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Myofibrillogenesis in the first cardiomyocytes formed from isolated quail precardiac mesoderm

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Abstract

De novo assembly of myofibrils was investigated in explants of precardiac mesoderm from quail embryos to address a controversy about different models of myofibrillogenesis. The sequential expression of sarcomeric components was visualized in double- and triple-stained explants before, during, and just after the first cardiomyocytes began to beat. In explants from stage 6 embryos, cultured for 10 h, ectoderm, endoderm, and the precardiac mesoderm displayed arrays of stress fibers with alternating bands of the nonmuscle isoforms of α-actinin and myosin IIB. With increasing time in culture, mesoderm cells contained fibrils composed of actin, nonmuscle myosin IIB, and sarcomeric α-actinin. Several hours later, before beating occurred, both nonmuscle and muscle myosin II localized in some of the fibrils in the cells. Concentrations of muscle myosin began as thin bundles, dispersed in the cytoplasm, often overlapping one another, and progressed to small, aligned A-band-sized aggregates. The amount of nonmuscle myosin decreased dramatically when Z-bands formed, the muscle myosin became organized into A-bands, and the cells began beating. The sequential changes in protein composition of the fibrils in the developing muscle cells supports the model of myofibrillogenesis in which assembly begins with premyofibrils and progresses through nascent myofibrils to mature myofibrils.

Keywords: Myofibrillogenesis; Cytokinesis; α-actinin; Nonmuscle myosin II; Muscle myosin II; Titin; Premyofibril; Nascent myofibril; Mature myofibril; Sarcomere; Ectoderm; Precardiac mesoderm; Endoderm

Introduction

One of the earliest steps in cardiomyogenesis is the assembly of contractile proteins into organized structures capable of contraction. This process requires the polymerization and assembly of several different proteins into three types of filaments that interact with one another and become organized into sarcomeric units that comprise the myofibrils of the cardiac myocytes (Rhee et al., 1994; Sanger and Sanger, 2001a). Despite many studies, there are still unanswered questions about the sequence of events that are required for myofibril assembly. One question yet to be resolved is whether mature myofibrils arise from premyofibrils, that contain both nonmuscle and muscle isoforms of sarcomeric proteins (Sanger et al., 2000), or whether the major myofilaments form independently and become organized into sarcomeric units without reference to preexisting premyofibrils (Holtzer et al., 1997; Ehler et al., 1999; Rudy et al., 2001).

Many of the studies of cardiac myofibril formation have been based on the use of cells isolated from hearts of 5- to 10-day-old avian embryos (Sanger et al., 1984, 1986a; Wang et al., 1988; Rhee et al., 1994; Holtzer et al., 1997; Wu et al., 1999). Embryonic cardiomyocytes in culture possess several properties of embryonic cardiomyocytes in vivo: they contract; they undergo mitosis and cytokinesis; they migrate; and they assemble myofibrils (Conrad et al., 1991, 1995; Dabiri et al., 1999a; Sanger and Sanger, 2001b). Despite offering superior resolution of sarcomeric structure, cardiomyocyte cultures of 5- to 10-day embryonic chick hearts also present disadvantages. The cells contain

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mature myofibrils when they are isolated, and although new myofibrils form in culture, it is difficult to be certain of the sequence of events of myofibrillogenesis and to determine whether or not myofibrils are undergoing assembly or disassembly without observing live cells. This is technically very challenging, although GFP-linked probes show promise. The results of such studies support the premyofibril model (Dabiri et al., 1997; Ayoob et al., 2000; Sanger and Sanger, 2001b).

A system of observing myofibril formation in cardiomyocytes de novo is provided by explants of avian mesoderm tissue (Lash et al., 1987; Linask and Lash, 1993; Imanaka-Yoshida et al., 1998; Rudy et al., 2001). When precardiac mesoderm tissue is isolated from stage 5–7 quail or chicken embryos (Hamburger and Hamilton, 1951) and placed in culture, it develops within 20 h to form beating cells (Lash et al., 1987; Linask and Lash, 1993; Imanaka-Yoshida et al., 1998; Rudy et al., 2001).

In a recent study employing immunofluorescence, Rudy et al. (2001) fixed mesoderm explants at different times to study myofibrillogenesis. They were unable to detect any signs of premyofibrils in these cultures, i.e., fibrils with closely spaced bands of sarcomeric α-actinin and nonmuscle myosin IIB. No nonmuscle myosin IIB was detected in the precardiac mesoderm at all, leading them to conclude that the model proposing independent assembly of I-Z-I complexes and thick muscle myosin II filaments (Holtzer et al., 1997) was valid for the initial assembly of myofibrils in the first cardiomyocytes in the embryo rather than the premyofibril model (Gregorio and Antin, 2000; Rudy et al., 2001).

Since the mesoderm cells divide and therefore presumably have nonmuscle myosin II in their cleavage furrows, as do older generations of embryonic cardiomyocytes undergoing cell divisions (Conrad et al., 1991, 1995), it prompted us to examine mesoderm explant cultures further with a view to determine whether there is evidence for premyofibrils in the first cardiomyocytes formed from precardiac mesoderm cells. Our results demonstrate that the first cardiomyocytes contain premyofibrils composed of mini-sarcomeres with the sarcomeric isoform of α-actinin and the nonmuscle isoform of myosin II. Beating first-generation cardiomyocytes were found to undergo mitosis and cytokinesis. These cells were fixed and determined to contain nonmuscle myosin II in their cleavage furrows. Moreover, inhibition of the formation of premyofibrils with ML- 7 inhibited the normal assembly of mature myofibrils in a reversible manner. Similar reversible inhibition results were obtained with older embryonic cardiomyocytes. Biochemical analyses of these more abundant cells revealed that ML-7 induced an increase in the soluble pool of nonmuscle myosin IIB; removal of the inhibitor led to an increase in the cytoskeletal fraction of this important premyofibril protein.

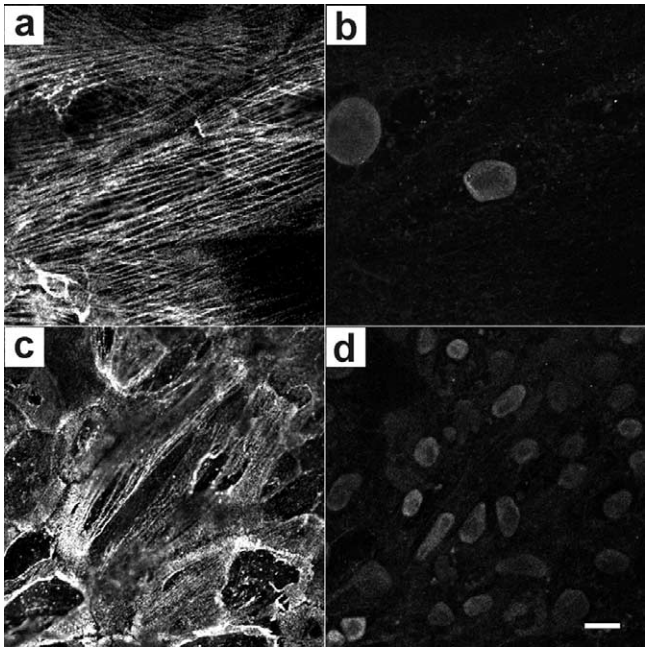

Fig. 1. Endoderm (a, b) and ectoderm (c, d) explants, separated from stage 6 precardiac mesoderm and cultured for 24 h, were stained with antibodies against two isoforms of nonmuscle myosin II. The stress fibers in these cells react in a banded pattern with antibodies against nonmuscle myosin IIB (a, c), but do not react with nonmuscle myosin IIA (b, d). Bar, 10 microns.

Materials and methods

Explant culture of precardiac regions

Fertile quail eggs (Coturnix, Japanese Quail) were supplied by Truslow Farms (Chestertown, MD). Dissections of precardiac cell layers were performed as described previously with some modifications (Linask and Lash, 1993). The stages of the embryos were determined by the criteria of developmental stages of chicken embryos (Hamburger and Hamilton, 1951). The heart-forming regions from stage 5–7 embryos were dissected in Hanks' Balanced Salt Solution, and the three germ layers, ectoderm, mesoderm, and endoderm, were separated in 0.25% chymotrypsin (Worthington Diagnostic Systems Inc., Freehold, NJ). The mesoderm layer was explanted onto coverslips coated with fibronectin (Gibco-BRL, Life Technologies, Inc., Gaithersburg, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco-BRL, Life Technologies, Inc.) in a humidified 5% CO_2 at 37°C. For inhibitor studies, stage 7 explants were allowed to attach for 2 h, and then exposed to 30 μM ML-7 (Sigma, St. Louis, MO) for another 17 h before fixation and immunostaining. ML-7 is an inhibitor of the formation of myosin filaments formed from nonmuscle myosin II (Burridge and Chrzanowska-Wodnicka, 1996). Sister control explants were fixed and stained at 19 h. Sister treated explants were washed free of the inhibitor with

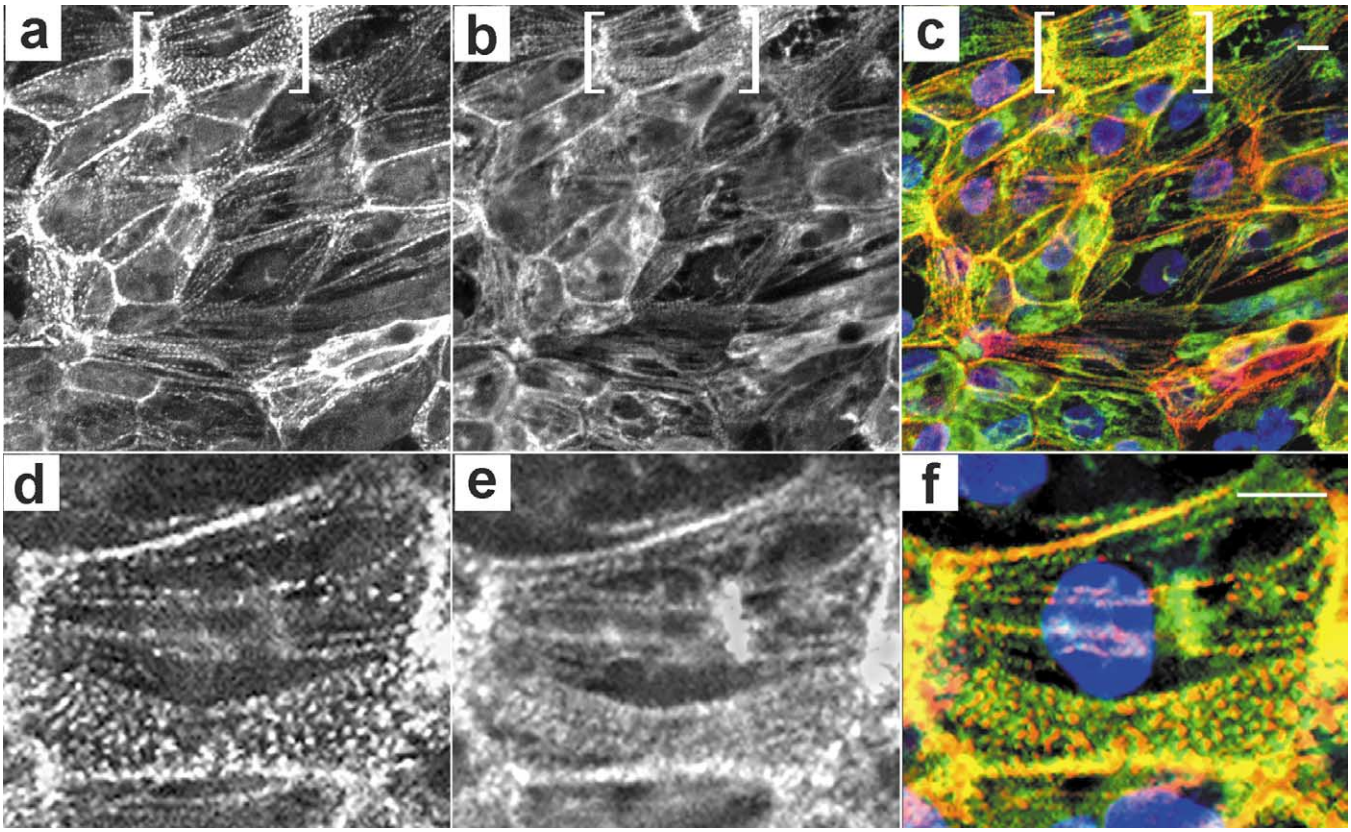

Fig. 2. Precardiac mesoderm explants (a–f) from a stage 6 embryo, cultured for 17 h, were fixed and stained for confocal microscopy with antibodies to sarcomeric α-actinin (a, d; red in c, f) and nonmuscle myosin IIB (b, e; green in c, f), and with DAPI (blue in c, f). There is a higher density of premyofibrils along the periphery of the cells (a–c). (d–f) A higher magnification view of a cell (bracket) in (a–c). The banded patterns of sarcomeric α-actinin and those of nonmuscle myosin IIB sometimes alternate with one another (f). In other areas, especially at the edges of the cells, the two different antibody bands appear to overlap (yellow color in f). This may be due to overlapping premyofibrils or to our inability to resolve small bands of fluorescence that are spaced 0.5–0.8 microns apart. Bar, 10 microns.

several rinses of normal medium and cultured for 2 more h before fixation and immunostaining.

Eight-day-old cardiac myocytes culture

Cardiac myocytes were isolated from 8-day-old chicken embryos (Dabiri et al., 1999b) and cultured in Minimum Essential Medium (MEM), 10% fetal bovine serum, and 1% penicillin/streptomycin. The experimental cells were cultured as follows: (a) 3 days in control medium before fixation and immunostaining; (b) control medium for 2 days, treated with ML-7 30 μM for 24 h, then fixed and processed for immunostaining; (c) control medium for 2 days, inhibitor for 1 day, then rinsed several times with control medium to remove the ML-7, cultured for another 24 h in control medium, and then fixed and processed for immunostaining; (d) 4 days in control medium before fixation and immunostaining. Larger dishes, 100 mm in diameter, containing 8-day-old embryonic chick cardiomyocytes were cultured under the three different culture conditions described above (a–c). Instead of fixing these cells, the cells were processed as described in the next section for biochemical analysis for the distribution of nonmuscle myosin IIB in the soluble and bound fractions.

Immunoblot analysis of nonmuscle myosin IIB in the soluble and insoluble fractions of detergent-extracted cardiomyocytes

Cells were permeabilized with 0.05% Triton X-100 in a buffered solution of protease, kinase, and phosphatase inhibitors as described by Kolega (1997). The extract was collected and mixed 3:1 with 4 $\times$ Laemmli buffer (63 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 30 μM Bromophenol blue; Laemmli, 1970). This is termed the soluble fraction. The dish was gently rinsed three times with PBS (phosphate-buffered saline) buffer. Three volumes of permeabilization buffer and one volume 4 $\times$ Laemmli buffer were added to the dish and all the attached cells were collected by using a scraper. This is termed the pellet fraction. Equal amounts of protein from each fraction were loaded on 8% SDS–PAGE gel. The separated proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) and

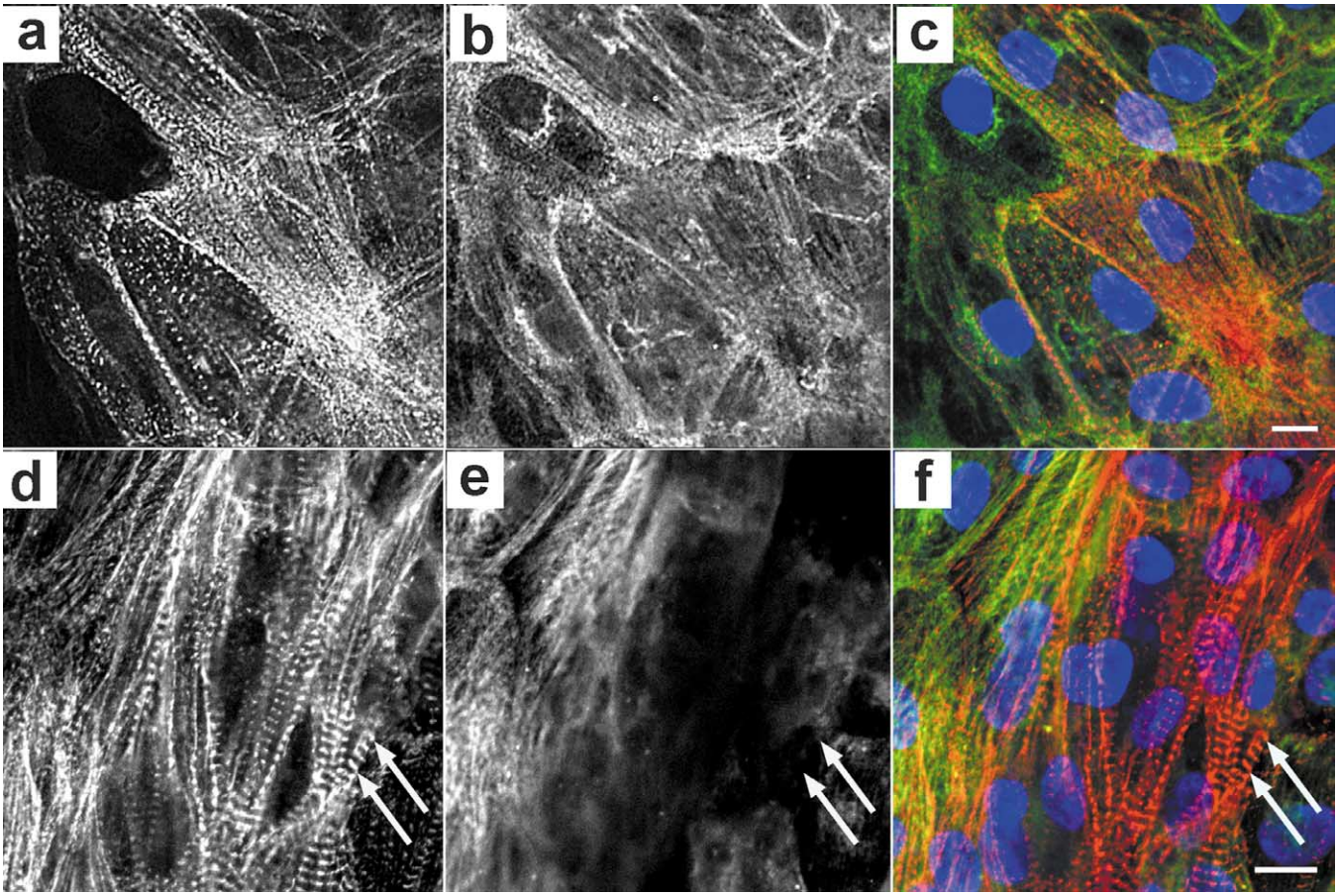

Fig. 3. Confocal images of precardiac mesoderm explant cultured for an intermediate period of time (24 h) (a–c) and a longer period (45 h) (d–f), fixed and stained with antibodies against sarcomeric α-actinin (a, d; red in c, f) and non muscle myosin IIB (b, e; green in c, f) and DAPI (blue in c, f). (a–c) In the intermediate stage, fibrils containing nonmuscle myosin IIB (b) have small bands of α-actinin (a). (d–f) Myofibrils with Z-bands (arrows in d, f indicate the Z-bands of mature myofibrils) staining positively for sarcomeric α-actinin do not stain with nonmuscle myosin IIB antibody (arrows in e). Bar, 10 microns.

probed with anti-nonmuscle myosin IIB and muscle myosin II antibodies, followed by horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham Life Science) as described by Chowrashi et al. (2002). The protein probed was then visualized by the enhanced chemiluminescence system (ECL; Amersham).

Antibodies

The monoclonal immunoglobulin (Ig) G antibody against sarcomeric α-actinin was purchased from Sigma (St. Louis, MO). Monoclonal anti-titin IgM antibody (9D10; a region of titin near the A-I junction; Wang et al., 1988), and anti-nonmuscle II IgG antibodies (CMII23 and CMII25) were obtained from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Monoclonal anti-nonmuscle myosin IIB (IgM) was purchased from Chemicon (Temecula, CA). Monoclonal muscle specific myosin antibody was used as previously described (Sanger et al., 1986). Rhodamine and fluorescein phalloidins, to localize actin filaments, were purchased from Molecular Probes (Eugene, OR). DAPI was obtained from Sigma. Fluorescently labeled secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Immunofluorescence

The cultures were fixed at various times (ranging from 10 to 48 h following explantation) in 3% paraformaldehyde in PBS buffer for 15 min at room temperature and permeabilized with 0.1% IGEPAL CA-630 (Sigma) in PBS for 5 min. Cells were incubated with primary antibodies and secondary antibodies for 1 h at 37°C and with DAPI for 20 min at room temperature in humid chambers (Dabiri et al., 1999b). Fluorescently labeled phalloidin was applied for 30 min at room temperature. After staining was completed, cells were washed in standard salt solution, then in distilled water, and mounted in Mowiol (Calbiochem, La Jolla, CA).

Microscopy

Confocal images were taken with a Zeiss LSM 510 microscope using a Zeiss 63 ×, 1.4 N.A. water immersion

objective. For recording fluorescein-labeled antibodies and phalloidin, the 488-nm argon–ion laser band was used for excitation with a 510- to 530-nm band-pass filter. For rhodamine-labeled antibodies and phalloidin, the 543-nm helium–neon band and 585-nm long-pass filter were used. For DAPI, the 364-nm UV laser band and a 400- to 435-nm band-pass filter were used. Images were processed with the Zeiss LSM510 image processing system and Adobe Photoshop (San Jose, CA). Live and fixed older cardiomyocytes were observed with phase-contrast microscopy on a Nikon Diaphot 200 microscope with 40× or 100× objectives as described by Dabiri et al. (1999b). Images were acquired with a Hamamatsu (Bridgewater, NJ) Orca-100 cooled CCD and processed with Adobe Photoshop.

Results

Nonmuscle myosin II B expression in endoderm and ectoderm

In vertebrates, there are at least two isoforms of nonmuscle or cytoplasmic myosin II (IIA and IIB) that differ from one another in the chromosomal location of their genes, in their mRNA coding sequence, polypeptide sequence, and tissue distribution (Katsuragawa et al., 1989; Kawamoto and Adelstein, 1991; Murakami et al., 1991; Shohet et al., 1989; Simons et al., 1991). Endoderm and ectoderm were separated from precardiac mesoderm and placed in explant cultures to determine what type of nonmuscle myosin II was present. Both the ectoderm and endoderm stained with only the nonmuscle myosin IIB antibodies (Fig. 1). The ectoderm and endoderm, as expected, did not stain with any muscle-specific antibodies. Both embryonic layers displayed typical stress fiber arrangement of their nonmuscle myosin IIB (Fig. 1a and c). Cardiac fibroblasts, isolated from 8-day-old embryonic quail hearts, expressed both nonmuscle myosin IIA and IIB in the same cytoplasm (data not shown). The same is true in similar preparations of fibroblasts from 8-day-old embryonic chick hearts (Rhee et al., 1994).

Myofibrillogenesis in mesoderm explants

Mesoderm tissue from the precardiac region of stage 5–6 quail embryos was used in explant culture to investigate steps in the assembly of myofibrils in the first cardiomyocytes formed from mesoderm. The three layers of the explants: endoderm, mesoderm, and ectoderm, were separated, and the mesoderm was cultured for varying lengths of time. Differentiation into cardiomyocytes was detected with antibodies directed against muscle isoforms of α-actinin, titin, and muscle myosin II. In addition, antibodies directed against nonmuscle isoforms of myosin II were used to determine whether both nonmuscle myosin II and muscle-specific proteins coexisted in single cells in an arrangement similar to that seen in cardiomyocytes cultured from older embryonic hearts.

Cells cultured from mesoderm explanted from stage 6 quail embryos first showed fibrils with muscle-specific anti-α-actinin reactivity after 10 h in culture as was first reported by Imanaka-Yoshida et al. (1998) (Fig. 2a and d). The α-actinin was arranged as small periodic bands in the fibrils that were also composed of actin filaments as determined by phalloidin staining (not shown). The mesoderm cells in these early cultures were all positive for the nonmuscle isoform of myosin IIB. In the mesoderm cells that expressed muscle-specific α-actinin, the nonmuscle myosin IIB was localized in small periodic bands (Fig. 2b and e) that often appeared to alternate with the bands of α-actinin (Fig. 2c and f). These fibrils are considered to be premyofibrils as defined by Rhee et al. (1994). Although structurally similar to stress fibers, they are fundamentally different in isoform composition, having both muscle and nonmuscle protein isoforms, whereas stress fibers have only nonmuscle isoforms.

The cells in the precardiac mesoderm explants were linked to one another via junctions, and in early cultures, muscle-specific anti-α-actinin staining sometimes appeared in just one of a pair of connected cells (data not shown). The cardiomyocytes retained their junctional connections and began to beat in culture approximately 24 h following explantation. All cells in an aggregate of beating cells expressed muscle α-actinin that localized primarily in Z-bands (Fig. 3a and c). By 45 h, the cardiomyocytes were packed with mature myofibrils (Fig. 3d and f). Accompanying the increase in mature myofibrils in these cardiomyocytes was a decrease in fibrils that stained with the nonmuscle myosin IIB antibody (Fig. 3b and c vs e and f).

Muscle myosin II was not detected in explants that were in culture for fewer than 17 h; however, at about 17 h incubation, before beating had begun in explants, muscle myosin II could be seen in unbanded fibrils (Fig. 4a and c). In the same cells, nonmuscle myosin IIB was present in fibrils in a periodic pattern (Fig. 4b and c). The initial arrays of muscle myosin II were parallel to the fibrils containing nonmuscle myosin IIB, and sometimes the two myosins appeared to be in the same fibrils (Fig. 4a–c); these fibrils containing two different types of myosin II are the nascent myofibrils as defined by Rhee et al. (1994).

By 24 h in culture, when small groups of cells within an explant began to beat, cells with A-bands composed of muscle myosin II (mature myofibrils; Rhee et al., 1994) could be detected (Fig. 4d and f). Fibrils with small bands of nonmuscle myosin IIB ran parallel to the muscle myosin II filaments forming A-bands, but no nonmuscle myosin IIB staining was detected in the A-bands (Fig. 4e and f). However, in other areas of these same cardiomyocytes, unstriated muscle myosin II rodlets were closely apposed to or in the same fibrils containing banded nonmuscle myosin IIB (Fig. 4d). By 48 h of culture, the cardiomyocytes contracted vigorously and were larger and full of mature myofibrils

(Fig. 4g and i). Fibrils with bands of nonmuscle myosin IIB were greatly decreased in these cells (Fig. 4h and i). The A-bands present in the cells were well defined with pseudo-H zones in the middle (Fig. 4g and i), in contrast to the forming A-bands present at 24 h that were also about 1.6 microns in length, but with no sign of a pseudo-H zone or well-defined edges indicative of uniform alignment of the thick filaments (Fig. 4d and f).

To discover why fibrils with nonmuscle myosin IIB were not detected in a previous study of precardiac mesoderm explants (Rudy et al., 2001), explants of a similar stage were stained with the same antibody applied at the 1/100 dilution used (Rudy et al., 2001). Staining of mesoderm cells with this antibody, together with anti- titin antibodies to mark cardiomyocytes, revealed an absence of fibrils containing nonmuscle myosin IIB (data not shown). When the hybridoma supernatant containing the antibody was used undiluted, nonmuscle myosin IIB-positive fibrils were visible in the titin-positive cardiomyocytes (data not shown).

Cell division in cardiomyocytes in mesoderm explants

Time-lapse observations of contracting explants showed that individual cells in a group of beating cells would stop beating, enter mitosis, and divide, and then spread and resume beating. These observations indicate the explanted mesoderm cells and their progenitors, i.e., the first cardiomyocytes, are healthy in culture, and retain properties thought to exist in cells in the intact heart. Sister cultures were fixed and stained to determine which myofibril proteins localized in the cleavage furrows (Fig. 5a–h). Consistent with the lack of contractions in the mitotic and cleaving cells was the absence of mature myofibrils in the mitotic cells (Fig. 5). Actin, nonmuscle myosin IIB and the sarcomeric isoform of α-actinin were localized in the cleavage furrows, but titin was not. At the end of cytokinesis (Fig. 5f and h), premyofibrils were observed in the spreading edges of the two daughter cells.

Reversible inhibition of myofibrillogenesis

Mesoderm cultures

To determine whether nonmuscle myosin IIB filaments were required for myofibrils to form, precardiac mesoderm explants were exposed to ML-7, an inhibitor of the phosphorylation of the light chains of nonmuscle myosin II (Saitoh et al., 1987). In the presence of ML-7, nonmuscle myosin II assembly into filaments was inhibited, and the nonmuscle myosin II in stress fibers disassembled as do most of the stress fibers (Saitoh et al., 1987; Burridge and Chrzanowaska-Wodnicka, 1996). In contrast, muscle myosin II molecules assemble by a phosphorylation-independent step. After precardiac explants had been in culture for 2 h to permit attachment of the explant to the substrate, the medium was exchanged for one containing ML-7. The normal distribution of fibrils with myosin IIB was diminished (Fig. 6b). Most of the first cardiomyocytes had lost their premyofibrils in the presence of ML-7 after 17 h (Fig. 6b). No contractions were detected in these inhibited cultures. Counterstaining of these cardiomyocytes with the muscle myosin II-specific antibody revealed that most cells had unassembled accumulations of muscle myosin II (Fig. 6a). A few cardiomyocytes were present with some fibers of nonmuscle myosin IIB (Fig. 6b). Unstriated bundles of muscle myosin II were associated with these remaining nonmuscle IIB fibers (Fig. 6a), i.e., nascent myofibrils (Rhee et al., 1994). Comparable aged cardiomyocytes at 19 h had contractions, A-bands, and mature myofibrils (Fig. 6c). When the ML-7 inhibitor was removed from the inhibitor-treated explant cultures, contractions returned within several hours. Fixing and staining of these reversed cultures revealed A-bands and mature myofibrils (Fig. 6d). These experiments suggest that the assembly of mature myofibrils was dependent on the presence of nonmuscle myosin IIB in premyofibrils.

Eight-day-old embryo cultures

To analyze the effect of ML-7 on the state of nonmuscle myosin IIB in treated cells, cardiomyocytes were cultured from 8-day-old embryos to provide a sufficient number of cells for extraction and Western blot analysis. In the older cardiomyocytes, myofibrils were present when the cells were isolated (Rhee et al., 1994), and new myofibrils formed in culture at the spreading edges of the cells with mature myofibrils concentrated in the center regions of the cells (Fig. 7). After exposure to 30 μM ML-7 for 1 day, all signs of premyofibrils were absent, and the widths of the Z-bands in the mature myofibrils were diminished (Fig. 7b) compared with the control cultures of the same age (Fig. 7a). Removal of the inhibitor led to the reformation of premyofibrils and an increase in the number and diameter of the mature myofibrils in the reversed cells (Fig. 7c). Control cultures that had never been exposed to ML-7 had more mature myofibrils than reversed inhibitor-treated cardiomyocytes of the same age (Fig. 7d).

To determine whether ML-7 induced a change in the distribution of nonmuscle myosin IIB in these cells, control, inhibitor-treated, and reversed cells were separated into detergent-soluble and -insoluble fractions. SDS-PAGE and Western blots probed with anti-nonmuscle myosin IIB showed that nonmuscle myosin IIB was enriched in the supernatant fraction of ML-7-treated cells, in contrast to control cultures in which the amount of nonmuscle myosin IIB was greater in the pellet than in the supernatant. (Fig. 8). When cells treated with ML-7 were reversed, the nonmuscle myosin IIB was again in excess in the insoluble fractions (Fig. 8). The ML-7 treatment had no effect on the distribution of muscle myosin II in the insoluble vs soluble fractions (Fig. 8b). Under all conditions, all the detectable muscle myosin IIB was present in the pellet fraction, indicating that, ML-7 did not affect the distribution of muscle myosin II in mature myofibrils. This biochemical observation was

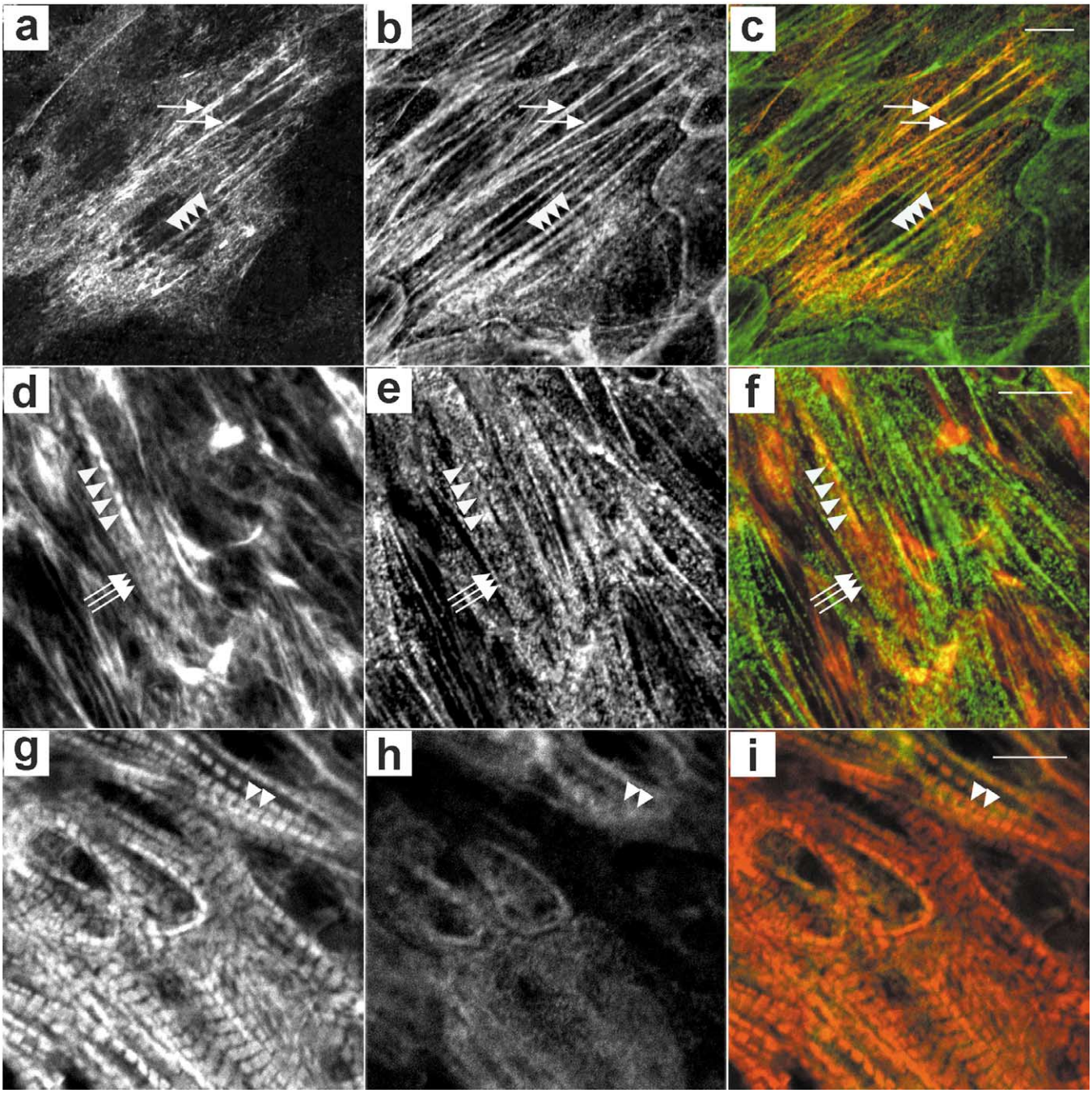

Fig. 4. Precardiac mesoderm explants were stained with antibodies specific for two different isoforms of myosin: muscle myosin II (a, d, g; red in c, f, i) and nonmuscle myosin IIB (b, e, h; green in c, f, i). The explants were cultured for 17 h. (a–c), 24 h (d–f), and 48 h (g–i). The arrows and arrowheads are in the same positions on the individual images in each set of culture times (a–c; d–f; and g–i) to aid in the comparison of the localization of the two different myosins. (a–c) Confocal images show that, at 17 h in culture, both myosins can be colocalized in linear arrays of variable lengths (arrows, a–c) or in short bands (arrowheads, a–c). (d–f) After 24 h in culture, forming A-bands (about 1.6 microns in length) of muscle myosin II are present in some fibrils (arrowheads, d). Nonmuscle myosin IIB at the same position in the cell (arrowheads, e) does not have a comparable pattern of localization. In places where nonmuscle myosin IIB is in banded fibers (arrows, e), the muscle myosin II is not in the same fiber (arrows, d). (g–i) Well-defined A-bands (1.6 microns in length) are prominent by 48 h in culture (arrowheads, g). Compare the forming A-bands in (d) with the mature A- bands in (g). The level of nonmuscle myosin IIB antibody staining is very low in the areas of cells that are populated with mature myofibrils (h). Bar, 10 microns.

consistent with the patterns of assembling and mature myofibrils in Fig. 7. See Fig. 7b for the presence of mature myofibrils, as well as the absence of premyofibrils and nascent myofibrils in the presence of ML-7.

Discussion

Myofibril architecture is highly conserved in vertebrate striated muscle (Sanger and Sanger, 2001a), and it has been

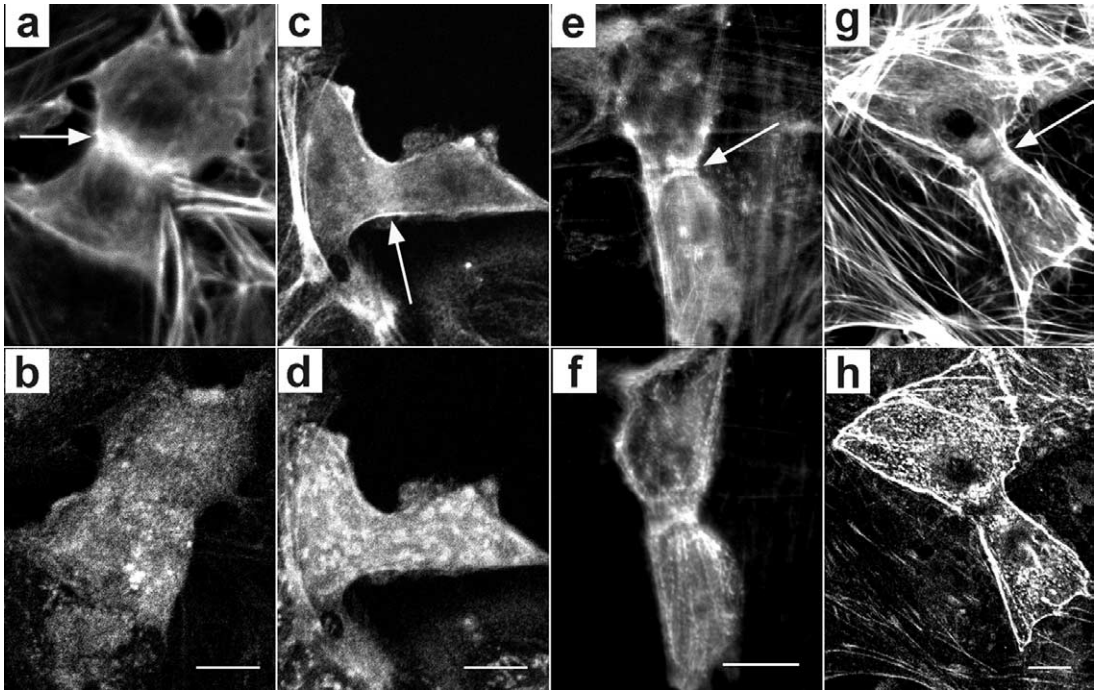

Fig. 5. Cardiomyocytes in division in precardiac mesoderm explanted at stage 6 and fixed after 24 h in culture. The explants were double-labeled with (a) phalloidin and (b) titin; (c) nonmuscle myosin IIB (CMII23) and (d) titin; (e) nonmuscle myosin IIB and (f) sarcomeric α-actinin; (g) phalloidin and (h) sarcomeric α-actinin. F-actin (a, g), sarcomeric α-actinin (specific muscle cell marker) (f, h), and nonmuscle myosin IIB (c) are localized in the cleavage furrows, but titin (specific muscle cell marker) is not (b, d). A few premyofibrils can be detected in one of the spreading cells stained with the anti-sarcomeric α-actinin antibody (f, h). Bars, 10 microns.

proposed that the myofibril assembly processes might be conserved as well (Sanger et al., 2002). Myofibrillogenesis requires that filaments of different types become aligned in precise arrays that are optimized for contraction. It is not clear exactly how this is achieved, but there are two models that can be tested.

The premyofibril model of myofibrillogenesis was based first on observations of fixed cultures of cardiomyocytes isolated from 5- to 10-day-old chick embryos (Rhee et al., 1994). Fibrils containing repeating bands of nonmuscle myosin IIB and muscle-specific α-actinin, formed at the edges of spreading cardiomyocytes (Fig. 9). These fibrils were termed premyofibrils since they appeared to align next to each other and fuse in groups of three or four to form nascent myofibrils in from the edges of the cells (Rhee et al., 1994). Nascent myofibrils, a transient state between premyofibrils and mature myofibrils, contain a core of repeating units of nonmuscle myosin II and the sarcomeric isoform of α-actinin with titin and muscle myosin II present in nonstriated patterns (Fig. 9). Mature myofibrils form by the elimination of nonmuscle myosin II and the alignment of the muscle myosin II filaments into A-bands with M-band protein additions (Fig. 9). Similar premyofibril patterns have been detected in adult rat cardiomyocytes that assemble myofibrils in culture (LoRusso et al., 1997). There is also evidence from observations on live embryonic cardiomyocytes transfected with green fluorescent protein (GFP)-α-actinin that premyofibrils were deposited in the spreading regions of the cardiomyocytes. These premyofibrils could be observed, in a time-lapse recording of the same living cells, to fuse with adjacent premyofibrils, with the distance between the bands of α-actinin increasing to form mature myofibrils (Dabiri et al., 1997). An increase in α-actinin band spacings was first detected in cultured skeletal muscle cells microinjected with fluorescently labeled α-actinin (Sanger et al., 1986a,b).

An alternative model, based on fixed cells in similar chick cultures, postulates that actin filaments, together with α-actinin and titin, form scattered I-Z-I units that align to form linear arrays of I-Z-I bodies (Lu et al., 1992; Holtzer et al., 1997). Full-length muscle myosin II filaments are postulated to form independently from the I-Z-I-bodies and subsequently to interdigitate with these units to form sarcomeres (Holtzer et al., 1997). This putative assembly model is independent of nonmuscle myosin II, and nonmuscle myosin II was reported to be absent from these cardiomyocytes (Lu et al., 1992; Holtzer et al, 1997). This was based, however, on staining with an antibody specific for nonmuscle myosin IIA, whereas only nonmuscle myosin IIB is present in these cells (Rhee et al., 1994) where it also functions in the cleavage furrow when the myocytes divide (Conrad et al., 1991, 1995).

Recently, another investigation of myofibril formation addressed the question of the existence of premyofibrils in precardiac mesoderm cultures, in order to distinguish between the two models (Rudy et al., 2001). A key difference

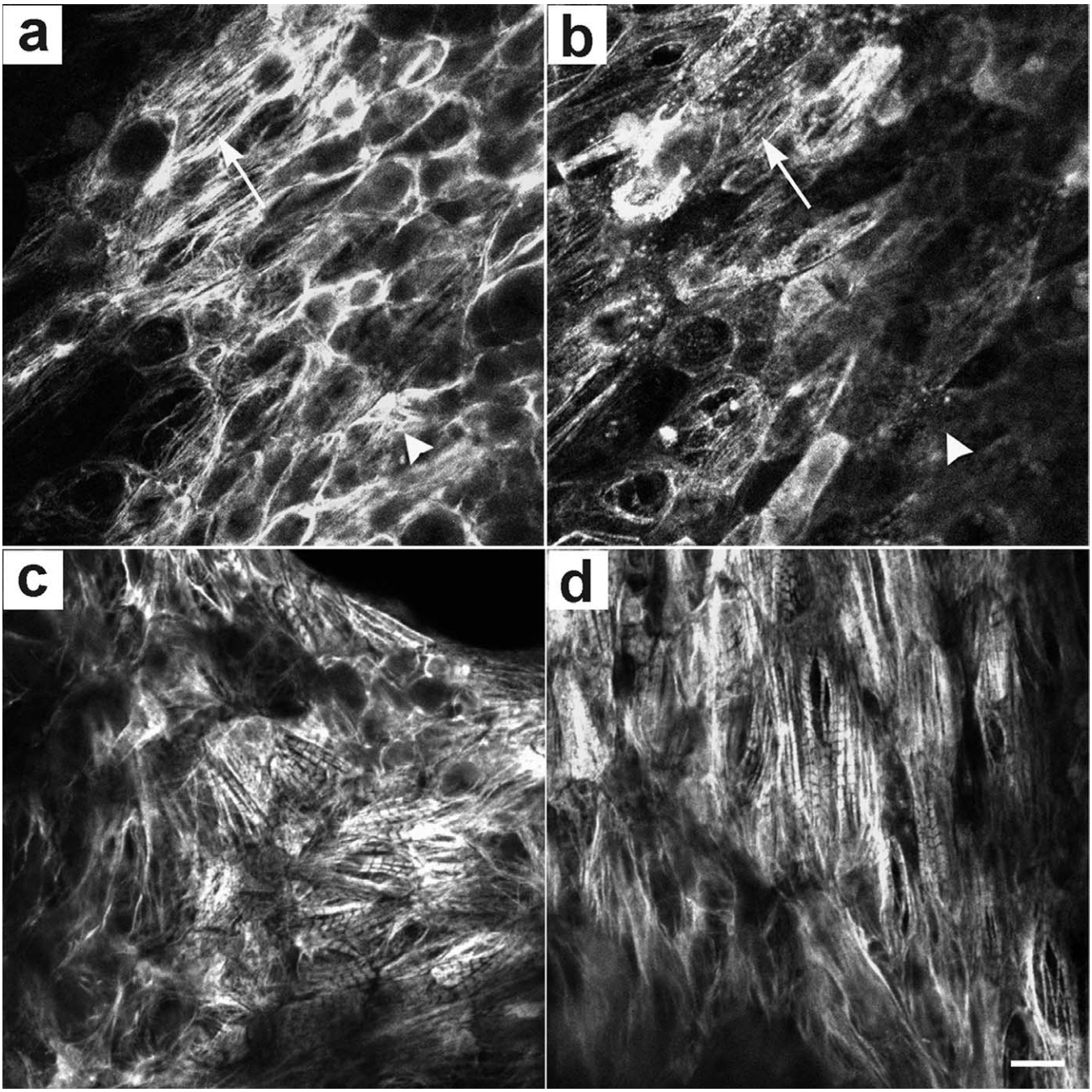

Fig. 6. (a, b) Precardiac mesoderm from a stage 7 explant cultured for 17 h in the presence of 30 μM ML-7 was inhibited from forming mature myofibril. The explant was stained with (a) muscle myosin II and (b) nonmuscle myosin IIB antibodies. Fibers with both isoforms of myosin are present in some cells (arrow in a and b). Muscle myosin II is found in unorganized accumulations in many cells (arrowhead in a). (c, d) Cardiomyocytes stained with muscle specific myosin II antibodies. (c) Sister control cultures of the same age as in (a, b) exhibited normal mature myofibrils. (d) Explants placed in control medium after 17 h in ML-7, developed into contractile tissue containing mature myofibrils with A-band localization of muscle myosin II (d). Bar, 10 microns.

in the two models is the requirement that nonmuscle myosin II be present as a defining component in premyofibrils, and conversely, the absence of nonmuscle myosin II characterizes the model of myofibril formation that proposes that independently assembled I-Z-I bodies and muscle myosin filaments form sarcomeric units (Lu et al., 1992; Holtzer et al., 1997; Gregorio and Antin, 2000). In support of this model, Rudy et al. (2001) in their study of precardiac mesoderm cultures were unable to detect nonmuscle myosin II in precardiac mesoderm cells and were also unable to detect sarcomeric α-actinin in premyofibril arrays. In an earlier study of myofibrillogenesis in avian precardiac mesoderm cultures, however, fibrils with closely spaced bands of α-actinin were shown to be present prior to muscle

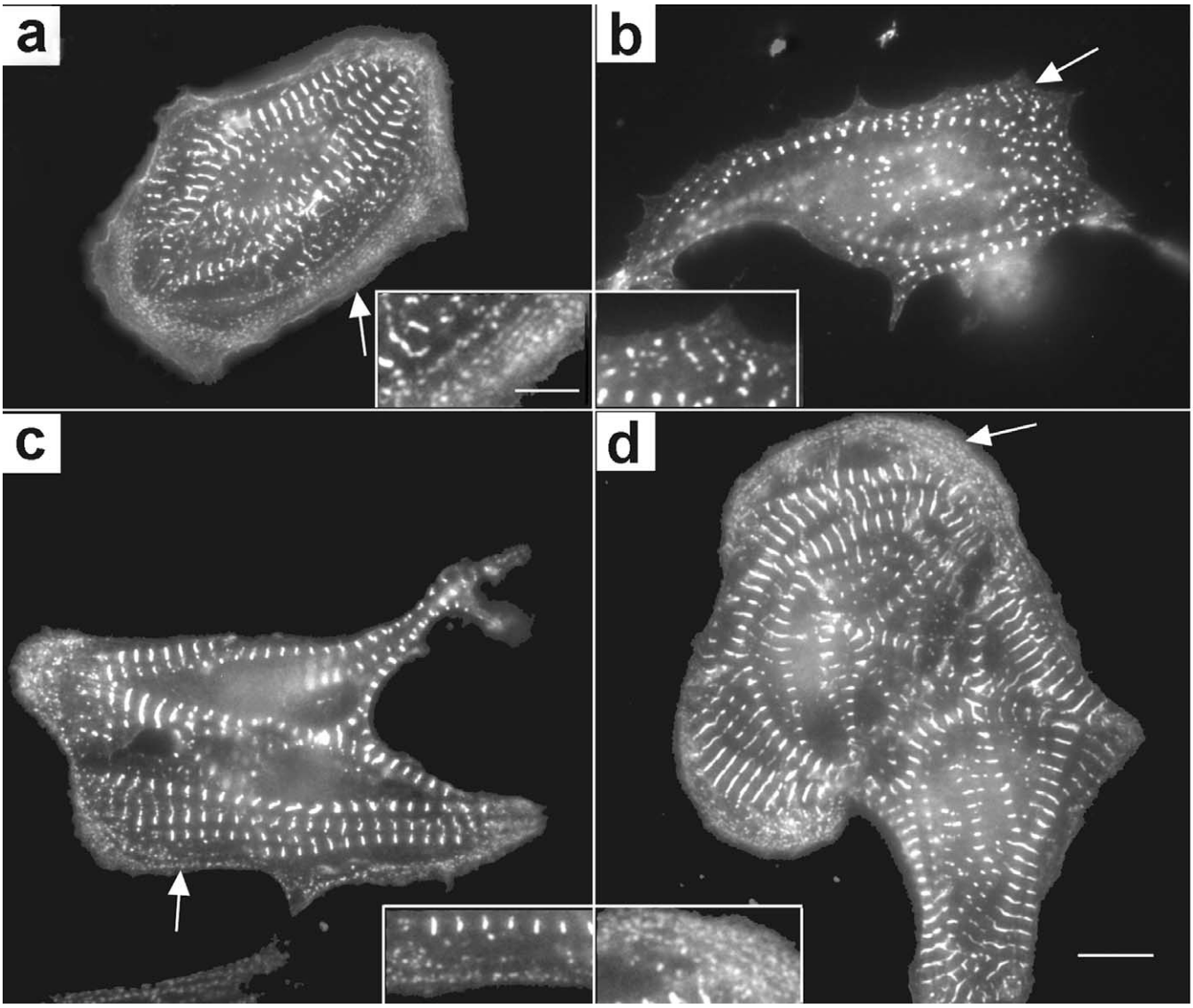

Fig. 7. Cardiomyocytes from cultures of 8-day-old embryonic hearts stained with sarcomeric α-actinin. (a). Control cardiomyocyte in culture for 3 days. Note the central accumulation of mature myofibrils and the peripheral presence of premyofibrils. Insert shows premyofibrils present in area marked with an arrow at a higher magnification. (b) Cardiomyocyte in control medium for 2 days followed by 1 day in medium containing ML-7. Note the loss of the premyofibrils at the edges of the cell and the presence of thin mature myofibrils in the cell. Insert shows absence of premyofibrils in area marked with an arrow. (c). Cardiomyocyte was present in control medium for 2 days followed by 1 day in ML-7 medium, and then placed in control medium for 1 day. Note the reformation of the premyofibrils at the edge of the cell, and that the Z-bands of the mature myofibrils are wider than those of the ML-7 treated cell in (b). Insert shows premyofibrils present in area marked with an arrow at a higher magnification. (d). Control cardiomyocyte in culture for 4 days. Note the accumulation of mature myofibrils and the peripheral regions of premyofibrils. Insert shows premyofibrils present in area marked with an arrow at a higher magnification. Bar, 10 microns in main images (a–d); Bar in inserts, 5 microns.

formation in the mesoderm cells; however, antibodies to nonmuscle myosin II were not used in that study (Imanaka-Yoshida et al., 1998).

The data reported in this paper demonstrate that nonmuscle myosin IIB is present in the first cardiomyocytes formed from precardiac mesoderm. In fact, nonmuscle myosin IIB is present in the ectoderm and endoderm as well as the mesoderm cells. The discrepancy with the study by Rudy et al. (2001) can be explained by the dilution of the anti-nonmuscle myosin IIB in that study, as we also found no reactivity when the antibody was similarly diluted 1:100 (data not shown). Fibrils showed positive anti-nonmuscle myosin IIB staining when the antibody was used as undiluted hybridoma supernatant. Furthermore, it has been shown that cardiomyocytes in cultures from embryonic hearts divide and nonmuscle myosin IIB is concentrated in their cleavage furrows (Conrad et al., 1991, 1995). We found that the same nonmuscle myosin IIB antibodies stained the furrows of dividing cardiomyocytes in these explants (Fig. 5). We found also that the sarcomeric isoform of α-actinin is in bands that alternate with bands of nonmuscle myosin IIB (Fig. 2). This matches images of pre-

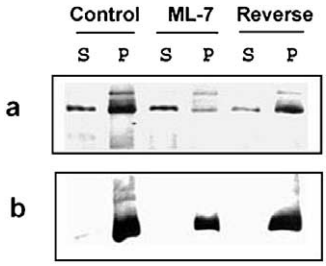

Fig. 8. Western blot of polyacrylamide gels stained with antibodies to (a) nonmuscle myosin IIB and (b) muscle myosin II heavy chains. In untreated control cultures, nonmuscle myosin IIB was present at higher concentration in the insoluble pellet (P) than in the detergent soluble fraction (S). In ML-7-treated cultures, the opposite was the case. When the ML-7 was removed (Reverse), the nonmuscle myosin IIB was again enriched in the pellet fraction. (b) Muscle myosin II was concentrated in the pellet fractions under all conditions.

myofibrils that we have seen in 5- to 10-day-old avian embryonic cardiomyocytes in this report (Fig. 7) and in previous studies (Rhee et al., 1994; Sanger and Sanger, 2001c) as well as in hypertrophying adult cardiomyocytes (LoRusso et al., 1997). After cytokinesis in precardiac mesoderm cells, premyofibrils were detected in the spreading daughter cells (Fig. 5f and h).

Nonmuscle myosin II light chains must be phosphorylated in order for the nonmuscle myosin II hexamers (two heavy chains and four light chains) to form filaments (Burridge and Chrzanowska-Wodnicka, 1996). The kinase inhibitor ML-7 will prevent this phosphorylation and cause myosin filaments to disassemble (Burridge and Chrzanowska-Wodnicka, 1996). The inhibition of myofibril assembly by ML-7 in this study is consistent with the idea that premyofibrils with filaments of nonmuscle myosin IIB are precursors of mature myofibrils. Premyofibril formation was reduced (Figs. 6a and 7b) and the muscle myosin II filaments that formed aggregated and did not assemble into myofibrils (Fig. 6b). The mature myofibrils in older embryonic cardiomyocytes treated with ML-7 appeared to be thinner when compared with control cells in culture for the same period of time (compare Fig. 7a with 7b). Removal of the ML-7 from the precardiac explants led to the reassembly of premyofibrils and the resultant mature myofibrils, all within a few hours. Ferrari et al. (1998) used this same inhibitor to inhibit assembly of myofibrils in Xenopus skeletal myocytes, suggesting that nonmuscle myosin II might be involved in the assembly process. The ML-7-induced loss of filamentous nonmuscle myosin IIB, however, may have other effects. It may lead to the disarray of the myocyte cytoskeleton as well, disrupting links of the Z-bodies to the cell membrane that are necessary for signaling and membrane attachment (Burridge and Chrzanowska-Wodnicka, 1996). Nevertheless, it should be noted that the inhibitor

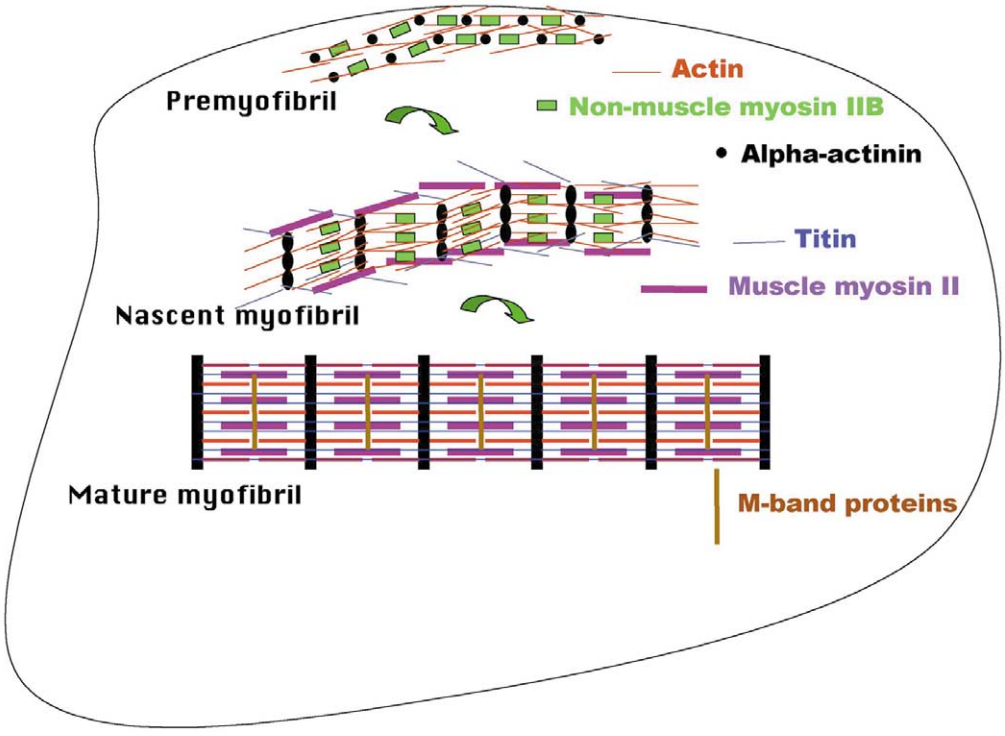

Fig. 9. A three-step diagram for myofibrillogenesis, *Premyofibrils*, composed of overlapping actin filaments and alternating bands of sarcomeric α-actinin and non-muscle myosin II, form at the edge of a cardiomyocyte. A *nascent myofibril* forms when premyofibrils fuse together at the level of their α-actinin-containing Z-bodies and recruit titin and muscle myosin II filaments. The nascent myofibril contains two different types of myosin IIs, with the muscle myosin II filaments present in overlapping arrays. *Mature myofibrils* form by the alignment of the muscle thick filaments into A-bands, the addition of C-proteins and M-band proteins. Nonmuscle myosin II is not present in the mature myofibril.

does not disrupt the alignment of the Z-bands of mature myofibrils (Fig. 7).

Since cardiomyocytes isolated from 5- to 10-day-old avian embryos already have myofibrils, the question arises as to how different myofibrillogenesis is in these cells as compared with myofibrillogenesis in the first cardiomyocytes formed from precardiac mesoderm. Both cardiomyocytes assemble myofibrils, disassemble myofibrils prior to cell division, and reassemble their myofibrils after cell division. In addition, the common effect of ML-7 on both the first cardiomyocytes and these older embryonic cardiomyocytes supports the idea that myofibrillogenesis follows similar paths. The diagram in Fig. 9 summarizes how premyofibrils could be involved in the assembly of mature myofibrils at the spreading edges of cardiomyocytes, e.g., after cytokinesis and migrating cardiomyocytes.

In summary, the patterns of myofibrillogenesis are the same in the two types of embryonic cardiomyocytes, the first cardiomyocytes formed from the precardiac mesoderm and the subsequent embryonic generations of cardiomyocytes. Use of precardiac mesoderm cultures that produce the first cardiomyocytes does have several advantages over older embryonic cardiomyocytes. First, the first generation cells do not have preexisting myofibrils at the initiation of culture. In the older embryonic cardiomyocytes, living cells must be studied to be able to discern remodeling and disassembly from de novo assembly of myofibrils (Dabiri et al., 1997). Second, the formation of the first cardiomyocytes presents an ideal experimental system for the determination of the various signal transduction pathways that are responsible for the differentiation of mesoderm into cardiomyocytes and the assembly of the first myofibrils in the forming heart.

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