*MYOGENESIS: FUSION, MYOSIN SYNTHESIS, AND THE MITOTIC CYCLE**

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Multinucleated skeletal muscle fibers form by the fusion of cells.[1-8] Whether myogenic cells fuse after or before synthesizing myosin and actin depends upon the age of the embryo. Both *in vivo* and *in vitro* mononucleated myoblasts from 3-day chick embryos show typical striated myofibrils. Later in development these myoblasts fuse to form multinucleated myotubes.[9] Mononucleated myoblasts are relatively rare in 11-day chick embryos. In the older muscle, synthesis of contractile proteins is confined primarily to multinucleated myotubes. When mononucleated myogenic cells, obtained by trypsinizing 11-day embryonic muscle, are cultured, myosin and actin are synthesized primarily after the cells fuse to form myotubes.[8, 10, 11, 13, 14]

Fusion is initiated by the recognition of homotypic cells, and is presumably mediated by unique molecular markers on the cell surface.[10] Recognition is followed by further interactions leading either to breakdown of the intervening membranes, as in phagocytosis, or to a more subtle rearrangement of membranes as may occur in fertilization.[12] Fusion occurs between mononucleated cells, between mononucleated cells and multinucleated myotubes, and between nascent multinucleated myotubes. Inspection of individual cells for evidence of DNA synthesis and myosin synthesis reveals that those mononucleated cells which incorporate H^3-thymidine fail to bind antibodies against myosin and actin. Conversely, mononucleated cells or multinucleated myotubes which bind antibody have diploid nuclei and fail to incorporate H^3-thymidine.[8, 10, 13, 33] These and related observations[10, 14] lead to the proposition that presumptive myoblasts repress pathways leading to DNA synthesis and withdraw from the mitotic cycle prior to translating for myosin. The experiments to be described support this view and suggest additional correlations between myogenesis and the mitotic cycle.

Materials and Methods.—Mononucleated myogenic cells were obtained from 3-day somites or 11-day chick breast muscle as described.[3, 8] One milliliter of the suspension was layered over coverslips with, or without, plasma clots, in Leighton tubes at concentrations ranging from 1.5×10^5 to 1×10^6 cells/ml. After varying periods the coverslips were removed and the adhering cells prepared for microscopy. Culture media consisted of either 8:1:1 (Eagle's MEM:horse serum:embryo extract) or 2:2:1 (Simm's salt solution:horse serum:embryo extract). Radioautography followed procedures described.[8, 10] Colchicine (10^{-6} *M*) in medium was used to arrest cells in metaphase. The presence or absence of myofilaments was determined with fluorescein-labeled antibodies against chick myosin[1, 5, 10] and with polarization optics.

In the following discussion "presumptive myoblasts" or "myogenic cells" denotes proliferating mononucleated cells which have not yet synthesized myosin. "Myoblasts" are mononucleated cells which have synthesized myosin. Cells with two

or more nuclei are "multinucleated myotubes." Only cells whose multinucleated state is beyond doubt are scored as fused.

Results.—Mononucleated cells from 11-day embryonic muscle cultured in 8:1:1 behave differently from those cultured in 2:2:1.[10] To define these differences better, aliquots from the same cell suspension were grown in each of the two media. The results of these experiments are shown in Figures 1 and 2 and Table 1. Mitotic activity is higher, rate of fusion greater, and the appearance of myofilaments earlier in cells reared in 8:1:1 as compared to cells reared in 2:2:1. Many cells go through more than one mitotic cycle in 24 hr. In two different experiments there were 2.0 and 2.3 times as many cells after 80 hr in culture in 8:1:1 as in 2:2:1. Note that though there are more cells in 8:1:1 cultures, the proportion of nuclei in myotubes to the total number of nuclei is the same after 120 hr in both series. Medium 8:1:1 in this period does not differentially promote mitotic activity of myogenic over the nonmyogenic cells (e.g., fibroblasts). With time, however, as mononucleated cells continue to proliferate and formation of new myotubes ceases, the proportion of nuclei in myotubes to the total number diminishes. The earlier appearance of myofilaments in 8:1:1 is probably due to the indirect consequence of the medium's promotion of mitosis and fusion, rather than to the direct stimulation of the synthesis of myosin and actin.

Many small myotubes in 2-day cultures contain nonstriated myofilaments which bind antibody and are positively birefringent. Myotubes in 3-day cultures may be over 2 mm in length, over 50 μ in girth, and contain hundreds of diploid, postmitotic nuclei within a common sarcoplasm. At this time striated myofilaments are observed with antibody-treated material as well as with the polarizing microscope. Both nonstriated and striated myofilaments are detected hours earlier with the antibody technique than with polarizing optics. Unlike cultures prepared from 3-day somites,[5, 8] cultures from 11-day muscle exhibit, during the first 3 days in culture, relatively few mononucleated, spindle-shaped cells which syn-

TABLE 1

RATE OF FUSION AND TIME OF APPEARANCE OF MYOFIBRILS IN THE TWO MEDIA (1.5×10^5 CELLS/ML)

Medium		Age of Culture When Fixed (hr)								
		15	26	37	49	61	73	96	119	140
8:1:1	Av. no. of nuclei in one myotube*	2.1 (2–10)	2.7 (2–8)	3.3 (2–12)	—	—	—	—	—	—
	Nonstriated myofibrils†	—	—	+	+	+++	(2 to more than 100)			
	Striated myofibrils†	—	—	—	—	++	+++++	+	+++	++++
								++	++	++++
2:2:1	Av. no. of nuclei in one myotube*	2.2 (2–10)	2.5 (2–7)	2.7 (2–12)	2.8 (2–8)	3.1 (2–12)	9.7 (2–34)	—	—	—
	Nonstriated myofibrils†	—	—	—	—	—	+	++	(2 to more than 100)	
	Striated myofibrils†	—	—	—	—	—	—	—	+++	+++
									++	+

* The average number is based on counts made of 200 myotubes.
† Observed under the polarizing microscope. + = approximately 25%; ++ = approximately 50%; +++ = approximately 75%; ++++ = approximately 100%.

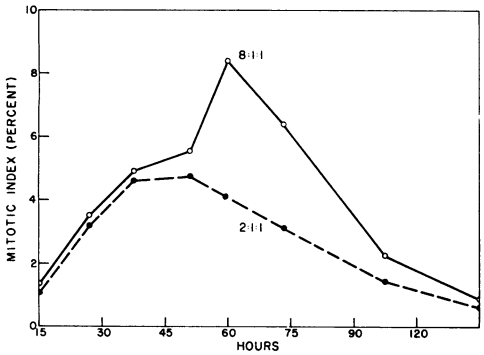

FIG. 1.—Comparison of the mitotic activity of cells grown in 8:1:1 with aliquots from the same cell suspension grown in 2:2:1; 1.5 × 10^5 cells/ml were plated on plasma clots. The mitotic index equals the number of cells in mitosis/total number of mononucleated cells × 100%. Each point is the average of five fields (100 cells/field) selected at random from four different coverslips. The decline in mitotic activity is due to the fact that these cultures were not fed after the 50th hr.

thesize myosin, or have birefringent myofibrils. Small numbers of elongated myoblasts do, however, appear in cultures 4–10 days old. *In vitro* myotubes differ from *in vivo* myotubes in that the former are relatively hypernucleated. The exceedingly rapid accumulation of myosin in *in vitro* myotubes may be a reflection of their hypernucleated condition.[10]

Often cells in mitosis adhered to, but were never incorporated into, myotubes. This observation led to testing the proposition that the activity of cell membranes required for fusion is blocked during cell division. Cells were exposed to colchicine for 4 hr, washed 3 times in normal medium, and then either immediately fixed or grown for an additional period in normal medium before being fixed. The increase in numbers of metaphase-arrested (MA) cells after removal from the colchicine (Table 2) confirms the observation that the inhibitor is probably bound by cells in interphase.[15] In all series MA cells were scattered throughout the cultures, but in not a single instance had an MA cell fused with another MA cell nor was an MA cell incorporated into a myotube. Large numbers of MA cells were collected by a 10-min trypsinization of 25-hr cultures which had been exposed to colchicine 10 hr

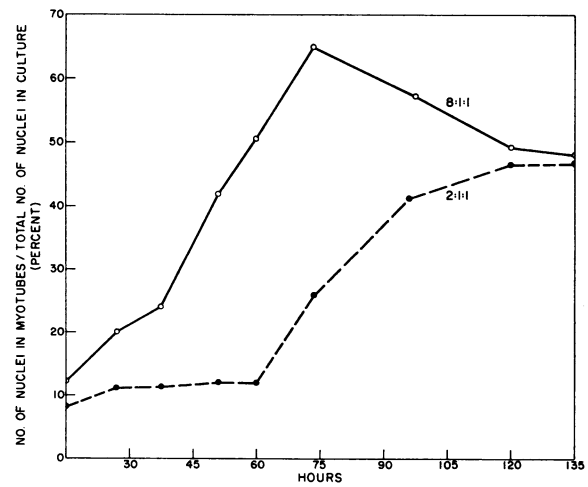

FIG. 2.—The same slides used for Fig. 1 were analyzed for their rate of fusion into myotubes. Two fields in the middle of each coverslip were selected, and the number of nuclei in myotubes per 1000 nuclei in or out of myotubes was counted.

TABLE 2

PER CENT OF MONONUCLEATED CELLS ARRESTED IN MITOSIS AFTER 4 HR EXPOSURE TO COLCHICINE

Age of culture treated with colchicine (hr)	Hours after Removal from Colchicine				
	0	4	15	24	48
15–19	3.3	8.6	86.2	81.4	3.1
	(2–5)	(7–11)	(72–93)	(76–88)	(2–5)
25–29	16.7	30.7	92.4	21.4	13.2
	(13–20)	(21–34)	(79–97)	(17–28)	(9–17)
40–44	5			73.7	3.5
	(1–7)			(61–82)	(2–6)
120–124	0.7	1.3		70.1	11.6
	(0–2)	(0–3)		(62–81)	(6–18)

* The fragmenting effects of colchicine on myotubes[24, 34] will be described elsewhere.

previously. Then 1×10^6 MA cells were spun down in centrifuge tubes into pellets. The pellets were placed in Leighton tubes 10 hr later and allowed to spread. Dense, mulberry-like clusters of MA cells were observed in these cultures 24 hr later, but the cells did not fuse. Fusion occurred in control cultures treated similarly, but not exposed to colchicine. In another experiment 1×10^6 MA cells were added to normal cultures 15, 30, 45, or 102 hr old. In not a single instance was an MA cell incorporated into the normal myotubes which formed in these mixed cultures.

The observation that nuclei in myotubes are diploid and do not incorporate H^3-thymidine and that cells in M (mitosis) are excluded from fusing led to testing the proposition that as cells begin to synthesize DNA (i.e., enter S), they are incapable of fusing and remain so until the following G1. Three-, 4-, and 5-day cultures were exposed to H^3-thymidine for 4 hr and immediately fixed. The duration of the pulse was based on the observation that the average S period for these cells is 5 hr, G2 1.2 hr, and M 1 hr.[16] Accordingly, the majority of labeled cells immediately following a 4-hr exposure should be in some phase of S, a smaller number in G2, and still a smaller number in M. The smallest number of labeled cells should have gone through M and entered the following G1. Labeled mononucleated cells were common. In some cultures as many as 45 per cent of all mononucleated cells were labeled, indicating a degree of synchrony of mitotic activity. In no instance was a labeled nucleus found within a myotube. This finding suggests that cells in S and G2, as well as in M, cannot fuse.

If cells in S, G2, and M cannot fuse, they must fuse in G1. To test whether cells fuse immediately after emerging from mitosis, the following experiments were performed: Early 3-day cultures were pulsed $^1/_2$ hr with H^3-thymidine and fixed either immediately or after 4, 8, 12, or 20 hr in cold medium. While a few labeled nuclei were in myotubes in cultures removed from H^3-thymidine 8 hr previously, many more were in the myotubes in cultures removed from the label 12 and 20 hr previously.[16] A minimum of 5–8 hr elapses between the end of the mitosis and the completion of fusion.

To correlate more rigorously the binding of antibody with stages of the mitotic cycle, the same cells were prepared both for radioautography and fluorescence microscopy or for radioautography and polarization microscopy. In both instances, though the fine cytology is somewhat obscured by the emulsion, it is possible to check whether an individual cell which has incorporated H^3-thymidine has bound antibody or has birefringent myofibrils. Cultures ranging from 2 to 8 days

were pulsed with H^3-thymidine for 30 min, 1 hr, 4 hr, or 6 hr. In no case in which a nucleus incorporated the isotope did the cytoplasm react typically with the antibody[25] or display birefringent myofibrils. Though the majority of labeled cells must have been in S, many must have been in G2 at the end of the incubation with H^3-thymidine. This suggests that cells in G2, as well as cells in S, were not synthesizing myosin.

Normal cells in mitosis and MA cells from all series were inspected for myosin and myofibrils. No normal cell in any stage of mitosis or MA cell bound the antibody or exhibited birefringent myofibrils. Interestingly, in the cytoplasm of MA cells there are striking circular or figure-eight bands whose birefringent properties, however, are different from those of myofibrils.

In another series of experiments cells from 3-day somites were exposed to H^3-thymidine for 30 min and then grown in excess cold thymidine. Cultures were fixed after 2, 6, 8, 10, and 12 hr. Cells with both grains above their nuclei and with antigen in their cytoplasm were not observed in the 2-, 6-, or 8-hr series but were found in the 10- and 12-hr series. Apparently the presumptive myoblast must pass through a "critical" division before one or both daughter cells are able to initiate the synthesis of myosin in the following G1.

Discussion.—One of the central problems in cell differentiation is the determination of how many different kinds of molecules a single cell can synthesize concurrently.[17, 18] A closely related problem is whether a cell can synthesize any of its molecules during any phase of the mitotic cycle. An old speculation, periodically revived, is that individual embryonic cells not only have the potential, but do synthesize many different kinds of molecules (e.g., a single cell makes concurrently myosin, DNA, chondroitin sulfate, hemoglobin, etc.), only to lose this metabolic versatility as the cells mature.[19] Experiments tracing myosin and actin synthesis in single cells in salamander and chick embryos do not support this view.[2, 4, 5] The experimental evidence is that normally only cells committed to myogenesis ever actually translate for myosin.

Interactions between cell surfaces leading to fusion are not expressed during S, G2, or M. One possible explanation is that as myogenic cells enter S, their surfaces undergo alterations which preclude participation in the events of fusion. This block is released only after the daughter cells enter G1. Whether this surface alteration is an adaptation unique to myogenic cells, or whether other cells undergo changes in their surfaces as they progress through the mitotic cycle, remains to be determined. Normal cells display different metabolic and surface properties in M from those exhibited in G1, S, or G2. It is not clear whether the blocking of fusion of MA cells is due to the physiological state of mitotic arrest or due to side effects of colchicine.[20–24]

An alternative explanation is that at no stage in the mitotic cycle can replicating presumptive myoblasts fuse. In the course of replication there must occur a "critical" division and then one or both daughter cells would withdraw from the cycle and acquire, for the first time, a cell surface compatible with fusion. How the acquisition of the capacity to fuse is correlated with the initiation for the translation of myosin is unknown.

The 5–8-hr period after mitosis, before fusion is completed, or before myosin is

detected, raises interesting questions. While the time for fusion probably varies, nevertheless in many instances it occurred in less than 2 hr. Assuming that fusion requires the last 2 hr of the 5–8-hr period, there are still 3–6 hr in G1 to account for. Is this 3–6 hr early in G1 obligatory, and is this the period in which the cell puts into effect its decision to withdraw from the mitotic cycle? This period in G1 might be required for the cell to differentiate a surface with the properties required for fusion. Last, this latent period in G1 might reflect the time required to activate and assemble the myosin-synthesizing machinery of the myoblast.

Myoblasts or myotubes differentiating in these cultures, or in clones from a single cell,[30] are derived from presumptive myoblasts or myogenic stem cells which are not themselves synthesizing myosin.[10, 33] There are now several reports of tumor cells *in vitro* transmitting their capacities to synthesize somatic molecules through *many* subcultures.[31, 32] It will be of interest if cloned, normal, myogenic stem cells can be propagated through *many* subcultures and continue to throw off cells which no longer divide, but which fuse and synthesize myosin and actin.

The reliability of many of the above interpretations rests on the sensitivity of the labeled-antibody technique in detecting myosin and on the capacity of the polarizing microscope to detect aligned molecules. A single cardiac[25] or skeletal[1, 10] myoblast is readily identified among thousands of presumptive myoblasts in 2-day chick embryos. The antibody does not react with nonmyogenic cells,[5] nor is it bound by mitotic spindle proteins,[25, 26] cilia, or flagella.[27] Myosin filaments have not been recognized under the electron microscope before they could be detected with fluorescence microscopy *in vivo* or *in vitro*.[28, 29] Indeed, myosin-containing cells are more readily identified with labeled antibodies than with EM microscopy, but this may be a problem of sampling.

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