Introduction

The development and proliferation of undifferentiated cells into muscle is called myogenesis while the more specific process of myofibril assembly is called myofibrillogenesis. The assembly of myofibrils from their constituent proteins is a complicated process and the mechanisms involved are just beginning to be understood at the cellular level. During differentiation of myoblasts, there is a dramatic shift in protein expression, resulting in the synthesis of muscle-specific proteins. These myofibrillar proteins subsequently associate into the highly organized supramolecular structure called the myofibril. The mechanisms by which the thin and thick filament components are aligned and interdigitated are poorly understood. Some of the mechanisms involved in the formation of thin and thick filaments reside in the domains on the constituent proteins. Both myosin and actin will polymerize in vitro to form filamentous structures, yet the resulting filaments have highly variable lengths in contrast to those found in the myofibril. Thus, there must be some cellular mechanism which dictates the organization and final filament lengths that are observed in the sarcomere. This mechanism likely involves a higher-level protein template or foundation upon which the nascent filaments form and subsequently interdigitate, giving rise to the myofibril.

The ultrastructure of the mature myofibril and cartoons of the arrangements of its constituent contractile and structural proteins are shown in Figure 1. A few features of this diagram represent observation from more recent studies. These include the spanning of titin from the Z-line to the middle of the A-band (Fürst et al., 1988); the binding of nebulin along the thin filament (Wang and Wright, 1988); capping of the actin filaments at the Z-line by the protein Cap Z (Casella et al., 1987); and the localization of zeugmatin at or near the Z-line (Maher et al., 1985). At right, we describe the processes involved in the assembly of this structure with emphasis on the mechanisms thought to be involved in this process. Other reviews on this subject can be found in Fischman (1986) and Epstein and Fischman (1991) and a review of the genetic and hormonal regulation of myogenesis can be found in the preceding articles of this publication.

Histogenesis of Skeletal Muscle

Cells committed to become skeletal muscle, called presumptive myoblasts, originate from the somites of the mesodermal germ layer (see Stockdale, 1992 and references cited therein). During development, these cells are motile and migrate from the somite to regions which develop into muscle. Figure 2 shows the various stages of muscle cell development. The presumptive myoblasts give rise to myoblasts, which are spindle-shaped cells. These cells subsequently cease cell division, begin synthesis of myofibrillar proteins and assembly of nascent myofibrils, and then fuse to form myotubes.
During the development of muscle fibers from myotubes, there are gross morphological changes which occur and these are shown in Figure 3. Initially, the myotube is rather amorphous with centrally located nuclei. The first myofibrils form at the cell periphery and continue to form until they fill the entire cell. The myofibrils are initially poorly aligned at the Z-disc, and during development they align to form the transverse striations characteristic of the muscle fiber. Accompanying this morphological transition is the migration of the nuclei from the center of the cell to the periphery. Some examples of these morphological changes are shown in the electron micrographs in Figures 4 and 5. A well-developed fiber is shown in the lower portion of Figure 4 with mature appearing myofibrils and peripherally located nuclei. Younger myotubes are adjacent to this developed fiber and have the characteristic centrally-located nuclei and sub-sarcolemmal myofibrils. An enlargement of the marked area is shown in Figure 5, and it demonstrates a very small diameter myofibril in close apposition to the sarcolemma.

An interesting feature of myofiber development is the formation of primary myotubes followed by secondary myotube formation, the latter using the primary myotube as a template. Primary myotube formation occurs by the apparently simultaneous fusion of myoblasts (usually 3-4), and this occurs early in the embryonic development of muscle. Soon afterwards, secondary myotubes form by the fusion of myoblast along the primary myotube. A unique feature of secondary myotube formation is its initiation only near the motor endplate region of the primary myotube (Duxson et al., 1989). During this development, the primary myotube and the developing secondary myotube are encased within the same basal lamina and coupled by gap junctions (Ontell, 1977). Growth and development of the secondary myotube occurs by a random fusion of myoblasts along the length of the developing secondary fiber. Once the secondary fiber has grown sufficiently in length to reach the tendon, it develops its own basal lamina and is no longer intimately associated with the primary myotube.

**Expression of Muscle-Specific Proteins**

The MRF family of transcriptional regulators is involved in the process of converting undifferentiated cells into muscle (for review see: Mulvaney, this volume; Weintraub, 1993; Olson, 1992). These regulators, through a complex mechanism, cause the cells to cease division and activate the transcription of muscle-specific proteins. However, two muscle-specific marker proteins are expressed in mitotic myoblasts; these are desmin and a muscle-specific integral membrane protein, the o, integrin subunit (Kaufman et al., 1991, George-Weinstein et al., 1993). These proteins do not appear to be under the control of MyoD1 or myogenin (Kaufman et al., 1991) and can be used to distinguish which cells are destined to become primary or secondary fibers (George-Weinstein et al., 1993). In post-mitotic round myoblasts, the first myofibril-specific pro-
Electron micrograph of post-natal muscle. The sample was obtained from a 1 day-old rat and prepared by perfusion fixation with 4% formaldehyde, ethanol dehydration, embedding in LR White and thin sectioning. After staining, the section was fixed with glutaraldehyde, post-stained with uranyl acetate and lead citrate and then observed with an electron microscope at 50 KeV. Arrows mark Z-line and n denotes nuclei. Scale bar is 1 micron.

Some proteins show rather unique expression during muscle development. The non-muscle isoform of filamin disappears during early development of myofibrils and the muscle isoform re-appears after well-defined myofibrils have developed (Gomer and Lazarides, 1983; Price et al., 1994). The expression of intermediate filament proteins changes dramatically during myogenesis. Prior to fusion, vimentin is the major intermediate filament protein expressed; following fusion, vimentin expression decreases and desmin expression is increased (Gard and Lazarides, 1980). More recent experiments have found that there is transient expression of a newly-discovered intermediate filament protein called nestin (Sejersen and Lendahl, 1993). This protein is expressed early in development but is down regulated as is vimentin during maturation such that in the adult fiber, desmin is the major intermediate filament protein present (Sejersen and Lendahl, 1993).

During muscle development, there are also changes in the isoforms of many of the muscle-specific proteins. Myosin has received the most attention and studies have found that there are several developmental isoforms that occur during muscle maturation (Bandman, 1985). Isoforms of other contractile and structural proteins are present during development and this has been the subject of recent reviews (Bandman, 1992; Obinata, 1993).

While the various myofibrillar proteins may be present in the myoblast and young myotube, their organization into the mature myofibril is an elaborate process. This organization likely requires some sort of supramolecular structure to facilitate the organization of the various proteins into their final location in the adult myofibril.

**Role of Cell Adhesion Molecules in Myogenesis**

Both cell-extracellular matrix adhesion and cell-cell adhesion molecules are involved in myogenesis and may be involved in myofibril assembly. Cell-matrix interactions involve the integrins (see Hynes, 1992, for review). These proteins are dimers made of an \( \alpha \) and \( \beta \) subunit, and there are numerous isoforms of each subunit. The dimer is an integral membrane protein which has extracellular domains that bind to proteins of the extracellular matrix (fibronectin, laminin and collagen) and intracellular domains which bind to specific proteins of the cell cytoskeleton (talin). There are other cell adhesion molecules which are involved in cell-cell attachments (Edelman, 1986). Those relevant to myogenesis are neural...
cell adhesion molecule (NCAM), N-cadherin and M-cadherin. These various proteins are thought to be involved in the process of differentiation, myoblast fusion and possibly myofibrillogenesis.

**Cell-Extracellular Matrix Adhesion Molecules**

In muscle, integrins are present throughout development and in the mature tissue (Bozyczko et al., 1989). Occupancy of the extracellular matrix binding site of the αβ, integrin with laminin is important in proliferation of myoblasts (George-Weinstein et al., 1993) and, using a different receptor, in signalling the cessation of cell division (Menko and Beottiger, 1987). Specific integrins and their receptors are also involved in the formation of secondary myotubes upon existing primary myotubes (Rosen et al., 1992). As described above, one of the earliest markers for cells destined to become muscle is the α7 integrin subunit. Expression of the αA subunit is developmentally regulated during myogenesis. Recent studies have found that there are three isoforms with the α7, being the major form present in mitotic myoblasts while the αA and αC appear upon differentiation (Song et al., 1993). Integrins function likely involving signalling mechanisms using their coupling to the cell cytoskeleton (Hynes, 1992). An important aspect about this coupling is that it is involved in several aspects of myogenesis (proliferation, differentiation and myofibril assembly). In vivo, this coupling facilitates migration of presumptive myoblasts to the proper extracellular matrix environment and subsequent proliferation and differentiation in this matrix, these events likely involving genetic regulation through coupling of the integrins to the cytoskeleton (Enomoto et al., 1993; George-Weinstein et al., 1993; Menko and Beottiger, 1987). In developing muscle cells, this coupling is complex (Lowrey and Kaufman, 1989). The integrins are thought to be involved in myofibril assembly (Terai et al., 1989; Volk et al., 1990) but whether they play a pivotal role in myofibril formation is yet to be determined.

**Cell-Cell Adhesion Molecules**

Another group of cell-adhesion molecules is involved in cell-to-cell adhesion, a prerequisite for myoblast fusion. The interaction of specific cells is an important process in the histogenesis of muscle and other tissues, and specific membrane proteins are involved (for review see: Edelman, 1986; and Rutishauser et al., 1988). The process of myoblast fusion involves both calcium-dependent and independent cell adhesion molecules. The calcium-dependent adhesion proteins that are likely involved in myoblast fusion are M-cadherin (Donalies et al., 1991) and N-cadherin (Knudsen et al., 1990a). M-cadherin is a muscle-specific protein that is expressed at low levels in myoblasts and its expression increases upon differentiation of myoblasts into myotubes (Donalies et al., 1991). The neuronal cadherin (N-cadherin) also shows increased expression in fusing myoblasts and down-regulation in mature myotubes (Mege et al., 1992; Fredette et al., 1993). Treatment of myoblasts with antibodies to N-cadherin causes inhibition of both myoblast aggregation and subsequent fusion, suggesting that this protein plays a role in the process of myoblast fusion (Knudsen et al., 1990a; Mege et al., 1992).

Calcium-independent cell adhesion molecules may also be involved in myoblast fusion (Knudsen et al., 1990b). These molecules are called NCAM's (neuronal cell-adhesion molecules, for review see Rutishauser et al., 1988), and they show marked changes in isofrom expression and the extent of post-translational modification during the development of muscle. Antibodies against NCAM also inhibit myoblast aggregation (Knudsen et al., 1990b), suggesting this protein has a role in myoblast fusion. There are changes in the isofrom expressed and the types and extent of post-translational modifications during the transition from myoblast to myotube. In myoblasts, the 140 kD non-polysialylated transmembrane form predominates; while in myotubes, the 125 kD polysialylated lipid-linked membrane protein predominates (Covault et al., 1986). This latter isofrom is muscle specific (Barton et al., 1988) and transfection experiments demonstrate that its over-expression enhances myoblast fusion (Dickson et al., 1990).

An interesting feature of these cell adhesion molecules is that their level of expression and degree of post-translational modification changes to favor separation of developed myotubes. After development of secondary myotubes along primary myotubes, the secondary myotube separates and develops its own basal lamina. Thus, a change in the nature of cell-adhesion molecules must occur for this process to proceed. There is a decrease in the expression of N-cadherin during maturation of myotubes which likely decreases cell-cell adhesion (Mege et al., 1992; Fredette et al., 1993). There is also a change in the degree of sialation of NCAM's (Fredette et al., 1993). Highly polysialylated NCAM's favor cell separation (Rutishauser et al., 1988) and with maturation, the NCAM's become more polysialylated favoring separation of developed myotubes (Fredette et al., 1993). Thus, the cell-adhesion molecules are involved in the processes of initial cell fusion followed by cell separation.

**Methods Used to Study Myofibril Formation**

The methods normally used to study myofibril formation are inherently structural in nature. Both electron (see Figures 4 and 5) and light microscopic methods are employed to study the process of myofibrillogenesis from a structural standpoint. To define which proteins are involved in myofibrillogenesis, immunohistological techniques are usually employed in which antibodies are produced which detect specific myofibrillar proteins. The detection of these antibodies is either by immunogold techniques at the electron microscope level or with immunofluorescence techniques at the light microscope level (for example, see Figure 6). A major disadvantage of immunohistochemical methods is that the resulting images are only a snapshot of a dynamic process since the cells are chemically fixed prior to observation. Another disadvantage is that the cells are lyzed prior to or during fixation, which may result in the loss of unstable structures.

A different light microscopic method can be employed in which single cultured myoblasts/tubes are micro-injected with fluorescent conjugates of myofibrillar proteins followed by observation of the living cells with a highly sensitive fluorescence microscope. This approach has been termed fluorescent analogue cytochemistry (Wang, 1989) and has been used with cardiac and skeletal muscle cells in culture (Sanger et al.,...
Immunofluorescence of muscle cells with titin antibodies. Developing cultured cardiac muscle cells were stained with an anti-titin monoclonal antibody (9D10) and observed with phase contrast (A) and fluorescence (B) optics. The arrows mark stress-fiber-like structures and scale is 5 microns. Reproduced from Greaser et al., 1989 with permission of John Wiley & Sons.

1986; McKenna et al., 1986). A unique feature of this approach is that the temporal changes that occur during myofibril formation can be followed within the same cell for several hours to days. Also the specific distribution of two distinct proteins can be followed simultaneously. An example of this is shown in Figure 7 (A, B) in which a fibroblast was microinjected with both rhodamine tubulin and fluorescein alpha-actinin. The unique distribution of each of these cytoskeletal proteins is readily observed in the images. A requirement for this approach is that the injected fluorescent protein be readily incorporated into existing structures as if it were indistinguishable from the intrinsic proteins. This has been demonstrated for several myofibrillar and cytoskeletal proteins (Sanger et al., 1994). This exchange also occurs in cultured mature muscle cells (Imanaka-Yoshida et al., 1993). In some cases, the exchange occurs in vitro (Sanger et al., 1984).

The exchange of alpha-actinin in vitro in myofibrils is demonstrated in Figure 8. The myofibrils were incubated with fluorescein-labeled myosin subfragment 1 and rhodamine-labeled alpha-actinin and, at different times, separated from soluble fluorescent protein, fixed and imaged. The images were obtained using the same exposure conditions to demonstrate the change in fluorescence intensity over the incubation period. The fluorescein-S1 shows the same intensity over the incubation period while the rhodamine alpha-actinin fluorescence intensity increases. These studies demonstrate that rhodamine alpha-actinin exchanged with the native, unlabeled alpha-actinin in the Z-line while the fluorescein S1 did not.

Fluorescent analogue cytochemistry of a fibroblast and a cardiac myocyte. The fibroblast was injected simultaneously with rhodamine tubulin (A) and fluorescein alpha-actinin (B), and images were recorded from the live cells after incorporation of fluorescent proteins. A. Fibroblast tubulin pattern (rhodamine filter). B. Same cell, alpha-actinin pattern (fluorescein filter). C. A cardiac myocyte was injected with fluorescein alpha-actinin and the image was recorded from the cell after incorporation. Note both the similarities and differences in the alpha-actinin distribution in the fibroblast and myocyte.
This figure shows the dynamic nature of a structure generally assumed to be quite static.

The molecular genetics approach has recently been employed to determine whether specific myofibrillar proteins are essential for myofibrillogenesis (for review, see Epstein and Fischman, 1991). The Drosophila and C. elegans model systems have been used to study muscle protein mutants (see Epstein and Bernstein, 1992, for review). For example, Drosophila alpha-actinin mutants have abnormal Z-lines (Fytberg et al., 1990) and C. elegans vinculin mutants fail to develop functional muscle (Barstead and Waterson, 1990). Another molecular genetics approach is to transflect cells with cDNA encoding a truncated protein with altered function. Experiments using this approach have shown that the cytoskeletal protein desmin is not likely to be essential for myofibrillogenesis (Schultheiss et al., 1991), and the expression of carboxyl-end truncated alpha-actinin in myotubes results in the breakdown of myofibrils (Schultheiss et al., 1992). These methods will continue to define the specific protein players which are involved in the process of myogenesis.

Mechanisms of Myofibril Assembly

The true molecular mechanism or sequence of events resulting in myofibril formation is currently unknown. Most current models are based upon the involvement of the cell cytoskeleton acting as a template for the formation, alignment and possible registration of myofibrils in the mature muscle fiber. Thus a brief review of the cell cytoskeleton is helpful in understanding the nature of this structure followed by a description of how its components may be involved in myofibril formation.

The Cell Cytoskeleton and Its Role in Myofibrillogenesis

The cell cytoskeleton is made of an array of filamentous proteins which interconnect cellular organelles and attach to the cell membrane. These filaments are involved in the maintenance of cellular morphology, movement of organelles within the cell and cellular motility (for review, see Schlwa, 1986). There are three filament systems involved in the cytoskeleton: microfilaments, intermediate filaments and microtubules. Microfilaments are made of predominantly actin, and also contain alpha-actin, tropomyosin, caldesmon and myosin. When bundled and complexed with alpha-actinin, tropomyosin, caldesmon and myosin, the filaments are called stress fibers and they are contractile in nature (Kreis and Birchmeier, 1980). The proteins of the stress fibers are usually non-muscle isoforms. Intermediate filaments are made of vimentin and desmin in myoblasts and muscle cells and have a characteristic 10 nm diameter. Microtubules are made primarily of tubulin and have a diameter of 25 nm and a hollow core. Other proteins, the microtubule associated proteins (MAPS), are involved in organization and stabilization of microtubules (Hirokawa, 1994).

In mature muscle, the cytoskeleton can be divided into the inter-myofibrillar cytoskeleton and the intra-myofibrillar cytoskeleton. The inter-myofibrillar cytoskeleton is thought to maintain sarcomeric registration across the fiber through interconnections between myofibrils and between the peripheral myofibrils and the sarcolemma, while the intra-myofibrillar cytoskeleton maintains the contractile elements of the sarcomere in register (for review see Greaser, 1991). The various filament networks of the cytoskeleton found in non-muscle cells are relatively sparse in the inter-myofibrillar cytoskeleton of fully developed muscle, while in myoblasts and myotubes, they are more prevalent. Thus, these networks of filaments may be involved in myofibril formation by acting as an organizing center for myofibril assembly. The elements of the intra-myofibrillar cytoskeleton may also be involved in the myofibril assembly. These elements consist of muscle-specific proteins other than those described above for non-muscle cells.

Microtubules. The microtubules and their associated proteins are involved in cell division and are thought to aid in the maintenance of gross cell morphology. In myoblasts and myotubes, the cell morphology is elongate in nature; thus microtubules likely play a role in formation/maintenance of this morphology since treatment of myotubes with drugs that favor microtubule depolymerization results in rounding of the cells (Hill et al., 1986). Indeed, in myoblasts, microtubules are observed at higher levels near the cell periphery and in longitudinal orientation (Warren, 1974, Tassin et al., 1985). During development, there is a decline in the amount of tubulin (Cartwright and Goldstein, 1982). In mature muscle, microtubules are also found in predominantly longitudinal orientation and between myofibrils (Goldstein and Entman, 1979) with a greater density at myofibrils near the fiber surface (Kano et al., 1991).

Perturbations by drugs that modify the cell cytoskeleton, specifically the microtubule skeleton, result in changes in muscle cell morphology and development. For example, drugs which stabilize the microtubule network inhibit myoblast fusion and result in the normal spindle-shaped myoblast being star-shaped (Antin et al., 1981). Within these cells, there are striated myofibrils made of longitudinally aligned A-bands in which the actin filaments are replaced by microtubules, and poorly organized I-Z-I like structures form in other regions of the cell (Antin et al., 1981; Toyama et al., 1982). Conversely, drugs which destabilize microtubules change the normally elongated myotube into a round cell with poorly aligned myofibrils organized in a circular pattern (Toyama et al., 1982; Hill et al., 1986). These studies showed that A-bands and I-Z-I structures can develop independent of each other, suggesting that the simultaneous assemblage of A-band and I-Z-I structures as a sarcomere is not a prerequisite for myofibril formation (Antin et al., 1981; Toyama et al., 1982; Hill et al., 1986). However, correct myofibril formation requires the normal arrangement of microtubules.

For microtubules to be involved in myofibril formation, they must likely bind to a myofibrillar protein allowing for a connection between the microtubule and the developing myofibril. Recent in vitro experiments with fluorescently-labeled tubulin have shown that tubulin binds to the myofibril at its periphery. Figure 9 shows an image of a myofibril that was incubated with rhodamine tubulin at low concentration. The fluorescent image shows that tubulin binds to the myofibril in punctate spots, and the composite image of the phase and fluorescent images shows that the tubulin binds to the myofibril periphery. These punctate spots are likely short microtubules which may have formed on the myofibril periphery. These simple experi-
tubulin binding to myofibrils. Rhodamine tubulin (100 nM) was incubated with myofibrils followed by washing and preparation for imaging. The phase contrast (P) and rhodamine (R) fluorescence images were summed giving the P+R image. Note that the tubulin binds preferentially to the periphery of the myofibril and the fluorescence likely represents polymeric tubulin structures.


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ments demonstrate that there are tubulin binding sites on the surface of the myofibril and suggest that these sites may function in the formation or stability of myofibrils.

The role of microtubules in myofibril assembly is poorly understood at present. A working hypothesis is that the microtubule filament network functions as a template for the alignment of thick and thin filaments and stabilizes the nascent myofibril until it develops into the mature myofibril. Both biochemical and high-resolution microscopy studies are needed to define the role of microtubules in myofibril development.

Intermediate filaments. The intermediate filaments, made of vimentin and desmin in developing and adult muscle respectively, are thought to play a cytoskeletal role in mature muscle (Wang and Ramirez-Mitchell, 1983; Tokuyasu et al., 1983). During maturation of the myotube, there is a transition in the organization of intermediate filaments from a longitudinal to a lateral orientation (Bennett et al., 1979; Gard and Lazarides, 1980; Tokuyasu et al., 1984). The role of intermediate filaments is rather obscure but early hypotheses, based upon the temporal change in orientation, suggested that intermediate filaments are involved in registration of myofibrils. However, recent transfection experiments have led to the conclusion that intact intermediate filaments are not necessary for the formation and organization of myofibrils (Schultheiss et al., 1991). Other experiments with anti-sense desmin RNA have shown that inhibition of desmin expression prevents myoblast fusion and differentially inhibits myoD and myogenin, suggesting that desmin plays a role in the very early stages of myogenesis (Li et al., 1994).

A very interesting feature of intermediate filaments during myotube development is their change in orientation. This change in orientation either involves a replacement of the longitudinal intermediate filament subunits with new subunits that orient laterally or a re-organization of the existing subunits. Intermediate filaments are generally thought to be very inert structures because of their biochemical properties, but recent studies suggest that this filamentous structure is highly dynamic in living cells. When fluorescently labeled or biotin-labeled intermediate filament subunits are injected into cells, they rapidly incorporate into the existing intermediate filament network, demonstrating that the filamentous system is dynamic (Miller et al., 1991; Vikstrom et al., 1992). Recent studies have found that there is a muscle enzyme which ADP-ribosylates desmin resulting in decreased polymerization (Huang et al., 1993); inhibition of this enzyme slows differentiation and proliferation of myogenic cells (Kharadia et al., 1992). These studies suggest that intermediate filaments may play some sort of role in myogenesis and also provide a mechanism for re-organization of the filaments by altering their polymerization characteristics. Thus the role of this cytoskeletal element in myogenesis remains a mystery.

Stress fibers. The role of stress-fiber-like structures in myofibril assembly has received much attention in the last 10 years. This type of structure was first observed in fibroblasts and has been described as a rudimentary sarcomere (Gordon, 1978; Kreis and Birchmeier, 1980) because of its contractile nature and constituent contractile proteins (actin, tropomyosin, myosin, alpha-actinin). A unique feature of stress fibers is that they form connections with the cell membrane (for review, see Hilt and Juna, 1994). These connections are made through the binding of actin filaments to other proteins in a complex which forms the adherins-type junction found at cell-cell junctions and cell-matrix junctions (Burridge et al., 1987). Adherins junctions are also called focal adhesions or focal contacts when seen in cultured cells. The proteins involved in this complex are vinculin, talin, alpha-actinin, integrins and several other proteins (Geiger and Ginsberg, 1991). An example of fibroblast stress fibers is shown in the alpha-actinin image in Figure 7. Note that the alpha-actinin (Fig. 7B) is located at the cell periphery in a fibrous pattern (focal adhesions) and in the cell body as a string of punctate spots on the stress fibers. This type of junction and fiber may be important in myofibril formation as this process occurs first in the region just below the sarcolemma. Also shown in Figure 7 is an embryonic cardiac myocyte injected with alpha-actinin (Fig. 7C). The alpha-actinin is distributed throughout the cell in punctate spots and lines, and there is much less at the cell periphery. The arrangement of the alpha-actinin into Z-lines in the peripheral regions of the cell likely corresponds to more mature myofibrils in this region. This figure allows for a direct comparison of fibroblasts and myocytes in terms of their alpha-actinin distribution.

Some of the early studies which suggested a role for stress-fiber-like structures were the electron microscope studies of Peng et al., (1981). The elegant micrographs from these studies showed the presence of numerous stress fibers throughout the developing cells and strands of nascent myofibrils in which a transition from a defined sarcomeric structure to poorly aligned and undifferentiated filamentous structure was readily apparent. These observations led Peng et al., to propose that new myofibrils are formed from pre-existing bundles of thin filaments (stress fibers). Subsequent immunofluorescence studies (Dlugosz et al., 1984; Antin et al., 1986; Wang et al., 1986) supported the idea that stress-fiber-like structures are involved in myofibril formation. These studies showed that, early in culture, there is an abundance of stress-fiber-like structures throughout the cells, and, during development, these
structures are replaced sequentially by nascent myofibrils followed by well-developed myofibrils. Examples of stress-fiber-like structures are demonstrated in the phase-contrast image in Figure 6 and in the fluorescence image in Figure 10. In the latter figure, the actin filaments were stained with phallacidin and there are stress-fiber-like structures in the cell processes and striated fiber structures in the main cell body. Whether there was a metamorphosis of the stress-fiber-like structure into a myofibril was not apparent in the early immunofluorescence studies. A model was proposed by Dlugosz et al. (1984) for myofibril assembly in which the stress-fiber-like structure serves as a template for myofibril formation rather than a metamorphosis of the stress-fiber-like structure into a myofibril (at right).

Other studies of myofibril formation by immunofluorescence have led to the suggestion that the stress-fiber-like structure changes from a predominantly non-muscle isoform structure to a muscle-specific isoform structure (Lin and Lin, 1986; Greaser et al., 1989, Handel et al., 1989, Handel et al., 1991, Rhee et al., 1994). The diagram in Figure 11 (adapted from Handel et al., 1991) shows the transitions in immunofluorescence patterns for specific muscle proteins during myofibril assembly. These studies demonstrated the co-existence of muscle and non-muscle isoforms of tropomyosin and actin within stress-fiber-like structures and nascent myofibrils (Lin and Lin, 1986; Greaser et al., 1989, Handel et al., 1989, Handel et al., 1991). Additionally, co-existence of non-muscle and muscle myosin has been observed in nascent myofibrils (Rhee et al., 1994). Thus, these studies have led to the suggestion that the stress-fiber-like structure is transformed from predominantly non-muscle isoform to a muscle isoform structure rather than the stress-fiber-like structure acting as a template (Lin and Lin, 1986; Handel et al., 1989; Rhee et al., 1994). This transformation likely involves a change in the expression of actin and tropomyosin isoforms and a replacement of non-muscle isoforms with muscle isoforms by the process of dynamic exchange.

Another aspect of the transition in actin isoforms is the change in the polymerization status of actin. Studies on the actin pools (filamentous and non-filamentous) have shown that there is a dramatic change in the actin pool from the non-filamentous form to the filamentous form (see Obinata, 1993 and references cited therein) in developing muscle cells. This transition involves a change in both the expression of actin isoforms and actin-binding proteins which inhibit polymerization (Babcock and Rubenstein, 1983). The muscle-specific isoform of actin binds these proteins with lower affinity resulting in greater polymerization of this isoform into filaments than the non-muscle isoform. The net result is that this transition favors the replacement of the filamentous non-muscle isoform with the muscle isoform (Oshima et al., 1988).

A complicated feature of actin organization during myofibril assembly is the re-organization of the filaments in terms of both polarity and length. In stress-fiber-like structures, the lengths of the thin filaments are highly variable and appear to pass through the H-zone of nascent myofibrils (Peng et al., 1981). Similar interpretations can be made from immunofluorescence studies with anti-actin antibodies. The actin staining pattern appears continuous along the stress-fiber-like structure and the nascent myofibril and only later in development does the staining pattern appear continuous along the stress-fiber-like structure.
tein is involved in endocytosis, but these recent experiments with myotubes suggest that it may have a role in organization of the Z-line or actin filament lattice in the sarcomere of nascent myofibrils (Kaufman et al., 1990).

**Intra-myofibrillar cytoskeletal elements.** Several studies have addressed the role of titin in myofibril assembly. The unique size, structure and location of this protein in the myofibril has led to the hypothesis that it is an important protein in the assembly of myofibrils (Wang et al., 1988; Greaser et al., 1989; Fulton and Isaacs, 1991). The most pertinent feature of this protein is its apparent binding to both the Z-line and the thick filament (Furst et al., 1988), thus providing a mechanism to link the thick filament to the Z-line in the nascent myofibril.

Immunohistochemical studies suggest that the localization of titin into an organized periodic pattern precedes or is co-incident with that of myosin (Hill et al., 1986, Wang et al., 1988; Handel et al., 1991; Furst et al., 1989; Colley et al., 1990). An interesting feature of some of these studies is that, depending on the monoclonal antibody used, the organization of titin epitopes either preceded or was co-incident with myosin organization. Monoclonal antibodies specific to epitopes near the Z-line showed organization prior to those for epitopes in the A-band (Furst et al., 1989; Schultheiss et al., 1990). This has led to the proposal that titin first binds to the nascent Z-line, then another region of titin binds to a thick filament and interdigitates it into the nascent sarcomere (Rhee et al., 1994). The determination of the specific role of titin and its various domains in myofibril assembly awaits application of molecular genetics approaches with titin DNA.

The development of the A-band from its constituent thick filaments occurs during the later stages of myofibril assembly. As discussed earlier, myosin will associate to form thick filaments in vitro, yet the mechanisms for regulating the filaments final length are poorly understood. Some have proposed that titin may function as a regulator of thick filament length (Whitting et al., 1989), yet no direct evidence for this has been demonstrated. In fact, some of the immunofluorescence studies of myofibril assembly suggest that titin localized to the Z-body of the nascent myofibril prior to myosin-containing thick filaments (Schultheiss et al., 1990; Furst et al., 1990). The organization of thick filaments into the nascent sarcomere likely involves titin, as discussed earlier. The higher level organization of the thick filaments into the mature A-band likely involves other thick-filament associated proteins. The organized appearance of C-protein, myomesin and M-protein occurs relatively late in myofibril assembly and these proteins may be involved in the final stages of organization of the A-band (Schultheiss et al., 1990; Carlsson et al., 1990). How this process occurs is unknown at present.

**Models for Myofibril Assembly**

The previous discussion has reviewed the supramolecular processes involved in the assembly of the myofibril from its constituent proteins. At present, there are two working models to explain the assembly of the myofibril. Both models incorporate an essential role for the stress-fiber-like structures found in developing muscle cells, yet there are substantial differences in these models in terms of the mechanisms involved. The first model, based upon immunofluorescence studies, was developed by Holtzer and colleagues (Dlugosz et al., 1984) in which they proposed that the stress-fiber-like structure served as a template for the assembly of myofibrils. An adaptation of their model is presented in Figure 12. The assembly of myofibrils occurs in close apposition to existing Z-body type structures of the stress-fiber-like structures near the sarcolemma. Muscle isoforms of alpha-actinin and actin form nascent Z-bodies in this region and thick filaments interdigitate between these Z-bodies in a process likely involving titin. The Z-bodies and thick filaments further organize into A-bands and I-Z-I
Figure 12

STRESS-FIBER-LIKE-STRUCTURE MODEL

Illustration of the stress-fiber-like structure model for myofibril assembly. The various non-muscle (nm) and muscle proteins involved in the formation of the stress-fiber-like structure and the myofibril are shown from the initial stages (leftmost) to the mature myofibril (rightmost). Adapted from Dlugosz et al., (1984).

A distinguishing feature of this model is that the sarcomere length of the nascent myofibril is the same as that of the mature myofibril.

A second model is the premyofibril model of Sanger and colleagues (Rhee et al., 1994). This model was developed from data obtained from immunofluorescence studies (Lin and Lin, 1986; Handel et al., 1991; Rhee et al., 1994) and fluorescent analogue cytochemistry (Sanger et al., 1986). An adaptation of the model is shown in Figure 13. In this model, myofibril assembly is started at a structure called the premyofibril which is a stress-fiber-like structure located at the sarcolemma. During development, the non-muscle proteins of the premyofibril are replaced by muscle-specific isoforms (i.e. a metamorphosis of the stress-fiber-like structure into a myofibril) and there is a lengthening of the sarcomere from a premyofibril length of 0.3 - 1.4 microns to the mature myofibril length of 1.8 - 2.5 microns. Development proceeds from the premyofibril to the nascent myofibril to the mature myofibril. The premyofibril is made solely of non-muscle isoforms, the nascent myofibril contains both non-muscle and muscle isoforms, and the mature myofibril contains only muscle isoforms. There is a progressive narrowing of the Z-body into the Z-line during the transition from the premyofibril to the mature myofibril. This model assumes an essential role for titin in the incorporation of thick filaments into the nascent I-Z-I brushes. The distinguishing features of this model are that the stress-fiber-like structure (premyofibril) is transformed into a mature myofibril by replacement of non-muscle isoforms with muscle isoforms.

Illustration of the premyofibril model for myofibril assembly. The various stages of myofibril assembly are shown with the corresponding non-muscle (nm) and muscle proteins which are involved in the process. The myofibril develops from the premyofibril (uppermost) into the mature myofibril (lower most) by a metamorphosis of the premyofibril. Adapted from Rhee et al., (1994).
and that there is a lengthening of the sarcomere during the transition from the premyofibril to the mature myofibril.

**Conclusions**

The genesis of muscle from its embryonic progenitor cells involves several molecular and cellular processes. Commitment of progenitor cells to muscle involves transcriptional regulation through a number of regulatory factors. This regulation results in the expression of many muscle-specific proteins which form the sarcomeres of the myofibril. This structure bears the characteristic phenotype of striated muscle. Myofibrillar assembly is a dynamic process with assembly-disassembly, protein exchange, remodeling and repair both during the transition from the premyofibril to the mature myofibril. The process of muscle growth involves a greater rate of assembly than breakdown and therefore an understanding of the mechanisms involved in myogenesis has important economic implications for increasing the efficiency of nutrient conversion into muscle protein.

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**References**


Discussion

M. Koohmaraie: Lambs have recently been identified that carry a gene called the muscle hypertrophy gene. I'm interested in knowing more about this gene. The gene is silent during embryonic development and then is expressed a few weeks after birth, which makes it highly desirable, as opposed to the double-muscling gene. There seems to be a lot of similarity between this gene and the ski gene. Could this be the ski gene in lambs?

D. Mulvaney: I agree that there are a lot of similarities in muscle histochemistry changes seen in the ski transgenic mice in particular and perhaps in the ski transgenic pig also. Hypertrophy of the Type II fast fibers is probably similar to what's been observed in this lamb model. Until someone really examines the gene expression and some of these involvements, we won't know. It's certainly a nice parallel.

D. Cornforth: Is contraction involved in the assembly of myofibrils early on? In cell culture, these systems are able to undergo contraction right away.

D. Swartz: There is some thought that the actin and myosin interactions are important in getting the thick filament in the right place, through either contractile mechanisms or through weak binding stages, so the interaction is probably important. The difficulty is that there are several papers which suggest you have actin filaments (even though you are at 2.3 microns) that are cross polar. So it becomes a little more difficult to understand how that would work, because you have the improper polarity for the interaction. It is important to try something where you kill the contractile properties, either mutate myosin or reverse mutate actin, so it doesn't work contractile function-wise but it does form filaments. This would be an approach to test that idea.

Koohmaraie: I'd like to ask about the I-Z-I interaction or junction. Chemically, the Z-line can be removed. The Z-lines are gone and the myofibril is still intact. How is this possible?

Swartz: There's something there that comes off but we don't know what it is. There has been some interesting work from the Japanese in which they have suggested that α-actinin has phosphoinositol phosphate bound to it. They have antibodies specific for PIP2 and that binds specifically to the Z-line and is very tightly attached to α-actinin, and the α-actinin that is purified is there and it stays on when a gel is run with it. The point is that there is probably some lipid material that is not really seen in the gel, and it is not detected that way. There is some really old work suggesting using some specific types of detergent that can remove the Z-line (possibly digitonin), suggesting that there may be some non-proteinaceous elements that give phase density but aren't necessarily protein in nature. You can remove the phase density, but it doesn't necessarily mean you can remove the α-actinin. The inference is that α-actinin is what gives phase density. Z-lines can be removed and incorporate fluorescent α-actinin without regaining true phase density, but things still remain intact. So in summary, there is something else there other than α-actinin that gives the phase density, and that the α-actinin may still be there to keep things bundled and organized. We know they are more sensitive to any type of physical disruption, but they do stay essentially somewhat intact.

H. Swatland: It now seems very obvious that double muscling is due to the increased number of muscle fibers, but it wasn't obvious 20 years ago. I was fortunate to work with Nat Keefer at Texas A&M, and counted lots of muscle fibers. We showed that double muscling was due to an increase in numbers of muscle fibers. But the puzzling thing is that this is only in the post-otic segments; the segments after the first three segments of the head, the pre-otic segments which form the extra ocular muscles around the eye. They do not have any extra number of muscle fibers. This has always struck me as a bizarre thing. Somehow, if one could explain it, it could help explain the rest of the double-muscling phenomenon. So, does the panel have any ideas why the first three segments of the embryo don't have muscle hyperphasia?

None of the panel speakers were able to comment on this phenomenon.