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Role of M-Line Proteins in Sarcomeric Titin Assembly During Cardiac Myofibrillogenesis

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Abstract A rat polyclonal anti-M-line protein antiserum and three mouse monoclonal anti-titin antibodies (E2, F3, and A12) were used to study the spatiotemporal relationship between M-line proteins and titin during myofibril assembly in cultured chicken cardiomyocytes by immunofluorescence microscopy. In day 2 cultures, M-line proteins and titin were detected as punctate staining in most cardiomyocytes, which possessed many nonstriated fibrils. At a late stage (day 3 cultures), M-line proteins were incorporated into dot-like structures along nonstriated fibrils, while titin staining was continuous on these structures. As development progressed, M-line proteins were registered in periodic pattern in the mid-A band. In cardiomyocytes from day 5 cultures, the titin bands were separated by an unstained region, and achieved their adult doublet pattern. Thus, the organization of titin in the sarcomere appears to occur later than that of M-line proteins in the M-line. Our morphological data indicate that the early registration of M-line proteins in primitive myofibrils may guide titin filament alignment via interaction between M-line proteins and titin. In order to investigate the role of M-line proteins in the assembly of titin filaments, anti-M-line protein or anti-titin antibodies were introduced into cultured cardiomyocytes by electroporation to functionally bind the respective proteins, and the profile of myofibril assembly was examined. Cardiomyocytes from day 2–3 cultures with incorporated anti-M-line protein antibodies became shrunk, and exhibited defective myofibrillar assembly, as shown by the failure of titin to assemble into a typical sarcomeric pattern. Incorporation of anti-titin antibody E2, which recognizes the M-line end domain of titin, resulted in the failure of M-line proteins organized into the M-line structure, as shown by random, sporadic staining with anti-M-line protein antibody. These studies confirm the essential role of M-line proteins in the organization of titin filaments in the sarcomere and that the interaction between titin and M-line proteins is crucial to the formation of the M-line structure. J. Cell. Biochem. 71:82–95, 1998.

Key words: M-line proteins; titin; expression; antibody perturbation; immunocytochemistry; cardiomyocyte

Titin spans between the M-line and the Z-line in a half-sarcomere and is classified as M-line titin, A-band titin, I-band titin, or Z-line titin on the basis of immunofluorescence staining with specific titin monoclonal antibodies [Fürst et al., 1988; Whiting et al., 1989]. The interaction between titin and α-actinin is well documented [Wang and Jeng, 1992; Ohtsuka et al., 1997], the basis for titin insertion in the Z line. Developmental studies have shown Z-line titin to be colocalized with α-actinin aggregates, also known as primitive Z-lines, in cultured chicken cardiomyocytes [Schuletheiss et al., 1990], embryonic chick cardiomyocytes [Tokuyasu and Maher, 1987] and myotomes of mouse somites [Fürst et al., 1989] and it is concluded that insertion of the Z-line domain of titin on the primitive Z-line is crucial for the organization of titin in the sarcomere.

Little attention has been paid to the relationship between the assembly of M-line proteins and titin. The M-line traverses the center of the A-band and consists of several M-bridges connecting adjacent thick filaments [Luther and Squire, 1978]. Biochemically, three proteins, MM-creatine kinase (43 kD), M-protein (165 kD) and myomesin (185 kD or 190 kD in skeletal muscle or cardiac muscle, respectively) have been identified [Strehler et al., 1979, 1980; Grove et al., 1984, 1985]. Myomesin is expressed later than α-actinin and titin in cultured chicken skeletal myotubes [Lin et al., 1994], but appears at the same time as titin in

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human fetal skeletal muscle in which the incorporation of M-protein in striated myofibrils occurs later than myomesin [van der Ven and Fürst, 1997]. The simultaneous assembly of titin and myomesin in the sarcomere of developing skeletal myofibrils is based on the biochemical interaction between these two proteins. The head domain (C-terminal) of purified titin molecules is associated with myomesin and M-protein [Nave et al., 1989]. In addition, in an immunoelectron microscopic study, the C-terminal region of titin has been shown to enter the M-band [Oberman et al., 1996]. Phosphorylation of myomesin by cyclic adenosine monophosphate (cAMP)-dependent kinase blocks its association with titin [Obermann et al., 1997]. Thus, the interaction between M-line proteins and titin is important for titin incorporation into sarcomeres. However, the temporal and spatial relationship between M-line proteins and M-line titin in sarcomeric assembly is not reported in cardiomyocyte model.

During the development of skeletal and cardiac muscle cells, sarcomere formation is dependent on interaction between several muscle proteins; therefore, perturbation of these protein interactions may interfere with the process. Overexpression of the N-terminal fragment of titin in cultured chicken myotubes and cardiomyocytes results in the disassembly of myofibrils by interfering with the titin-α-actinin interaction [Turnacioglu et al., 1997]. In desmin null mice, both skeletal and cardiac muscles show disruption of muscle architecture and degeneration [Milner et al., 1996]. In addition, the association between the cytoskeleton and cell membrane plays a critical role in myofibrillar organization.When anti-N-cadherin antibody was added to neonatal rat cardiomyocyte cultures and anti-integrin antibody added to chicken myotube cultures to block the formation of the adherens junction and cytoskeletal–sarcolemmal linkages, respectively, myofibril assembly was inhibited [Goncharova et al., 1992; McDonald et al., 1995]. In this study, we are interested in examining the role of interactions between titin and M-line proteins in myofibril development.

In a previous study [Wang et al., 1991], we produced several mouse monoclonal anti-titin antibodies, E2, A12, and F3, directed against different epitopes in the M-line region, A-band and Z-line region, respectively. Using these together with rat anti-M-line protein antibodies, we examined the expression of M-line proteins and titin during sarcomeric assembly in cultured chicken cardiomyocytes. The introduction of specific antibodies into cells by microinjection, permeabilization and electroporation is a technique used to study the function of specific proteins [Chakrabarti et al., 1989]. In this study, we also introduced different antibodies directed against titin or M-line proteins into cultured cardiomyocytes by electroporation in order to evaluate the role of these proteins in sarcomere assembly.

MATERIALS AND METHODS

Purification of M-Line Proteins and α-Actinin

M-line proteins (a mixture of myomesin and M-protein) were purified from fresh chicken pectoralis muscle according to the procedure of Eppenberger and Strehler [1982]. The method used to prepare α-actinin was adapted from the procedure of Dr. Marion L. Greaser (personal communication).

Antibody Production

Wistar rats, aged 8 weeks, were immunized intraperitoneally with 100 µg of purified M-line protein or α-actinin in complete adjuvant and boostered with 100 µg of the same protein in incomplete adjuvant 2 weeks later. After four days late, the blood was collected from the tail vein and the antisera assayed for specificity by Western blotting.

Western Blotting

Embryonic cardiac muscle was homogenized in phosphate-buffered saline (PBS), dissolved in sample buffer and the proteins (50 µg per lane) electrophoresed on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels, then transferred to nitrocellulose paper according to Fritz et al. [1989] and Towbin et al. [1979]. Strips from the membrane were blocked with 5% nonfat milk in PBS at room temperature for 1 h, then incubated in a 1:1,000 dilution of rat anti-α-actinin and anti-M-line protein antibodies at 37°C for 2 h. After washes with PBS-0.1% Tween 20, the strips were incubated with peroxidase-conjugated goat anti-rat IgG (Sigma) and positive bands visualized using 4-chloro-1-naphthol as chromogen.

Cell Culture

Chick cardiomyocyte cultures were prepared from 7- to 8-day embryos according to the

method of Lin et al. [1989]. Ventricular tissues of the hearts were minced and single cells dissociated using 0.05% trypsin in Ca^{2+}, Mg^{2+}-free Hank's balanced saline solution (HBSS) for 8 min at 37°C. Dissociated cells in suspension were collected, mixed with cold nutrient medium (10% fetal calf serum [FCS], 100 IU/ml of penicillin, and streptomycin and 2 mM glutamine in minimal essential medium (MEM) containing Earle's salts; GIBCO, Grand Island, NY), and centrifuged at 1,000 rpm for 10 min. The cells were resuspended in the nutrient medium and preplated for 1 h to remove fibroblasts. The suspended cardiomyocytes were then plated on collagen-coated coverslips at a density of 2.0–3.5 $\times$ 10^5 cells per 35-mm tissue culture dish and maintained at 37°C in a humidified 5% CO_2 incubator. On the day following plating, the medium was replaced with glutamine-free nutrient medium, which was replaced twice weekly.

Immunofluorescence

Day 2–5 cultures were rinsed with PBS, fixed with 4% formaldehyde in PBS for 10 min at room temperature, washed three times with PBS for 5 min each, and then permeabilized using PBS containing 0.1% Triton X-100. After three washes, the cells were incubated with 5% nonfat milk in PBS for 30 min at room temperature, then stained with rat anti-M-line protein antiserum and one of the anti-titin antibodies, followed by an appropriate combination of secondary antibodies. After final washes, they were mounted in 60% glycerol in PBS containing 2% n-propylgallate (Sigma Chemical Co., St. Louis, MO) and examined with a Leica epifluorescence microscope using appropriate filters.

Cells electroporated with anti-M-line protein antiserum were stained for incorporated antibody and titin (antibodies E2, F3, or A12) and cells electroporated with E2 were stained for incorporated antibody and M-line proteins. For double-stained preparations, cells were incubated with different combinations of first antibodies and an appropriate pair of second antibodies.

Electroporation Experiments

Cardiomyocytes were grown on glass coverslips (18 $\times$12 mm) for 2 days before being washed twice in HEPES-buffered saline (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, pH 7.05) and transferred to an electroporation cuvette containing 1 ml of antibody solution (150 µl of antisera or ascites fluid plus 850 µl of HEPES-buffered saline). Three different antibodies—rat anti-M-line protein antiserum, mouse monoclonal anti-titin ascites (E2), and mouse monoclonal anti-pigment granule ascites (F5)—were used. Antibody F5 does not react with cardiomyocytes and acts as a control IgG. The electroporation cuvettes were shaken on ice for 5 min, then given a single brief electric pulse of 750 V/cm, generated by a GIBCO-BRL (Gaithersburg, MD) Cell Porator electroporation apparatus. The electrode distance was 0.4 cm, the capacitance 60 µF, and the resistance set at low Ω. After electroporation, the cells were left in the cuvette for another 15 min on ice, washed once in HEPES-buffered saline, then cultured in fresh medium for 2–4 h before processing for immunofluorescence staining.

RESULTS

The M-line protein preparation, purified from chicken skeletal muscle (Fig. 1a), contained both myomesin (185 kD) and M-protein (165 kD) (Fig.1b), and the antisera raised against the mixture showed good specificity, reacting only with myomesin (190 kD) and M-protein (165 kD) on immunoblots of embryonic hearts

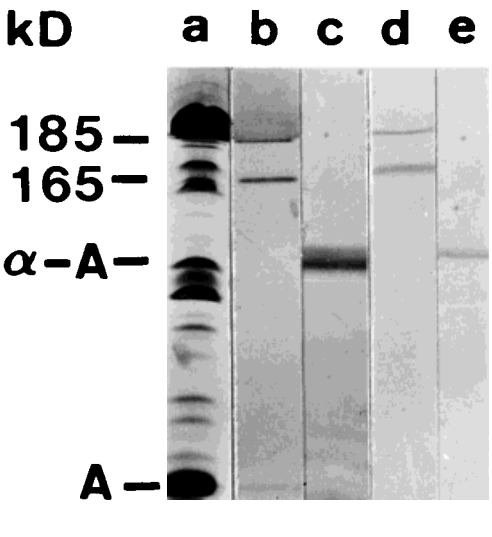

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Fig. 1. Characterization of rat anti-M-line protein antiserum and rat anti-α-actinin antiserum. **a:** Coomassie-blue-stained gel of adult skeletal myofibrillar proteins. **b:** Purified skeletal muscle myomesin (185 kD) plus M-protein (165 kD). **c:** Purified skeletal muscle α-actinin. **d:** Immunoblot of embryonic cardiac muscle proteins using anti-M-line protein antiserum. **e:** Immunoblot of embryonic cardiac muscle proteins using anti-α-actinin antiserum. α-A, α-actinin; A, actin.

(Fig. 1d). Purified skeletal muscle α-actinin (Fig. 1c) was used as the immunogen to generate rat anti-α-actinin antisera, which reacted only with α-actinin in embryonic hearts (Fig. 1e).

As it was easier to prepare stretched skeletal myofibrils for better observation of antibody localization than in cardiac myofibrils, the following immunofluorescence characterization was done on skeletal myofibrils. The staining patterns in both types of myofibrils were the same with each particular antibody used in the present study. The anti-M-line protein and anti-α-actinin antisera stained the M-line and Z-line, respectively (Fig. 2A–D). The anti-α-actinin antiserum was used to mark the position of Z-line. The specificity of three monoclonal anti-titin antibodies used in the present study was described by Wang et al. [1991]. They were antibodies E2, A12, and F3, recognizing different epitopes located near the M-line (Fig. 2E,F) and in the A-band (Fig. 2G,H) and I-band (Fig. 2I,J), respectively; all three stained a doublet of bands (called titin doublet) in the sarcomere.

Since trypsinization resorbed many myofibrils in cardiomyocytes, myofibrillogenesis occurred in day 2–5 cultures mostly represented the de novo assembly of new population of myofibrils and partially, regeneration from preexisting myofibrils [Lu et al., 1992]. Developing cardiomyocytes possessed many nonstriated fibrils that stained positively for α-actin and/or muscle tropomyosin but not for titin and muscle-specific myosin, while mature cells stained for all muscle proteins and contained well-organized myofibrils [Wang et al., 1988]. Cardiomyocytes at varying stages of differentiation were present in day 2–5 cultures. In day 2 cultures, cardiomyocytes contained many actin-positive nonstriated fibrils (Fig. 3A,C), and some of them were negative for M-line proteins (Fig. 3B) or titin (Fig. 3D). In some cells, staining for M-line proteins appeared as dotted pattern in the cytosol, whereas titin staining was amorphous and weak, as shown by immunostaining anti-titin E2 and A12 antibodies (Figs. 4A,B, 5A,B). In day 2–3 cardiomyocytes, M-line proteins were distributed as irregular dots on nonstriated fibrils, while the same structures exhibited continuous staining with anti-titin E2, A12, and F3 antibodies (Figs. 4C,D, 5C,D, 6A,B). In cardiomyocytes exhibiting typical M-line staining, all three anti-titin antibodies stained a single broad A-band pattern (Figs. 4E,F, 5E,F, 6C,D). For example, the width of the E2-, A12-, or F3-stained bands was 1.14 μm (Fig. 4F), 1.42–1.57 μm (myofibril 1 in Fig. 5F), and 1.42–1.85 μm (Fig. 6D), respectively. The typical doublet pattern, with characteristic spacing for each anti-titin antibody, similar to that obtained with mature adult myofibrils, was only seen in mature cardiomyocytes from day 3–5 cultures (Figs. 4G,H, 5G,H, 6E,F).

Since antibody F5, a mouse monoclonal anti-xanthophore pigment granules antibody, does not react with any component of cardiomyocytes, it was used as a control in electroporation experiments. Localization of the incorporated antibody was determined using a fluorescein- or rhodamine-conjugated second antibody. Incorporation of antibody F5 did not cause any change in cell number or morphology (Fig. 7A,B), indicating that the electroporation condition was suitable and did not cause cell damage. As shown by titin staining, F5-incorporated cells contained many myofibrils at various stages of assembly (Fig. 7C,D).

About 70–80% of cardiomyocytes incorporated anti-M-line protein antiserum. The incorporated antibody was distributed diffusely in the cytoplasm (Fig. 8E) or labeled the M-line of some myofibrils (Fig. 8C). Most cells shrank (Fig. 8B, control A) and showed incomplete assembly of myofibrils; staining for the M-line domain of titin with antibody E2 was diffuse (cell 1 in Fig. 8F) or labeled on disorganized sarcomeres (Fig. 8D, arrows). Cardiomyocytes without antibody incorporation in the same cultures exhibited a typical titin doublet staining pattern (star in Fig. 8C,D, cell 2 in Fig. 8E,F).

In the following experiment, we examined the assembly of M-line proteins when the M-line end of titin was blocked with anti-titin antibody E2 (reacts with the M-line domain of titin). Only 40–50% of cardiomyocytes incorporated E2 antibody, which had no obvious effect on cell morphology (Fig. 9A,B). In cells showing antibody incorporation (Fig. 9C), staining of the M-line protein was seen as random dots (cell 2 in Fig. 9D), while nearby cells without incorporated antibody showed typical M-line staining (cell 1 in Fig. 9C,D), suggesting that once the M-line domain of titin is bound to E2 antibody, M-line proteins fail to assemble into the M-line.

DISCUSSION

The purposes of the present study were to use an immunocytochemical method to investigate the spatiotemporal relationship between titin

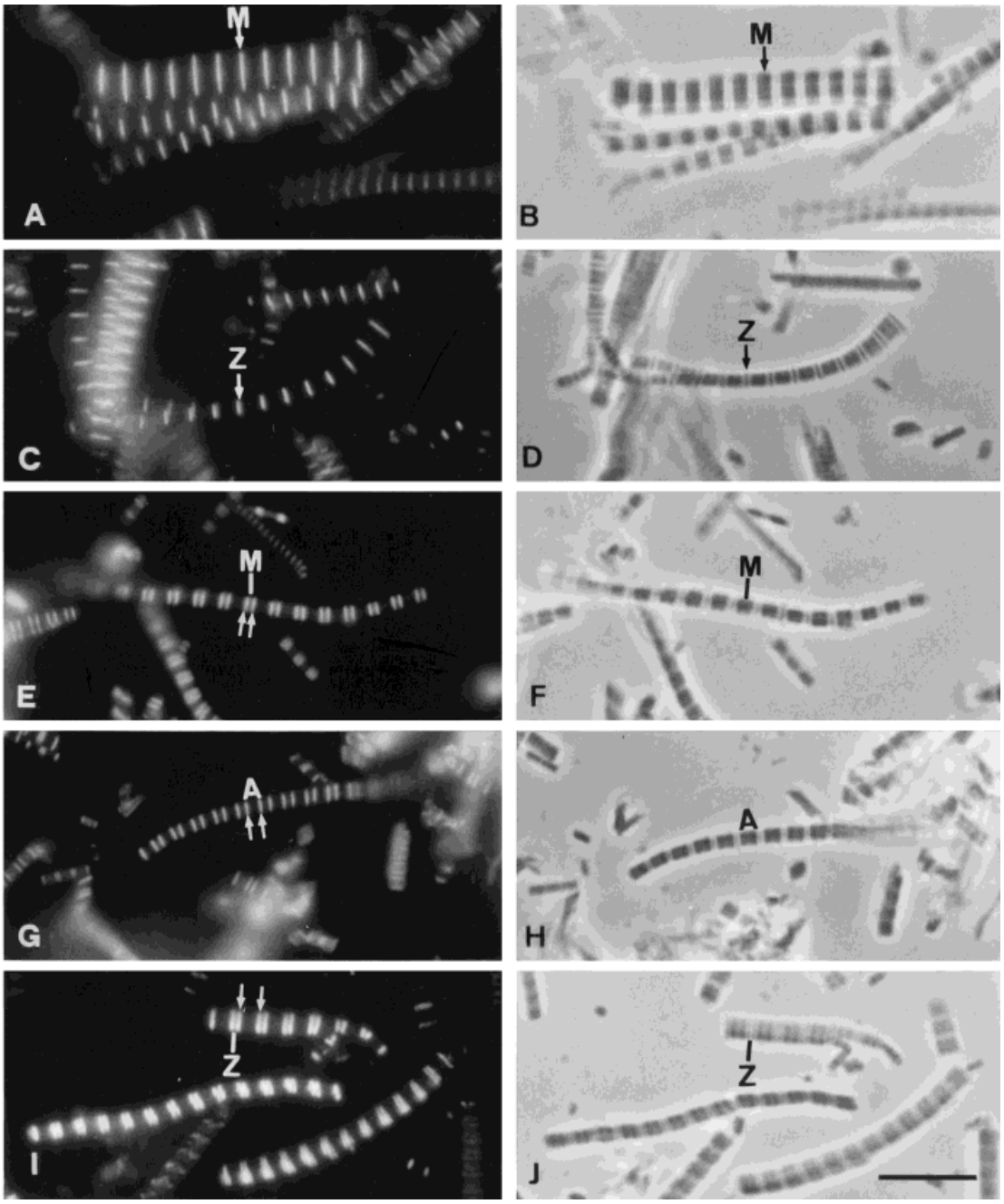

Fig. 2. Immunofluorescence staining of anti-M-line protein and anti-α-actinin antisera and anti-titin monoclonal antibodies on skeletal myofibrils. **B,D,F,H,J:** Phase pair of **A,C,E,G,I**, respectively. The anti-M-line protein antiserum stains the M-line **(A,B)**, while the anti-α-actinin antiserum stains the Z line **(C,D)**. Monoclonal anti-titin E2 stains a close doublet (arrows) on either side of the M-line **(E,F)**, A12 stains a doublet (arrows) near the A–I junction **(G,H)** and F3 stains a doublet (arrows) in the I-band **(I,J)**. M, M-line; Z, Z-line; A, A-band. Scale bar = 10 μm.

and its associated proteins, M-line proteins (M-protein and myomesin), and to examine the importance of M-line protein in maintaining the arrangement of titin, during cardiac myofibrillogenesis. Previous studies have shown that M-protein and myomesin are only detectable in the M-line region of striated myofibrils in cultured chicken cardiomyocytes [Eppenberger et al., 1981; Schultheiss et al., 1990] and chicken and human skeletal muscle cultures [Lin et al., 1994; van der Ven and Fürst, 1997]. This study demonstrated that M-line proteins appear in

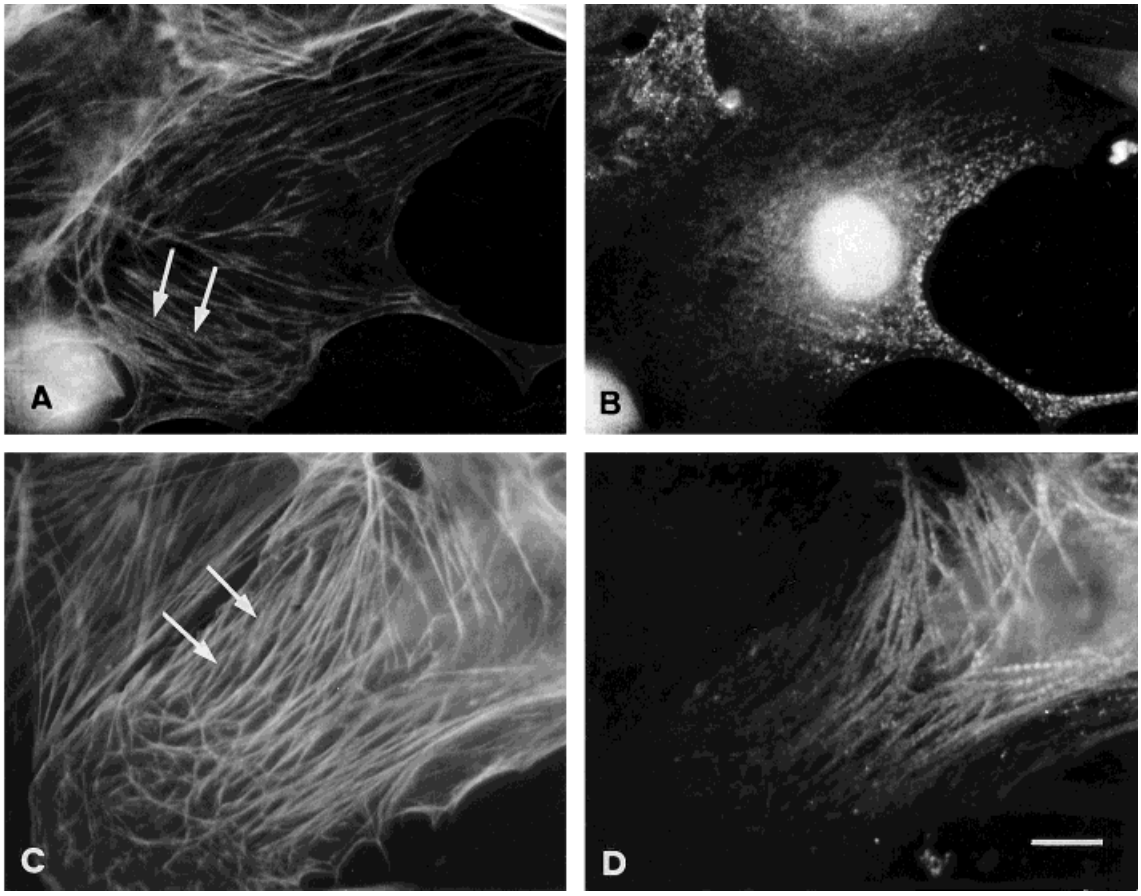

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Fig. 3. Distribution of M-line proteins and titin in nonstriated fibrils of day 2 cardiomyocytes. Cells are double-stained for actin with FITC-phallotoxin **(A,C)** and M-line proteins **(B)** or titin **(D)**. Some nonstriated fibrils are devoid of the staining for M-line proteins (**A**, arrows) or titin (**C**, arrows). Scale bar = 10 μm.

the cytosol as a punctate pattern, then gradually segregate on nonstriated fibrils as a random, dotted pattern and then finally become organized into the M-line structure as nonstriated fibrils transformed into mature myofibrils. This discrepancy between our study and that of other workers may be attributable to the poor sensitivity of the monoclonal antibodies used by previous investigators. In addition, our polyclonal rat antiserum recognizes both M-protein and myomesin, increasing the sensitivity of detecting the M-line structure. The present study also showed that M-line proteins are assembled into punctate structures as soon as they are synthesized. Some of these punctate structures contained both titin and M-line proteins by co-localization study, and possibly also myosin, since Issacs et al. [1992] reported that newly synthesized titin rapidly associates with myosin.

As shown by double label staining with rat anti-M-line protein antiserum and each of the monoclonal anti-titin E2, A12, and F3 antibodies, the assembly of M-line proteins into the M-line structure appears to occur before titin is assembled into the sarcomeres. We therefore suggest that M-line proteins may play a role in guiding the orientation or organization of titin during the formation of sarcomeres. In support of this hypothesis, a direct biochemical interaction has been demonstrated between titin and myomesin, and titin and M-protein [Nave et al., 1989; Vinkemeier et al., 1993; Obermann et al., 1997]. Phosphorylation of myomesin by certain kinases blocks its association with titin [Obermann et al., 1997]. Therefore, Obermann et al. [1997] suggest that a phosphorylation-controlled interaction in the sarcomeric cytoskeleton is related to the sarcomere formation.

A very interesting finding was the observation of the transitional single broad band seen with all three anti-titin antibodies before the appearance of typical titin doublets. This rea-

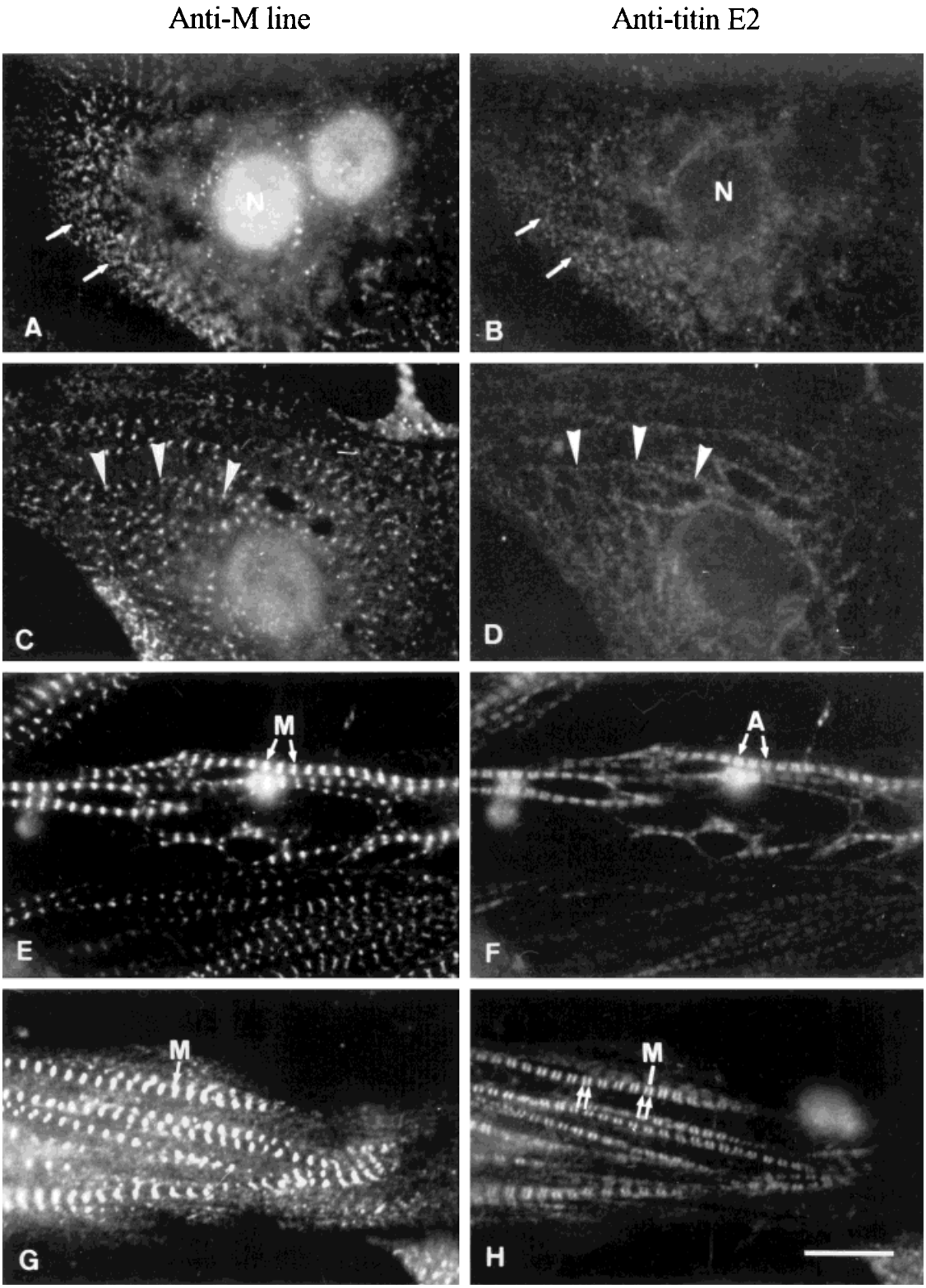

Fig. 4. Double immunofluorescence staining of day 2 cardiomyocytes with rat anti-M-line protein antiserum and mouse monoclonal anti-titin E2. **A,C,E,G:** Anti-M-line protein antiserum. **B,D,F,H:** E2 antibody. **A,B:** Both antibodies give punctate staining (arrows); staining for titin (E2) is much weaker. M-line proteins are distributed in a periodic pattern on primitive myofibrils (arrowheads, **C**), while staining with E2 is continuous (arrowheads, **D**). **E,F:** Anti-M-line protein antiserum stains the M-line (M), and E2 stains a broad A-band (A in **F**). **G,H:** E2 stains a doublet (arrows) on either side of the M-line. Scale bar = 10 μm.

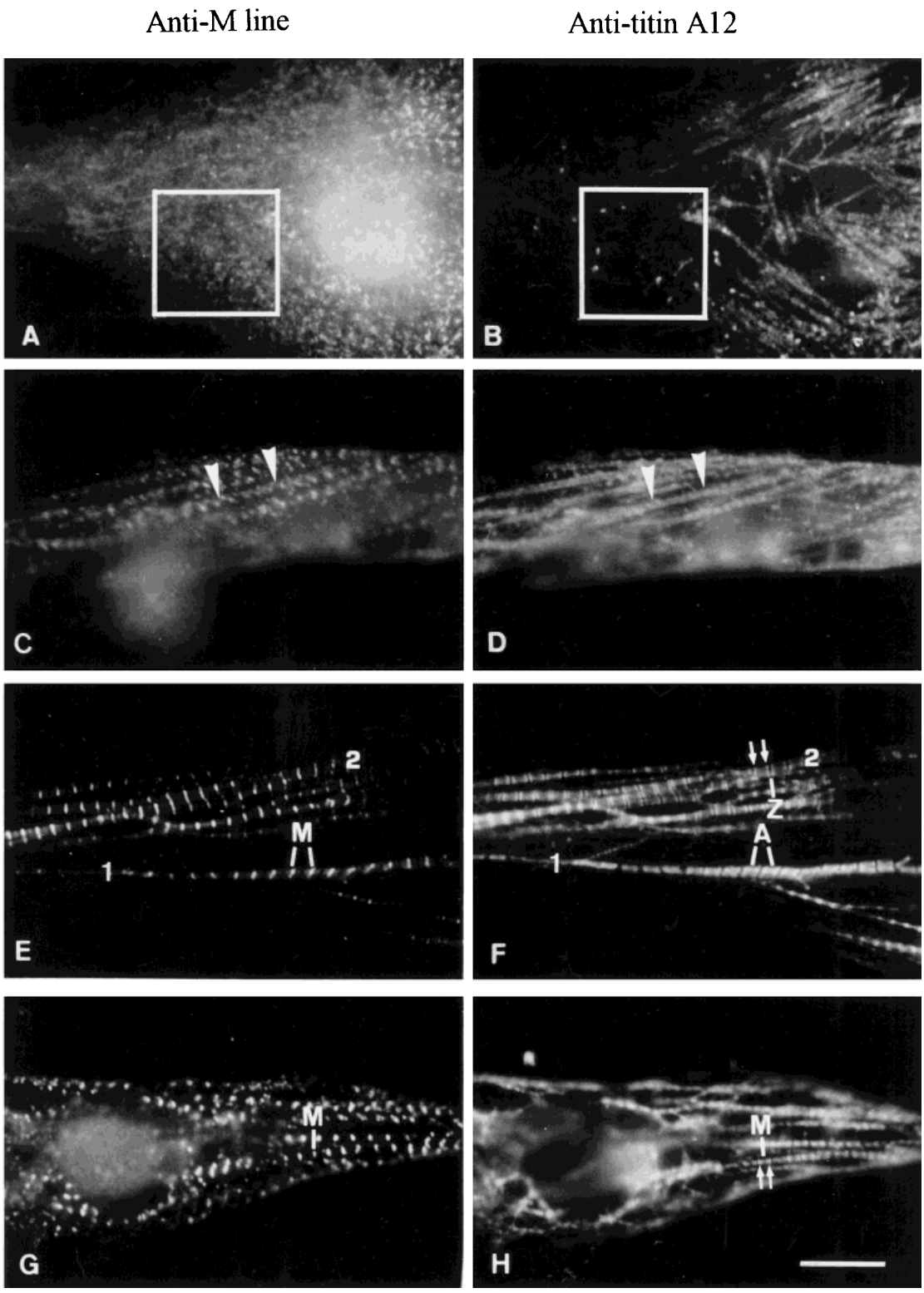

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Fig. 5. Double immunofluorescence staining of day 2 cardiomyocytes with rat anti-M-line protein and mouse monoclonal anti-titin A12 antibodies. **A,C,E,G:** Anti-M-line protein antiserum. **B,D,F,H:** A12 antibody. **A,B:** Punctate pattern of M-line proteins and continuous linear staining of titin (right half of the cell, **B**). Square, area with weak titin staining and punctate staining for M-line proteins. **C,D:** Periodic pattern for the M-line (arrowheads, **C**) and continuous pattern for titin (arrowheads, **D**). **E,F:** Anti-M-line protein antiserum stains the M-line (M) in both myofibril 1 and 2, whereas A12 antibody give a broad A-band stain **(A)** in myofibril 1, and clear doublet staining in myofibril 2. **G,H:** Typical M-line staining and titin doublet are observed. Scale bar = 10 µm.

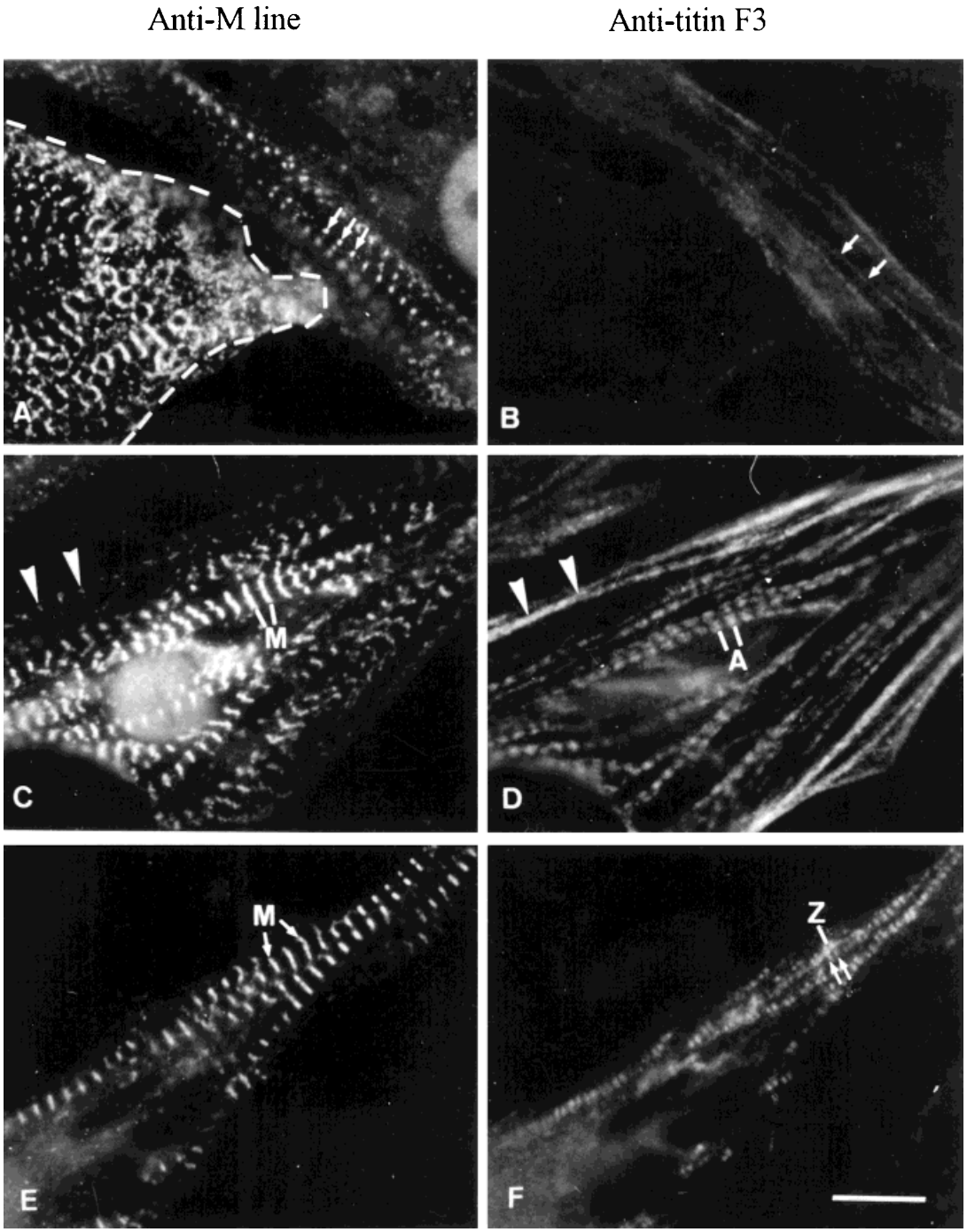

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Fig. 6. Double immunofluorescence staining of day 2 cardiomyocytes with rat anti-M-line protein antiserum and mouse monoclonal anti-titin F3 antibodies. **A,C,E:** anti-M-line protein antiserum. **B,D,F:** F3 antibody. Marked cell **(A)** shows staining for M-line protein but not for titin **(B)**. Periodic staining for M-line proteins and continuous staining for titin seen on the same nascent myofibril (arrowheads, **C,D**). **E,F:** Typical titin doublet (arrows, **F**) and M-line staining. Scale bar = 10 μm.

son may be due to a nonpolarized arrangement of titin molecules in early sarcomeres. With gradual rearrangement of the titin epitopes on the molecules into their polarized position, that is, bilateral symmetry to the M-line, mature myofibrils hence exhibited titin doublets, with development.

The M-line is composed of M-protein (165 kD) and myomesin (190 kD in cardiac muscle) [Strehler et al., 1979; Grove et al., 1984] and its function is to maintain the 3-D structure of adjacent myosin filaments in an A-band [Luther and Squire, 1978] and to provide the insertion site for titin filaments. The anti-M-line protein

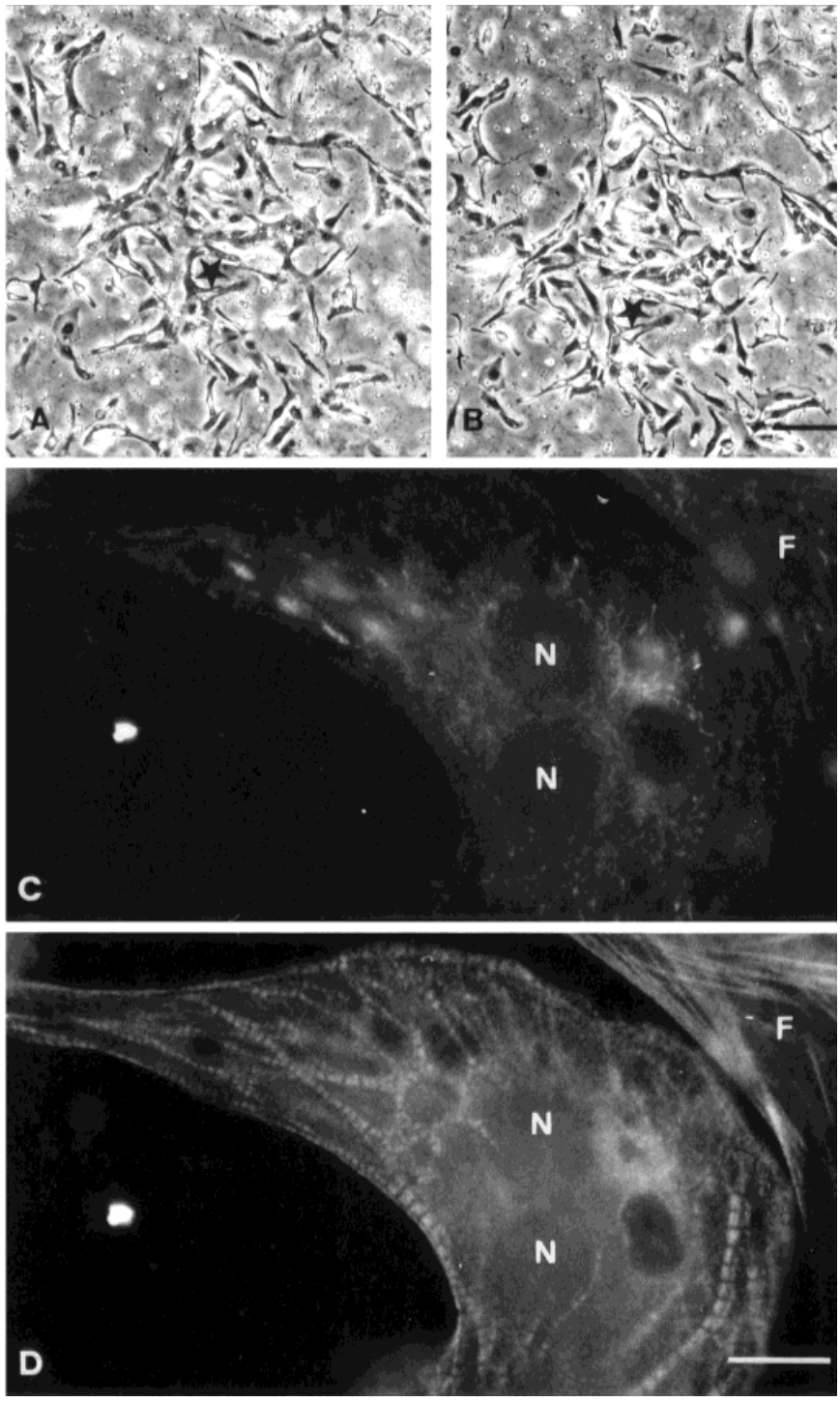

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Fig. 7. Immunofluorescence staining of day 2 cardiomyocytes with incorporated control mouse IgG (antibody F5). Phase images before **(A)** and after **(B)** electroporation; star, same region. The morphology of the cells is not affected by electroporation. **C,D:** Antibody F5-incorporated cells double-stained for mouse IgG and titin (anti-titin F3), respectively. The cells are full of normal myofibrils. N, nucleus; F, fibroblast. **A,B:** Scale bar = 100 μm; **C,D:** Scale bar = 10 μm.

antiserum used in this study reacts with both components. Not all cardiomyocytes showed morphological change in response to antibody incorporation. The extent of morphological change is dependent on the quantity of incorporated antibody. Most resistant cells were those incorporated fewer antibodies. The binding of anti-M-line protein antibody to M-line proteins

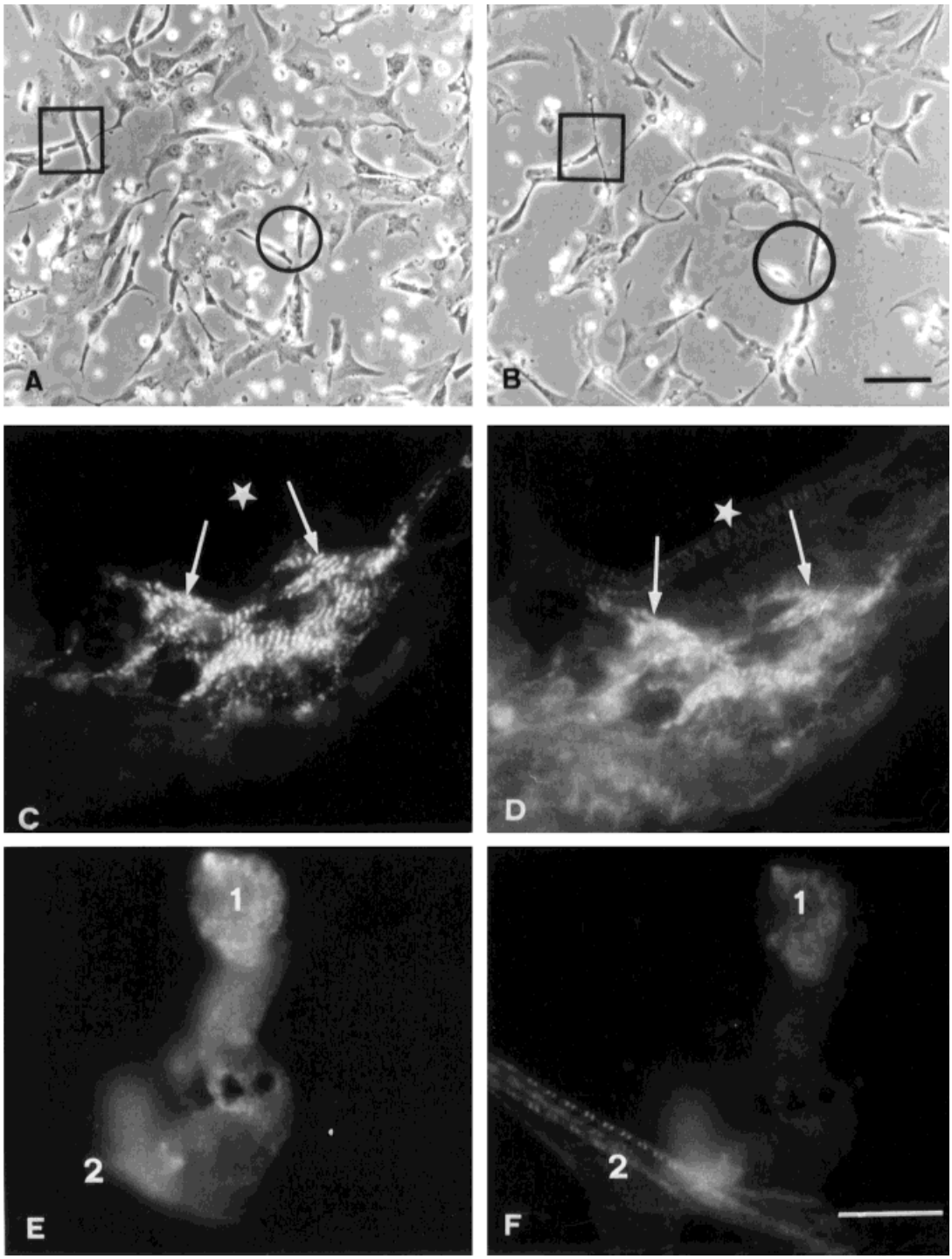

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Fig. 8. Effects of anti-M-line protein antiserum incorporation on day 2 cardiomyocytes. Phase images before **(A)** and after **(B)** electroporation. Cells appear slender and shortened after electroporation **(B)**. Square, circle, same areas of interest. **C,E:** Stained for incorporated anti-M-line protein antibody. **D,F:** Stained with anti-titin E2. In areas of antibody incorporation, titin is randomly organized (arrows, **C,D**), where, in areas devoid of incorporated antibody, it is arranged in a doublet pattern (star, **C,D**). **E:** A rounded-up cell (1) due to antibody incorporation, contrasting with a normal-looking cell, without antibody incorporation (cell 2). The myofibrils in these antibody-incorporated cells are shortened and less organized **(D,F)**. **A,B:** Scale bar = 100 μm. **C–F:** Scale bar = 10 μm.

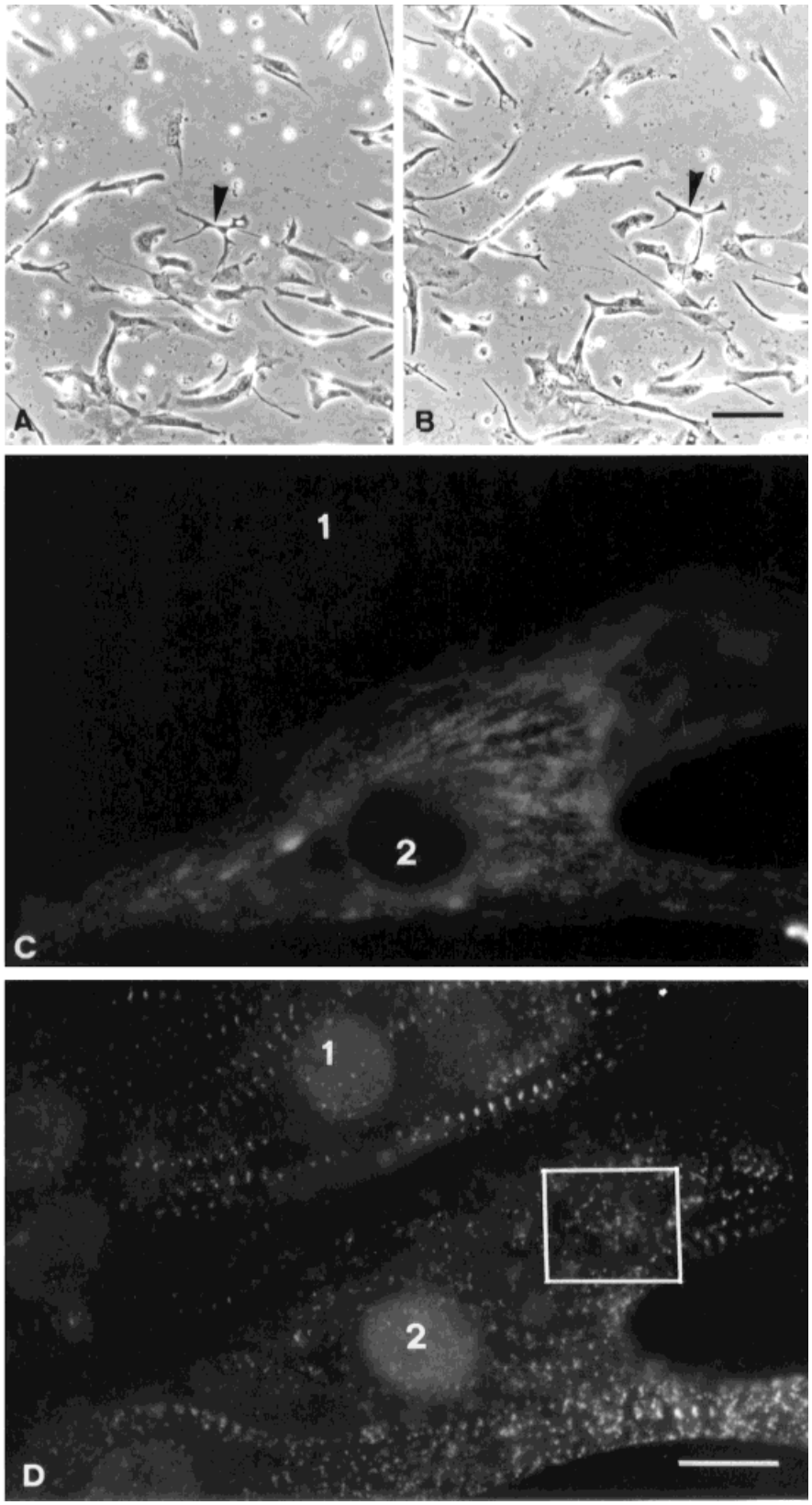

Fig. 9. Effects of anti-titin E2 antibody incorporation on day 2 cardiomyocytes. **A,B:** Phase images before and after electroporation. Cells after antibody incorporation show no significant change in morphology. Arrowheads, same cell. **C,D:** Same cell double-stained for incorporated E2 antibody and M-line proteins, respectively. In a cell (1) without antibody incorporation, M-line protein is arranged in a regular and periodic pattern (D), whereas, in a cell with incorporation (cell 2), the M-line protein is randomly organized in a dotted pattern (square, **D**). **A,B:** Scale bar = 100 μm. **C,D:** Scale bar = 10 μm.

either destroyed the M-line structure in primitive myofibrils or hindered the interaction between titin and the M-line, resulting in titin disorganization . Anti-titin antibody E2 specifically reacts with the M-line end of titin and incorporation of this antibody resulted in a punctate staining with anti-M-line protein antiserum, indicating a failure of M-line assembly.

The conclusion to be drawn from the above antibody perturbation experiments is that blockage of M-line proteins by antibody incorporation interrupts the assembly of titin into the sarcomere. Since titin is inserted in the M-line and Z-line, it is expected that disturbing the structural integrity of the M-line would result in titin disorganization, a hypothesis supported by our results.

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