

# Myofibrillogenesis in Skeletal Muscle Cells in the Presence of Taxol

Cornelia C. Siebrands, Jean M. Sanger, and Joseph W. Sanger\*

*Department of Cell and Developmental Biology, University of Pennsylvania  
School of Medicine, Philadelphia*

We address the controversy of whether mature myofibrils can form in the presence of taxol, a microtubule-stabilizing compound. Previous electron microscopic studies reported the absence of actin filaments and Z-bands in taxol-treated myocytes [Antin et al., 1981: *J Cell Biol* 90:300–308; Toyoma et al., 1982: *Proc Natl Acad Sci USA* 79:6556–6560]. Quail skeletal myoblasts were isolated from 10-day-old embryos and grown in the presence or absence of taxol. Taxol inhibited the formation of multinucleated elongated myotubes. Myocytes cultured in the continual presence of taxol progressed from rounded to stellate shapes. Groups of myocytes that were clustered together after the isolation procedure fused in the presence of taxol but did not form elongated myotubes. Actin filaments and actin-binding proteins were detected with several different fluorescent probes in all myofibrils that formed in the presence of taxol. The Z-bands contained both alpha-actinin and titin, and the typical arrays of A-Bands were always associated with actin filaments in the myofibrils. Myofibril formation was followed by fixing cells each day in culture and staining with probes for actin, muscle-specific alpha-actinin, myosin II, nebulin, troponin, tropomyosin, and non-muscle myosin II. Small linear aggregates of alpha-actinin or Z-bodies, premyofibrils, were detected at the edges of the myocytes and in the arms of the taxol-treated cells and were always associated with actin filaments. Non-muscle myosin II was detected at the edges of the taxol-treated cells. Removal of the taxol drug led to the cells assuming a normal compact elongated shape. During the recovery process, additional myofibrils formed at the spreading edges of these elongated and thicker myotubes. Staining of these taxol-recovering cells with specific fluorescent reagents reveals three different classes of actin fibers. These results are consistent with a model of myofibrillogenesis that involves the transition of premyofibrils to mature myofibrils. *Cell Motil. Cytoskeleton* 58:39–52, 2004. © 2004 Wiley-Liss, Inc.

**Key words:** microtubules; alpha-actinin; nebulin; non-muscle myosin II; muscle myosin II; titin; tropomyosin; troponin T; zeugmatin; premyofibril; mature myofibril; sarcomere

## INTRODUCTION

Myofibrils are components of both cardiac and skeletal muscle cells. A premyofibril model for myofibrillogenesis was proposed based on studies of cardiac muscle cells [Rhee et al., 1994; Sanger and Sanger 2002; Du et al., 2003a] and skeletal muscle cells [Sanger et al., 2002]. There are three proposed stages in the assembly of myofibrils: premyofibrils to nascent myofibrils to mature myofibrils. Premyofibrils are composed of alpha-actinin densities, termed Z-bodies, to which actin filaments are attached. Between the aligned Z-bodies are concentrations of non-muscle myosin II. The appearance of the premyofibrils is longitudinally striated due to the series of minisarcmeres, each minisarcmeres bounded by Z-

bodies and the mini-A-band of nonmuscle myosin II in between the Z-bodies. The actin staining is continuous in premyofibrils, indicating the actin filaments within the

Contract grant sponsor: NIH; Contract grant sponsor: MDA.

\*Correspondence to: Dr. Joseph W. Sanger, Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, 421 Curie Boulevard, BRB II/III, Philadelphia, PA19104-6058. E-mail: sangerj@mail.med.upenn.edu

Received 8 August 2003; Accepted 22 December 2003

minisarcomeres are overlapping. Nascent myofibrils are characterized by the presence of two isoforms of myosin II, the cytoplasmic or non-muscle myosin II and muscle myosin II filaments. The non-muscle myosin II is present in longitudinal bands while the muscle myosin staining is continuous, indicating that the muscle myosin II filaments are not yet arranged in mature A-bands. Mature myofibrils are devoid of non-muscle myosin II, and the muscle myosin II filaments are aligned to form A-Bands [Sanger et al., 2000, 2002].

This model has been criticized in several papers [Holtzer et al., 1997; Ehler et al., 1999; Gregorio and Antin, 2000; Rudy et al., 2001]. Holtzer et al. [1997] have stated that A-bands assemble independently of organized actin filaments and that non-muscle myosin II is not present in embryonic cardiac myocytes [Lu et al., 1992], the same cell type that was used in the studies by Rhee et al. [1994]. However, we were able to demonstrate that the antibody used to stain non-muscle myosin II in the Holtzer et al. studies [1997] recognized an isoform, i.e., nonmuscle myosin IIA, that, while present in cardiac fibroblasts, is absent in cardiomyocytes [Rhee et al., 1994; Sanger et al., 2000; Sanger and Sanger, 2002]. Gregorio and Antin [2000] and Rudy et al. [2001] reported that non-muscle myosin II could not be detected in cardiomyocytes formed in precardiac explants. Recently, we have demonstrated that non-muscle myosin IIB and sarcomeric alpha-actinin are present in premyofibrils and nascent myofibrils in the first cardiomyocytes formed in explant cultures of precardiac mesoderm [Du et al., 2003a]. Furthermore, Du et al. [2003a] reported that non-muscle myosin IIB and sarcomeric alpha-actinin, but not muscle myosin II or titin, were present in the furrows of cardiomyocytes undergoing cell division. Ferrari et al. [1998] demonstrated the importance of non-muscle myosin II for myogenesis in *Xenopus* skeletal muscle by reversibly blocking the activity of this protein. Moreover, transfected living cardiomyocytes were followed that deposited premyofibrils at the spreading edges of the cell and assembled mature myofibrils from the lateral fusion of three to four premyofibrils over a period of less than a day [Dabiri et al., 1997]. These live cell images demonstrated the alpha-actinin and actin molecules associated with the premyofibrils became part of the mature myofibrils.

Ehler et al. [1999] reported in their studies on myofibrillogenesis in early cardiomyocytes of the forming heart that they could not detect any signs of premyofibrils, i.e., ordered arrays of alpha-actinin enriched Z-bodies or aligned bands of non-muscle myosin II B. The distances between the detected dots of alpha-actinin resembled the distances in mature sarcomeres [Ehler et al., 1999]. The non-muscle myosin IIB localized in a diffuse pattern in these early cardiomyocytes [Ehler et al., 1999].

In a recent study, Du et al. [2003b] used deconvolution microscopy to examine identical staged cardiomyocytes, and were able to demonstrate premyofibrils that contained bands of non-muscle myosin IIB aligned between Z-bodies. In contrast to the studies of Ehler et al. [1999], Du et al. [2003b] were also able to detect nascent myofibrils in these early cardiomyocytes *in ovo*, i.e., fibrils containing banded arrays of non-muscle myosin II B and overlapping filaments of muscle myosin IIs. Mature myofibrils *in ovo* did not stain for non-muscle myosin, as was also the case in cardiomyocytes formed from precardiac mesoderm [Du et al., 2003a] and in cultured muscle cells, cardiac and skeletal [Rhee et al., 1994; Sanger et al., 2002]. Du et al. [2003b] have suggested that myofibrillogenesis follows the same pathway of assembly whether the cells are *in situ*, in precardiac explants, or in tissue culture.

Part of the evidence presented to criticize the premyofibril model of myofibrillogenesis are results from experiments that purported to show that aligned series of A-bands could form independently of actin filaments and Z-bands when muscle cells were examined after four continuous days in the presence of taxol, a drug that stabilizes microtubules and inhibits microtubular dynamics [Antin et al., 1981; Toyoma et al., 1982; Moncman and Wang, 1996; Holtzer et al., 1997]. These reports stating that microtubules could substitute for actin filaments are inconsistent with the three-step premyofibril model for the assembly of myofibrils [Rhee et al., 1994]. These taxol results suggested that there might be other pathways to assemble myofibrils, leading us to reexamine the effects of taxol on myofibrillogenesis. Populations of cells exposed to taxol from 1–4 days were fixed and stained with specific sarcomeric probes. We have demonstrated that actin and actin binding proteins are always associated with all assemblies of myosin filaments, and demonstrate that premyofibrils are associated with the areas of myofibril assembly in control, taxol-treated, and in taxol-reversed muscle cells. These current results support the premyofibril model for myofibrillogenesis proposed for both cardiac and skeletal muscle cells [Sanger and Sanger, 2002; Sanger et al., 2002; Du et al., 2003a].

## MATERIALS AND METHODS

### Isolation and Culturing of Cells

Myoblasts were isolated from breast muscles of 10-day-old quail embryos (Truslow Farms, Chestertown, MD) using procedures previously published by Dabiri et al. [1999]. The isolated cells were placed on collagen-coated cover slips at either 50,000 cells per ml (for control cultures) or at 200,000 cells/ml (for taxol-treated

cultures). Taxol at a concentration of 10  $\mu\text{M}$  (Sigma Corp., St. Louis, MO) was placed on the freshly plated cells. In a few cases, we also allowed cells to spread for 1 or 2 days before taxol was added. Taxol-treated cells were fixed 1–4 days after the start of drug treatment. Some taxol-treated cells were rinsed with normal medium after 4 days of treatment and allowed to recover in normal medium for 1–5 five days prior to fixation. Control quail muscle cells were fixed at corresponding ages of the taxol treated cells.

### Fixations, Antibodies, and Phalloidin

The control and taxol-treated quail cells were fixed with either a 4% paraformaldehyde solution followed by detergent extraction and quenching in ammonium chloride as described by Dabiri et al. [1999] or by plunging the cover slips into cold pure methanol ( $-20^{\circ}\text{C}$ ) containing 1 mM EGTA. Both paraformaldehyde and methanol fixed cover slips were rinsed several times with a low salt buffer [Dabiri et al., 1999].

Antibodies against sarcomeric alpha-actinin, nebulin, alpha-tubulin, and sarcomeric tropomyosin were purchased from Sigma Corp. (St. Louis, MO). Antibodies directed against actin (JLA20), troponin T (CT3), and zeugmatin (mAb20) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). The non-muscle myosin IIB antibody was ordered from Chemicon International (Temecula, CA). Fluorescein and rhodamine labeled secondary antibodies were shipped from the Jackson ImmunoResearch Laboratories (West Grove, PA). Most slides were counter-stained with either fluorescein (Molecular Probes, Eugene OR) or with rhodamine (Fluka Chemie AG, Ronkokoma, NY) phalloidin [Zhukarev et al., 1997]. The stained and rinsed cover slips were mounted in Mowiol that contained an antifade reagent [Ayoob et al., 2001].

The fixation method had to be checked because it affected the ability of the reagents to stain the different elements of the sarcomeres. Staining of muscle cells with the JLA actin IgM antibody only worked with methanol fixation. Methanol fixation resulted in the loss of sarcomeric alpha-actinin antibody staining. Methanol fixation rendered the cells incapable of being stained with phalloidin. Methanol fixation was best for microtubules; the microtubules were uniformly stained. In paraformaldehyde fixation, the microtubules were often beaded, almost speckled.

### Imaging

Fluorescent images were obtained with either a Nikon Diaphot 200 inverted microscope and Nikon Planapo 63 $\times$ , 1.4 NA or Plan Fluor 100 $\times$  1.3 NA lenses or a Zeiss (Thornwood, NY) LSM 510 microscope using a

Zeiss 63 $\times$ , 1.4NA. water immersion objective. The fluorescent images were assembled using Metamorph (Downingtown, PA) and Adobe Photoshop (San Jose, CA).

## RESULTS

### Control Myofibrillogenesis in Quail Muscle Cells

Myoblasts plated on collagen-coated glass cover-slips attached as round cells that stained lightly with actin and sarcomeric alpha-actinin probes after one day in culture (Fig. 1A,B). By the end of two days, the cells had elongated, and small bands of sarcomeric alpha-actinin were aligned along the fibers of actin (Fig. 1C,D). Some small myotubes that resulted from fusion of the myoblasts were detected at the end of two days of culture. On the third day in culture, fibers with small bands of alpha-actinin were localized along the lengths of narrow multinucleated myotubes. Myotubes elongate by additional fusion of myocytes and assemble mature myofibrils all along their lengths. By day 4 in culture, alpha-actinin was present in Z-bands in myofibrils that ran longitudinally along the myotubes (Fig. 1E,F). Nebulin antibodies stained the premyofibrils and nascent myofibrils at the elongating ends of the myotubes (Fig. 2) and the mature myofibrils in the central shafts of the myotubes (Fig. 3). Clearly striated patterns of nebulin were detected only in the mature myofibrils (Fig. 3). Staining of myotubes with tubulin antibodies revealed microtubules running all along the long axes of the myofibrils in these cells (Fig. 4).

### Taxol Inhibits Myotube Formation But Not Myofibrillogenesis

Myocytes cultured in the continual presence of taxol were inhibited in migration and cell division resulting in suppression of fusion except when cells were in aggregates when first cultured, as evidenced by the presence of cells containing up to five nuclei. These multinucleated cells did not elongate to form myotubes, but had a shape similar to the bi- and mononucleated taxol-treated cells (Fig. 5). By the end of one day in culture in the presence of taxol, the mononucleated cells were either rounded or elongated (Fig. 5A–D). The unfused myocytes were identified by their staining with sarcomeric alpha-actinin antibodies (Fig. 5). After two days in the presence of taxol, the myocytes were almost all bi- or mononucleated but had assumed a spiky appearance (Fig. 5E,F). As in control cultures, small bands of alpha-actinin were present along actin fibers in the cells (Fig. 5E). With increasing time in culture, some of the bands of alpha-actinin were spaced at two-micron intervals as in Z-band spacings in mature myofibrils (Fig. 5G,H).

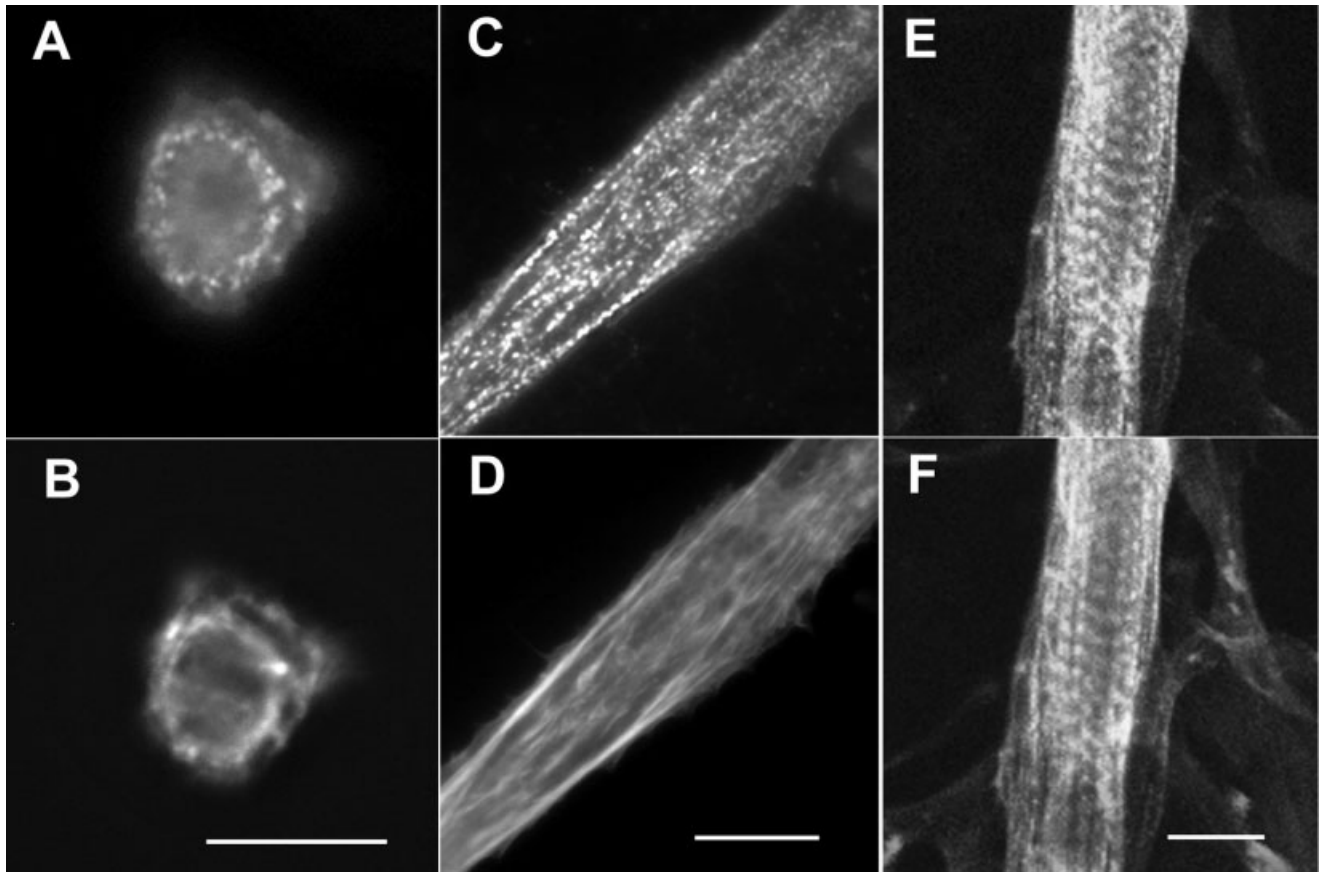


Fig. 1. Control skeletal myoblasts and myocytes stained with sarcomeric alpha-actinin (A,C,E) and (B,D,F) phalloidin after one (A, B), two (C, D), and four (E, F) days in culture. Small bands of alpha-actinin mark the Z-bodies of the premyofibrils (A, C) and the Z-bands of the mature myofibrils (E). Actin filaments are associated with both the premyofibrils and mature myofibrils. Bars = 10  $\mu$ m.

After 4 days in the presence of taxol, there was a marked increase in the number of myofibrils (Fig. 5 I,J). Actin filaments were associated with these forming myofibrils (Fig. 5B,D,F,H,J). The microtubules in both control myotubes (Fig. 4) and taxol-treated myocytes (Fig. 6) were closely associated with actin filaments in the assembling myofibrils.

#### Assembly of Myofibrils Occurs in the Presence of Taxol

Higher magnification of fixed and stained taxol-treated myocytes revealed that myofibrils formed in the presence of the drug. Figure 5 I and J illustrate a myocyte that has been stained with an anti-sarcomeric alpha-actinin antibody and then counter-stained with a fluorescent phalloidin probe used for the detection of fibrous actin. Actin filaments are co-localized with the myofibrils. The sizes of the sarcomeres are about 2  $\mu$ m in length. Figure 7 illustrates that these taxol-treated myocytes also had colocalizations of muscle myosin II (A-bands) and actin filaments. Other taxol-treated

cells were stained with titin antibodies to detect the position of the largest protein in the sarcomeres of the mature myofibrils that had formed after 4 days of taxol treatment. The zeugmatin antibody that was used is known to stain the Z-band region of titin [Turnacioglu et al., 1996, 1997]. This titin antibody stained the Z-band region of the taxol-treated myocytes (Fig. 8A). These cells were counterstained with an actin antibody that was of the IgM class (Fig. 8B). This combination of IgG and IgM antibodies permitted us to detect actin using a non-phalloidin approach. This actin antibody revealed that actin bands are in the same myofibrils stained by the zeugmatin antibodies (Fig. 8). Staining of other taxol-treated muscle cells with nebulin and fluorescent phalloidin revealed colocalization of actin filaments and nebulin molecules in the same mature myofibrils (Fig. 9). When other taxol-treated myocytes were stained with troponin T or tropomyosin antibodies, these two actin filament associated proteins were detected co-localized with the actin filaments (data not shown).

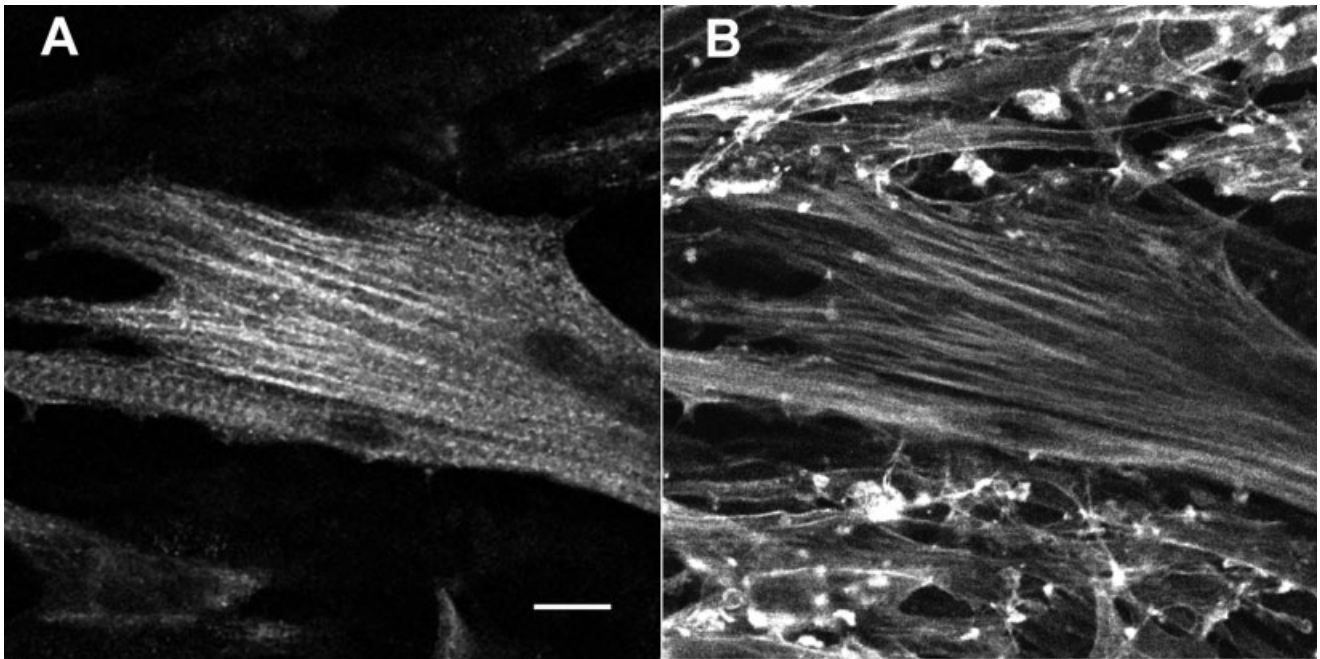


Fig. 2. End of a control quail 4-day-old myotube stained with nebulin antibodies (A) and fluorescently labeled phalloidin (B). Nuclei in the myotubes can be observed by their unstained images (A). Bar = 10  $\mu\text{m}$ .

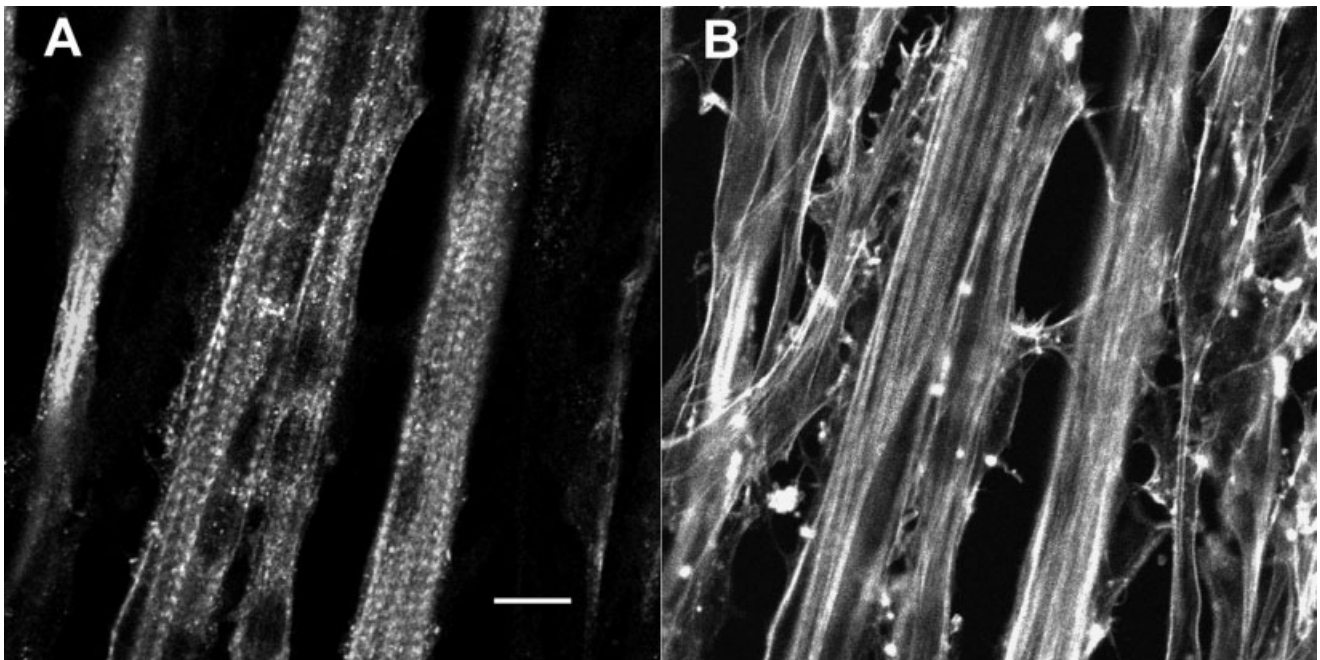


Fig. 3. Midsections of three control quail 4-day-old myotubes stained with nebulin antibodies (A) and fluorescently labeled phalloidin (B). Note the clear striations of nebulin in the myotubes (A). Bar = 10  $\mu\text{m}$ .

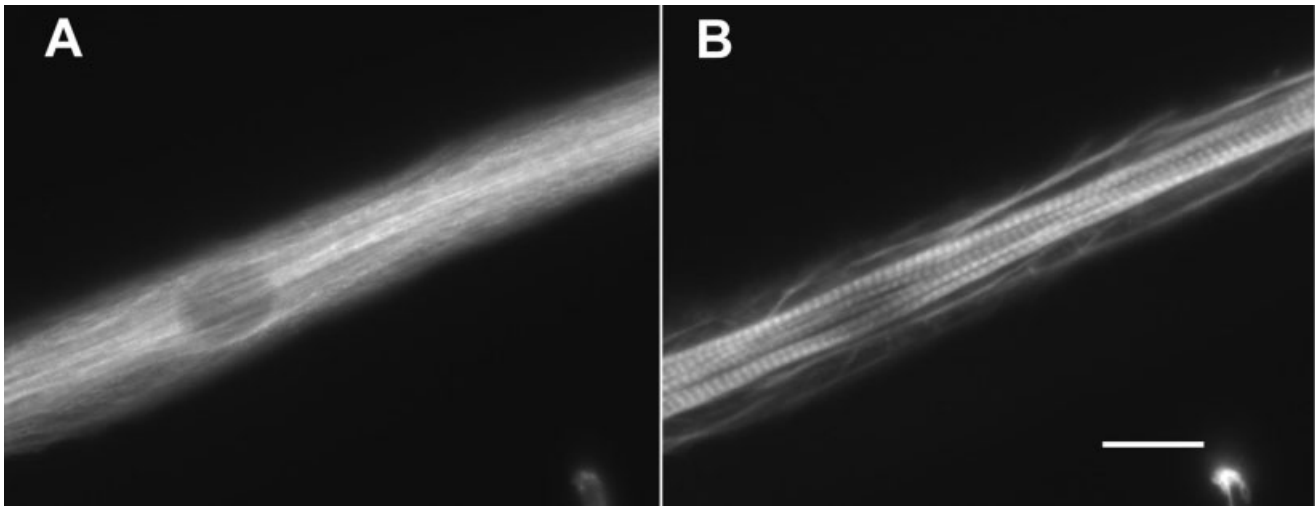


Fig. 4. Control myotube fixed and stained with antibodies against tubulin (A) and phalloidin (B). **A:** Microtubules are distributed along the long axis of this narrow 4-day-old myotube. **B:** A striated pattern of actin is present in the mature myofibrils. Bar = 10  $\mu$ m.

### Presence of Non-muscle Myosin II at the Ends of Taxol-Treated Cells

Fallon and Nachmias [1980] first reported the presence of two types of myosin IIs in control skeletal muscle cells: non-muscle myosin II and muscle myosin II. The non-muscle myosin II (also called cytoplasmic myosin II) was found to be concentrated at the leading edges of the myotubes in their studies. Taxol-treated skeletal muscle cells exhibited linear arrays of non-muscle myosin IIB at the tips and periphery of the extended spikes (see Fig. 10A). When the same cells were counterstained with a sarcomeric alpha-actinin antibody, alpha-actinin was detected in the areas occupied by non-muscle myosin IIB (see Fig. 10B). In areas where alpha-actinin staining was detected in Z-bands of mature sarcomeres, no staining of non-muscle myosin IIB was detected.

### Reversal From Taxol: Fusion and Myofibrillogenesis

After 4 days, taxol was removed from the cultures by exchanging it with several rinses of fresh muscle medium. Over the next 3 days in control medium, the cells lost their spiky appearance to form elongated short myotubes and the myofibrils realigned to course along the long axes of the cells (Figs. 11, 12). Staining of these elongating myotubes with sarcomeric alpha-actinin antibodies revealed the presence of short spacings of alpha-actinin at the spreading edges of the myotubes (Fig. 11A,C). Staining of these elongating and spreading myotubes with muscle myosin II specific antibodies and fluorescent phalloidin (Fig. 12A,B) revealed three different types of actin bundles: (1) actin fibers at the edge of the myotubes and no muscle myosin II staining, i.e.,

premyofibrils; (2) actin fibers and nonstriated arrays of muscle myosin II, i.e., nascent myofibrils; (3) actin fibers and aligned A-bands, i.e., mature myofibrils. The control myotubes at 7 and 8 days were much larger and full of mature myofibrils (data not shown). Similarly, the scattered microtubules in the taxol-treated myocytes also became aligned along the long axes of the reversed cells (data not shown). The number of reversed myocytes did not increase after the removal of taxol since they had become postmitotic cells during their exposures to taxol. These reversed cells were never able to increase to the sizes of myotubes that resulted from growth in 7 days of control medium. The control myotubes are thicker and longer since there has been no interference by taxol in cell division, migration, fusion, and elongation of the cells. These control myotubes are filled with abundant

Fig. 5. Pairs of taxol-treated cells stained with sarcomeric alpha-actinin antibodies (A, C, E, G, I) and phalloidin (B, D, F, H, J) after 1 (A to D), 2 (E, F), 3 (G, H) and 4 days (I, J) in culture. **A,B:** After 1 day, most of the cells are rounded and have small Z-bodies (A) that are associated with F-actin (B). **C,D:** A few cells spread after 1 day in taxol and exhibited banded arrays of Z-bodies (C) can be seen associated with the actin filaments (D). **E,F:** While a few rounded cells are still detected on both the second and third day of culture, the vast majorities of the cells have spread and exhibit both premyofibrils (arrow in E) and mature myofibrils. Actin filaments are associated with both the premyofibrils and mature myofibrils (F). **G,H:** After 3 days, the taxol-treated cells have grown longer with accumulations of mature myofibrils (arrows in G). Premyofibrils can be detected in the elongating arms of the cells. Actin filaments are part of both the premyofibrils and mature myofibrils at all times during exposure to taxol (H). **I,J:** By 4 days in the continual presence of taxol, a number of mature myofibrils have assembled (I). Actin filaments are associated with all of these mature myofibrils (J). Bars = 10  $\mu$ m.

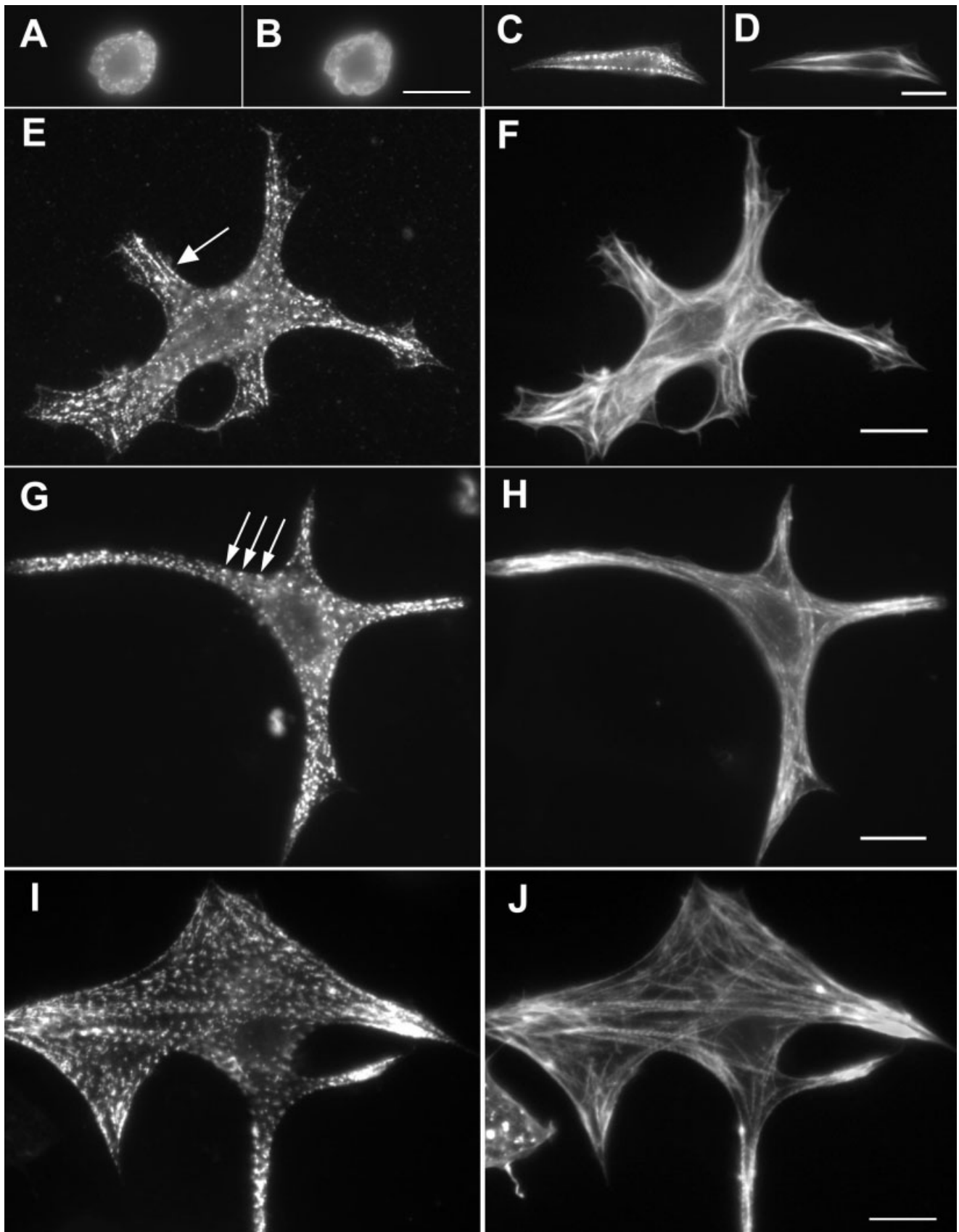


Figure 5.

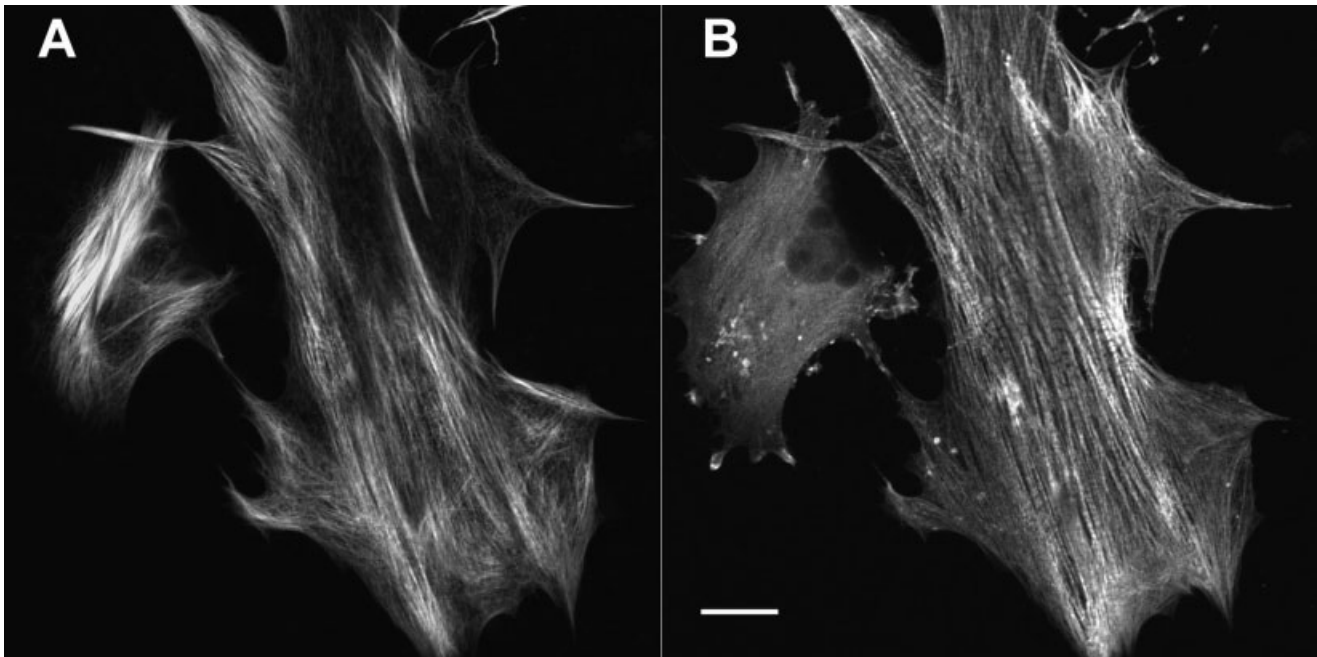


Fig. 6. Cells treated with taxol for 4 days and stained with anti-tubulin antibodies (A) and fluorescently labeled phalloidin (B). The fibroblast cell (left) and the myocyte (right) exhibit aggregates and individual microtubules (A). Bar = 10  $\mu$ m.

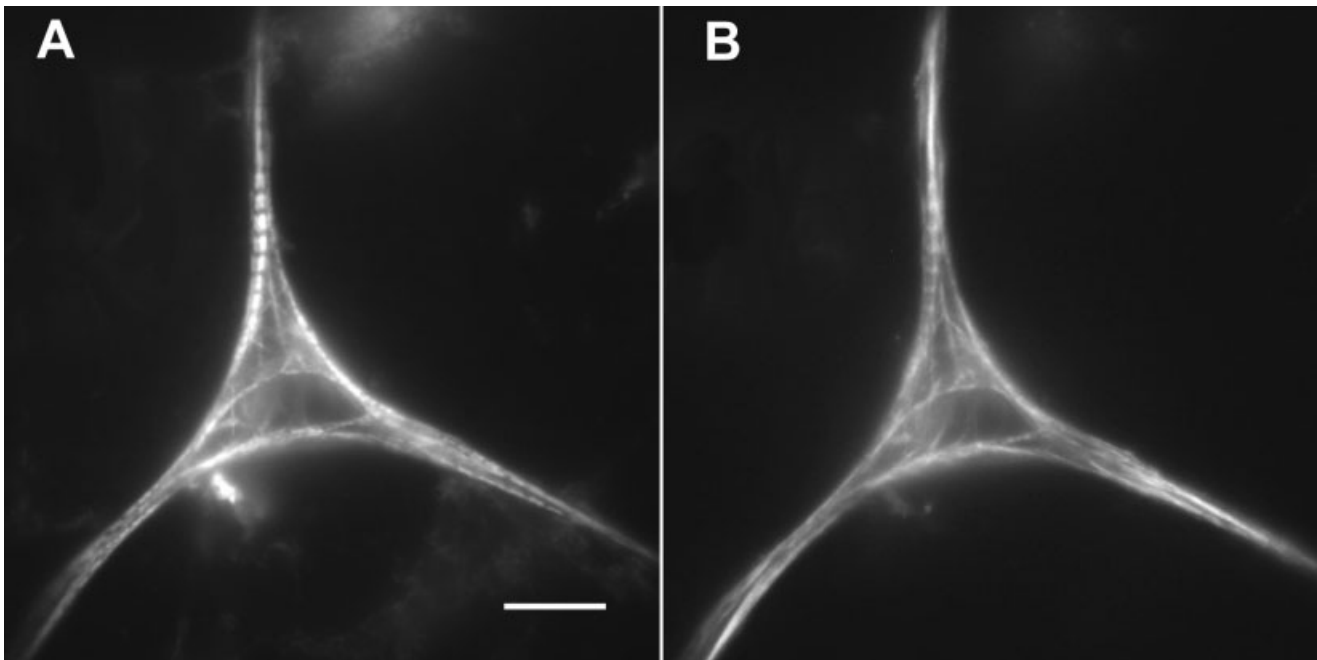


Fig. 7. Myofibrils that formed in a stellate muscle cell exposed to taxol for 4 days were fixed and stained with (A) an anti-muscle myosin II antibody and (B) phalloidin. The mature myofibrils that formed had normal distribution of A-bands and actin filaments. The Z-bands exhibit an extra concentration of actin staining (B). Bar = 10  $\mu$ m.



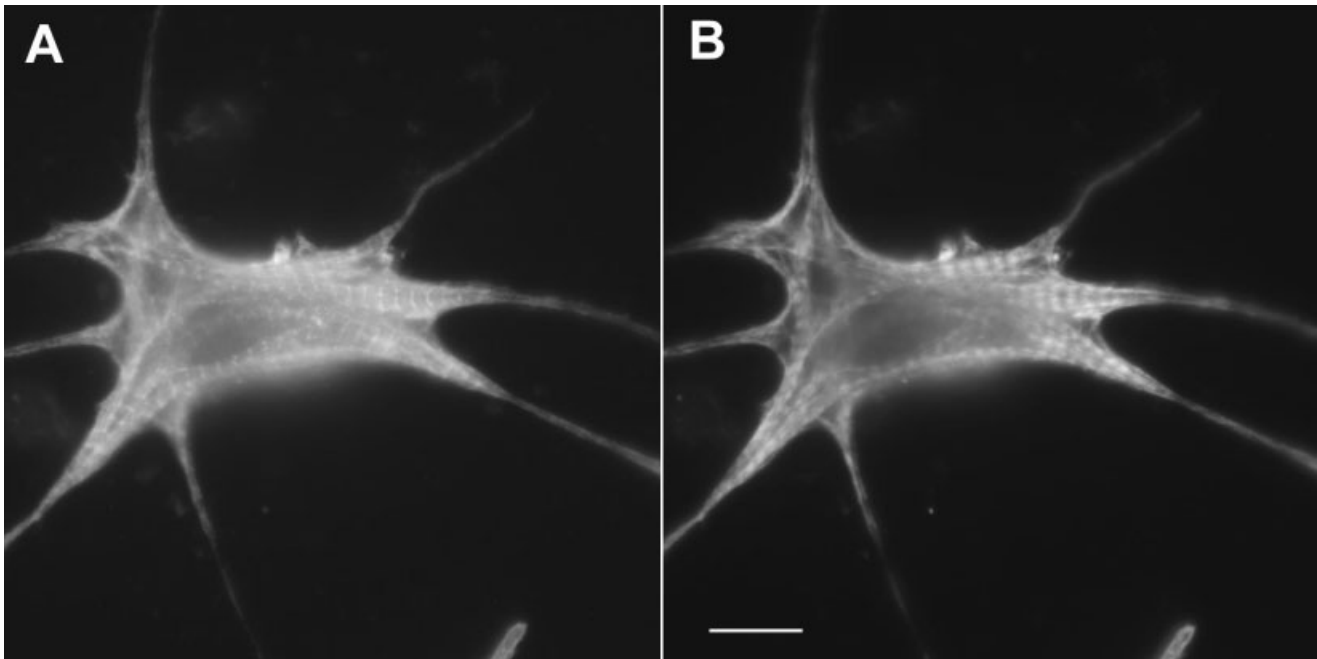


Fig. 8. Myofibrils that formed in a muscle cell exposed to taxol were fixed and stained with (A) an anti-titin antibody and (B) anti-actin antibody. The zeugmatin antibody that stains the Z-band region of titin, stains the Z-bands of the mature myofibrils in these taxol-treated cells. Actin filaments are associated with these Z-bands and the rest of the sarcomere. Bar = 10  $\mu$ m.

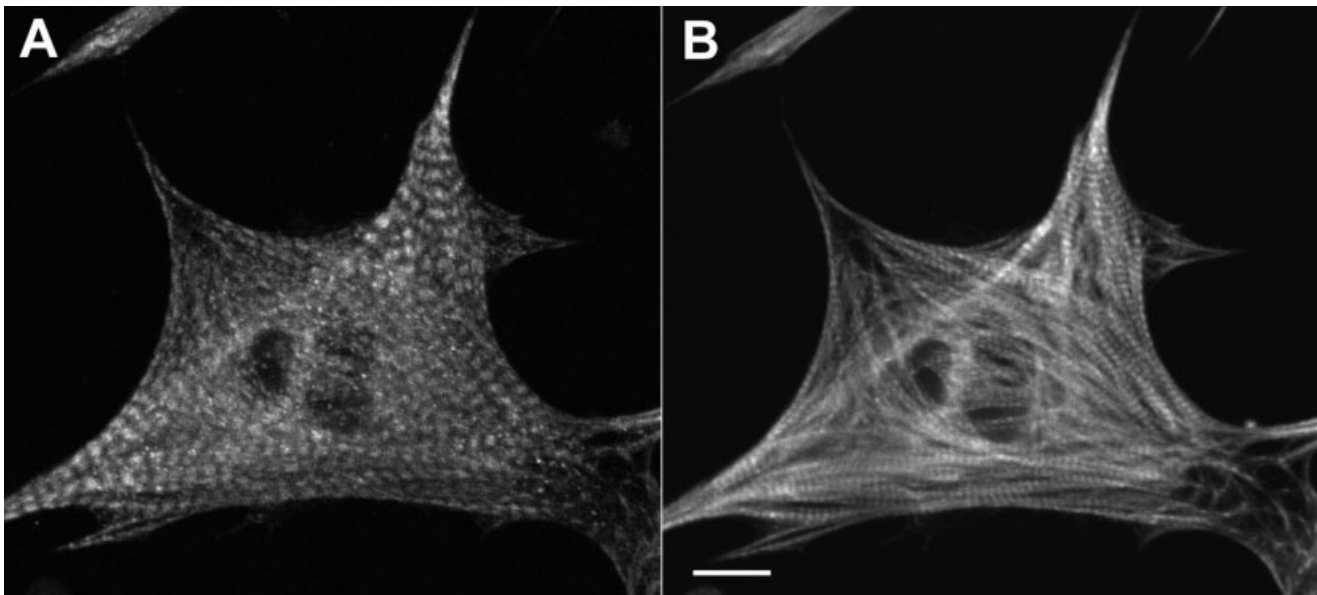


Fig. 9. Myocyte exposed to taxol for 4 days and then fixed and stained with nebulin antibodies (A) and then stained with fluorescently labeled phalloidin (B). Note the colocalization of nebulin (A) and the banded F-actin (B) in the mature myofibrils. Bar = 10  $\mu$ m.

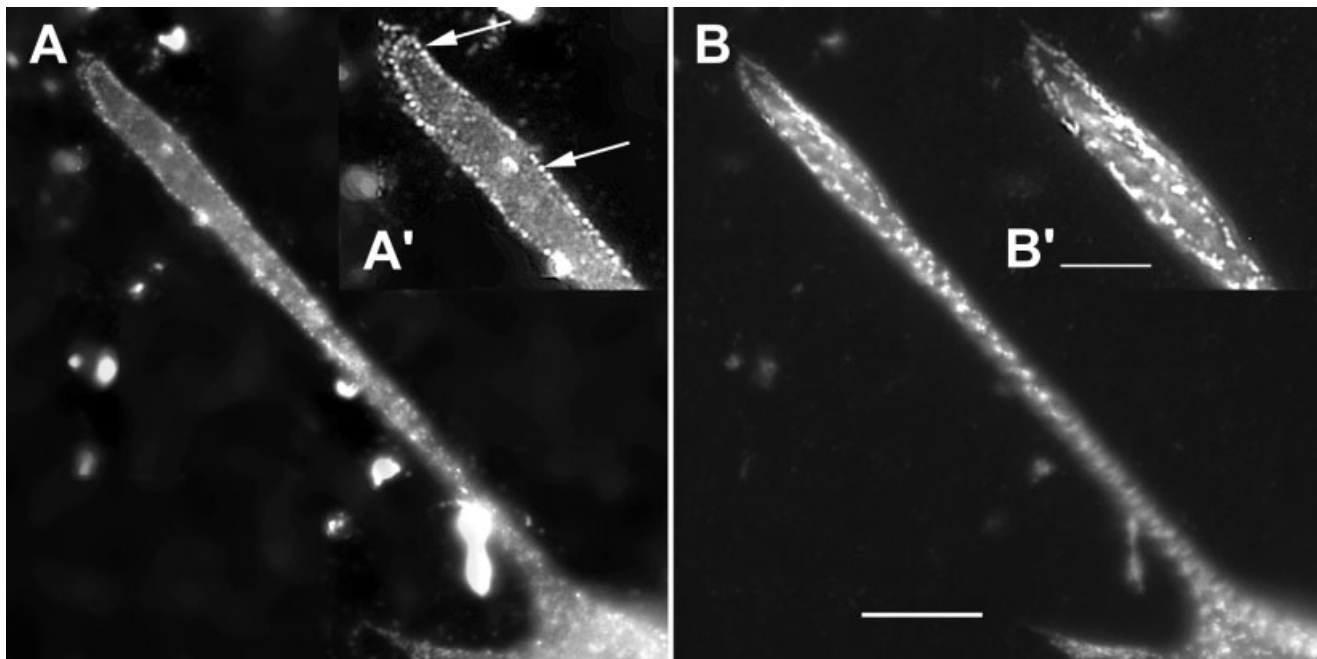


Fig. 10. Taxol-treated muscle cell stained with (A) anti-non-muscle myosin IIB and (B) sarcomeric alpha-actinin antibodies. **A, A', inset:** The non-muscle myosin II protein is localized around the periphery of the extension of the stellate muscle cell (*A'*, *arrowheads*). **B, B' inset:** The anti-alpha-actinin staining demonstrates the position of the mature actin

containing myofibrils as they enter the main part of the cell body at the lower right. Note that non-muscle myosin II is not present where mature myofibrils are concentrated, i.e., at the base of the cellular extension (*A, B*). Bar = (*A, B*) 10  $\mu\text{m}$ ; (*A', B'* insets) 5  $\mu\text{m}$ .

myofibrils all along their lengths. Microtubules are also abundant from their spreading ends to the interior of the myotubes.

## DISCUSSION

Microtubules are abundant in embryonic skeletal muscles [Fischman, 1967; Saitoh et al., 1988] but become less abundant in neonatal muscles and even less in adult skeletal muscle fibers [Cartwright and Goldstein, 1982]. Nevertheless, many of the remaining microtubules in adult skeletal muscle cells are aligned along the long axis of the peripheral nuclei near the long axis of the cell. A similar relationship was detected in adult cardiomyocytes [Goldstein and Entman, 1979; Cartwright and Goldstein, 1984]. Adult muscle cells are composed of myofibrils, soluble enzymes, organelles, intermediate filaments, and microtubules [Goldstein and Cartwright, 1982, 1985; Price, 1991; Boudriau et al., 1993; Chowrashi et al., 2002; Sanger et al., 2002]. Microtubules have long been known to be necessary for the elongation and maintenance of the early myotube shape in developing muscle [Warren, 1968, 1974]. The cross-linking of these microtubules with a MAP (Microtubule Associated Protein) to form a rigid cytoskeleton is also essential for the elongated structure of the myotube [Mangan and Olmsted, 1996].

Several reports had suggested that microtubules could take the place of actin filaments in the formation of myofibrils in avian muscle cultures [Antin et al., 1981; Toyoma et al., 1982; Gundersen et al., 1989; Moncman and Wang, 1996]. Avian skeletal myoblasts had been placed in taxol for 4 days and then analyzed [Antin et al., 1981; Toyoma et al., 1982]. In these experiments, the cells were fixed with osmic acid and examined in the electron transmission microscope for the distribution of the actin and myosin filaments. Thin filaments were reported to be missing in a number of the myofibrils. It is unclear when these myofibrils were assembled and if there had been actin filaments present initially in these sarcomeres. In our studies, taxol treated cells were examined for each of the 4-day's exposure to the drug using a variety of actin-binding probes. Moncman and Wang [1996] reported that actin polymers were dissociated from the nebulin molecules in the I-bands in the presence of taxol. They were not sure how the nebulin molecules remained extended in the I-bands in the absence of F-actin. They speculated that the N-terminal end of nebulin normally extending into the A-band was either trapped by titin filaments or interacted with muscle myosin filaments (thick filaments of the A-bands). Our staining results with both phalloidin and actin antibodies indicate that actin filaments are present in taxol-treated cells and

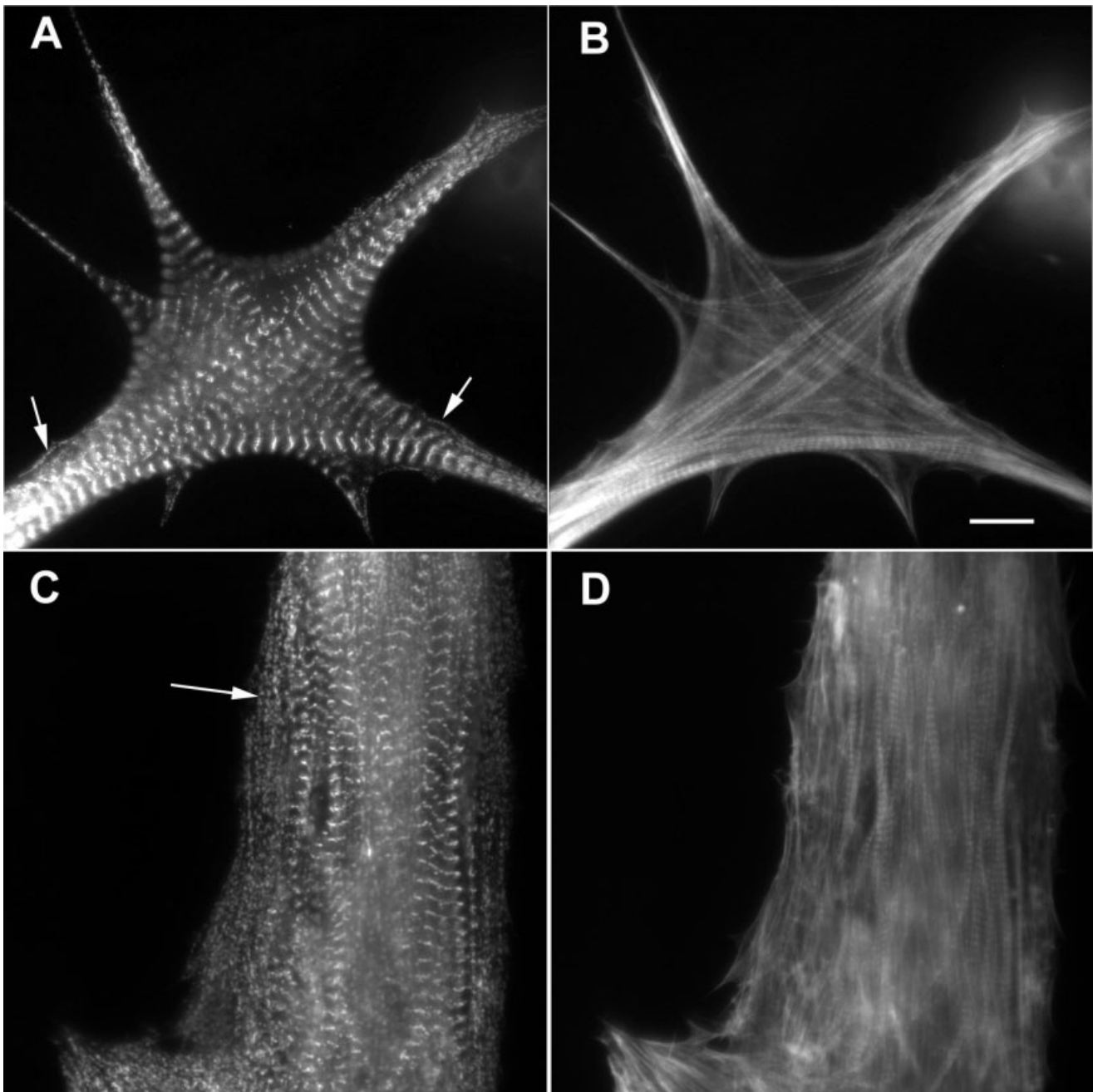


Fig. 11. **A,B:** Myocyte, 1 day after the removal of taxol, stained with sarcomeric alpha-actinin (A) and fluorescent phalloidin (B). The arms of the stellate cell are thicker than cells in taxol. The arms of the cell show signs of spreading (*arrows*). These areas contain actin fibers that have linear arrays of Z-bodies (A, *arrows*). **C,D:** Myocyte, 2 days after

the washout of taxol, stained with sarcomeric alpha-actinin (C) and fluorescent phalloidin (D). The cell has lost its stellate shape. The recovered cell has its premyofibrils (C, *arrow*) and mature myofibrils aligned along the long axis of the myotube. Bar = 5  $\mu\text{m}$ .

that the nebulin molecules are presumably associated with myofibrillar actin filaments.

It is clear from our experiments that actin filaments were always found associated with the myofibrils that formed in the presence of taxol. We confirmed some of the other published results on taxol-treated muscle cells,

i.e., the inhibition of long myotubes, the formation of spiky cells, and the association of A-bands along the edges of these cells [Antin et al., 1981; Toyoma et al., 1982]. However, in all cases, actin filaments, actin-associated proteins (tropomyosin and troponin), and Z-band proteins (titin, alpha-actinin) are found in myofibrils con-

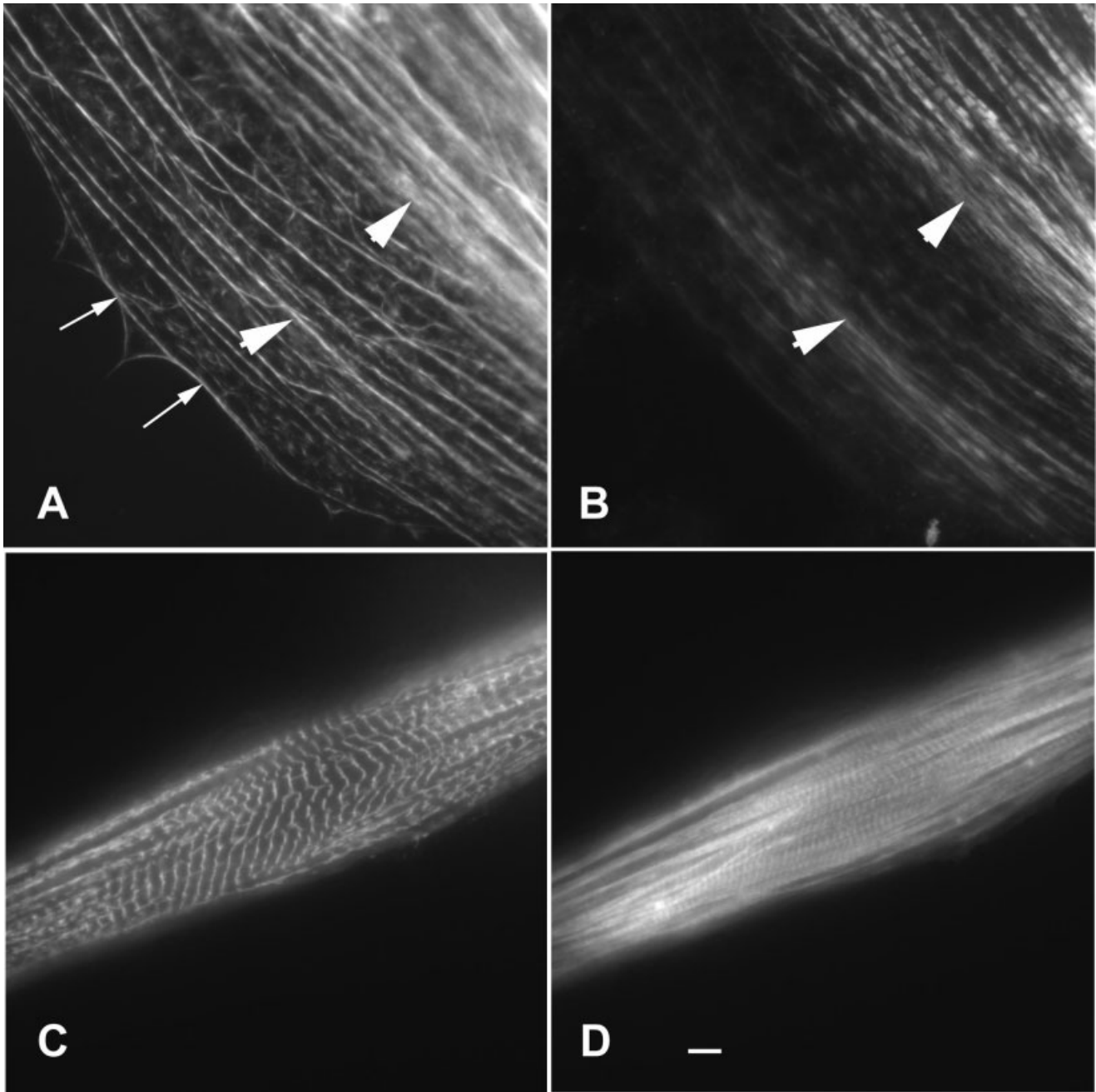


Fig. 12. **A,B:** Fluorescent images of 4-day taxol-treated cells that have been in normal medium for 5 days prior to fixation and staining. Reversed skeletal muscle cell fixed and stained with (A) fluorescent phalloidin and (B) muscle myosin II antibodies. There are three types of actin fibers in this recovered myotube: actin fibers with no muscle myosin II staining, i.e., premyofibrils (*arrows*, A); actin fibers with

non-striated arrays of muscle myosin II, i.e., nascent myofibrils (*arrowheads* in A and B), and actin fibers with A-bands of muscle myosin II, i.e., mature myofibrils (top right in B). **C,D:** Taxol reversed myotube stained with (C) sarcomeric alpha-actinin and (D) fluorescent phalloidin. The myotube is compact and contains mature myofibrils along its visible length. Bar = 5  $\mu$ m.

taining these A-bands formed in the presence of taxol. We detected actin molecules in these myofibrils using two independent ways: fluorescently labeled phalloidin and antibodies that react with thin filaments. Fluorescently labeled phalloidin was used on the fixed cells to

detect actin filaments in all myofibrils. Fluorescently labeled phalloidin has been available since 1979 for the staining of actin filaments but was not used in the previous studies of taxol-treated embryonic muscle cells reported by Antin et al. [1981] and by Toyoma et al.

[1982]. We have also used actin, tropomyosin, and troponin antibodies to detect the actin filaments in these myofibrils. Staining with the actin IgM antibody allowed us to use other sarcomeric antibodies that were IgGs in double immunofluorescent experiments. This actin antibody revealed actin bands in all myofibrils formed in the presence of taxol.

Electron microscopy was the only assay system utilized by Antin et al. [1981] and by Toyoma et al. [1982] for detecting thin filaments in taxol-treated cells. Interestingly, the Z-bands in these myofibrils were also absent in their electron micrographs [Antin et al. 1981; Toyoma et al. 1982]. The electron densities of Z-bands have been reported to be due to deposits of phosphorylase binding to alpha-actinin [Chowrashi et al., 2002]. In one light immunofluorescence image, normal alpha-actinin Z-band staining was detected in a taxol-treated myocyte [Antin et al., 1981]. Our light microscopic studies utilized fluorescent reagents and antibodies that have been developed since these two reports of taxol on skeletal muscle cells. In our immunofluorescent stainings, we found both normal staining of sarcomeric alpha-actinin and titin in these Z-bands. Occasionally, isolated uniform A-bands were also detected in the 4-day treated cells in the electron microscopic studies reported by Toyoma et al. [1982]. We did not detect any isolated A-bands in our studies of taxol-treated muscle cells. One can only speculate that the cells may have been degrading their myofibrils that had previously assembled. Kaneko et al. [1984] demonstrated elegantly that there is a two-step process in the disassembly of myofibrils in amphibian cardiomyocytes during cell division. First, the Z-bands and thin filaments disassemble, leaving linear arrays of A-bands in the former myofibril. The A-bands are then disassembled as the cardiomyocyte prepares for cytokinesis. The myofibrils are reassembled in the daughter cells [Kaneko et al., 1984; Du et al., 2003a]. These linear arrays of A-bands observed in mitotic cardiomyocytes appear very similar to those in the reports by Antin et al. [1981] and Toyoma et al. [1982]. The only difference between the A-band arrays in mitotic cardiomyocytes and the taxol-treated cells was the insertion of microtubules in the A-bands in the taxol cells. In the series of studies from the Goldstein laboratory, incomplete microtubules were detected within and outside sarcomeres [Goldstein and Cartwright, 1982, 1985]. The presence of taxol may induce these incomplete microtubules to increase their lengths throughout the sarcomeres and myofibrils.

In the premyofibril model, proposed for the assembly of myofibrils in both cardiac and skeletal muscle cells, actin filaments are considered to be an important component in assembly [Rhee et al., 1994; Dabiri et al., 1997]. Exposure of myotubes to latrunculin A, a binding

protein of monomer actin molecules, inhibited the formation of new myofibrils but did not affect the maintenance of mature myofibrils during the 1–2-h drug exposure [Sanger et al., 1998]. Microtubules did not substitute for the actin filaments in the assembly of new myofibrils in these latrunculin studies. Removal of this drug permitted the assembly of myofibrils to start. The first step was the formation of premyofibrils.

It is noteworthy that upon the removal of taxol, the stellate shape is gradually altered to form elongated myotubes. Accompanying this shape transformation is the formation of new myofibrils in the sides and ends of the new myotube. The staining of these recovering myocytes with specific fluorescent reagents reveals the presence of the three different steps in myofibrillogenesis, i.e., premyofibrils, nascent myofibrils, and mature myofibrils. These results support a common pathway for the formation of myofibrils in cardiac and skeletal muscle cells [Rhee et al., 1994; Sanger et al., 2002; Du et al., 2003a,b].

In summary, our experiments on skeletal myoblasts assembling myofibrils in the presence of taxol revealed normal mature sarcomeres containing actin filaments and actin binding proteins. These results do not support the hypothesis that microtubules can substitute for actin in the assembly of myofibrils. At the edges of these taxol-treated cells, over each of the 4 days of taxol treatment, we were able to detect premyofibrils. The detection of premyofibrils at the tips and sides of the elongating cells in taxol, in the reversal from taxol treatment and the subsequent assembly of mature myofibrils, supports the premyofibril model of myofibrillogenesis.

## ACKNOWLEDGMENTS

This work was supported by grants from NIH and MDA. We thank Dr. Andrea L. Stout and Lisa Gleason for their comments on this manuscript.

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