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How to build a myofibril☆

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Abstract

Building a myofibril from its component proteins requires the interactions of many different proteins in a process whose details are not understood. Several models have been proposed to provide a framework for understanding the increasing data on new myofibrillar proteins and their localizations during muscle development. In this article we discuss four current models that seek to explain how the assembly occurs in vertebrate cross-striated muscles. The models hypothesize: (a) stress fiber-like structures as templates for the assembly of myofibrils, (b) assembly in which the actin filaments and Z-bands form subunits independently from A-band subunits, with the two subsequently joined together to form a myofibril, (c) premyofibrils as precursors of myofibrils, or (d) assembly occurring without any intermediary structures. The premyofibril model, proposed by the authors, is discussed in more detail as it could explain myofibrillogenesis under a variety of different conditions: *in ovo*, in explants, and in tissue culture studies on cardiac and skeletal muscles.

Introduction

Myofibrillogenesis is a process that results in impressive structural conservation among vertebrate cross-striated muscles. Despite the differences in functional specialization that muscles acquire during development, the basic process of assembly of proteins into myofibrils appears to follow similar steps (Sanger *et al*., 2004). Building a sarcomere requires the formation of several different types of filaments, the association of additional proteins with the filamentous proteins, and the arrangement of the filaments into the sarcomeric subunits of myofibrils.

The myofibril is a scaffold for spatial distribution of the proteins that integrate force production and transmission. Myofibrils are connected to intermediate filaments, transverse tubules, and sarcoplasmic reticulum as well as to microtubules (Goldstein and Cartwright, 1982; Gundersen *et al*., 1989; Clark *et al*., 2002; Sanger *et al*., 2004). Near the periphery of the cell the Z-bands form costameric attachments that connect myofibrils with a cytoskeletal array of proteins beneath the sarcolemma (Ervasti, 2003). A number of metabolic enzymes and signaling molecules are also localized in different regions of the myofibril (Chowrashi *et al*., 2002; Knoll *et al*., 2002; Mitchell and Pavlath, 2002). Within the sarcomere, increasing numbers of proteins are being identified and their functions revealed (Faulkner *et al*., 2001; Sanger and Sanger, 2001a; Clark *et al*., 2002). Understanding how myofibrils assemble, therefore, will involve unraveling many interconnected processes.

In this report we present data predominately from observations and experiments on avian skeletal muscle cultures that support a premyofibril model for myofibrillogenesis, and we discuss aspects of alternative models that relate to this premyofibril model.

Materials and methods

Quail myoblasts were isolated from 9- and 10-day old embryos and grown in tissue culture on collagen-coated cover slips using techniques previously described (Dabiri *et al*., 1999a, b). The cells were fixed, permeabilized and stained for immunofluorescence using procedures detailed in Golson *et al*. (2004). Cells were stained with muscle specific alpha-actinin, nebulin or tropomyosin antibodies purchased from Sigma (St. Louis, MO, USA). Troponin T and zeugmatin antibodies were obtained from the Hybridoma Bank (Madison, WI, USA). Fluorescently labeled phalloidin (Alexa 488) was ordered from Molecular Probes, (OR, USA), and used as described by Zhukarev *et al*. (1997). The microtubule-stabilizing drug, taxol, was purchased from Sigma (St. Louis, MO, USA), and dissolved in dimethyl sulfoxide, and stored at 4°C. Before use it was diluted into warm muscle medium and added to muscle cultures on either the first or second day of culture. The drug was present in the cell cultures for as long as 4 days. Sister cultures of taxol-treated

☆In memoriam: This paper is dedicated to the memory of Professor Koscak Maruyama, a noted contributor in the field of muscle biochemistry.

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cells were fixed after each day of treatment. Some of the taxol-treated cultures were returned to control medium after 4 days of drug exposure by repeated rinsings of the cells with normal medium. Images were obtained using wide field microscopy (Nikon), and deconvolution fluorescence microscopy (API-Delta Vision). Images were assembled using Adobe Photoshop.®

Results and discussion

From the many reports on myofibrillogenesis in developing vertebrate muscle cells, there is general agreement that myofibrillogenesis takes place in stepwise processes, and that the first steps of myofibrillogenesis occur in association with the cell surface or sarcolemma (reviewed in Sanger *et al*., 2004). The procession of specific creation and assembly events leading to the whole mature myofibril remains to be discovered. In the meantime many pieces of the puzzle are being uncovered, giving rise to models that attempt to assemble the pieces into the whole picture.

Theories of myofibrillogenesis

Template model

This model of myofibrillogenesis proposes that the components of the future myofibril, dense components of the Z-band, thin and thick filaments, are recruited to the surfaces of stress fibers or stress fiber-like structures in the developing muscle cell (Figure 1), with each stress fiber-like structure serving as a temporary template for the elements necessary to form one myofibril (Dlugosz *et al*., 1984). At the completion of assembly, the stress fiber template disappears and reassembles to serve as a template for a new assembling myofibril.

It is now known that the stress fiber-like structures in muscle cells are composed of a number of muscle specific proteins, i.e., alpha-actinin, tropomyosin, troponins, and tropomodulin (Rhee *et al*., 1994; Almenar-Queralt *et al*., 1999) with nonmuscle or cytoplasmic myosin II the only nonmuscle protein isoform present. A potential problem in this template model is that stress fibers themselves are sarcomeric in their own organization, and presumably form in the absence of a template for their own assembly (Sanger and Sanger, 1980; Sanger *et al*., 1983; Langanger *et al*., 1986). The sarcomeric bands of the stress fiber-like structures are also smaller than the 2–2.5 μm mature myofibril sarcomeric sizes, so it is difficult to see how they would provide the template spacing information for myofibrils (Sanger *et al*., 1986a). Observations of living muscle cells transfected with GFP-muscle alpha-actinin demonstrated that the initial fibers formed and fused with one another to form mature myofibrils (Dabiri *et al*., 1997). These observations are inconsistent with a template model of myofibrillogenesis.

Independent subunit assembly

A second model of myofibrillogenesis proposes that the elements of the future I-Z-I bands, i.e., I-Z-I bodies, assemble in scattered arrays in an area separate from the assembly site where thick filaments form (Figure 2). Titin would then join or stitch the scattered I-Z-I bodies and thick filaments together and promote their assembly into mature myofibrils (Figure 2). Myofibrils were then predicted to elongate by the end on assembly of thick filaments and I-Z-I bodies (Lu *et al*., 1992). This model was first proposed for myofibrillogenesis in cardiac muscle cells (Lu *et al*., 1992), and then extended to skeletal muscle cells (Holtzer *et al*., 1997). Nonmuscle myosin II was not detected in association with the I-Z-I bodies in cardiac muscle cells (Lu *et al*., 1992) as it was in a previous report from the same laboratory in stress fiber-like structures in both cardiac and skeletal muscle cells (Fallon and Nachmias, 1980; Dlugosz *et al*., 1984). The anti-platelet nonmuscle myosin antibody used by Lu *et al*. (1992) was directed against the IIA isoform of nonmuscle myosin II, and this isoform is not present in cultured avian cardiomyocytes (Rhee *et al*., 1994; Conrad *et al*., 1995). Subsequently Rhee *et al*. (1994)

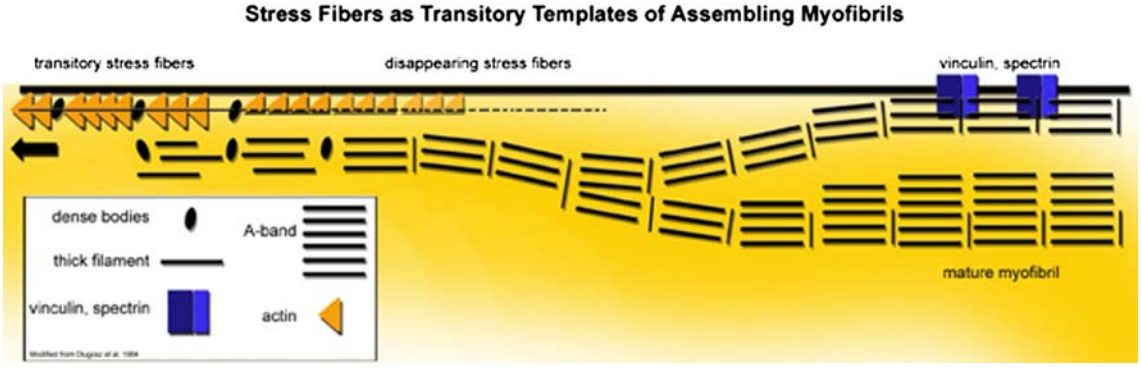

Fig. 1. This template model summarizes important elements of how stress fibers composed of nonmuscle proteins can serve as a temporary template for the formation of a single myofibril. Elements of the myofibril, alpha-actinin densities, thin and thick filaments lacking the ability to directly assemble into mature myofibrils, are instructed by information in the stress fibers into myofibrils (modified from Dlugosz *et al*., 1984).

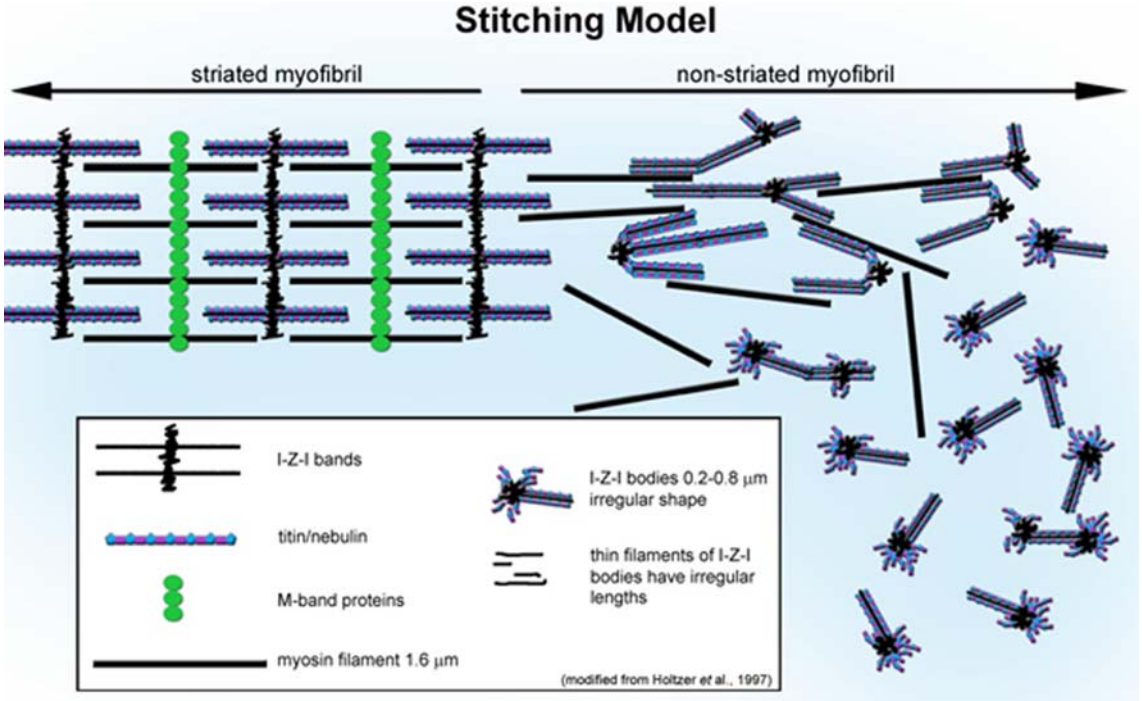

Fig. 2. This stitching model proposed by Holtzer *et al.* (1997) postulates that I-Z-I bodies and thick filaments first assemble independently of each other. Titin molecules stitch together the scattered I-Z-I bodies and thick filaments into elongating mature myofibrils (modified from Holtzer *et al.*, 1997).

demonstrated with antibodies directed against the IIB isoform of nonmuscle myosin that nonmuscle myosin was present in association with the I-Z-I bodies in cardiomyocytes. Rhee *et al.* (1994) also demonstrated that the anti-platelet nonmuscle myosin IIA antibody did not cross-react with the IIB in cardiomyocytes. The nonmuscle myosin antibody prepared by Fallon and Nachmias (1980) against nonmuscle myosin IIs puri-

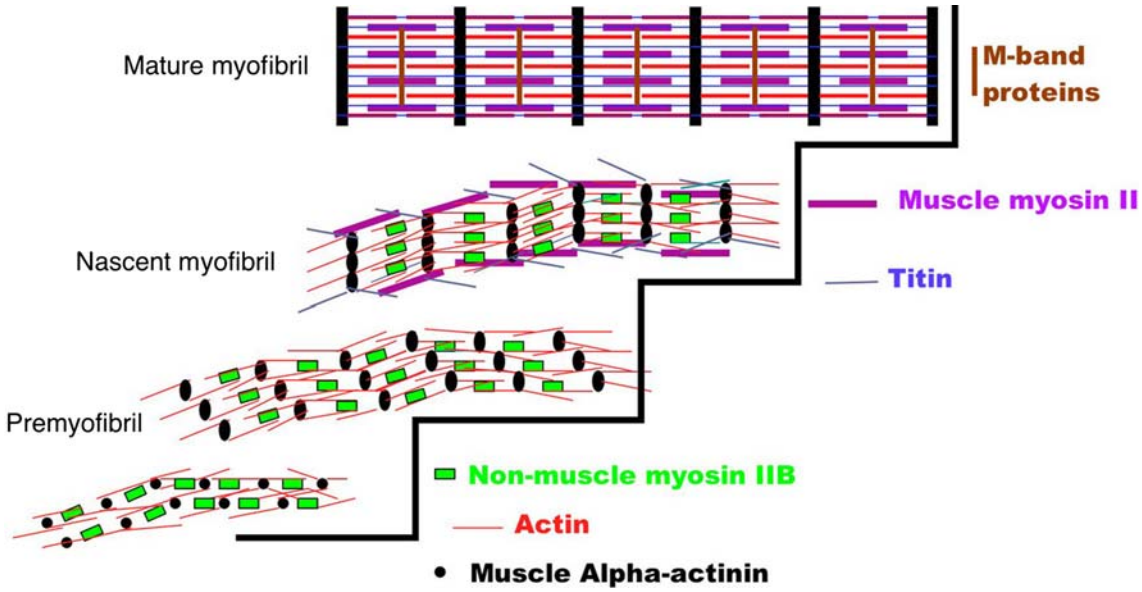

Fig. 3. Diagram of the transition of Z-bodies in premyofibrils and nascent myofibrils to Z-bands in mature myofibrils. In this premyofibril model, assembly begins at the edges of muscle cells with *premyofibrils* composed of minisarcomeres that contain sarcomeric proteins in the α-actinin enriched z-bodies and thin filaments of actin (and associated proteins: tropomyosin, and troponins). Nonmuscle myosin II filaments are present in the minisarcomeres of the premyofibrils. Z-bodies in adjacent fibrils begin to align in *nascent myofibrils*, forming beaded Z-bands that gradually become linear Z-bands or Z-lines in *mature myofibrils*. Titin molecules and muscle myosin II thick filaments are also present in nascent myofibrils. The thick filaments in the nascent myofibrils are not aligned, but are in an overlapped relationship. M-band proteins are recruited to the mature myofibrils, thick filaments become aligned into A-bands, while nonmuscle myosin II proteins are absent (modified from Du *et al.*, 2003a, b).

fied from a rat lymphoma cell line, and used by Dlugosz *et al*. (1984), may have been an anti-IIA isoform that also cross-reacted with the nonmuscle myosin IIB isoform of nonmuscle myosin present in cardiomyocytes. Hematopoietic cell lines are now known to contain the IIA isoform of nonmuscle myosin II, but not the IIB isoform (Buxton *et al*., 2003). Recently a third isoform of nonmuscle myosin, IIC, has been discovered in hematopoietic, neural, and muscle cells (Tullio *et al*., 1997; Buxton *et al*., 2003; Golomb *et al*., 2004) raising further questions about the isoform specificity of the antibody prepared by Nachmias and Fallon (1980), and the roles of the different nonmuscle myosin IIs, i.e., IIA, IIB, and IIC) in myofibrillogenesis.

Premyofibril model of myofibrillogenesis

This premyofibril model is illustrated in Figure 3. Premyofibrils are considered to consist of minisarcomeres whose boundaries are marked by Z-bodies containing muscle alpha-actinin. Nonmuscle myosin II filaments interdigitate with actin filaments that contain muscle isoforms of troponins and tropomyosin. The barbed ends of the actin filaments are embedded in the Z-bodies of the premyofibrils. In the earliest steps, the premyofibrils associate at the level of their Z-bodies. As the premyofibrils begin to align and grow in width, titin and muscle myosin II appear in fibrils (Figure 3). The fibrils at this stage have been termed nascent myofibrils (Figure 3) (Rhee *et al*., 1994). The muscle myosin II antibody staining is in a continuous linear pattern along the nascent myofibrils, a result possibly caused by overlapping thick filaments (Rhee *et al*., 1994; Du *et al*., 2003a, b). In the progression from nascent myofibrils to mature myofibrils, the Z-bodies transform from aligned Z-bodies to Z-lines or Z-bands (Figure 3), the muscle myosin II filaments align into A-bands, and nonmuscle myosin II is no longer detected (Figure 3) (Rhee *et al*., 1994; Du *et al*., 2003a, b). It is unclear how this loss of nonmuscle myosin II is accomplished as nascent myofibrils are transformed into mature myofibrils. C- and M-band proteins are present in the A-bands of the mature myofibrils, and may be responsible for the final alignment of the thick filaments (Figure 3) (Rhee *et al*., 1994; Sanger and Sanger, 2002). One of the surprises of studying myofibrillogenesis in living cells was that myofibrils did not elongate by the serial addition of sarcomeres, but by the deposition of premyofibrils that grew and fused laterally with existing myofibrils resulting in elongated myofibrils in a matter of hours (Dabiri *et al*., 1997). These observations of living cells are incompatible with both the stress fiber template model and independent subunit assembly model, both models being based on studies of fixed and stained cells.

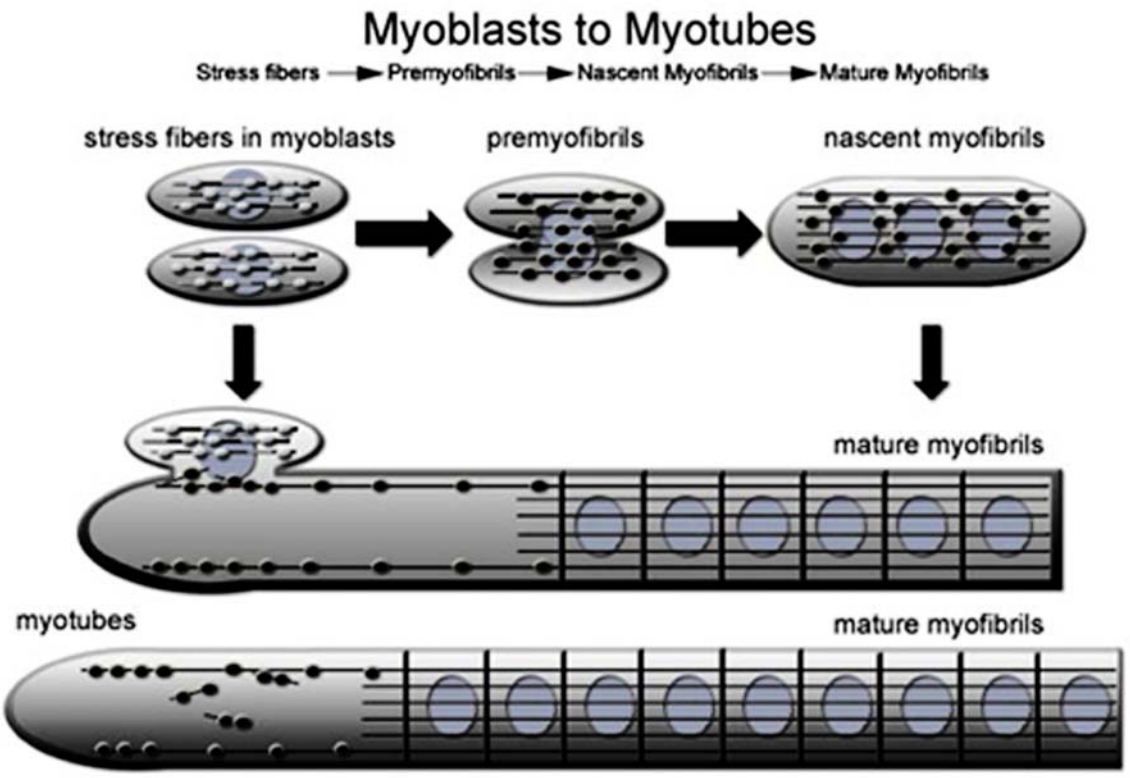

Fig. 4. Diagram summarizing the different steps in fusing of myoblasts to a myotube. Stress fibers are present in myoblasts. Stress fibers are composed of nonmuscle proteins organized into minisarcomeres marked by the presence of nonmuscle alpha-actinin in densities (light circles) along nonstriated actin fibers. The initiation of myofibrillogenesis is marked by the expression of a number of muscle specific proteins including muscle alpha-actinin (dark circles). The stress fibers undergo a transition in their constituents as sarcomeric proteins incorporate into them to be transformed into premyofibrils, then nascent myofibrils, and finally mature myofibrils.

Nonmuscle myosin II is an important component of premyofibrils and nascent myofibrils (Figure 3). Phosphorylation of the nonmuscle myosin light chains is required for nonmuscle myosin molecules to form polymers or filaments (reviewed in Du *et al*., 2003a, b). Inhibition of the phosphorylation activity of nonmuscle myosin II light chain kinases by specific nonmyosin light chain kinase inhibitors, e.g., ML-7, leads to the inhibition of myofibrillogenesis (Ferrari *et al*., 1998; Du *et al*., 2003a, b). Du *et al*. (2003 a, b) reported that treatment of cardiomyocytes formed in precardiac explants with ML-7 led to the accumulation of overlapping thick filaments. Removal of this kinase inhibitor led to resumption of myofibrillogenesis and reorganization of the accumulated thick filaments into A-bands of mature myofibrils. Cultured embryonic cardiomyocytes exposed to this inhibitor showed a loss of pre- and nascent myofibrils and an inhibition of myofibrillogenesis. In extracts of the cultures in the soluble fraction, there was an accumulation of nonmuscle myosin heavy chains. Removal of the kinase inhibitor led to redistribution of nonmuscle myosin heavy chains into the cytoskeletal fraction, the reformation of premyofibrils and nascent myofibrils, and the accumulation of mature myofibrils. These experimental results support an important role for nonmuscle myosin II in maintaining the integrity of premyofibrils and nascent myofibrils, transitional stages predicted in only the premyofibril model of myofibrillogenesis (Figure 3).

Direct assembly of myofibrils

There is a recent report on myofibrillogenesis in the myotubes of the myotomes of the zebrafish embryo in which no intermediate stages of myofibrils have been detected (Costa *et al*., 2002). This study suggested the intermediate stages of myofibrillogenesis have been detected only in muscle cells in tissue culture. Elements of a spontaneous assembly model of myofibrillogenesis have been cited in other studies on myofibrillogenesis. Thus appearances of the elements of the Z-band, detected using alpha-actinin antibodies, have been reported to appear at the mature Z-band separations of 2–2.5 μm (Ehler *et al*., 1999). In contrast to these reports, there are other studies in intact hearts and skeletal muscle cells where precursor states of myofibrillogenesis have been reported (Fischman, 1967; Kelly, 1969; Peng *et al*., 1981). As fluorescent imaging techniques are applied to living embryos, it should be possible to resolve this controversy (Sanger and Sanger, 2001b).

Myofibril formation in avian cultures

The assembly of myofibrils in skeletal muscle cells can be readily studied in isolation from the body by obtaining myoblasts from avian embryonic breast muscles and growing them in culture, where the cells will fuse with one another to form myotubes, and assemble myofibrils *de novo* as diagrammed in Figure 4 (Dabiri *et al*., 1999a, b). Prior to the synthesis of muscle-specific proteins, an actin/myosin cytoskeleton is present in myoblasts as stress fibers composed of minisarcomeres of nonmuscle proteins homologous to those in muscle; e.g., alpha-actinin, tropomyosin and myosin II (also called cytoplasmic myosin II; Fallon and Nachmias, 1980). The edges of the minisarcomeres are bounded by dense bodies composed of nonmuscle alpha-actinin in which the barbed ends of the thin filaments are embedded (Sanger and Sanger, 1980; Sanger *et al*., 1983). The nonmuscle myosin II filaments are positioned between each pair of dense bodies (Langanger *et al*., 1986; Mittal *et al*., 1987).

Mononucleated myocytes can either fuse with one another to form the first myotubes, or fuse with existing myotubes that have elongated and widened (Figure 4). In the latter case, there would be an inflow of soluble muscle isoforms of sarcomeric proteins into the fusing myocyte (Figure 4). In the initial stage of myofibrillogenesis in skeletal muscle, one of the earliest muscle-specific proteins detected in a sarcomeric banding pattern is alpha-actinin (Figure 5a, b). The result is fibrils at the sides (Figure 5c, d) and ends (Figure 6) of growing myotubes that resemble stress fibers structurally, but that contain muscle-specific alpha-actinin (as well as a number of other muscle isoforms of actin binding proteins, i.e., muscle tropomyosins and troponins). Support for the idea that isoform replacement could occur without disassembly of the stress fibers present in myoblasts is found in studies in which alpha-actinin purified from vertebrate smooth and cross-striated muscles, and labeled with fluorescent dyes, was shown to incorporate into the dense bodies of stress fibers of nonmuscle cells, as well as into the Z-bands of muscle cells (Feramisco *et al*., 1979; Sanger *et al*., 1984, 1986a, b; McKenna *et al*., 1985).

Z-bands

Z-band formation has been followed during myofibrillogenesis in fixed cells with electron microscopy (Kelly, 1969), and immunofluorescence (Dlugosz *et al*., 1984; Sanger *et al*., 1984; Rhee *et al*., 1994). These data indicate that Z-bodies form near the surfaces of the developing muscle cell. They align, then fuse, and finally undergo a major change in shape to form Z-lines or Z-bands (Figure 4) (Kelly, 1969; Rhee *et al*., 1997). Studies in live muscle cells, either microinjected with fluorescently labeled alpha-actinin or transfected with plasmids encoding Green Fluorescent Protein (GFP) ligated to muscle alpha-actinin, confirm this view that the Z-bodies align laterally and fuse with one another to form Z-lines or Z-bands (Sanger *et al*., 1984; McKenna *et al*., 1985; Sanger *et al*., 1986a, b; Dabiri *et al*., 1997; Sanger *et al*., 2002; Golson *et al*., 2004; Wang *et al*., 2005a, b). The spacing between the aligned Z-bodies along the fibrils varies from 0.3 to 1.2 μm, as opposed

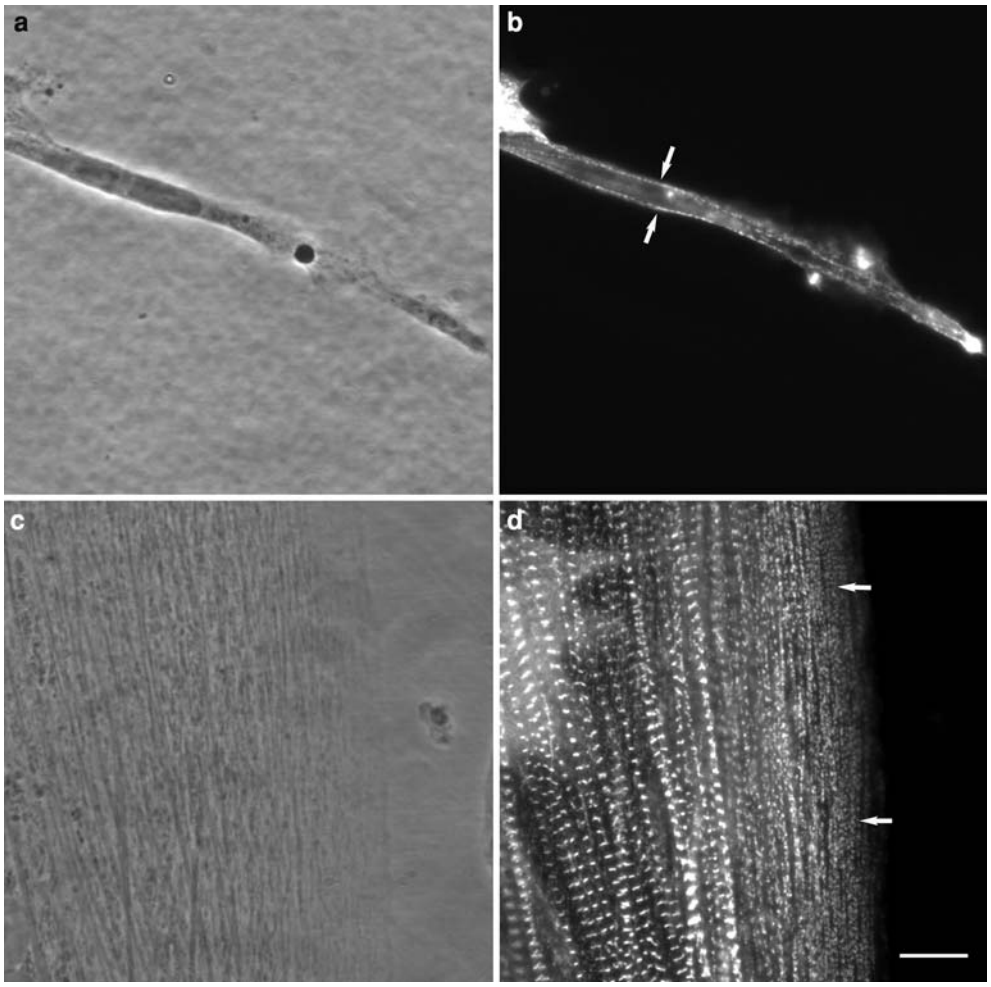

Fig. 5. Phase contrast (a, c) and immunoofluorescent (b, d) images of muscle cells stained with anti-muscle alpha-actinin antibodies (b, d). (a, b) At the end of one day in tissue culture, single banded fibers, i.e., premyofibrils, (arrows in B) are found along the two lateral edges of a binucleated myotube. (c, d) By the end of 3 days of culture, myotubes have increased in length and diameter. Nevertheless, premyofibrils are still deposited along the spreading sides of the myotube (arrows in d). This process enables the myotube to get thicker by the lateral assembly of new myofibrils. The longitudinal spacings between the alpha-actinin densities (Z-bodies) in the premyofibrils increase towards the central region of the myotube, marked by the Z-bands of the mature myofibrils. Bar = 10 μm.

to the 2–2.5 μm spacings between the Z-bands (Sanger *et al*., 1986a, b, 2002). In live cells expressing fluorescently tagged alpha-actinin molecules, the growth of spacings between Z-bodies in minisarcomeres to the full spacings of mature sarcomeres occurs over a period of 24 h (Sanger *et al*., 1986a, b; Dabiri *et al*., 1997; Sanger *et al*., 2002; Golson *et al*., 2004).

In contrast to the localization of alpha-actinin, which is banded all along the length of the actin bundles in the myotube (Figure 6), the initial localization of the Z-band domain of titin is not organized in small bands along the actin fibers at the growing tips of myotubes (Figure 7a, b). There is a low level of titin antibody staining in the area at the myotube ends where the premyofibrils are present, but fibril banding begins at a distance removed from the tip (Figure 7a–d). In previous studies using spreading cardiomyocytes in tissue culture, Rhee *et al*. (1994) found that banded titin antibody staining was correlated with the appearance of the muscle specific isoform of myosin II. This could suggest a role for titin in integrating muscle myosin filaments and actin filament systems (Figure 8) (Rhee *et al*., 1994; Holtzer *et al*., 1997).

Although alpha-actinin is one of the major proteins in the Z-band, the Z-band is home to a growing list of proteins (Faulkner *et al*., 2001; Sanger and Sanger, 2001 a; Clark *et al*., 2002), a majority of which are alpha-actinin binding proteins (Figure 8), e.g., the enzyme phosphorylase (Maruyama *et al*., 1985; Chowrashi *et al*., 2002). Future work will be needed to determine if all currently known Z-band proteins are present in the initial precursors of the Z-bands, i.e., the Z-bodies, or if there are gradual additions of proteins as the Z-bodies fuse and metamorphose into Z-lines (Kelly, 1969; Dabiri *et al*., 1997; Wang *et al*., 2005a, b).

A-band formation and role for actin

In the independent subunit assembly model (Figure 2), the actin filament system and the muscle myosin filament system form in isolation from one another in

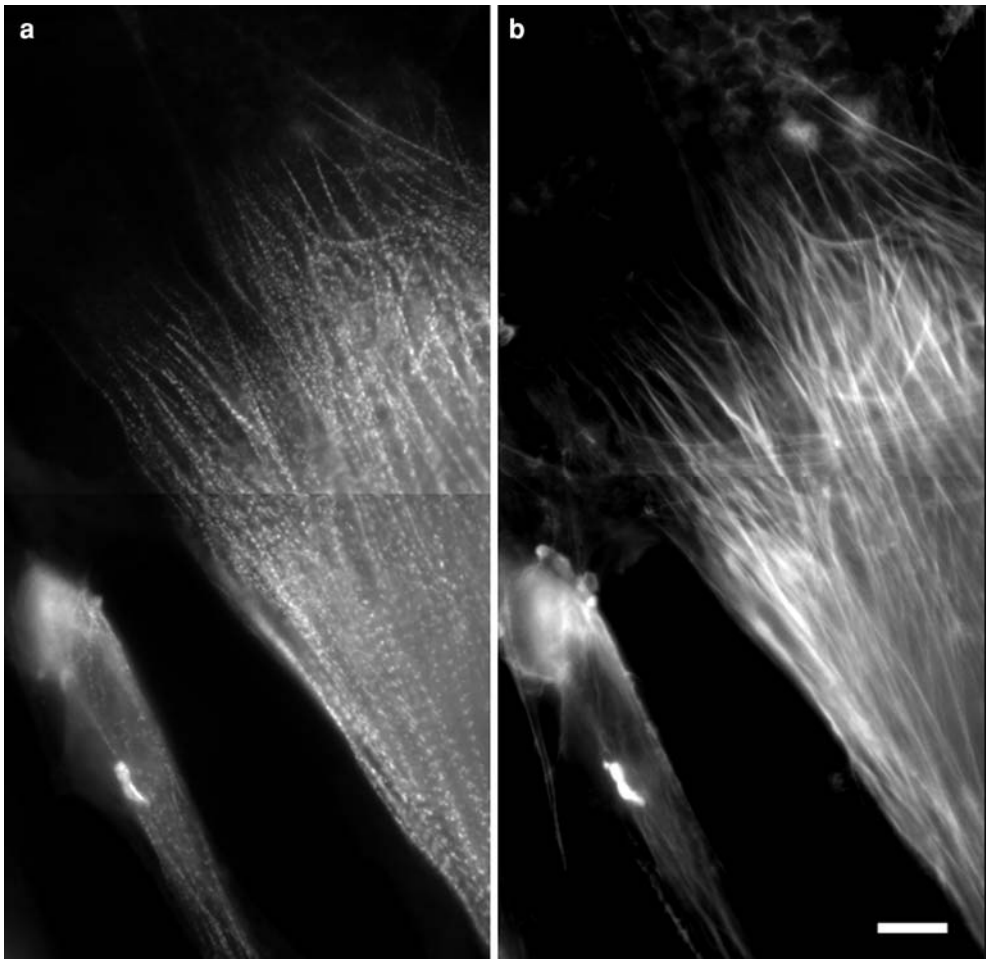

Fig. 6. (a, b) An elongating end of a myotube stained with (a) muscle specific alpha-actinin antibodies, and (b) fluorescently labeled phalloidin. Note that alpha-actinin is present in banded arrays on all of the actin bundles in the myotube. The spacings between the linear arrays of alpha-actinin increase as the fibers project from the spreading tip of the myotube to the area where the Z-lines or Z-bands of the mature myofibrils are present. Bar = 10 μm.

arrays identical to their mature appearance (Holtzer *et al*., 1997). Actin filaments attached to alpha-actinin bodies spaced at 2 μm intervals (I-Z-I brushes) are judged to be segregated initially from bands of 1.6 μm muscle myosin filaments (Holtzer *et al*., 1997). This would suggest that myosin filaments do not require interactions with actin filaments to form A-bands. Evidence that this indeed might occur comes from reports that actin filaments can be replaced by microtubules in the assembly of myofibrils (Antin *et al*., 1981; Toyama *et al*., 1982). In these studies myoblasts were cultured in the presence of taxol for 4 days, fixed, and examined in the electron microscope. Serially aligned A-bands were present that lacked any interdigitating thin filaments. Z-bands were also absent in these taxol-treated cells. These electron microscopic images of aligned A-bands resembled those images reported by Kaneko *et al*. (1984) where disassembled myofibrils from mitotic cardiomyocytes were analyzed. Reexamination of the effects of taxol on myofibrillogenesis during each of the 4 days of treatment revealed that actin filaments are indeed present in myofibrils formed in the presence of taxol (Siebrands *et al*., 2004). Instead of the typical multinucleated elongated myotubes, two types of muscle cells form in the presence of taxol: mononucleated stellate cells and some flattened cells termed myosheets containing up to ten nuclei (Antin *et al*., 1981; Toyama *et al*., 1982; Siebrands *et al*., 2004). Myofibrils in both types of cells contained thin filaments as judged by positive staining with a variety of thin filament probes, i.e., antibodies and phalloidin, (Siebrands *et al*., 2004). Figure 9 illustrates a muscle cell after 4 days of taxol treatment costained with fluorescently labeled phalloidin and antibodies to the actin-binding protein, nebulin. Removal of the taxol restored the elongated myotube shape, parallel microtubules, and myofibrils spanning the length of the cell (Figure 10). These results support the view that microtubules and their cross-linking proteins are necessary for the elongated shape of myotubes (Warren, 1968, 1974; Saitoh *et al*., 1988; Mangan and Olmsted, 1996), but since actin filaments were not eliminated from the cells, the idea that A-bands can form in isolation from actin filaments requires new evidence. At the periphery of the recovering cells, muscle specific myosin II antibodies and phalloidin staining revealed

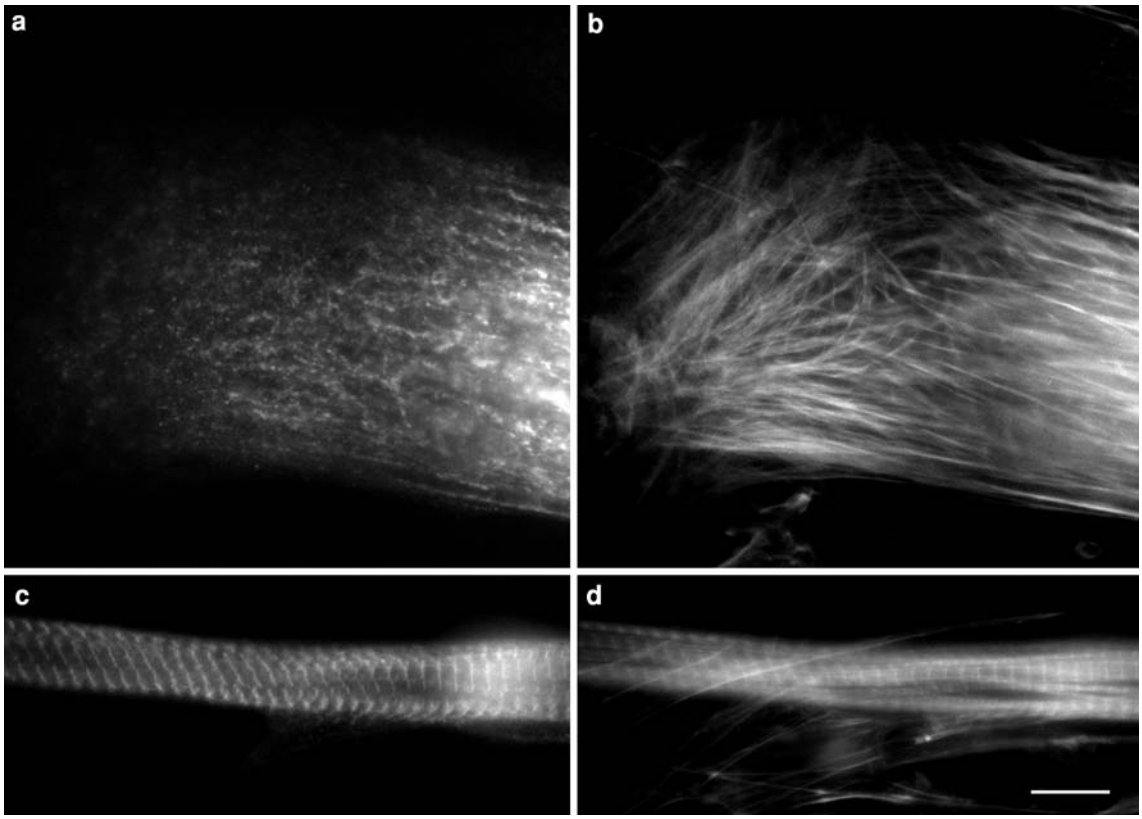

Fig. 7. (a–d) Quail myotubes stained with (a, c) an anti-titin antibody (anti-zeugmatin directed against an epitope of the Z-band region of titin), and (b, d) fluorescently labeled phalloidin. There are three patterns of titin staining in the myotubes: diffuse staining in the spreading end of the myotube (a, b); association with some of the actin fibers in the ends of the myotube (a, b); Z-band staining in the shaft of the myotube (c, d) These titin/F-actin images in combination with those alpha-actinin/F-actin images in Figure 3 indicate that many of the F-actin fibers with short alpha-actinin spacings do not have titin associated with them. Bar = 10 μm.

three types of fibers consistent with the premyofibril model of myofibrillogenesis: actin fibers unassociated with muscle myosin II (premyofibrils), actin fibers associated with unbanded bundles of myosin filaments (nascent myofibrils), and actin filaments associated with A-bands (mature myofibrils) (Siebrands *et al.*, 2004). These results are consistent with the premyofibril model of myofibrillogenesis (Siebrands *et al.*, 2004).

Myofibrillogenesis in culture vs. in vivo

Studying myofibrillogenesis in cultures derived from avian embryonic tissue has the advantage that cells

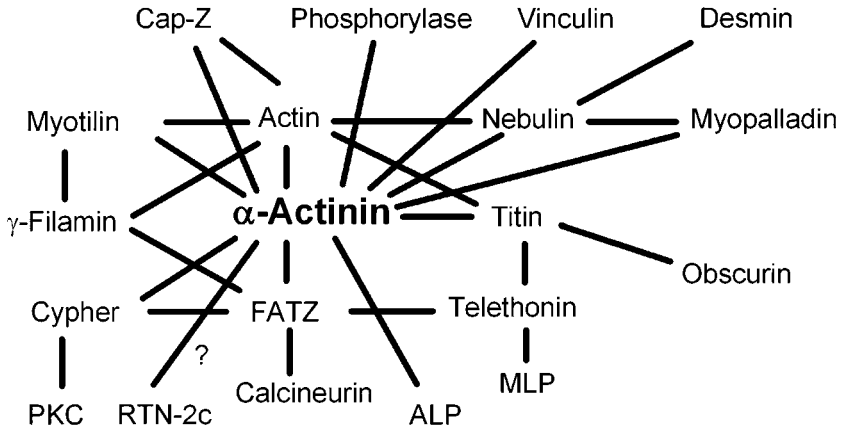

Fig. 8. This stick diagram of some of the proteins localized in the Z-bands of mature myofibrils emphasizes the important linking role of alpha-actinin. Alpha-actinin molecules not only bind actin filaments, nebulin and titin molecules, central elements of the sarcomere, but other elements, directly and indirectly, of the muscle cell, i.e., intermediate filaments, sarcoplasmic reticulum, costameres, signaling molecules and a metabolic enzyme.

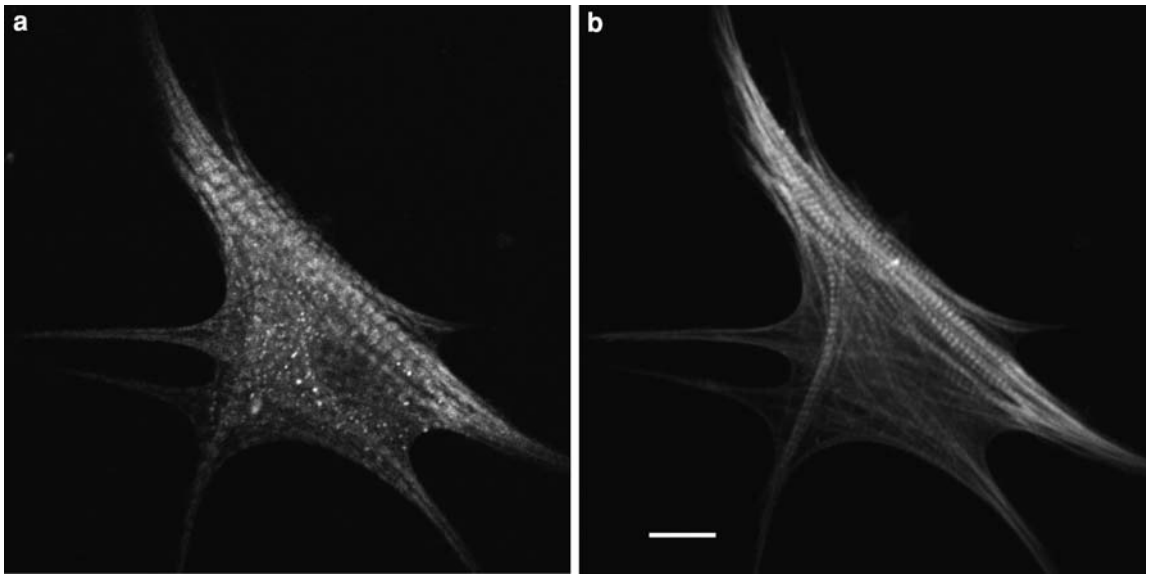

Fig. 9. This skeletal muscle cell has assembled myofibrils in the presence of taxol. The cell has been stained with (a) nebulin antibodies and (b) fluorescently labeled phalloidin. Note that the sarcomeres contain normal distributions of nebulin containing thin filaments. The taxol has induced the cell to assume a spiky appearance. Bar = 10 μm.

can be microinjected or transfected with fluorescent probes specific for myofibril proteins, allowing myofibrillogenesis to be followed in live cells with wide-field or confocal microscopy that allows subcellular resolution of structures. Skeletal muscle stem cells can be isolated and placed in tissue culture where they begin as myoblasts, and fuse to form myotubes assembling myofibrils. The disadvantage of comparable cardiac muscle cultures is that myofibrils are already present in the isolated 5–10 day-old embryonic cells as new myofibrils form at the spreading edges of the cells in culture (Dabiri *et al.*, 1997, 1999a, b). Several investigators have expressed concern that the formation of myofibrils in the first generation of cardiomyocytes might follow a different pathway when there are no myofibrils previously present (Ehler

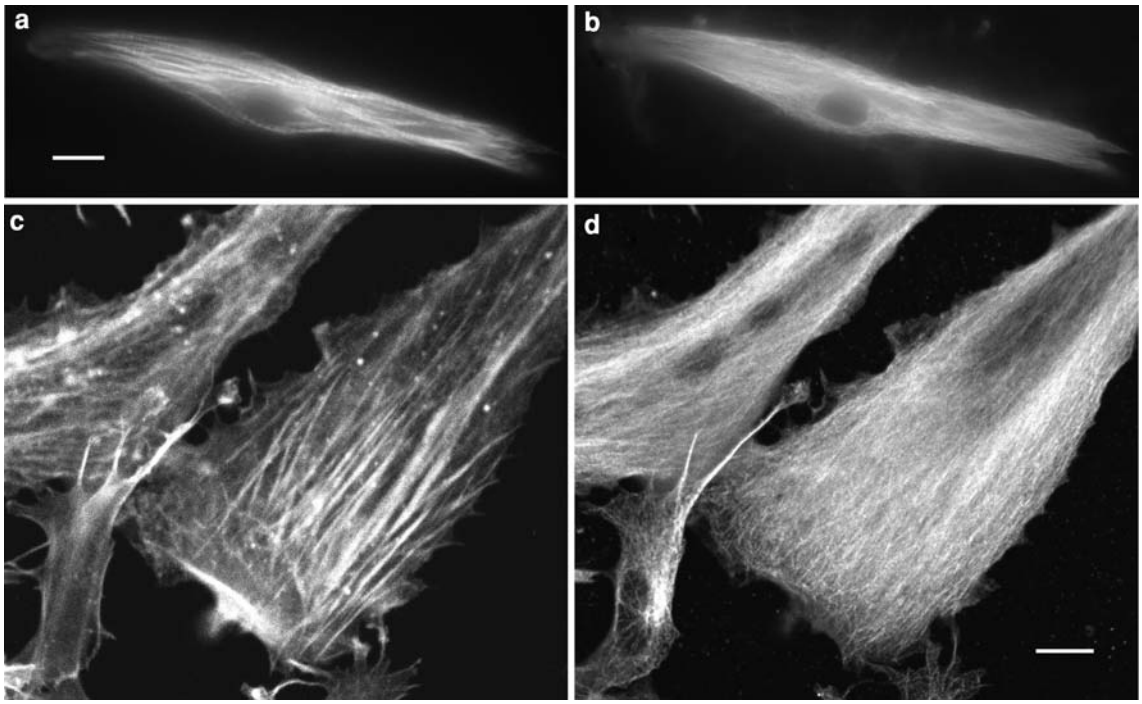

Fig. 10. (a, b) Fluorescent images of a cell treated for 4 days with taxol, and then placed in normal medium for 3 days prior to fixation and staining. Reversed skeletal muscle cell fixed and stained with (a) fluorescent phalloidin and (b) anti-tubulin antibodies. The former taxol induced star-shape of the cell is lost in normal medium as the reversed cells assume a more typical, although short, myotube shape. (a) Myofibrils and (b) microtubules are distributed along the length of the myotubes. (c, d) Control myotube, 7 days old, reveals larger myotubes with (c) phalloidin and (d) anti-tubulin staining. Bars = 10 μm.

et al. 1999; Gregorio and Antin, 2000; Rudy *et al*., 2001). However, only in the first generation of cardiomyocytes do myofibrils form in the absence of other myofibrils. After the first cardiomyocytes form in the area of the precardiac mesoderm, these new cardiomyocytes will divide almost daily until birth to generate an increasing number of cardiomyocytes needed for heart development (Kelly and Chacko, 1976). Moreover, an extensive immunological study of the first cardiomyocytes formed in explants isolated from the avian precardiac mesoderm revealed identical patterns to those detected in cultures of older cardiomyocytes (Du *et al*., 2003a). These timed developmental studies supported the three step premyofibril model for the first cardiomyocytes as well. An extension of these studies to the first cardiomyocytes formed in hearts *in situ* produced similar results supporting the premyofibril model for myofibrillogenesis (Du *et al*., 2003b).

Cardiomyocytes have also been isolated from adult rat and cat hearts and placed in tissue culture (Claycomb and Palazzo, 1980; Imanaka-Yoshida *et al*., 1996; Schaub *et al*., 1997). These cells in their initial day in culture do not display any signs of myofibrillogenesis. However, after approximately a day in culture, the edges of the cells begin to spread and the first signs of assembly are detected: premyofibrils with their banded arrays of nonmuscle myosin IIB, and Z-bodies staining for the presence of sarcomeric alpha-actinin (LoRusso *et al*., 1997). These results support a similar premyofibril model of myofibrillogenesis in both adult and embryonic cardiomyocytes.

Conclusions

There are several different theories about how myofibrils may be assembled in vertebrate cross-striated muscles (Figures 1–3). The premyofibril model (Figure 3) is supported by numerous observations and experiments in cardiac and skeletal muscle cells *in situ*, in precardiac explants, and in tissue-cultured myocytes. It is now clear that in addition to myofibrils, stress fibers are clearly sarcomeric in organization (Langanger *et al*., 1986; Mittal *et al*., 1987), and there is growing evidence that even cleavage furrows of dividing cells may be composed of sarcomeric structures (Sanger and Sanger, 1980, 2000, 2001b; Conrad *et al*., 1995). Sarcomeric structures seem to have appeared quite early in the evolution of eukaryotic cells. The appearance of novel proteins may have given unusual properties to stress fibers in myoblasts, leading to the myofibrils of cross-striated muscles. More work is needed to determine how the muscle cells keep the nonmuscle myosin IIs of the premyofibrils and cleavage furrows from copolymerizing with the muscle myosin IIs of the nascent and mature myofibrils (Rhee *et al*., 1994; Conrad *et al*., 1995). We will also need to determine how the myriad number of proteins in the Z-band are assembled in the transition of Z-bodies in the premyofibrils and nascent myofibrils to the typical Z-lines or Z-bands of mature myofibrils (Wang *et al*., 2005a, b). Titin appears to be involved in connecting the thick filaments to the Z-bodies of nascent myofibrils (Rhee *et al*., 1994; Holtzer *et al*., 1997), and it has long been an article of faith that titin molecules determine the length of the thick filaments in mature myofibrils. It is important to obtain evidence to support this hypothesis. Just as stress fibers are not needed as templates to form myofibrils, it may turn out that A-band assemblies are a property of the muscle myosin molecules.

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