated, Mg\textsuperscript{2+}-dependent adenosinetriphosphatase activity that is coupled to the active accumulation of Ca\textsuperscript{2+} by the SR membranes. A nerve impulse passing along the T-tubules effects some as yet unknown change in the SR membranes (the T-tubule does not open directly into the lateral cisternae) that causes these membranes to lose some of the Ca\textsuperscript{2+} they have accumulated. This efflux of Ca\textsuperscript{2+} from SR membranes triggers contraction. It is obvious, therefore, that both the T-system and the SR

system of skeletal muscle cells have vitally important physiological roles, and the sequence of development of these structures is of considerable interest.

Myogenesis

The unique and highly ordered structure of the myofibril can be used in three different ways to study skeletal muscle cell development ultrastructurally: 1) attempts can be made to determine the stage in development when thick and thin filaments first appear in embryonic muscle cells; since thick and thin filaments are not observed in large quantities in other cells, it is assumed that they are formed only in muscle cells; 2) assembly of thick and thin filaments into their characteristic interdigitating array can be monitored; and 3) attempts can be made to
Figure 5. A schematic of a muscle cell (fiber) showing the extensive nature of T-tubules and sarcoplasmic reticular membranes in a skeletal muscle cell. T-tubules can be visualized as numerous channels that may actually pass through the entire diameter of a muscle cell. From Porter and FranZini-Armstrong (1965) and reproduced by permission of W. H. Freeman and Company and the authors. Copyright (1965) by Scientific American, Inc. All rights reserved.

determine when in muscle cell development the Z-disk first appears and how it is incorporated into the myofibril (e.g., is the Z-disk formed first and thin filaments then stuck into it like pins into a cushion, or is the interdigitating thick and thin filament array assembled first and the Z-disk then added to it?). Unfortunately, as will become evident during the remainder of this review, it has not been possible by ultrastructural studies alone to obtain unequivocal answers to objectives 1 and 3; this is principally because the electron microscope can detect myosin, actin, and Z-disk proteins only after they have been assembled into structures that resemble the filaments or Z-disks seen in mature muscle. As a result of this restriction, studies using only the electron microscope to monitor muscle cell development have encountered three problems: 1) it has been impossible with electron microscope studies to determine whether myosin and actin are synthesized very early in muscle cell differentiation but are not assembled into filaments until some later event triggers such assembly; 2) embryonic muscle cells contain several kinds of filaments in addition to thick and thin filaments, and careful examination is required to distinguish among these different kinds of filaments; 3) it currently seems that the embryonic precursor of the Z-disk is a much more diffuse and amorphous structure than the “mature” Z-disk, and it is extremely difficult to determine when Z-disks first appear in embryonic muscle cells. Biochemical methods, possibly involving antibody binding or selective extraction of proteins, are needed to supple-
ment the ultrastructural evidence in these areas. In spite of these few shortcomings, however, ultrastructural studies have provided a great deal of information about the processes that occur during differentiation and development of skeletal muscle cells, and it is now possible to describe a sequence of events that cells experience during differentiation to mature skeletal muscle cells. This sequence of events is outlined in Scheme I.

**SEQUENCE OF EVENTS**

![Diagram of sequence of events]

**DEFINITIONS**

**Mesoderm** - one of the three primary germ layers of the embryo

**Presumptive Myoblast** - a mononucleated cell committed to becoming a muscle cell but incapable of fusion or of synthesizing contractile proteins; difficult to distinguish from other cells by ultrastructural examination alone.

**Myoblast** - a mononucleated cell capable of fusion and of synthesizing myofilament proteins; bipolar in shape.

**Myotube** - an elongated, multinucleated cell formed by fusion of myoblasts; nuclei do not divide mitotically and are centrally located; contains myosin and actin filaments at the periphery of the cell and may contain sarcomeres depending upon stage of development.

**Myofiber** - a mature skeletal muscle cell filled with myofilaments and with nuclei lying next to the sarcolemma; neither myofibers nor nuclei within myofibers divide mitotically.

Scheme I. Events and nomenclature associated with differentiation and development of skeletal muscle cells.

It has been well established that almost all muscle tissue (including cardiac and smooth muscle) differentiate from mesoderm, the middle of the three primary germ layers of the embryo. The only known exceptions to this generalization are the smooth muscles of the iris in the eye and those of sweat and mammary glands; these muscles are ectodermal. Although Holtzer (1970) has suggested that no naive, uncommitted, or undifferentiated cells exist at the molecular level, it is presently impossible to recognize unequivocally those cells destined to form muscle tissue before they reach the myoblast stage. Even at the myoblast stage, identification usually is based on the bipolar or spindle shape of myoblasts compared with fibroblasts and other mesodermal cells, and myoblasts can be identified with certainty only if they contain myosin and actin filaments. Tissue culture studies have indicated, however, that cells committed to becoming muscle cells but structurally difficult to distinguish conclusively from other cells committed to becoming fibroblasts, etc., exist before the myoblast stage. These cells are called presumptive myoblasts (Scheme I). Presumptive myoblasts undoubtedly have undergone considerable differentiation from the early mesodermal cell and probably also must pass through several additional stages of differentiation before becoming recognizable as myoblasts. Since virtually nothing is known of events that distinguish these additional stages, however, they are not included in Scheme I.

**The Myoblast.** Myoblasts generally appear in muscle cell cultures or in sections of embryonic muscle as spindle-shaped, mononucleated cells containing numerous ribosomes (figures 6 and 7). Myoblasts derived from somites will occasionally possess thick and thin filaments, but myoblasts derived from leg or limb muscle rarely exhibit thick and thin filaments. The reason for this difference between limb and somitic myogenesis is unknown, but it may simply indicate that fusion to myotubes (Scheme I) occurs earlier in the life cycle of limb myoblasts than it does in somitic myoblasts (Königsberg, 1965). Biochemical experiments involving incorporation of labeled thymidine into nuclear DNA have demonstrated that those myoblasts containing thick and thin filaments do not undergo mitosis. Indeed, numerous experiments have now demonstrated that cells containing myosin (thick) and actin (thin) filaments do not exhibit mitotic activity, and it is generally assumed that a cell must withdraw from the mitotic cycle before it can begin bulk synthesis of the contractile proteins. It must be admitted, however, that until recently this conclusion depended primarily on ultrastructural identification of thick and thin filaments only in those cells that do not incorporate labeled thymidine. Since myosin and actin molecules cannot be detected ultrastructurally before assembly into filaments, it was possible that myosin and actin synthesis could be initiated before withdrawal from the mitotic cycle, but that assembly of myosin and actin into filaments was delayed until after mitotic activity had ceased. The recent experiments by Paterson and Strohman (1972) and Yaffe and Dym (1973), however, have made it unlikely that bulk synthesis of contractile proteins occurs before withdrawal from the mitotic cycle.

Since myoblasts can be conclusively identified only if they contain myosin and actin filaments, and since such myoblasts have, of course, ceased mitotic activity, it was natural to define myoblasts as postmitotic cells capable of synthesizing contractile proteins and of fusing with other similar postmitotic cells (Bischoff and Holtzer, 1969; Fischman, 1972; Holtzer and Bischoff, 1970; Ishikawa, Bischoff and Holtzer, 1968). The recent results of Stockdale...
and O’Neill (O’Neill and Stockdale, 1972a; 1972b; Stockdale and O’Neill, 1972), however, indicate that cells exist that are capable of fusing but that can also be induced by changing environmental conditions to undergo one or more additional mitotic cycles. Consequently, mitotic ability may not be a distinguishing feature of myoblasts, and until some reliable criteria for clearly defining myoblasts becomes available, it may be necessary to admit that myoblasts do not irreversibly withdraw from the mitotic cycle until after fusion or synthesis of contractile proteins has been initiated.

**Fusion.** The first event that can consistently be detected ultrastructurally and that is unique to myogenesis is fusion of myoblasts to form myotubes (Scheme I). Because it can be observed ultrastructurally and because it seems to be the “committed” step in myogenesis, myoblast fusion has been studied extensively. In spite of this lavish attention, however, the nature of the events that cause fusion remains unclear. Biochemical studies coupled with microscopic examination have shown that mitotically active myogenic cells fuse only in the G1 phase of the cell cycle (Scheme II). Holtzer and coworkers (Bischoff and Holtzer, 1969; Holtzer and Bischoff, 1970) have argued that the mitosis immediately preceding the G1 period in which fusion occurs is an unusual mitosis (termed a “quantal mitosis”) that produces a cell committed to fusion. O’Neill and Stockdale (1972a,b), on the other hand, suggest that competent myoblasts in G1 are not committed to fusion but may undergo one or more additional mitotic cycles, depending on environmental conditions. The factors controlling withdrawal of myoblasts from the mitotic cycle and fusion of these myoblasts to form myotubes is of critical importance to animal sci-
Figure 7. Electron micrograph of a mononucleated, spindle-shaped cell in a 24-hr. culture of embryonic chick skeletal muscle. This cell is presumably a myoblast or presumptive myoblast. B and C are enlargements of the upper and lower squared-off areas, respectively. One portion of this mononucleated cell, shown in B, exhibits the cytological characteristics of a cell synthesizing proteins for export since it contains extensive rough-surfaced endoplasmic reticulum (RER). Another region of this same cell, shown in C, contains mainly free ribosomes, which are more typical of a myoblast. A large number of 10-nm filaments (IF) are evident in the region of the cell containing membrane-bound ribosomes (B), but relatively few 10-nm filaments are seen in the region having free ribosomes (C). Microtubules and microfilaments are present throughout the cell. From Fischman (1972) and reproduced by permission of Academic Press, Inc. and the authors. A. X8400. B. X16,800. C. X16,800.

![Diagram of cell cycle](image)

Scheme II. Relation of the cell cycle to fusion of myoblasts.

- M = mitosis
- G1 = first gap phase; synthesis of RNA and protein
- S = synthesis of DNA
- G2 = second gap phase; synthesis of RNA and protein
- Fusion

Entists, since it seems probable that the total number of cells constituting mature muscles is determined by these factors. Whether cells competent to fuse are also committed to fuse remains an open question, but it currently seems likely that some as yet unknown events that occur in a critical mitotic cycle of a myoblast program that myoblast for fusion.

Ultrastructurally, the onset of fusion is heralded by an end-to-end alignment of myoblasts (figure 6). Alignment is accompanied by a very close approach of the surface membranes of adjacent cells. These surface membranes gradually fuse and then disappear altogether (figures 8 and 9). Fusion is followed by a gradual confluence of the cytoplasm of the two cells, although immediately after fusion, cytoplasm of the newly formed cell sometimes takes on a mosaic appearance in the electron microscope, presumably because the cytoplasm of
the two fusing cells has not yet completely blended (figure 9). Fusion of myoblasts produces elongated, multinucleated cells called myotubes. Extensive structural studies have shown that myoblasts may fuse with other myoblasts, that myoblasts may fuse with myotubes (figure 8), and that myotubes may fuse with other myotubes (figure 9). Neither myoblasts nor myotubes, however, will fuse with fibroblasts or with other “nonmuscle” cells. Indeed, presumptive myoblasts are incapable of fusing with either competent myoblasts or myotubes. Although it is obvious that fusion is an extremely cell-specific process, it is not known whether fusion proceeds randomly among competent myoblasts and myotubes or whether the extent of fusion is controlled by size of the myotube, et cetera.

An impressive amount of evidence has been accumulated to demonstrate that nuclei in normal myotubes have no mitotic capacity. Therefore, the multinucleated nature of mature muscle cells originates directly from fusion of embryonic myoblasts, and the number of nuclei in a newly formed myofiber is equal to the number of myoblasts that fused to form that myofiber. Although the number of nuclei in a muscle cell may increase later during hypertrophy of the cell, this increase does not cause an increase in total number of muscle cells. The available evidence suggests that myoblast fusion ceases about the time of birth in domestic animals; this means that a newly born calf or pig contains all the skeletal muscle cells that it will ever possess, and growth of skeletal muscle subsequent to birth occurs through enlargement of existing cells. If it were suggested in a very elementary way that delaying myoblast fusion for one mitotic cycle (or delaying the critical mitotic cycle that produces myoblasts copme-
tent to fuse for one mitotic cycle) would produce twice as many myoblasts and that these myoblasts would in turn result in twice as many muscle cells in the newborn animal, the importance of myoblast fusion and its regulation to production of muscle in domestic animals becomes obvious.

Almost immediately after fusion, synthesis of contractile proteins increases dramatically, and thick and thin filaments are observed very quickly after myotube formation. Ostensibly, the process of fusion triggers a signal for bulk synthesis of myosin and actin, although since myosin and actin filaments can also be observed in mononucleated myoblasts, it is clear that fusion is not an obligatory antecedent to synthesis of contractile proteins. Newly formed thick and thin filaments evidently self-assemble in the cytoplasm of the myotube to form hexagonal myofibril arrays similar to but somewhat less highly ordered than those observed in mature skeletal muscle cells (figures 8, 9, 10 and 11). The forces responsible for self-assembly of thick and thin filaments are not known. Moreover, even though the process of myofibril assembly is amenable to ultrastructural study, the mechanism of that assembly remains unclear.

For example, it is not known whether thick filaments or thin filaments are formed first and serve as nucleation sites for assembly. Neither is it known whether Z-disks and M-lines are involved in myofibrillar assembly. It has been observed, however, that myofibrils generally
form first at the periphery of the cell; this results in elongated tubes filled with myofibrils at their periphery but having "open" areas of cytoplasm and nuclei in their center (figure 11). Hence the name, myotube. As myotubes mature, additional myofibrils are formed until the entire interior of the myotube is filled with myofibrils leaving only space for the nuclei in the center of the cell (figure 12). At this stage, the nuclei begin to migrate toward the periphery of the cell until they come to lie immediately under the sarcolemma; this is their location in mature skeletal muscle cells, and at this stage, the myotube becomes a myofiber (Scheme 1). Both the physiological significance and the forces responsible for the outward migration of nuclei in skeletal muscle myotubes are completely unclear. Such outward migration occurs only in skeletal muscle cells, and the nucleus remains in the center of mature cardiac and smooth muscle cells (cardiac and smooth muscle cells are usually mononucleated in contrast to skeletal muscle cells).

Although myofibril assembly from thick and thin filaments can be studied ultrastructurally, such studies have been complicated by the presence of at least two kinds of filaments or tubules in addition to thick (14 to 16 nm in diameter) and thin filaments (6 to 8 nm in diameter). Microtubules, approximately 200 nm in diameter are frequently observed both in myoblasts and in newly formed myotubes (figure 10, 13 and 14). Microtubules are almost invariably oriented parallel with the long axis of the myotube and some investigators believe that microtubules may be responsible for maintenance of the elongated shape of the myotube. It is also possible that microtubules form a cytoskeleton that directs assembly of myofibrils because myofibrils, even in the earliest stages of their formation, are always oriented parallel with the long axis of the myotube. Treatment of myotubes with agents that degrade microtubules (such as colchicine or col-
chicine) causes myotubes to collapse into spherical structures called myosacs (figure 15). Rudimentary myofibrils are also completely disoriented by colcemide (figure 15), even though neither the hexagonal array of the individual myofibrillar elements nor the synthesis of myosin and actin is affected by colcemide. These two observations support a cytoskeletal role for microtubules.

In 1968, Ishikawa et al. (1968, 1969) discovered that a fourth class of filaments, in addition to microtubules, thick filaments, and thin filaments, exists in myotubes. This fourth class of filaments is about 10 nm in diameter and is distributed ubiquitously through the cell (figure 13). Ishikawa et al. (1968) called these 10 nm filaments "intermediate filaments." Although intermediate filaments were often up to 2.0 μm in length, they exhibited no preferential orientation in the cell and frequently ran perpendicularly to developing myofibrils (figure 14). Microtubule-disrupting agents do not affect intermediate filaments; rather intermediate filaments frequently appear more numerous after treatment with colcemide (figure 15). Several ultrastructural studies in the 1960's found that actin filaments seemed prevalent in early stages of myotube development and suggested that actin may therefore be synthesized at an earlier stage than myosin. Since the diameter of intermediate filaments is only slightly larger than the diameter of thin filaments, the unrecognized presence of intermediate filaments may account for some of these early ultrastructural findings. Alternatively, "actin-like" filaments (actin-like in the sense that they are of the same diameter as actin filaments and will bind heavy meromyosin like actin filaments) have been observed in many cells, including presumptive myoblasts, that presumably do not synthesize contractile proteins. Recent studies (Schroeder, 1973) have
Figure 12. This electron micrograph shows a well-developed myotube from a 16-day culture of embryonic chick skeletal muscle. Although the nuclei (N) are still centrally located, this is a terminally differentiated fiber in most other respects. Well-defined myofibrils having a cross-striated pattern identical to myofibrils in mature muscle are observed throughout the cell. Cells of this kind contract continuously in the absence of innervation. From Holtzer et al. (1973) and reproduced by permission of the Cold Spring Harbor Laboratory and the authors. X4200.

indicated that actin-like filaments exist in the contractile rings of all mitotically dividing cells, and Holtzer et al. (1973) have suggested that animal cell genomes may contain two regions that transcribe for actin. One region is transcribed in all cells, including blastula and gastrula cells early in embryonic differentiation; actin coded by this region of the genome is found in the contractile ring and in fibroblasts, presumptive myoblasts, and many other "non-muscle" cells. The other region coding for actin is transcribed only in muscle cells; this region becomes available for transcription only after a particular kind of mitosis has occurred and is transcribed at the same time that genes for the other contractile proteins are transcribed. Whatever their genetic origin, these "actin-like" filaments are also present early in myotube development, and their presence, too, could account for the ultrastructural findings that actin seems to be synthesized before myosin. Biochemical and structural studies have shown that neither microtubules nor intermediate filaments will bind heavy meromyosin, and it is very unlikely that either microtubules or intermediate filaments contain actin or myosin. The physiological significance of the intermediate filaments remains unknown.

The process of fusion can be observed in three-dimensions by use of scanning electron microscopy. Figures 16 and 17 show two striking examples of myoblasts adhering to the surface of myotubes and presumably in the process of fusing. Although fusion must certainly depend on unique properties of the surface membranes of competent cells, the scanning electron microscope does not yet possess resolving power sufficient to detect these unique surface properties.

Z-Disk Development. Development of Z-
Figure 13. An electron micrograph of a 6-day culture of embryonic chick skeletal muscle that shows a mononucleated cell (MB) lying next to a myotube (MT). Microtubules (mt) are observed in the myotube coursing parallel with developing myofibrils (mf). Intermediate or 10 nm filaments (f) are also observed both in the mononucleated cell and in the myotube, but these filaments are not so precisely aligned parallel to the myofibrils. The plasmalemmas of the mononucleated cell and the myotube run parallel and these cells may be in the process of fusing. Ribosomes (r), nucleus (n), dense granules (dg), rough-surfaced endoplasmic reticulum (er). From Shimada (1971) and reproduced by permission of the Rockefeller University Press and the author. X10,600.

disks has long been of interest because Z-disks form connections between adjacent sarcomeres in mature muscle, and it might therefore be supposed that Z-disks are formed early in development and serve as organizing centers for subsequent assembly of thin and thick filaments into myofibrils. It has been difficult, however, to study Z-disk development because, although Z-disks have a distinct and unique appearance in mature skeletal muscle, they are not so easily recognized in embryonic muscle. The narrow, fibrillar, Z-disks observed in mature skeletal muscle seem to differentiate from amorphous, broadened, darkly staining areas located approximately in the center of I-bands in early myofibrils (figure 18). These broad, amorphous, darkly staining areas, in turn, seem to be derived from coalescence of darkly staining amorphous "Z-bodies" (figure 19). The origin of Z-bodies is unknown, although Hagopian and Spiro (1970) have observed that Z-bodies in cardiac muscle occur in greatest numbers near the periphery of the cell, and that in some instances, direct connections can be seen between Z-bodies and the outer cell membrane (figure 20). Several other investigators (Heuson-Stiennon, 1965; Kelly 1969; Rash, Biese and Gey, 1970) have also suggested that Z-disks are ultimately derived from densely staining material associated with the inner surface of the sarcolemma. Although myofibrils seem to be attached to the surface membrane of myotubes, the relationship of these attachments to mature Z-disks remains
Figure 14. This electron micrograph of a 4-day culture of embryonic chick skeletal muscle shows some of the first myofibrils that form in a myotube. Thick and thin filaments seem oriented parallel with the long axis of the myotube at all stages of development. On the other hand, intermediate or 10 nm filaments (arrows) are randomly oriented and in one instance are almost perpendicular to the developing myofibril. Microtubules (Mt) are seen running parallel with the developing myofibrils. From Holzer et al. (1973) and reproduced by permission of the Cold Spring Harbor Laboratory and the authors. X30,000.

unknown. Moreover, although some authors (Heuson-Stiennon, 1965; Rash et al., 1970) have suggested that primitive Z-bodies appear very early in muscle development before assembly of thick and thin filaments into sarcomeres, other authors state (Fischman, 1970; 1972; Fischman and Zak, 1971) that Z-disks do not appear until after thick and thin filaments have assembled into individual hexagonal arrays and that Z-disks are therefore necessary only for longitudinal growth of the myofibril. Because of the difficulty in conclusively identifying embryonic Z-disks in the first stages of their development, it seems unlikely that the disagreement about the stage at which Z-disks appear in myogenesis will be resolved without the use of biochemical studies attempting to detect Z-disk proteins by immunochemical techniques.

Development of the Sarcoplasmic Reticulum and T-System. Development of the sarcoplasmic reticulum and T-system has not been studied as extensively as formation of the myofibril. T-tubules have been suggested to originate as caveolae of the plasmalemma of the myotube (Kelly, 1971; Warren and Porter, 1969). These caveolae appear somewhat later in development of the myotube than either thick or thin filaments or the sarcoplasmic reticular membranes do. The caveolae proliferate and gradually push into the interior of the myotube (figure 21). The extended caveolae usually lie parallel or at an oblique angle to myofibrils early in their development, and they do not assume their transverse orientation until later. Membranes of the sarcoplasmic reticular system develop from granular endoplasmic reticular membranes which in turn probably develop from the outer membrane of the nuclear envelope (Ezerman and Ishikawa, 1967). The sarcoplasmic reticular membranes do not form
terminal cisternae and fenestrated collars around myofibrils until fairly late in development. Consequently, the organization of thick and thin filaments into myofibrils is not determined by a membranous sarcoplasmic reticular scaffolding (Fischman, 1967; 1970; 1972). Rather, the sarcoplasmic reticular membranes appear to form around the already developed myofibrils. Lough et al. (1972) have shown that sarcoplasmic reticular membranes possess Ca²⁺ accumulating ability soon after they are formed in tissue cultures, and before spontaneous contractions occur.

Although T-tubule development lags behind development of the sarcoplasmic reticulum in vivo (Kelly, 1971), T-tubules and sarcoplasmic reticular membranes seem to develop in parallel in cultured cells (Ezerman and Ishikawa, 1967; Ishikawa, 1968).

**General Comments on Myogenesis.** Before discussing ultrastructural observations on growth of myofibers, it should be pointed out explicitly that tissue culture studies have demonstrated that the myogenic events described in the preceding paragraphs all occur in cell cultures and therefore in the complete absence of innervation. Consequently, the information for differentiation of myoblasts, fusion, synthesis of myosin and actin, and formation of myofibrils is all programmed into the presumptive myoblast, and innervation is not necessary for development of myofibers.

**Growth of Muscle Cells**

As indicated earlier in this review, postnatal muscle growth of mammals occurs almost entirely by enlargement of existing multinuclea-
Figure 16. A scanning electron micrograph of multicellular chains (MC), some of which are ostensibly myotubes (MT), seen in a 48-hr. culture of chick embryonic skeletal muscle. Spindle-shaped and rounded myogenic cells (MB) have fibroblastic cells (F) lying among them. The inset shows an enlargement of the area enclosed by the square. A myotube (MT) is seen with a cell, presumably a myoblast (MB) adhering to it and possibly in the process of fusing with it. The three-dimensional effect seen in scanning electron micrographs emphasizes the tubular nature of myotubes. From Shimada (1972) and reproduced by permission of Academic Press, Inc. and the author. X600. Inset X2700.

Compared muscle cells (hypertrophy) and not by an increase in the number of muscle cells. The very small postnatal increase in number of muscle cells observed in some species occurs immediately after birth and can be regarded as an extension of embryonic muscle differentiation. For example, marsupials, such as the kangaroo, are born at a very early stage in their development, and the number of muscle cells in marsupials increases rapidly for the first 100 days or so beyond birth. Further muscle growth then occurs entirely by hypertrophy of the existing fibers. Mammals, on the other hand, are born at a relatively late stage in their development, and the number of muscle cells in mammals either does not increase at all postnatally or increase slightly only for the first few days after birth. Postnatal muscle growth, therefore, differs from embryonic or prenatal muscle growth in at least two fundamental ways: 1) postnatal muscle growth is due principally to hypertrophy (increase in cell size), whereas embryonic muscle growth originates from both hypertropy and hyperplasia (increase in cell number); 2) postnatal muscle growth is greatly influenced by innervation and circulating hormones (such as insulin or growth hormone), whereas embryonic muscle can differentiate and develop in the complete absence of innervation.

Goldspink (1972) has shown that the postnatal increase in skeletal muscle cell size is due almost entirely to an increase in the number and size of individual myofibrils within the cells; increase in the amount of cytoplasm evidently contributes very little to skeletal muscle cell growth. Numerous investigators (Cheek et al., 1971; Moss, 1968; Williams and Goldspink, 1971; Winick and Noble, 1965) have reported that the total number of nuclei
within each multinucleated skeletal muscle cell also increases during growth, even though nuclei within the multinucleated cells do not divide. Consequently, three principal questions exist concerning postnatal growth of skeletal muscle: 1) What is the origin of new nuclei that appear in multinucleated skeletal muscle cells during growth? 2) What is the mechanism used to increase the number of myofibrils in growing skeletal muscle cells? Do new myofibrils begin to assemble de novo in the center of the cell and then grow in length until they extend from one end of the cell to the other, or is some other mechanism used? 3) How do myofibrils grow longitudinally without having to divide and form a gap large enough to insert a new sarcomere? The last three sections of this review will discuss the current evidence relating to these three questions.

Satellite Cells and the Postnatal Increase in Skeletal Muscle Nuclei. In 1961, Mauro (1961) described the presence of small, mononucleated, fusiform cells, which he called satellite cells, lying between the basement membrane and the plasmalemma of multinucleated, skeletal muscle cells (figure 22). Satellite cells lie so close to the larger multinucleated skeletal muscle that they appear to be part of the multinucleated cell when viewed in the light microscope. Consequently, satellite cells can be identified conclusively only in the electron microscope. Since Mauro's discovery, numerous studies (Church, 1969; MacConnachie, Enesco, and Leblond, 1964; Moss and Leblond, 1970; 1971; Shafiq, Gorycki and Mauro, 1968) have reported that satellite cells will incorporate labeled thymidine into their nuclei, and that satellite cells therefore possess mitotic capabilities (figure 23). Moreover, the careful studies of Moss and Leblond (1971) show that satellite cells also possess the ability to fuse with mature skeletal muscle cells and thereby add one
Figure 18. Peripheral region of a myotube in newt larval limb muscle differentiating in vivo. A well-formed myofibril containing a primitive Z-disk is observed lying interior to a mixture of nonregistered thick and thin filaments. Several Z-bodies (Zb) which may be precursors of the Z-disk also are seen in the region containing the nonregistered filaments. From Kelly (1969) and reproduced by permission of the Wistar Institute Press and the author. X67,800.

Figure 19. A portion of a mesenchymal cell in an area of muscle formation in a newt larval limb bud. No thick and thin filaments are evident in this cell, but numerous free ribosomes and randomly oriented 10 nm filaments (arrows) are seen. Peripherally located skeins of finer filamentous elements (S) that might be precursors of Z-bodies are also evident. From Kelly (1969) and reproduced by permission of the Wistar Institute Press and the author. X33,400.
nucleus to the skeletal muscle cell's complement of nuclei. Consequently, it seems probable that satellite cells are the origin of the new nuclei that appear in multinucleated skeletal muscle cells during growth.

The origin of satellite cells remains uncertain. Some investigators believe that satellite cells represent dedifferentiated myoblasts that failed to fuse during embryonic development but that retain the capacity to re-differentiate and form a myoblast. This view is supported by the finding that the proportion of satellite nuclei to skeletal muscle nuclei decreases about eightfold between birth and maturity in the rat (Goldspink, 1972). Such a decrease would be expected if satellite cells were remnants of embryonic myoblasts and were therefore not replaced after they fused with multinucleated muscle cells. On the other hand, satellite cells seem particularly numerous in injured areas of muscle tissue, and some studies have suggested that satellite cells originate from multinucleated skeletal muscle cells themselves (Hess and Rosner, 1970; Reznik, 1969). According to these latter studies, multinucleated skeletal muscle cells respond to an injury or trauma by isolating a nucleus and a small envelope of its surrounding cytoplasm and then pinching this nucleus off to form the satellite cell. Once removed from the multinucleated cell, the pinched-off nucleus ostensibly regains its capacity for mitotic division, and it divides to form more myoblast-like cells, which can then fuse to replace or regenerate the injured multinucleated cell. Whatever the origin of satellite cells, they seem to be responsible both for the limited capacity of skeletal muscle to regenerate and for the new nuclei that appear during muscle growth. Cheek et al. (1971) and Goldspink (1972) have suggested that a limit exists as to how much cytoplasmic area a single nucleus can maintain and that an increase in number of nuclei is a necessary prerequisite to an increase in muscle cell size.
Increase in Number of Myofibrils in Growing Skeletal Muscle. There are at least two possible ways that the number of myofibrils can increase in growing skeletal muscle. First, newly assembled thick and thin filaments could combine with Z-disk proteins to form a sarcomere, and a new myofibril could be assembled de novo by adding more sarcomeres to this first sarcomere until the myofibril extends the entire length of the cell. This is essentially the same process used to form myofibrils in embryonic muscle. Since mature, skeletal muscle cells already possess myofibrils, however, they also possess an alternative method for forming additional myofibrils that is not available to an embryonic muscle cell just beginning myofibril assembly. This second method involves longitudinal splitting of one or more of the existing myofibrils to form two daughter myofibrils.

If myofibrils proliferated by the first of these two possible methods, single sarcomeres and short segments of three, four, or five sarcomeres should be observed in growing muscle cells. Such segments have not been seen in electron micrographs of growing muscle. On the other hand, longitudinal splitting of myofibrils has been observed frequently in growing muscle (Goldspink, 1970; 1971; 1972). Myofibril splitting seems to commence at the Z-disk and then spread longitudinally (figure 24). Extensive studies by Goldspink (1970) have shown that the number of myofibrils in mouse biceps brachii muscle may increase about 15-fold during postnatal growth. Moreover, Goldspink (1972) finds that myofibrils in fast growing muscles such as mouse biceps brachii form two distinct classes with respect to diameter. The smaller myofibrils are about 15 to 20 μm in diameter and are not observed splitting, whereas the larger myofibrils are approximately 40 μm in diameter and can often be observed in the process of splitting longitudinally. Goldspink (1972) suggests that when a myofibril reaches a critical size (40 μm
in diameter for mouse biceps brachii), it splits to form two smaller myofibrils. The force for such longitudinal splitting may originate from stress placed on the Z-disk by the oblique pull of actin filaments during a contraction (Goldspink, 1971). This stress may cause the Z-disk to tear near its center, and spreading of this tear longitudinally will then split the entire myofibril. Because very rapid contractions may produce greater stresses on Z-disks than slow contractions, this mechanism would account for the observation that myofibrils in rapidly contracting muscles have small diameters, whereas myofibrils in slowly contracting muscles tend to be larger in diameter.

Increase in Length of Myofibrils. The limbs of most animals approximately double in length during postnatal growth. This increase in limb length must be paralleled by an increase in length of myofibrils in the muscles attached to these limbs. Myofibrils can lengthen in either one of two ways: 1) the individual sarcomeres can lengthen; or 2) the number of sarcomeres constituting a myofibril can increase. Experiments on mouse biceps brachii muscle (Goldspink, 1968; 1972) have shown that average sarcomere lengths in this muscle increase from 2.3 μm in the newborn animal to 2.8 μm in the adult. This increase is due entirely to a decrease in amount of overlap of interdigitating thick and thin filaments, and not to an increase in lengths of the filaments themselves. Sarcomeres at the ends of multinucleated skeletal muscle cells are always shorter than those near the center. This postnatal increase in sarcomere length, however, can only account for approximately 25% of the total increase in myofibril length during muscle growth (Goldspink, 1968). Most of the total increase in myofibril length is due to addition of new sarcomeres. Goldspink’s measurements (Goldspink, 1972) indicate that the number of sarcomeres per myofibril in mouse soleus muscle increases substantially during postnatal growth. Most of this increase in sarcomere numbers occurs during the first 3 weeks after birth, when growth rate of the limbs is greatest, and increases in myofibril length after 3 weeks of
Figure 23. An electron microscope radioautograph of muscle from a 30-g rat 100 hr. after injection of thymidine-\(^3\)H. A satellite cell (S) is identified by the space (arrows) that separates it from the myofibril-containing cytoplasm and by its location under the basement membrane (BM) of the mature muscle cell. A nucleus (N) that contains no silver grains is observed within the muscle cell in direct contact with the myofibrils. The satellite cell nucleus is overlaid with silver grains, which indicates that it has incorporated thymidine-\(^3\)H during DNA synthesis prior to mitosis, and that it therefore is mitotically active. From Moss and Leblond (1970) and reproduced by permission of the Rockefeller University Press and the authors. X28,300.

Age are due principally to increases in sarcomere length.

The mechanism by which new sarcomeres are added to myofibrils that extend continuously from one end of a muscle to the other remains a fascinating problem. Experiments in which labeled adenosine was injected into young rats have shown that most of the adenosine remaining with myofibrils isolated from these rats was bound to actin (Griffin, Williams and Goldspink, 1971). Consequently, intracellular location of the labeled adenosine could be used to determine the site of the most recently synthesized actin, and hence the site of formation of new sarcomeres. Such experiments showed that new sarcomeres were being added at the ends of multinucleated skeletal muscle cells. This result is consistent with the fact that the terminal sarcomeres of myofibrils are invariably shorter than those in the middle; presumably, the terminal sarcomeres are the most recently formed, and they have not yet had time to lengthen. These findings, however, still provide no clues as to the mechanism by which sarcomeres are added to continuous myofibrils.

One possible mechanism for addition of sarcomeres to myofibrils has been proposed by Legato (1970), who suggests that Z-disks are the centers for production of new sarcomeres in cardiac muscle. According to Legato, sarcomereogenesis begins as hypertrophy of the Z-disk (figure 25). The Z-disk widens until it is as wide as an entire sarcomere; at this stage the accumu-
lated Z-substance is gradually replaced with thick and thin filaments until a new sarcomere, bordered on either edge by the formerly hypertrophied Z disk, has been formed (figure 25). Legato substantiates her ingenious hypothesis with electron micrographs purporting to show sarcomeres in each stage of the scheme she proposes for sarcomerogenesis (figure 26, 27 and 28). Legato's proposal provides for addition of new sarcomeres without interrupting myofibril continuity and hence ability of the myofibril to function, but if Legato's mechanism also applies to skeletal muscle, it is somewhat surprising that hypertrophied Z-disks are not observed more frequently than has been reported. Additional studies are needed to determine whether Z-disk hypertrophy has an integral role in sarcomerogenesis, or whether it is a response of myofibrils to cell injury or trauma.
Figure 25. Diagrammatic representation of a possible mechanism for elongation of myofibrils in cardiac muscle. Z-disks are indicated, and the cross-hatched area represents an enlarged Z-disk. Parallel, vertical lines in the middle of the sarcomere represent M-lines. From Legato (1970) and reproduced by permission of Academic Press, Inc. and the author.
Figure 26. This electron micrograph of cardiac muscle shows Z-disks just in the initial stages of hypertrophy. The very subtle swelling of the Z-disk (arrows) represents the earliest stages in Z-substance accumulation. From Legato (1970) and reproduced by permission of Academic Press, Inc. and the author. X25,800.
Figure 27. Accumulation of Z-substance (Z) has nearly reached its maximum in this electron micrograph of cardiac muscle. The hypertrophied Z-disk extends nearly the length of one sarcomere, and is longer than the adjacent A-band (A). When this hypertrophied Z-disk is replaced by thick and thin filaments, the resulting myofibril will be one sarcomere longer than it previously was. This micrograph is represented by Stage II in figure 25. From Legato (1970) and reproduced by permission of Academic Press, Inc. and the author. X33,500.
Figure 28. This electron micrograph of cardiac muscle illustrates the last step in elongation of myofibrils, when the hypertrophied Z-disk is replaced by primary filaments (PM). Remnants of the hypertrophied Z-disk are still evident. This stage in sarcomerogenesis is represented by Stage III in figure 25. From Legato (1970) and reproduced by permission of Academic Press, Inc. and the author. X36,300.

Literature Cited


