Scanning electron microscopic studies of myoblasts from 11- to 13-day-old chick embryonic breast muscle cultured on collagen-coated glass coverslips showed six stages of development into multinucleated myotubes: (1) growth of flattened, spread-out cells for 20–30 hr following initiation of monolayer cultures; (2) extension of microprocesses (1–150 μm) from cells that have become spindle shaped; (3) contact and adherence of microprocesses from adjacent cells; (4) thickening of fused processes; (5) approximation of the cells; and (6) coalescence of the cells to form a spindle-shaped myotube. When the calcium-ion concentration in the growth medium was lowered—either by increasing the concentration of ethylene-glycol-bis (aminoethyl ether) *N, N'*-tetraacetate (EGTA) or by decreasing the concentration of free calcium ion used—the number of microprocesses present on the cells was reduced. Presumably, however, these microprocesses could still fuse together, provided that the calcium-ion concentration was greater than 160 μM. Indirect immunofluorescence assay with actin-specific antibody indicated that actin is a major component of the myoblasts' microprocesses. Cytochalasin B (5μg/ml) caused the microprocesses to retract within 15 min and the myoblasts to round up and detach from the glass substrate. This was presumably caused by the action of the drug on actin filaments.

MUSCLE & NERVE 1:219–229 1978

ACTIN-CONTAINING MICROPROCESSES IN THE FUSION OF CULTURED CHICK MYOBLASTS

HELENA L. HUANG, PhD, ROBERT H. SINGER, PhD, and ELIAS LAZARIDES, PhD

Presumptive muscle cells—myoblasts—can be removed from embryonic chicken breast and grown in tissue culture.[5,8] Initially, these single cells proliferate in culture on a glass coverslip, exhibiting no morphologic characteristics that serve to distinguish them as muscle cells. After one and one-half days in culture, however, they become bipolar, orient in a linear head-to-tail fashion, and then fuse to form multinucleate syncytia, or myotubes. Concurrent with the fusion, proteins which are important to muscle contraction are rapidly synthesized: actin, myosin, tropomyosin, and myoglobin, as well as enzymes associated with contraction, appear in quantity over a matter of a few hours.[7,8,25,27,35] In previous studies, fusion of myoblasts—defined by observation with phase-contrast microscopy—was used as the critical marker for differentiation. Fusion of single cells into multinucleate syncytia was considered the catalytic event that initiated the genetic program leading to expression of the muscle phenotype. The fusion event was interpreted as the conclusive morphologic indication of muscle "differentiation" and was considered proof that cells undergoing such fusion in a tissue culture were indeed muscle cells.

Recent biochemical information has challenged the idea that fusion itself initiates differentiation. First, single myoblasts prevented from fusion by insufficient calcium in the medium can still produce the differentiated isozymic form of myosin,[22,32,40] thus demonstrating that fusion is not an obligatory event for the expression of "muscle" genes. In our current studies investigating the appearance of the message for actin, we attempted to establish a precise temporal relationship between activation of muscle genes and fusion. In pursuing this problem on the early appearance of specific messengers, we found it essential to define the morphology of fusion in greater detail and at a higher resolution than had been accomplished in previous investiga-

From the Department of Anatomy, University of Massachusetts Medical School, Worcester (Dr. Singer and Dr. Huang); and the Division of Biology, the California Institute of Technology, Pasadena (Dr. Lazarides).

Supported by a grant from the Muscular Dystrophy Association to Dr. Singer, by a postdoctoral fellowship to Dr. Huang, and by NIH grant 11329.

The authors wish to thank Kevin Byron for his assistance in preparing tissue culture.

Address reprint requests to Dr. Singer at the Department of Anatomy, University of Massachusetts Medical School, Worcester, MA 01605.

Received for publication November 27, 1977; revision received March 6, 1978.

tions. We therefore conducted this study on myoblasts using scanning electron microscopy (SEM).[36] With the study's results, sensitive biochemical analyses can now be more precisely correlated with morphologic events in fusion. The results indicate that, through actin-containing, 0.1-μm-diameter microprocesses which are not resolvable by phase-contrast microscopy, the fusion of myoblasts may occur earlier than had been surmised.

MATERIALS AND METHODS

Cells. Primary cultures of normal chick breast muscle from 11- to 13-day-old embryos (SPAFAS, Norwich, CT) were grown on collagen-coated glass coverslips, as described by O'Neill and Stockdale.[24] Excised breast muscles were first rinsed in Eagle's balanced salt solution (EBSS); they were then minced to small pieces and trypsinized with constant shaking in 0.2% trypsin in EBSS at 37°C for 20 min. Trypsinization was stopped by adding horse serum to a final concentration of 5%. The cell suspension was decanted and centrifuged at 500 g for 5 min. The cell pellet was washed once with complete growth medium (CGM)—i.e., Eagle's minimum essential medium (EMEM) enriched with 10% horse serum (HS) and 1% chick embryo extract (EE). After resuspending in CGM, the cells were filtered through a 10-μm-pore-size nylon mesh filter (Tetko, Lancaster, NY) into a 100-mm Falcon tissue-culture plate. They were then incubated at 37°C in a humidified 5% CO_2–95% atmosphere incubator (Hotpack, Philadelphia, PA). Preplating was done so that the fibroblasts would settle down and stick to the plate while the myoblasts remained afloat.[31] The supernatant from the preplate was then withdrawn to a sterile capped tube and mixed with a vortex mixer, and its cell content was counted. The cells were usually plated at 2×10^6 cells per 100-mm plate. Four or more 22-mm round glass coverslips were placed in each plate after having been coated with 0.5 mg/ml collagen solution (Worthington Biochemical, Freehold, NJ). These were then drained and dried overnight in a laminar-flow hood before being used. Twenty-four hours after plating, the medium was replaced with fresh medium. Preparation of embryo extract consisted of pressing decapitated 11-day-old chick embryos through a 50-ml disposable syringe diluted 1:1 with EMEM, and centrifuging the suspension obtained at 1,000 g for 10 min to remove cell debris. The supernatant was pipetted off and stored in 1-ml aliquots at -80°C until used.

Low-Calcium Medium. Calcium-ion concentration in the growth medium was regulated either by addition of various concentrations of ethylene-glycol-bis (aminoethyl ether)*N,N'*-tetraacetate (EGTA) to CGM 20 hr after the myoblasts were first plated, according to the method of Paterson and Strohman,[27] or by use of calcium-free EMEM supplemented with reduced amounts of horse serum. The free calcium ion in the growth medium, the horse serum, and the embryo extract was individually determined in a Fiske calcium titrator (Uxbridge, MA) using $CaCO_3$ as the standard.

Scanning Electron Microscopy. Glass coverslips bearing cultured chick myoblasts were removed from tissue-culture dishes at various intervals after plating and were placed upright in a small staining jar filled with 0.12 M phosphate buffer (PB), pH 7.4, containing 2.5% dextrose. The cells were gently rinsed with two changes of the same buffer at room temperature, fixed for 30 min in 0.5% paraformaldehyde–2.5% glutaraldehyde in 0.12 M PB, and postfixed for 30 min in 2% osmium tetroxide in 0.12 M PB. They were then dehydrated, first through changes of increasing concentration of ethanol for 5 min each (25%, 35%, 50%, 70%, 80%, 95%, and 100% twice) and then through a series of isoamyl acetate/absolute ethanol for 5 min each (25%, 50%, 70%, 85%, and 100%). The coverslips were stored in 100% isoamyl acetate until the cells were critical-point dried using liquid CO_2 in a Samdri PVT-3 apparatus (Biodynamic Research, Rockville, MD), according to the method of Anderson.[4] The specimens were coated with gold–palladium in a Denton DV-200 high-vacuum evaporator equipped with a tilting omni-rotary table (Cherry Hill, NH) or in a Hummer II sputter-coating apparatus (Technic, Alexandria, VA). The micrographs were taken on an ETEC autoscan microscope (Hayward, CA) at 20 kV and at a 45° tilt using Polaroid P/N 55 films.

Cytochalasin B. Cytochalasin B (Aldrich Chemical, Milwaukee, WI) was dissolved in dimethylsulfoxide (DMSO) to a concentration of 1 mg/ml for a stock solution (after the method of Sanger[33]). The final concentration of cytochalasin added to the cultures was 5 μg/ml. DMSO was added to control cultures without cytochalasin.

Indirect Immunofluorescence. Actin extracted from chicken-gizzard smooth muscle was purified through preparative sodium dodecyl sulfate (SDS) slab gel electrophoresis and was used as an antigen to induce actin-specific antibodies from rabbit, as previously described.[15,16] When this antibody preparation was characterized and compared with the antibodies induced against mouse fibroblast or calf-thymus actin by indirect immunofluorescence techniques, it was found to be indistinguishable from the others in its ability to specifically stain the actin filaments (I band) of chicken

myofibril.[17] This reactivity was lost when the antisera were first preincubated with polymeric actin.

The presence of actin in the cultured muscle cells was detected by a modified indirect immunofluorescence technique, as previously described.[17] The cells grown on glass coverslips were first washed gently and briefly in phosphate-buffered saline (PBS), fixed in 3.7% formaldehyde, and rinsed three times in PBS. They were then treated in 1:1 acetone–water for 3 min, in acetone for 5 min, in acetone–water for 3 min, and in PBS for 3 min. The coverslips were drained and placed horizontally in well-leveled and -soaked filter paper. The specimens were immediately covered with 20-μl diluted antisera and were then incubated at 37°C for 50 min, washed three times in PBS, and reincubated for 1 hr with fluorescein-labeled goat antirabbit IgG (Miles Laboratories, Elkhart, IN). After incubation, the coverslips were rinsed in PBS and distilled water; they were then prepared for viewing and photographing by a Zeiss microscope (PM II) fitted with epifluorescence optics. Kodak Plus-X film was used to take photomicrographs with a 63× oil-immersion objective.

RESULTS

Morphology of the Muscle Cells during Differentiation. Scanning electron microscopy revealed that the myoblast surface underwent rapid and extensive changes in configuration during the process of differentiation. Over the course of the differentiation process, the morphologic features of the cell cultures were tabulated. This tabulation revealed the presence of an intermediate stage taking place prior to cell fusion. This stage, wherein the cells developed long microprocesses by which they contacted other single cells, was transitory and appeared to lead to the fusion of the single cells into myotubes. The characteristics of these developmental stages are explicated further below.

Twenty hours after the primary culture was plated, about 80% of the cells were flattened (fig. 1), and the cytoplasm was thinly spread with a centrally located nucleus (fig. 2). The surfaces of the cells were generally smooth. Between 20 and 40 hr after plating, the myoblasts became spindle-shaped and highly elongated filamentous processes—which we have termed *microprocesses*—projecting from all surfaces of the cell body (figs. 3, 4, and 5). By 40 hr after plating, most of the myoblasts had acquired an abundance of microprocesses (fig. 3), which subsequently continued to increase in number (figs. 6 and 7). At this point, microprocesses could be traced between adjacent cells without apparent interruption, suggesting either fusion of processes at their tips or intimate membrane contact.

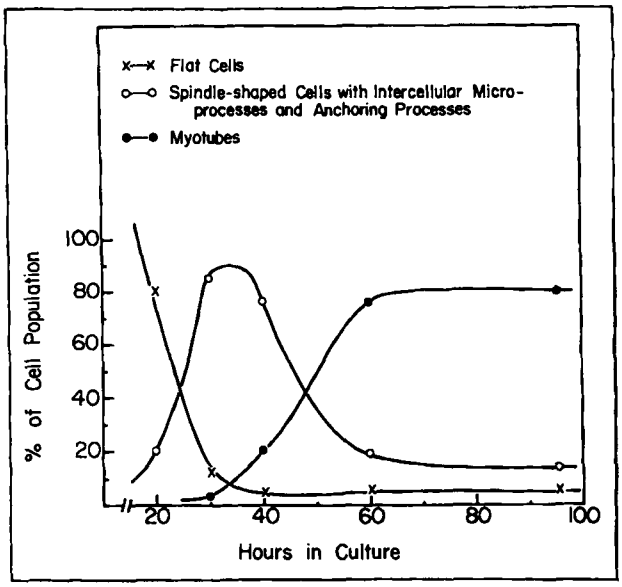

Figure 1. Physical characteristics of primary chick myoblasts at different stages of growth. Chick myoblasts grown on glass coverslips removed at various hours after plating were prepared for viewing by SEM. For each time interval noted, at least 100 randomly selected cells were scored and examined. Repeated experiments yielded similar results. The percentage of myotubes on each specimen corresponded closely with the rate of fusion as determined by light microscopic examination of Giemsa-stained plates of myoblast fixed at various time intervals.

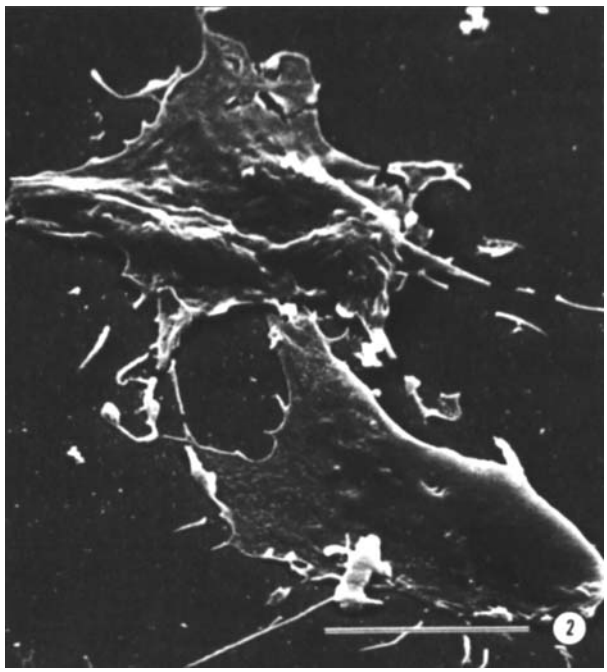

Figure 2. Scanning electron micrograph of chick-breast myoblasts at early stage of growth. Twenty hours after culturing, prior to the 24-hr medium change, the cell surface appears to be flat and smooth and the cytoplasm thinly spread. Bar = 10 μm.

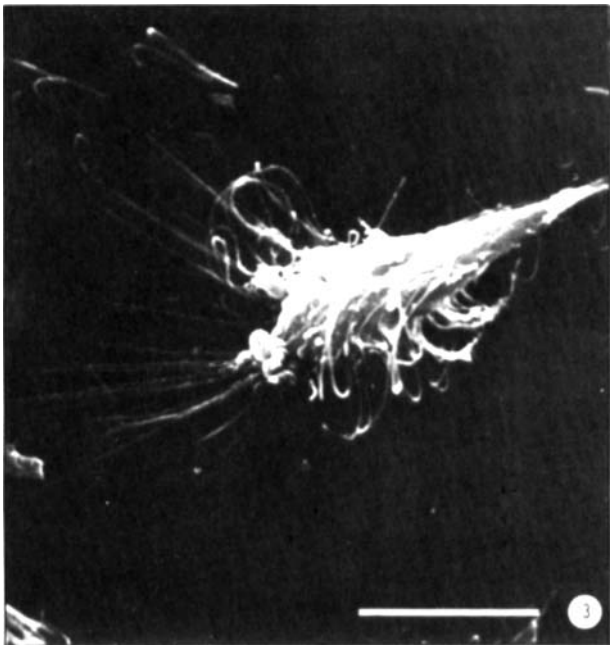

Figure 3. A myoblast 45 hr after plating. The cell becomes spindle shaped, with microprocesses extending from all sides of the cell body. Bar = 10 μm.

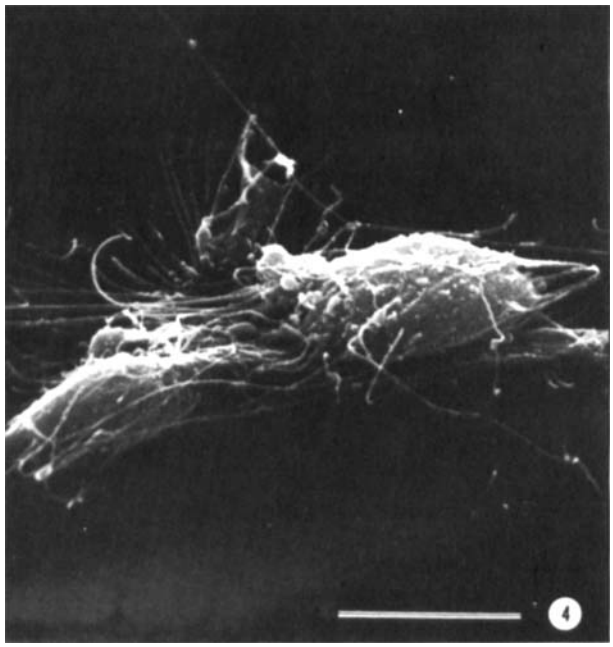

Figure 4. Two 45-hr myoblasts seen in close contact. The microprocesses from each cell extend to reach and touch the adjacent cell. Bar = 10 μm.

The exact region along the interconnecting process where fusion or membrane contact occurred is not known. However, time-lapse cinematography, as well as continual light-microscopic examination as performed in our studies and in other investigations,[29] seems to indicate that microprocesses from two adjacent prefusion myoblasts extend toward each other, make contact, and may eventually either fuse or break away. When fusion does occur, however, it appears to do so via these microprocesses. In many of our cultures, single rather than multiple processes served to connect two cells; transmission electron microscope observations indicate that fusion can occur at a single site.[19] Often, however, many connections could be seen between adjacent cells (figs. 6 and 7).

In addition to contacts made end to end by way of microprocesses arising from the polar ends of the spindle-shaped myoblasts, connections by laterally originated processes were also observed between myoblasts. Such lateral connections that apparently lead to fusion have been reported by other investigators using the light microscope.[29] The myoblasts also sought out and attached to myotubes (figs. 5, 8, and 9). Once formed, the myotubes themselves appeared to contain no microprocesses, but their surfaces contained irregularities (such as blebs, ridges, and short extrusions) in varying numbers (fig. 9). The microprocesses connecting myoblasts to myotubes were of various lengths, some short and thick and others long and thin, suggest-

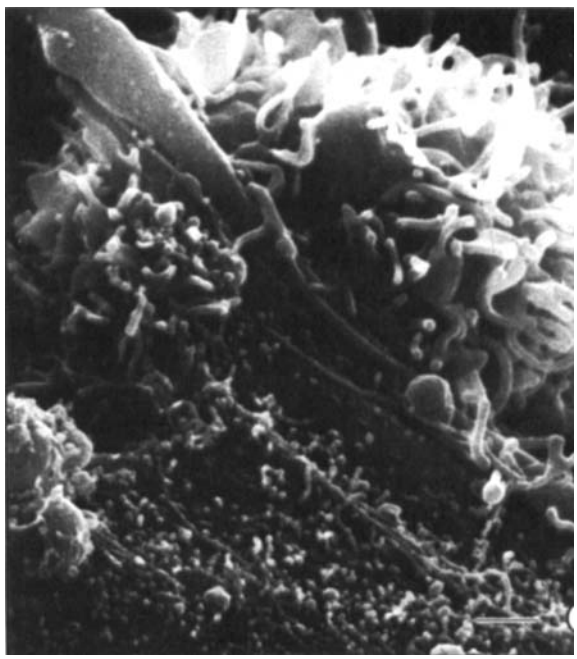

Figure 5. Myoblasts at a late stage of differentiation. After 65 hr, the cells become covered with microvilli and microprocesses. However, these processes appear to be shorter and stouter as compared to those seen on the 45-hour myoblasts. Bar = 1 μm.

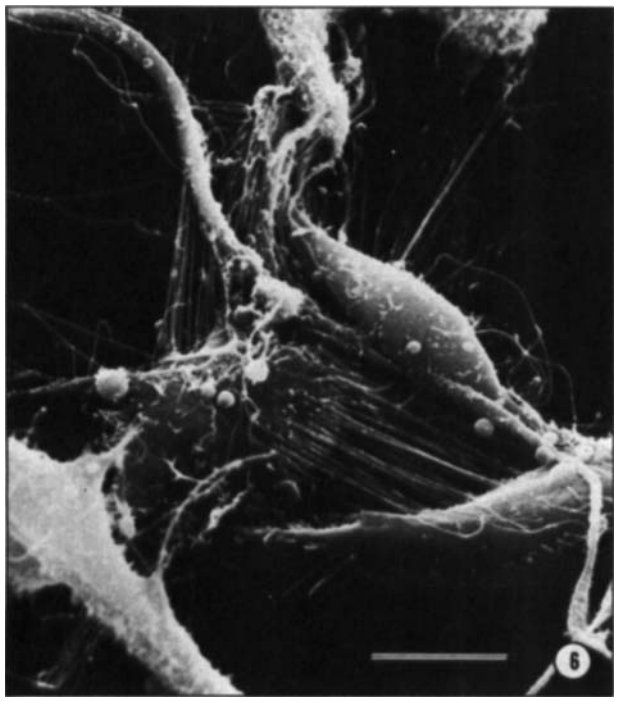

Figure 6. Early stage of fusion. Prefusion myoblasts 65 hr after plating are spindle shaped and are covered with microprocesses which extend toward neighboring cells, effecting a shorter distance between cells. Bar = 10 μm.

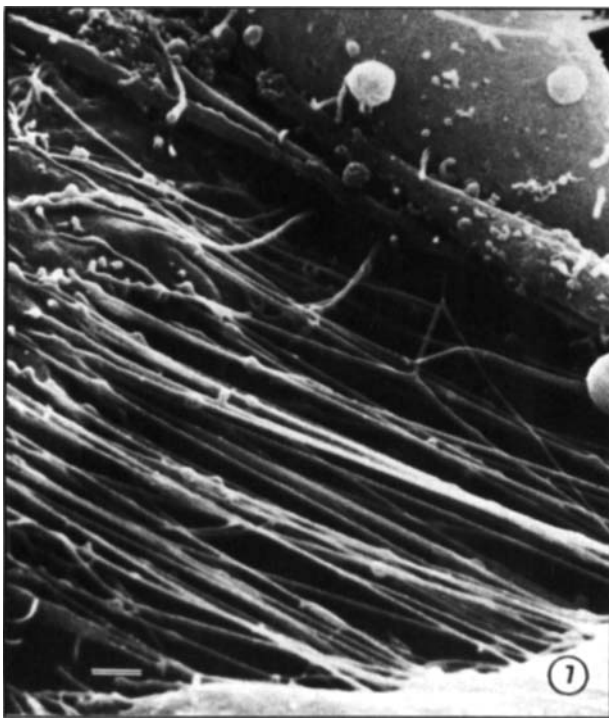

Figure 7. A closer look at the prefusion microprocesses. Higher magnification of the microprocesses seen in figure 6 shows their varying thickness and how they project from one cell to anchor onto the neighboring cells. Bar = 1 μm.

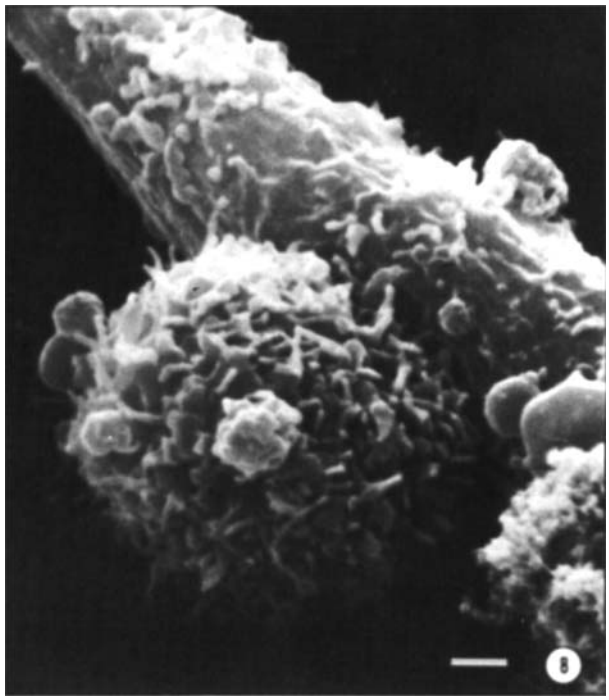

Figure 8. Thickening of microprocesses and approximation of the cells. Sixty-nine hours after culturing, a myoblast is seen in close apposition to a myotube. The microprocesses have become thicker, which may in turn draw the two partners closer to one another. Bar = 1 μm.

ing that the myoblasts are drawn to the myotube by gradual shortening of the connection (figs. 5, 8, and 9). Moreover, myoblasts were seen in close contact with myotubes (fig. 8), which may be interpreted as a further stage in the fusion process. Occasionally, a craterlike structure with prominent lips or with a ruffled border was observed (fig. 9)—a structure which may mark the position where the myoblast fused with the myotube. This presumed stage of fusion may be very transitory, since it was less frequently seen. However, differentiation continued in the cultures for several days. Between 60 and 96 hr, the total nuclei in the myotube did not increase significantly; yet myotubes merging together to form larger syncytia were commonly seen.

Within 64 hr of plating, about 60%–80% of the myoblasts had fused into myotubes (fig. 1). Chick breast muscle is significantly lower in fibroblasts than is thigh muscle. Furthermore, by preplating the primary chick breast cells for 30 min, we were able to eliminate residual fibroblasts with up to 90% efficiency—that is, on the fourth day after plating the cells, we counted less than 10% of total nuclei in flat-mononuclear cells, while the remaining nuclei were found in the multinucleated myotubes. Therefore, any surface morphologic

changes observed during the process of differentiation could be attributed with relative confidence to myoblasts. The time of the fusion, as revealed in our cultures, affirms the stages reported by O'Neill and Stockdale[24] for cells similarly plated at low density. Plating at low density allowed for greater control over the time of fusion as well as good separation of cells for viewing under the SEM.

The Effect of Calcium Deficiency on the Cultures. When the calcium-ion concentration in the culture medium was progressively lowered by adding increasing amounts of EGTA to chelate the free divalent ion, or by utilizing calcium-free growth medium and lowering the amount of horse serum (2.5 mM Ca^{++}), fusion as seen by light microscopy was reduced by at least 90%, which is consistent with the findings of others.[27,34] When viewed under the SEM, there was a reduction of microprocesses on the cell surface (figs. 10 and 11). However, fusion of microprocesses was not blocked until the calcium concentration fell below 160 μM. By light microscopy, failure of fusion appears to occur at a higher concentration.

The morphology of the microprocesses in calcium-deficient medium differed from that of the microprocesses in normal medium. Occasional microprocesses detectable only by SEM were seen connecting cells over a distance of 150 μm (fig. 12). In such cases, the elongated processes persisted without the shortening and thickening that occurred in the normal cultures; indeed, these microprocesses were much in evidence after six days of culture in calcium-deficient medium, at a time when myotube formation was fully completed in control cultures. Another difference observed was the presence of overlapping but unconnected processes with distinct individual surfaces.

The Presence of Actin in the Microprocesses. Actin was visualized using antibodies specific for actin in indirect immunofluorescence. Figures 11, 13, and 14 show that there was extensive actin in the microprocesses as well as in the cell body. Even the thinnest microprocesses (0.1 μm) were revealed, and showed beading as well as other size irregularities (fig. 13). The beading was characteristic of newly forming processes. No break in the fluorescence of the connecting microprocesses could be found; instead, the process, once in contact, appeared to be one continuous actin-rich cytoplasmic bridge.

The drug cytochalasin B is believed to cause disorganization of actin filaments by disrupting actin-membrane interaction.[19] It also inhibits myoblast fusion.[13] When cytochalasin was applied to myoblasts in culture, it resulted in the regression of unconnected microprocesses within 5 min (figs. 15 and 16). Some of the microprocesses from adjacent cells—which may have already fused—remained intact after addition of the drug (fig. 15). Many processes, however, appeared to collapse into the membrane—leaving craters or, in some cases, a completely smooth membrane (fig. 16).

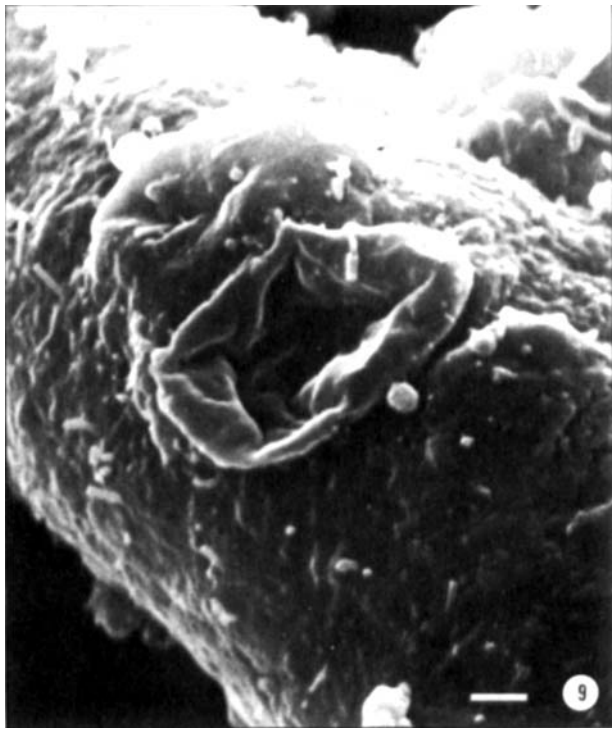

Figure 9. Fusion. The complete fusion of a myoblast into a myotube as seen on the same coverslip as figure 8. The cell content of the myoblast is emptied into the myotube, forming a craterlike structure with prominent ruffled borders. Bar = 1 μm.

DISCUSSION

The nestling and entangling involvement of microprocesses between two neighboring cells, as well as the long extensions of a lone myoblast apparently "in search" of another myoblast, was captured by SEM. The results revealed morphologic stages during the differentiation of muscle that had not been detected by light microscopy. It seems possible to divide these events into six stages, as follows:

1. Cellular multiplication takes place, during which the cells are flat (fig. 2).
2. The cells become spindle shaped and form microprocesses from all surfaces, predominantly at the polar ends (fig. 3).
3. The microprocesses of adjacent myoblasts contact one another or the surfaces of newly formed myo-

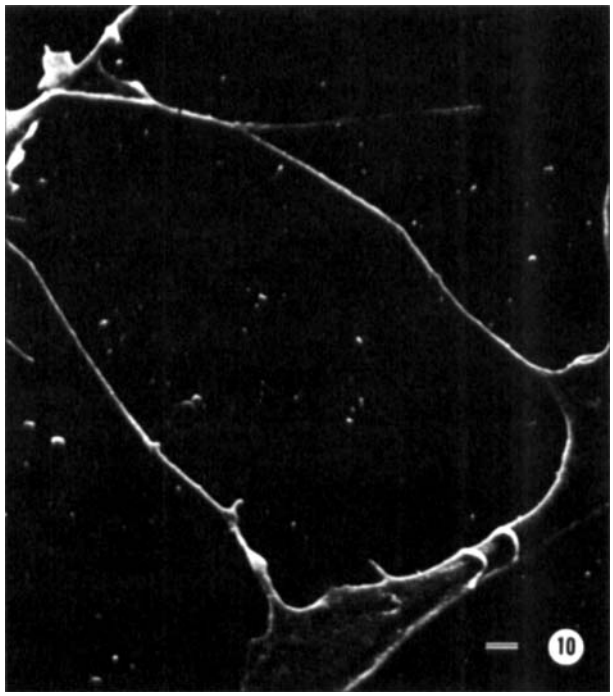

Figure 10. Myoblasts grown in low-calcium-ion concentration. After 26 hr of culturing in 160 μM Ca++, there is a definite reduction of microprocesses seen on the cell surface. Bar = 1 μm.

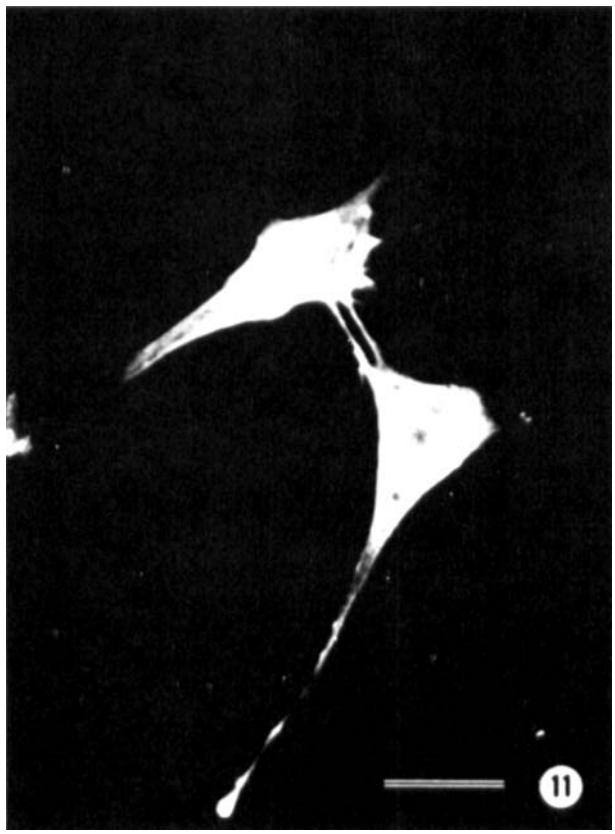

Figure 11. Indirect immunofluorescence of 40-hr chick-breast myoblasts with antiactin antibodies. Actin is present in both cell bodies and microprocesses. Bar = 10 μm.

tubes—at which point they either become thicker, drawing together the connected cells, or break contact and reform.

4. Fusion eventually takes place, presumably occurring at a microprocess's region of contact with a cell or myotube surface (fig. 4).
5. The microprocesses shorten (figs. 6 and 8).
6. The cells combine to form a single syncytium (fig. 9).

This last event represents the fusion stage as seen in light microscopy, and it occurs about 45–60 hr after plating.

The most important stage is the formation of long actin-containing filamentous microprocesses, approximately 0.1 μm in diameter, from two adjacent cells that contact with each other. We interpret this point of contact as the first potential locus of cellular fusion. Since the SEM allows visualization of cell surfaces only, these data offer no proof that the cytoplasm of two cells is continuous throughout a joining microprocess. Serial sections of embedded cells yielding longitudinally transected microprocesses did not afford such proof, as these microprocesses were never fully within a plane of section. Present work is directed toward providing proof for the fusion of microprocesses from adjacent cells. At this time, however, fusion of the microprocesses must remain conjecture, based as it is on circumstantial evidence that processes connecting two cells did not pull apart during the action of cytochalasin or low-calcium medium (both of which cause the rapid retraction of a microprocess unlinked to another microprocess).

A method of transmission microscopy that utilizes triton to dissolve the membranes of these cells, leaving only cytoskeleton, shows that actin cables continue from one cell to the next via these microprocesses (J Pudney and RH Singer, unpublished observations). This fusion, should it occur at this time, is followed by a change in diameter and by a shortening of the connecting bridge, suggesting that the bridge also connects cells together to effect the final syncytium. Data not presented here, using time-lapse cinematography, show that microprocesses thicken, pulling two cells together to effect a fusion. Moreover, they may also function to intensify the molecular "conversation" between the interacting cells, thereby ensuring complete fusion. This *apparent* fusion between microprocesses occurred in our cultures before myotubes

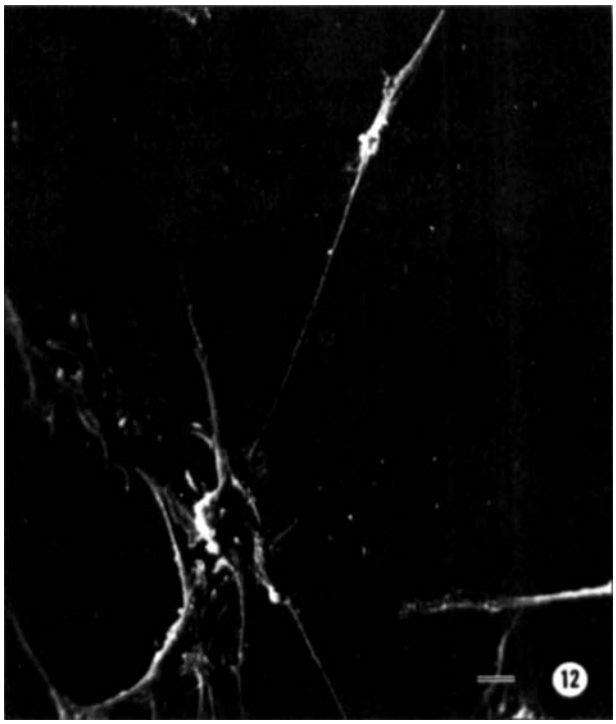

Figure 12. Myoblast grown for six days in low calcium. Microprocesses of 150 μm or even longer are often seen on the myoblasts. However, no fusion as seen in figure 9 is observed on the coverslips of myoblasts grown at 160 μM calcium for six days. Bar = 10 μm.

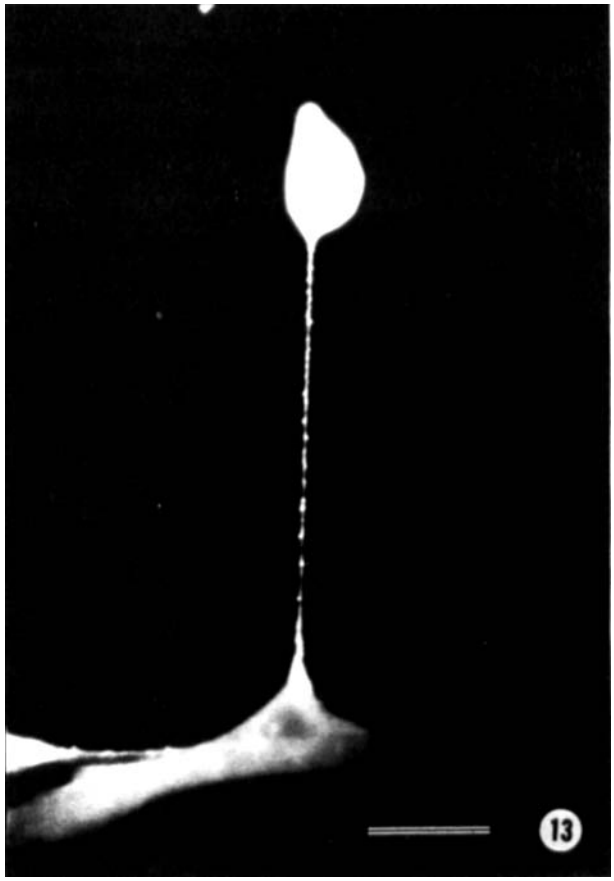

Figure 13. Actin seen in the very thin microprocesses. Actin can be visualized as the beading effect between two contacting 40-hr myoblasts. Bar = 10 μm.

were observable under the light microscope. Thus, the first occurrence of fusion might be defined as the fusion of microprocesses from adjacent cells which are potentially quite far apart (e.g., 200 μm) and which, by virtue of their spatial separation, would appear to be unfused under the light microscope. Hence, some biochemical characteristics might be attributable to single cells that are, in fact, conjoined.

The same logic follows from the effect of calcium-deficient medium on myoblasts, presumably preventing their fusion. Calcium-deficient medium (160 μM) does inhibit fusion subsequent to the addition of this medium. In a calcium-deficient concentration of 160 μM, however, when light microscopy would interpret the cells as unfused, the SEM reveals apparently fused microprocesses of adjacent cells. Indeed, the deficiency in calcium augments the proportion of long processes in the culture and inhibits the pulling together of the cells, thus making it difficult to draw conclusions on the state of fusion. Hence the time frame for the fusion of cultures, as well as the definition of fusion, might be revised to encompass all of these SEM results. Such results could affect conclusions that have previously been drawn using light-microscopy fusion as a time marker for the activation of specific differentiated muscle gene products.

Microprocesses have also been observed in other types of eukaryotic cells cultured in vitro. Cornell[9] showed long "microvilli" in mouse embryo cells using high-power light microscopy which revealed cellular contacts by way of these microvilli. Transmission electron microscopy further revealed that these processes contained microfilaments, rarely microtubules. More recently, Albrecht-Buehler[1] described similar projections in 3T3 cells in culture and proposed a sensory role for them. The measurements he obtained for his "filopodia" were 30 μm or less.

Microprocesses protruding from the surface of eukaryotic cells have been designated variously as microvilli, microextensions, microspikes, filopodia, and retractile fibrils;[10,23,39] they have also been credited with many cellular-surface functions, such as cell-substrate attachment, spreading and retraction of cytoplasmic content, phagocytosis and absorption, locomotion, and virus-induced cell fusion.[1,11,12,18,30,42] Hence it is reason-

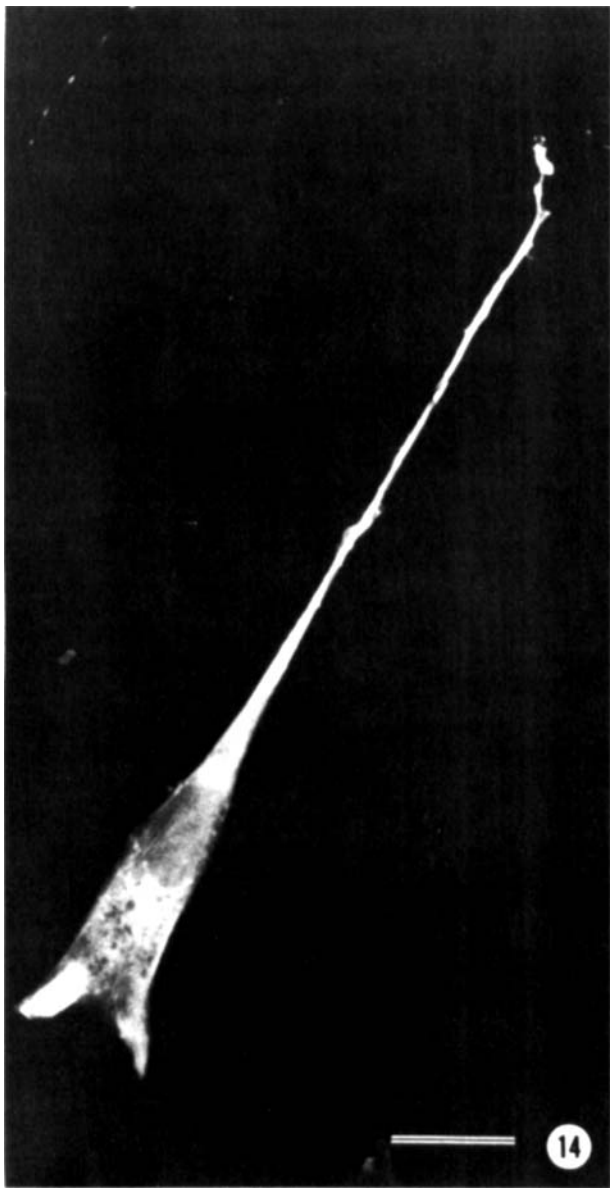

Figure 14. Differential staining for actin. The microprocesses of a 40-hr chick myoblast appear to be more prominently stained for actin than the cell body. Bar = 10 μm.

able to propose that the microprocesses we depict play a prime role in the fusion of muscle cells.

The role of these processes becomes clear when one is considering the development of myoblasts into muscle, a process that hinges on the interaction between cells. No myoblast can become a myotube without fusing with another myoblast. Thus these long filamentous structures are obvious mechanisms for contacting another distinct myoblast. Two myoblasts may be as much as 300–400 μm apart and yet may still be able to fuse. The processes thus provide the physical

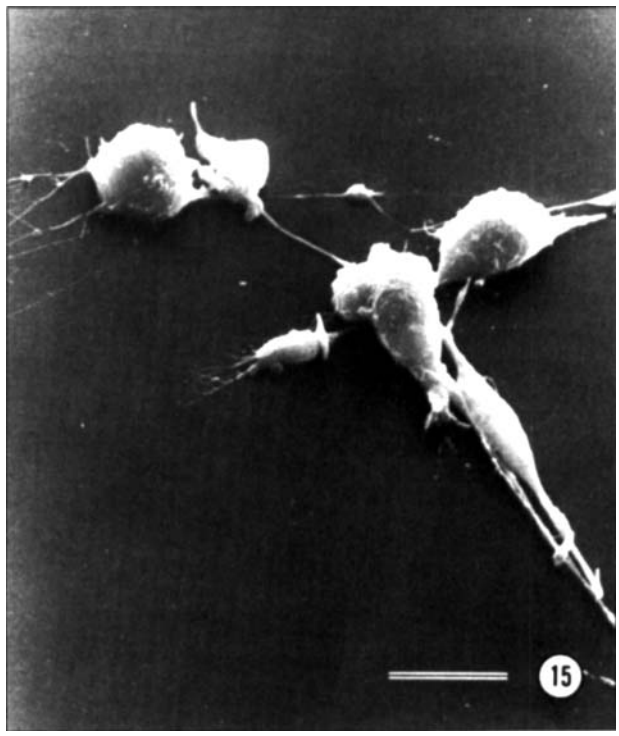

Figure 15. Myoblast in cytochalasin B. Scanning electron micrograph of a 45-hr chick myoblast after 15 min in cytochalasin B, 5 μg/ml. Bar = 10 μm.

means by which myoblast membranes are brought into contact, especially at low cellular density. Once fusion has been initiated in this way, the processes may serve to pull the two cells together, a theory that appears to be substantiated by our initial observations on the fusing of cells using high-resolution Nomarski optics. Bayne and Simpson[6] have seen similar processes at a stage before fusion in lizard muscle. Powell[29] has seen long processes extended by prefusion spindle-shaped myoblasts in time-lapse cinematography.

It is interesting to consider the role of actin in mediating these processes. Although the evidence does not eliminate other proteins from involvement in the formation of these processes, actin is the major protein component of these cells and is synthesized in large amounts at this time.[14,26] Our results indicate a large amount of actin mRNA just before fusion, perhaps involved in the synthesis of actin for the microprocesses. Electron microscopists have seen microfilaments in developing myoblasts.[3,37] The presence of myosin at this time[22,32,40] raises the possibility of an interaction between actin and myosin, perhaps within the microprocess, forming a "micromuscle" which pulls the cells together once fusion of these processes has occurred. These processes may be reminiscent of the microvilli in the intestinal brush border, where the

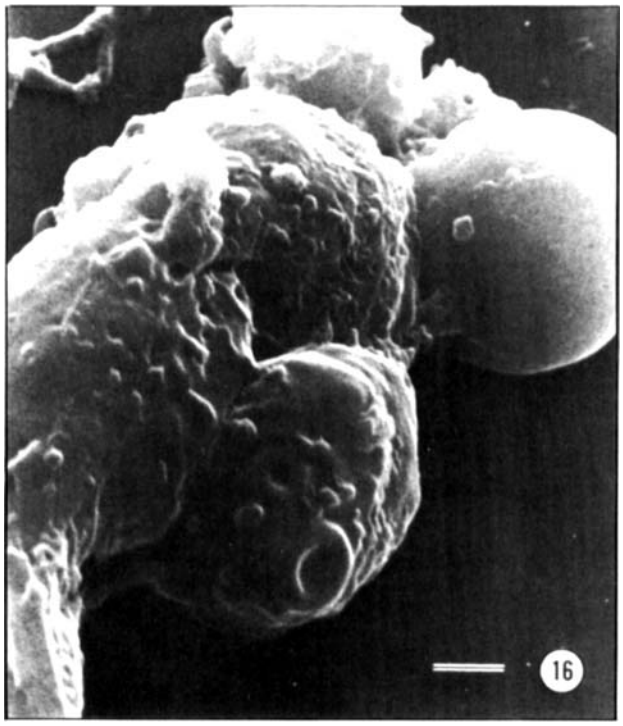

Figure 16. Collapse of microprocesses in cytochalasin B. One of the most prominent features of cytochalasin treatment consists of the smooth, craterlike recesses seen on the myoblast, possibly resulting from the rapid return or collapse of the microprocesses upon addition of the drug. Bar = 1 μm.

movement of the villi is mediated by an actomyosin interaction at the base of the villus.[21,28]

Cytochalasin is believed to act on microfilaments (actin), particularly those that are membrane associated.[33] It has been shown to interfere with the structure of microvilli,[20] as these structures withdraw into HeLa cells within 4 min of exposure to the antibiotic. Purified actin has also been reported to interact with cytochalasin,[38] although this interaction may not be analogous to that in situ. Gelatin of actin is also inhibited by cytochalasin.[41] Miranda et al[20] have shown a rearrangement—but not a disappearance—of microfilamentous bundles in the presence of cytochalasin.

Our results on the effect of cytochalasin on the microprocesses of myogenic cells reinforce the conclusion that these structures are actin mediated, and that they participate in the fusion process. Inhibition of fusion is reported in the presence of cytochalasin.[13] Fibroblasts are less sensitive than muscle cells in their response to cytochalasin; in the presence of 5 μg/ml of the drug, fibroblasts remain attached to the substrate, whereas most muscle cells round up and detach in this concentration.[2,13,33] The large quantity of actin in the differentiating myoblasts might account for this specificity. It is possible, then, that the stage of myoblast development morphologically characterized by the microprocesses indicates that a time previous to fusion may exist when the augmentation of specific gene products, such as actin, controls or promotes further differentiation.

REFERENCES

1. Albrecht-Buehler G: The function of filopodia in spreading 3T3 mouse fibroblasts. In Goldman R, Pollard T, Rosenbaum J (Editors): *Cell Motility*. New York, Cold Spring Harbor Laboratories, 1976, pp 247–264.
2. Albrecht-Buehler G: Filopodia of spreading 3T3 cells. Do they have a substrate-exploring function? *J Cell Biol* 69:275–286, 1976.
3. Allen ER, Pepe FA: Ultrastructure of developing muscle cells in chick embryo. *Am J Anat* 116:115–148, 1965.
4. Anderson RF: Techniques for preservation of three-dimensional structures in preparing specimens for electron microscopy. *Trans NY Acad Sci* 13:130–133, 1951.
5. Askanas V: Tissue culture approach in studying normal and diseased muscle. In Milhorat AT (Editor): *Exploratory Concepts in Muscular Dystrophy II*. New York, Excerpta Medica Foundation, 1974, pp 639–649.
6. Bayne EK, Simpson SB: Lizard myogenesis in vitro: a time-lapse and scanning electron microscopy study. *Dev Biol* 47:237–256, 1975.
7. Coleman JR, Coleman AW: Muscle differentiation and macromolecular synthesis. *J Cell Physiol* 72:suppl 1:19–34, 1968.
8. Cooper WG, Konigsberg IR: Dynamics of myogenesis in vitro. *Anat Rec* 140:195–206, 1961.
9. Cornell R: In situ observations on the surface projections of mouse embryo cell strains. *Exp Cell Res* 57:86–94, 1969.
10. Dalen H, Scheie P: Microextensions on Chang's liver cells as observed throughout their division cycle. *Exp Cell Res* 57:351–358, 1969.
11. Follett EAC, Goldman RD: The occurrence of microvilli during spreading and growth of BHK 21/c13 fibroblasts. *Exp Cell Res* 59:124–136, 1970.
12. Harris J, Watkins JF, Ford CE, Shoefl G: Artificial heterokaryons of animal cells from different species. *J Cell Sci* 1:1–30, 1966.
13. Holtzer H, Croop J, Dienstman S, Ishikawa H, Somlyo AP: Effects of cytochalasin B and colcemide on myogenic cultures. *Proc Natl Acad Sci USA* 72:vol 2:513–517, 1975.
14. Kessler-Icekson G, Singer RH, Yaffe D: The capacity of polyadenylated RNA from actinomycin D–treated myogenic cells to direct protein synthesis in a cell free system. Unpublished paper, 1978.
15. Lazarides E: Immunofluorescence studies on the structure of actin filaments in tissue culture cells. *J Histochem Cytochem* 23:501–528, 1975.
16. Lazarides E: Actin-a-actinin and tropomyosin interaction in the structural organization of actin filaments in non-muscle cells. *J Cell Biol* 68:202–219, 1976.
17. Lazarides E, Weber K: Actin antibody: the specific visualization of actin filaments in non-muscle cells. *Proc Natl Acad Sci USA* 71:2268–2272, 1974.
18. Lesseps RJ: Cell surface projections: their role in the aggregation of embryonic chick cells as revealed by electron microscopy. *J Exp Zool* 153:171–182, 1963.
19. Lipton BH, Konigsberg IR: A fine structural analysis of the fusion of myogenic cells. *J Cell Biol* 53:348–364, 1972.
20. Miranda AF, Godman GC, Tanenbaum SW: Action of cytochalasin D on cells of established lines. II. Cortex and microfilaments. *J Cell Biol* 62:406–423, 1974.

21. Mooseker MS: Brush border motility. Microvillar contraction in triton-treated brush borders isolated from intestinal epithelium. *J Cell Biol* 71:417–433, 1976.
22. Moss PS, Strohman RC: Myosin synthesis by fusion-arrested chick embryo myoblasts in cell culture. *Dev Biol* 48:431–437, 1976.
23. O'Neill CH, Follett EAC: An inverse relation between cell density and the number of microvilli in cultures of BHK 21 hamster. *J Cell Sci* 7:695–709, 1970.
24. O'Neill MC, Stockdale FE: A kinetic analysis of myogenesis in vitro. *J Cell Biol* 52:52–65, 1972.
25. O'Neill MC, Strohman RC: Changes in DNA polymerase activity associated with cell fusion in cultures of embryonic muscle. *J Cell Physiol* 73:61–68, 1969.
26. Paterson B, Roberts BE, Yaffe D: Determination of actin messenger RNA in cultures of differentiating embryonic chick skeletal muscle. *Proc Natl Acad Sci USA* 71:4467–4471, 1974.
27. Paterson B, Strohman RC: Myosin synthesis in cultures of differentiating chick embryo skeletal muscle. *Dev Biol* 29:113–133, 1972.
28. Pollard TD, Fujiwara K, Niederman R, Maupin-Szamier P: Evidence of the role of cytoplasmic actin and myosin in cellular structure and motility. In Goldman R, Pollard T, Rosenbaum J (Editors): *Cell Motility.* New York, Cold Spring Harbor Laboratories, 1976, pp 689–724.
29. Powell J: Development of normal and genetically dystrophic mouse muscle in tissue culture. *Exp Cell Res* 80:251–264, 1973.
30. Rajataman R, Rounds DE, Yen SPS, Rembaum A: A scanning electron microscopic study of cell adhesion and spreading in vitro. *Exp Cell Res* 88:327–339, 1974.
31. Richler E, Yaffe D: The in vitro cultivation and differentiation capacities of myogenic cell lines. *Dev Biol* 23:1–22, 1970.
32. Rubinstein J, Chi J, Holtzer H: Coordinated synthesis and degradation of actin and myosin in a variety of myogenic and non-myogenic cells. *Exp Cell Res* 97:387–393, 1976.
33. Sanger JW: The use of cytochalasin B to distinguish myoblasts from fibroblasts in culture of developing chick striated muscle. *Proc Natl Acad Sci USA* 71:3621–3625, 1974.
34. Shainberg A, Yagil G, Yaffe D: Control of myogenesis in vitro by Ca++ concentration in nutritional medium. *Exp Cell Res* 58:163–167, 1969.
35. Shainberg A, Yagil G, Yaffe D: Alteration of enzymatic activities during differentiation in vitro. *Dev Biol* 25:1–29, 1971.
36. Shimada Y: Scanning electron microscopy of myogenesis in monolayer culture: a preliminary study. *Dev Biol* 29:227–233, 1972.
37. Shimada Y, Fischman DA, Moscona AA: The fine structure of embryonic chick skeletal muscle cells differentiated in vitro. *J Cell Biol* 35:445–453, 1967.
38. Spudich J: Effect of cytochalasin B on actin filaments. *Cold Spring Harbor Symp Quant Biol* 37:585–593, 1972.
39. Taylor AC, Robbins E: Observations on microextensions from the surface of isolated vertebrate cells. *Dev Biol* 7:660–673, 1963.
40. Vertel BM, Fischman DA: Myosin accumulation in mononucleated cells of chick muscle cultures. *Dev Biol* 48:438–446, 1976.
41. Weihing R: Cytochalasin B inhibits actin-related gelatin of HeLa cell extracts. *J Cell Biol* 71:303–307, 1976.
42. Wessells NK, Spooner BS, Ash AF, Bradley MD, Luduena MA, Taylor EL, Wrenn JT, Yamada KM: Microfilament in cellular and developmental processes. *Science* 171:135–143, 1971.
43. Wolpert L, Gingell D: Cell surface membrane and amoeboid movement. In Miller F (Editor): *Aspects of Cellular Motility, vol 22, Cambridge Soc Exp Biol Symposium.* Cambridge, Cambridge University Press, 1968, pp 169–196.