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Myofibrillogenesis in Skeletal Muscle Cells

Joseph W. Sanger, PhD; Prokash Chowrashi, PhD; Nathan C. Shaner, BA; Simon Spalthoff, MD; Jushuo Wang, PhD; Nancy L. Freeman, PhD; and Jean M. Sanger, PhD

How are myofibrils assembled in skeletal muscles? The current authors present evidence that myofibrils assemble through a three-step model: premyofibrils to nascent myofibrils to mature myofibrils. This three-step sequence was based initially on studies of living and fixed cultured cells from cardiac muscle. Data from avian primary muscle cells and from a transgenic skeletal mouse cell line indicate that a premyofibril model for myofibrillogenesis also holds for skeletal muscle cells. Premyofibrils are characterized by minisarcomeres bounded by Z-bodies composed of the muscle isoform of alpha-actinin. Actin filaments are connected to these Z-bodies and to the mini-A-bands composed of nonmuscle myosin II filaments. Nascent myofibrils are formed when premyofibrils align and are modified by the addition of titin and muscle myosin II filaments. Mature myofibrils result when nonmuscle myosin II is eliminated from the myofibrils and the alpha-actinin rich Z-bodies fuse as the distance between them increases from 0.5 μm in premyofibrils to 2 to 2.5 μm in the mature myofibrils.

From the Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA.

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Reprint requests to Joseph W. Sanger, PhD, Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, 421 Curie Boulevard, Philadelphia, PA 19104–6058.

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List of Abbreviations Used

CDNA	complementary deoxyribonucleic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
YFP	yellow fluorescent protein

Adult Skeletal Muscles

Muscle in cardiac and skeletal muscle cells is composed of interdigitating filaments that are organized in a basic repeating unit, the sarcomere (Fig 1). Actin and titin filaments attach to the boundaries of the sarcomere, the Z-bands, home to an increasing number of newly discovered proteins,[19,30] one of which is involved with a limb muscular dystrophy.[21] The actin filaments and the titin filaments bind to the ordered arrays of thick myosin II filaments in the A-band (Fig 1). An ultrastructural view of longitudinal sections of skeletal muscle reveals bundles of myofibrils, which are approximately a half micrometer wide and contain a series of sarcomeres with an average rest length of approximately 2.5 μm (Fig 2). In skeletal muscle cells, also called muscle fibers, the myofibrils run from one end of the cell to the other end. The muscle fibers that comprise a muscle bundle rarely run the length of the bundle.[11,12] In fact, of approximately 1000 individual cells dissected from a sartorius mus-

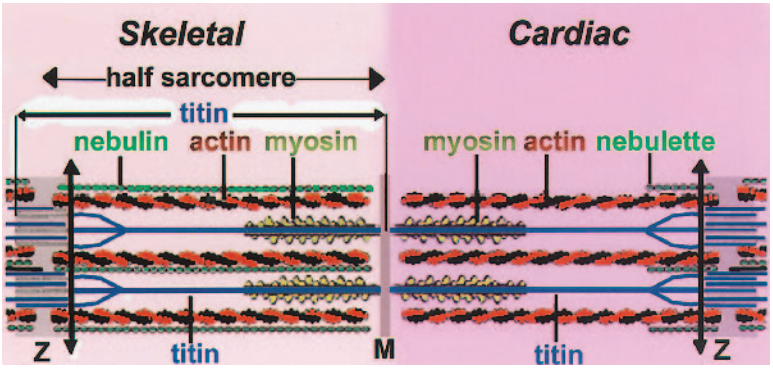

Fig 1. A model of a hybrid sarcomere comprising a left half of a skeletal sarcomere and a right half of a cardiac sarcomere is shown. The Z-bands mark the boundaries of one sarcomere, and are the sites in the sarcomere where the ends of the actin filaments and titin filaments are embedded. Both of these filaments bind alpha-actinin in the Z-bands. The actin filaments are complexed with several different proteins including tropomyosin, troponins, and nebulin. In skeletal muscle sarcomeres, nebulin extends all along the length of the thin filament, whereas in cardiac muscle, the nebulin isoform, nebulette extends from the Z-band to just a few tenths of a micron along the actin filaments. In both muscle types, the 1.6-μm long myosin filaments are aligned into arrays called A-bands that are approximately 1.6 μm in length. Six titin filaments connect each half of a myosin filament to the end of the sarcomere, extending from the M-band to the Z-band. The C-terminal ends of the two sets of titin filaments in the same sarcomere overlap in the M-band, and the N-terminal ends of the titin filaments from adjacent sarcomeres overlap in the Z-bands.[10,17] Several additional proteins cross-link the myosin filaments in the M-band. (Modified with permission from Sanger JW, Sanger JM; Fishing out proteins that bind to titin. J Cell Biol 154:21–24, 2001.)

cle bundle of a domestic cat, only seven extended the full 10 cm length of the bundle.[12] Most of the fibers were in the range of 1 to 3 cm in length. Muscle fibers with an average diameter of 50 μm have approximately 600 myofibrils in cross section, and if they were 1 cm in length, they would contain approximately 2.4 million sarcomeres.

The sarcomeres serve as a scaffold for membranes and many metabolic enzymes. Regulation of contraction of the sarcomeres is controlled by an extensive array of the membranous

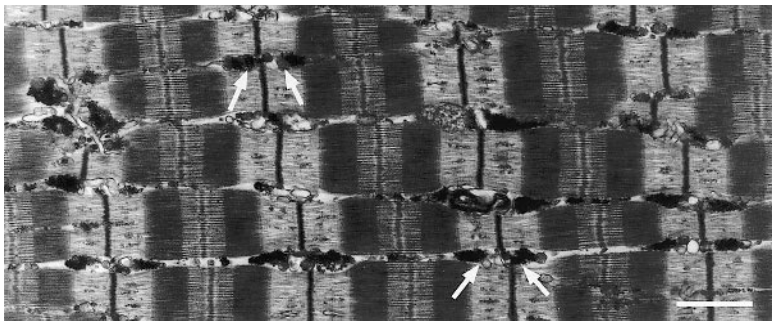

Fig 2. A transmission electron micrograph of a longitudinal section of a skeletal muscle cell or fiber (rabbit psoas) shows the sarcomeres are arranged longitudinally to form myofibrils that are aligned laterally. Deposits of glycogen (arrows) are found near the Z-bands. Bar = 1 μm

transverse tubules and sarcoplasmic reticulum.[9] Enzymes required for muscle function are present in bound and soluble form in the muscle cell.[14] Creatine kinase is a component of the M-bands of mature myofibrils.[35] Phosphorylase is a component of the Z-bands.[3] Several glycolytic enzymes, for example, aldolase, GAPDH, and phosphofructokinase bind to thin filaments via actin and tropomyosin-troponin binding sites.[8,13–15,18,31]

Model for the Study of Myofibrillogenesis

Myoblasts, the stem cells of embryonic skeletal muscle, undergo a series of divisions to form myocytes that fuse to form multinucleated myotubes (Fig 3) in which myofibrils form (Fig 4). Fusion is not necessary for the assembly of myofibrils, because under certain experimentally-induced conditions, myocytes can be prevented from fusing and a few myofibrils will form within the elongated structures.[22,25] In contrast to skeletal muscle cells, embryonic cardiac muscle cells can undergo mitotic divisions, resulting either in two daughter cardiac muscle cells or a binucleated muscle cell.[4]

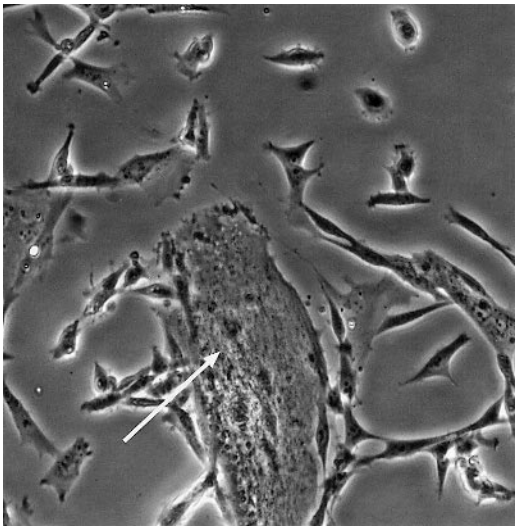

Fig 3. Mononucleated stem cells of myoblasts can undergo a series of divisions resulting in postmitotic daughter cells, myocytes, that fuse with one another to form a multinucleated myotube (arrow).

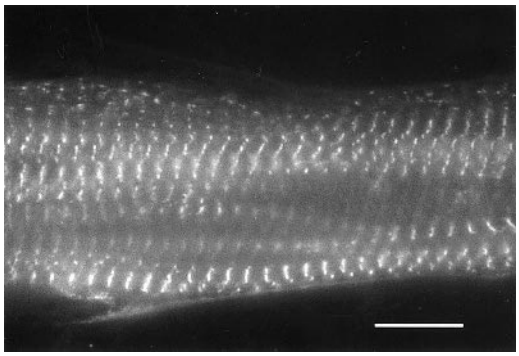

Fig 4. A fluorescent light micrograph of a myotube with mature myofibrils is shown. The myocytes previously had been infected with a virus encoding the cDNA for alpha-actinin ligated to the cDNA for YFP. The YFP-alpha-actinin is localized in the Z-bands of the sarcomeres. Two Z-bands in series mark the limits of one sarcomere. Bar = 10 μm

A popular model system for the study of myofibrillogenesis has been cultured avian embryonic myoblasts.[5,6] Myoblasts isolated from the breast muscle of chick or quail embryos from 10-day-old fertilized eggs are treated with a low level of trypsin for approximately 10 to 30 minutes to separate the muscle cells. The digested tissue is spun down in a tabletop centrifuge to form a pellet of cells that is resuspended in culture medium and filtered through a 50-μm filter so that only single cells pass through.[5] The filtrate contains myoblasts, myocytes, and fibroblasts. By preplating the cells for 1 hour, the more adherent fibroblasts are decreased in the isolated cell preparation, and the unattached cells, enriched for myoblasts and myocytes are collected and plated on collagen-coated tissue culture dishes. The authors recorded myoblasts and myocytes as they attached during the first day in culture and began to migrate and divide during the second and subsequent days in culture. As the myocytes migrate toward one another they fuse with one another and form small elongated myotubes during the third day of culture (Fig 3). During the next several days, addi-

tional myocytes fuse to form wider and longer myotubes, and by 5 days, the multinucleated myotubes undergo spontaneous irregular contractions. Under certain conditions, the cross striations of the myofibrils can be detected in living myotubes using phase contrast microscopy. However, to observe the formation of myofibrils in these cells, fluorescent reagents that target specific regions of the sarcomeres and myofibrils must be used.

Alpha-Actinin as a Probe for Studying Myofibrillogenesis

Alpha-actinin is an actin-binding protein that is localized in all Z-bands of the sarcomeres. As such, it is a good marker for sarcomeres. When a fluorescent dye is chemically linked to alpha-actinin and the protein is injected in trace amounts into muscle cells, it allows the Z-bands to be observed with light microscopy in live cells.[24,26–28] The cDNA for alpha-actinin also can be inserted into viruses or plasmids that encode the bioluminescent protein, GFP, responsible for the bioluminescence found in several different marine animals (the bioluminescent jellyfish).[1] When myocytes are infected with the virus (Fig 4) or transfected with the plasmid (Fig 5), the cells express the fusion protein, alpha-actinin-GFP. Near the middle of transfected myotubes, arrays of Z-bands are detected in the myotubes (Fig 3, 5B, 6). At the ends (Fig 5) and sides (Fig 6) of elongating myotubes, fibrils with smaller periodic bands are detected. By following the same myotube, the authors were able to show that the short periodic bands grew further apart along the fibrils, that adjacent strands fused to form wider fibers, and that the fluorescent bands fused to form the typical linear Z-bands of mature myofibrils[26,27] (Fig 7). The deposition of new premyofibrils and the annealing of short premyofibrils into longer premyofibrils that moved toward the interior of the myotube (Fig 8) also was seen in embryonic cardiomyocytes in tissue culture.[7] The fusion of Z-bodies to form solid Z-bands typical of mature myofibrils implies a dynamic activity of the Z-band components that permits remodeling.

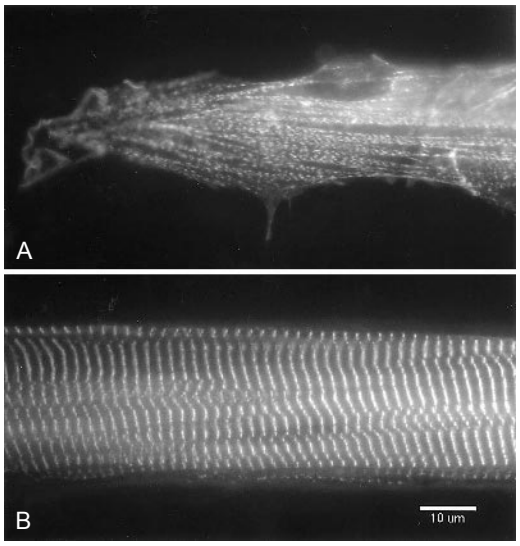

Fig 5A–B. (A) The end of a quail myotube formed from myocytes that had been transfected with a plasmid encoding YFP-alpha-actinin before fusion is shown. Closely spaced Z-bodies of YFP-alpha-actinin are found in the premyofibrils at the end of the myotube. The Z-bodies mark the boundaries of the minisarcomeres that comprise a premyofibril. (B) A midsection of the same myotube in Figure 5A is shown with fluorescent Z-bands aligned in well-organized mature myofibrils. Bar = 10 μm

Premyofibril Model

Figure 9 shows a model that explains results on living and fixed cells stained with antibodies directed against alpha-actinin, nonmuscle myosin II, muscle myosin II, and titin. This model, called the premyofibril model, indicates thin fibers along the membrane surfaces at the ends and sides of a growing myotube. The thin fibers are termed premyofibrils. Alpha-actinin is concentrated along the fibrils in small aggregates called Z-bodies. Actin filaments are attached to these Z-bodies and to nonmuscle myosin II filaments. The Z-bodies mark the boundary of the repeating subunits of the premyofibrils. Just as the Z-bands of myofibrils mark the boundaries of a sarcomere, so too, the Z-bodies mark the boundaries of the minisarcomeres of the premyofibrils. During the course of a few hours, the beaded Z-bands

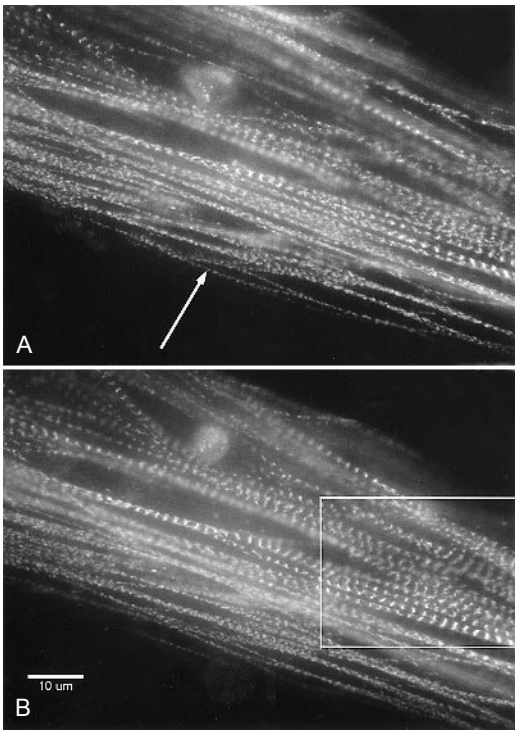

Figure 6A–B. Two different points of one region of a myotube transfected with a plasmid encoding YFP-alpha-actinin are shown. (A) The presence of premyofibrils (arrow) on the edge of this myotube can be seen. (B) The same live myotube is shown 1 hour later. The boxed area indicates an area where Z-bodies of the myofibrils have fused to one another to form the linear Z-bands of mature myofibrils. Bar = 10 μm

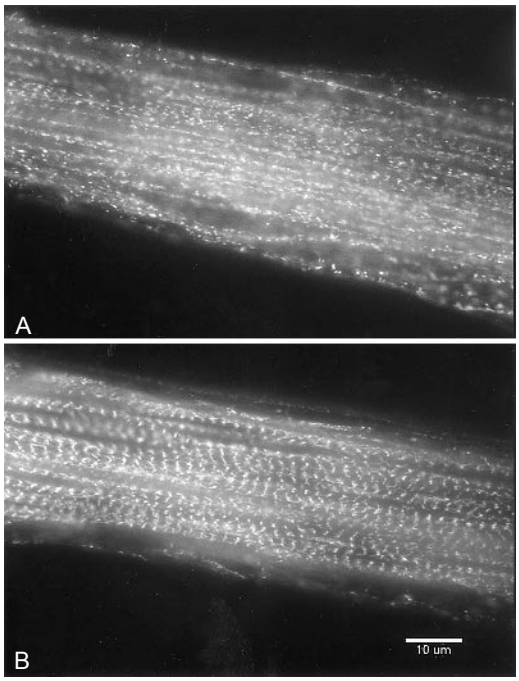

Fig 7A–B. A myotube expressing YFP-alpha-actinin is shown at (A) Time 0 and (B) 6 hours later. Many of the Z-bodies of the premyofibrils aligned and fused to form the Z-bands of a mature myofibrils. Bar = 10 μm

transform into the linear Z-lines observed in muscles from adults.[7]

Staining of premyofibrils with muscle-specific proteins reveals that muscle-specific actin and alpha-actinin are present, but muscle-specific myosin II and titin are absent.[20] Intermediate in time of formation and in structure between premyofibrils and mature myofibrils, are fibrils that are called nascent myofibrils. Titin is present in the nascent myofibrils, and muscle-specific myosin II and nonmuscle myosin II are present. The nonmuscle myosin II is present in periodic repeats in the nascent myofibrils, whereas the muscle myosin II is not (Fig 9). In mature myofibrils, nonmuscle myosin II is absent and myosin II filaments are aligned into the A-bands that can be seen clearly in electron micrographs (Fig 2). How the nonmuscle myosin II molecules are eliminated from the nascent myofibril as it transforms into the mature myofibril is not known. The components of the sarcomeres also turn over with half-lives of 5 to 7 days.[37] How they are able to exchange without affecting the integrity of the myofibrils and the contractility of the muscle cells is the subject of current research. The importance of titin for the integrity of mature sarcomeres has been shown in muscle cells transfected with fragments of titin.[2,32,33]

The association of the premyofibrils with the plasma membrane provides a surface on which the initial components can be anchored and organized. Most mature myofibrils lose the close association with the plasma mem-

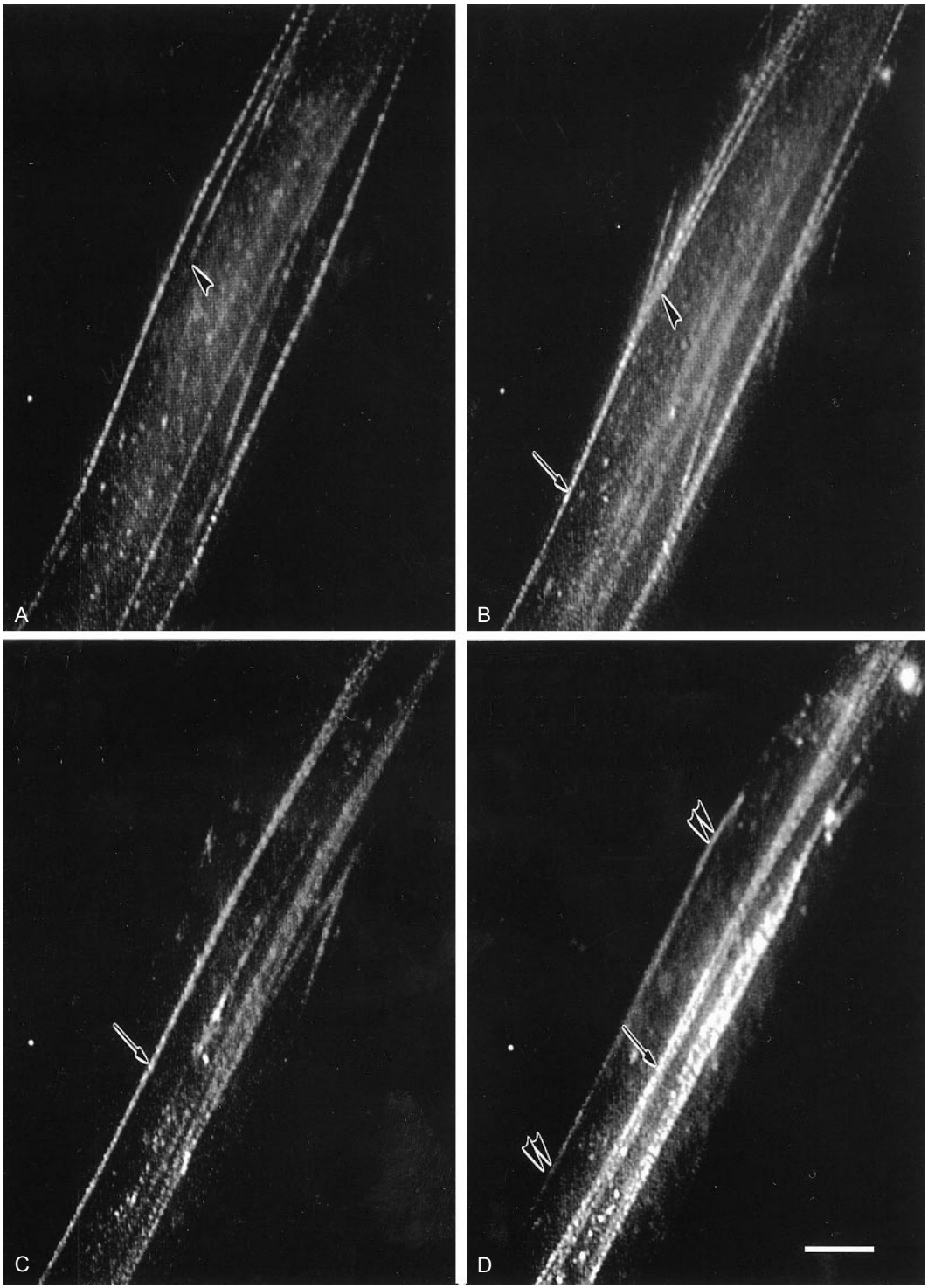

Fig 8A–D. Four points of a living myotube that was injected with fluorescently labeled alpha-actinin are shown. (A) The end of a premyofibril (arrowhead) is shown. (B) The end of the premyofibril (arrowhead) in Figure 8A has fused with a premyofibril (arrow) near the edge of the myotube. (C) The premyofibrils have fused (arrow). (D) The annealed premyofibril (arrow in Fig 8C) has moved to a position where it fused with other fibers (arrow). A new premyofibril was formed at the edge of the myotube in the last interval (double arrowheads). (A) Time 0; (B) 3 hours 55 minutes; (C) 8 hours 57 minutes; (D) 23 hours 17 minutes. Bar = 5 μm

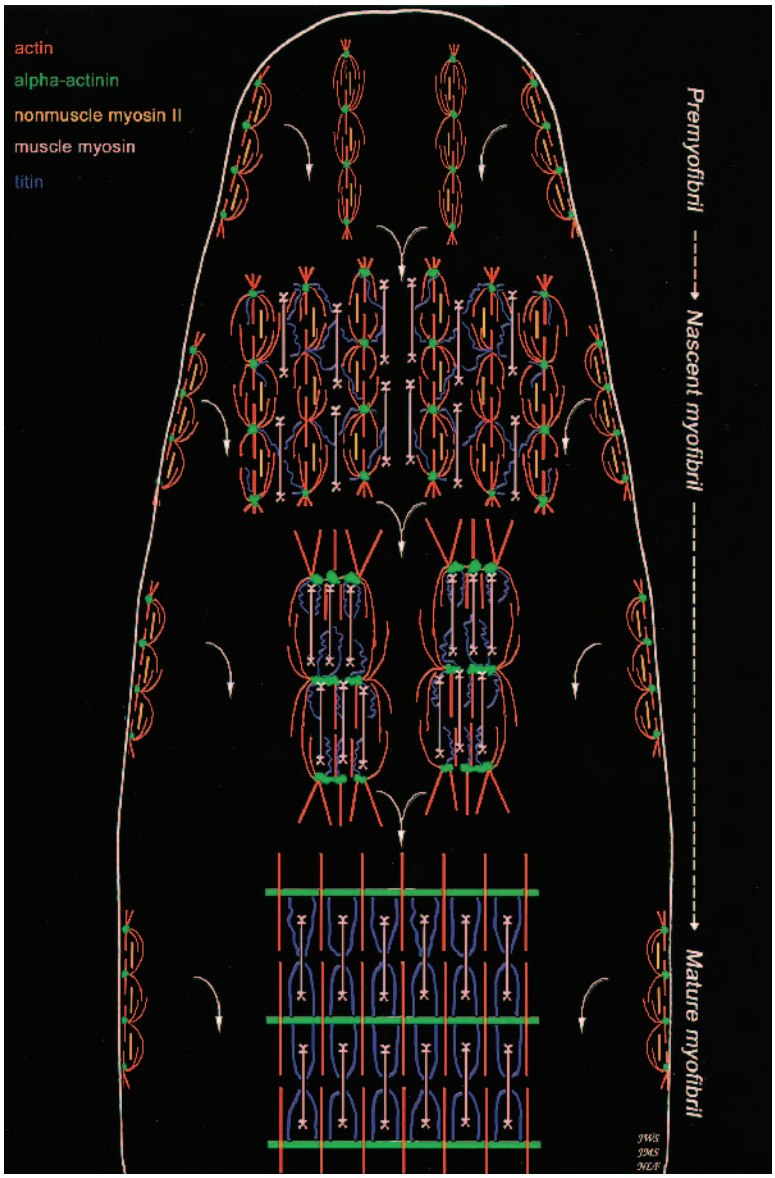

Fig 9. A model of myofibrillogenesis in an elongating and widening skeletal muscle myotube is shown. Premyofibrils, composed of minisarcomeres, are deposited at the ends of elongating and widening myotubes. Premyofibrils are composed of minisarcomeres, the ends of which are alpha-actinin-rich Z-bodies to which actin filaments are attached. Mini-A-bands composed of nonmuscle myosin II filaments are localized between the Z-bodies. As groups of premyofibrils begin to align with one another, titin and muscle-myosin II filaments are recruited to form nascent myofibrils. The mature myofibrils form from the fusion of the Z-bodies into Z-bands, the elimination of the nonmuscle myosin II filaments and the alignment of the muscle-myosin filaments into A-bands.

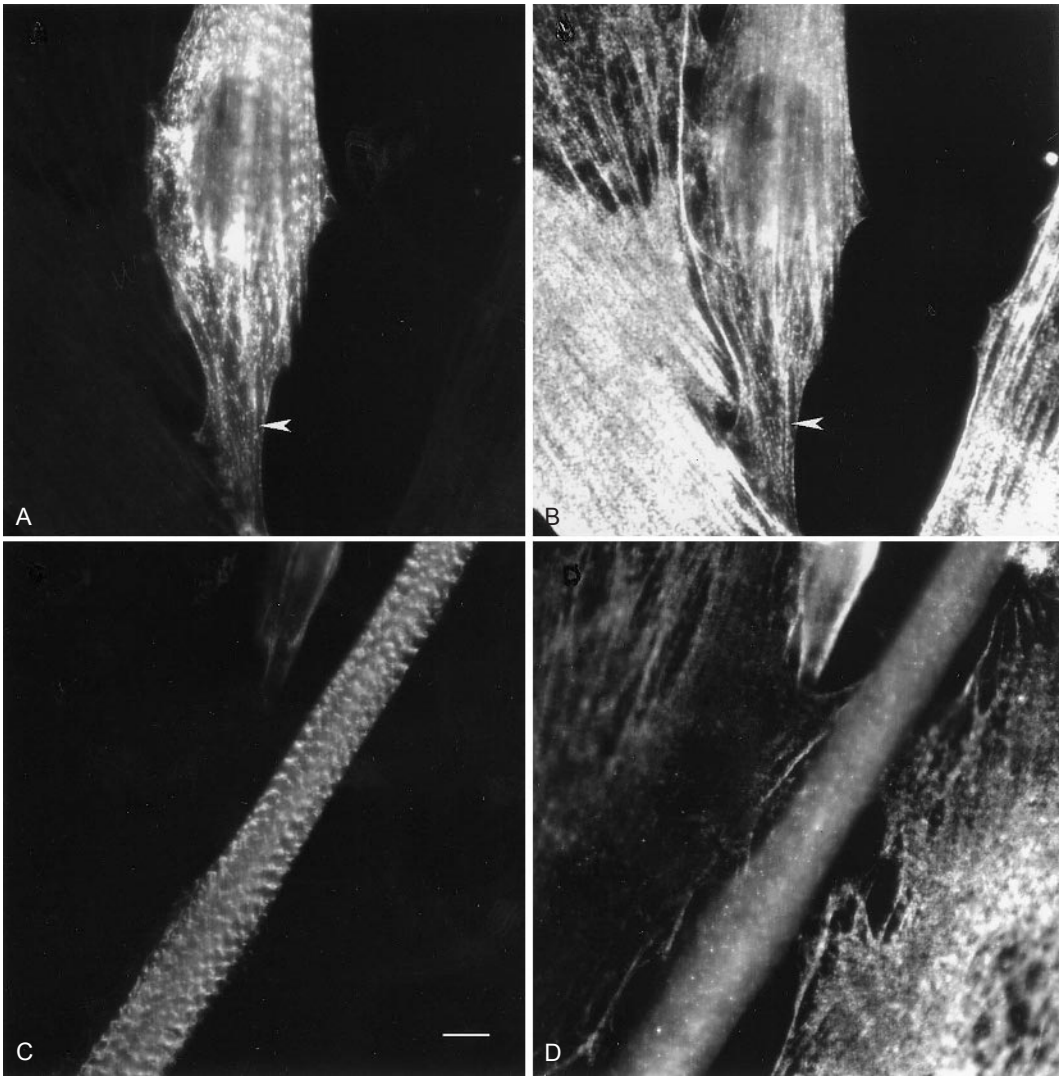

Fig 10A–D. Mouse myoblasts grown at 39° C are induced to form myofibrils. The myoblasts down-regulate the synthesis of alpha-actinin and upregulate the synthesis of the muscle isoform of alpha-actinin. (A) A myotube formed after 2 to 3 days incubation at 39° C was fixed and stained with an antibody specific for the muscle isoform of alpha-actinin. Premyofibrils (arrow) are present at the end of the myotube. (B) The fibrils in the same cell also reacted with an antibody directed against nonmuscle myosin IIA indicating that these were premyofibrils (arrowhead). Only one of the myosin-positive cells in (B) reacted with sarcomeric alpha-actinin antibody (Fig 10A). The other myoblasts have not yet made the genetic switch to the muscle differentiation program. (C) Z-bands in a myotube in the culture stained positively with muscle specific alpha-actinin are shown. (D) The same myotube in (C) showed only a diffuse distribution of antinonmuscle myosin II antibodies along the mature myofibrils. Bar = 5 μm

brane seen with premyofibrils and nascent myofibrils. Nevertheless, the mature myofibrils retain a less extensive association with the cell surface via the indentations of the surface membranes, that is transverse tubules, at their Z-bands or A-I bands.

Myofibrillogenesis in a Mouse Cell Line from Skeletal Muscle

How general is this premyofibril model of myofibrillogenesis in skeletal muscle cells? The model originally was developed using embryonic chick muscle cells.[20,26] This model also was tested by using a transformed mouse cell line. Myogenic cell lines isolated from limb muscles of transgenic mice will grow at 33° C as mononucleated cells but when shifted to 39° C, the cells continue to grow, but also will fuse with one another to form myotubes that contain myofibrils that stain positively for neonatal muscle myosin II.[16] Myotubes were not examined in that study to determine how myofibrils formed.[16] To test the premyofibril model in mouse myotubes, cells from this cell line was grown in culture to determine whether premyofibrils could be detected. A field of mouse cells, growing at the temperature where muscle formation is inhibited, was stained with an antibody specific for nonmuscle alpha-actinin and counterstained with a fluorescent phalloidin probe to reveal the filamentous actin in these cells. Alpha-actinin antibody staining shows that the fibers in the cell have linear arrays of dense bodies such as are found in the stress fibers of nonmuscle cells.[34] The phalloidin probe indicates that actin filaments are distributed along the length of these fibers in a nonperiodic manner. Staining of these cultures with a muscle-specific alpha-actinin antibody revealed the absence of any mature myofibrils. When sister cultures of these mouse transformed cells were grown at 39° C, the mononucleated cells that fused with one another to form myotubes contained mature myofibrils that could be detected by staining the myotubes with a muscle-specific antialpha-actinin antibody (Fig 10A). The alpha-actinin was concentrated in the Z-bands of the component sarcomeres of the myofibrils. The same antibody stained the growing ends of the myotubes in a premyofibril pattern (Fig 10A). Counterstaining of these myotube ends with a nonmuscle myosin II antibody revealed alternating bands of this protein between the bands of alpha- (Z-bodies) at the ends of the myotubes (Fig 10B). When mature myofibrils are present in the myotubes (Fig 10C), no nonmuscle myosin II is associated with these structures (Fig 10D), as expected from the premyofibril model theory for the assembly of mature myofibrils (Fig 8). Interestingly, these myotubes did not stain with a nonmuscle alpha-actinin antibody. Raising the temperature from 33° to 39° C causes a halt in the synthesis of the nonmuscle isoform of alpha-actinin in muscle cells and switches on the synthesis and accumulation of the muscle isoform of alpha-actinin. The experiments on this muscle cell line are consistent with a premyofibril model of formation of myofibrils in mouse skeletal muscle cells.

Experiments on primary embryonic avian myoblasts and a transformed mouse skeletal muscle cell line, support a three-step model for the formation of mature skeletal myofibrils: premyofibrils to nascent myofibrils to mature myofibrils (Fig 9). This model originally was proposed for the formation of mature myofibrils in cardiac muscle cells.[7,20,23,29] It should not be surprising that the same mechanism may apply for both types of cross-striated muscles, cardiac and skeletal, because their mature myofibrils are so similar in structure (Fig 1). Nevertheless, one must remember the quote from John Williams in The Compleat Strategyst. "The invention of deliberately oversimplified theories is one of the major techniques of science . . . "[36] Future work will be needed to determine how oversimplified this premyofibril model may be.

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