Fusion between myoblasts and adult muscle fibers promotes remodeling of fibers into myotubes *in vitro*

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Summary

Muscle satellite cells are residual embryonic myoblast precursors responsible for muscle growth and regeneration. In order to examine the role of satellite cells in the initial events of muscle regeneration, we placed individual mature rat muscle fibers *in vitro* along with their satellite cells. When the satellite cells were allowed to proliferate, they produced populations of myoblasts that fused together to form myotubes on the laminin substrate. These myoblasts and myotubes also fused with the adult fibers. When they did so, the fibers lost their adult morphology, and by 8 days *in vitro*, essentially all of them were remodeled into structures resembling embryonic myotubes. However, when proliferating satellite cells were eliminated by exposure to cytosine arabinoside (araC), the vast majority of fibers retained their adult shape. Addition of C2C12 cells (a myoblast line derived from adult mouse satellite cells) to araC-treated fiber cultures resulted in their fusion with the rat muscle fibers and restored the ability of the fibers to remodel, whereas addition of either a fibroblast cell line or a transformed, non-fusing variant of C2C12 cells, or addition of conditioned medium from C2C12 cells, failed to do so. These results imply that myoblast fusion is responsible for triggering adult fiber remodeling *in vitro*.

Key words: satellite cells, myoblast, muscle fiber, myotube, fusion, rat muscle.

Introduction

Injured vertebrate skeletal muscle regenerates from its satellite cells, a reserve population of myoblast precursors located between the sarcolemma and the basal lamina of muscle fibers (Mauro, 1961; review, Campion, 1984). Additional evidence implicates satellite cells in the normal growth process in adult muscle (Campion, 1984), a process that apparently does not require an injury 'trigger'. Satellite cells proliferate in response to muscle injury as well as in the enlargement of already-existing adult muscle fibers. In an injury response, the resulting myoblasts fuse with each other to form new immature fibers called myotubes (reviews: Allbrook, 1981; Carlson and Faulkner, 1983). In the normal growth of mature fibers without injury, satellite cells fuse with the preexisting fibers (Moss and Leblond, 1971). Mature fibers that survive an injury may also participate in the regeneration process. Multinucleated sprouts, resembling embryonic myotubes, are often seen at the ends of mature fibers in histological sections of injured mammalian skeletal muscle (e.g. Clark, 1946; Walton and Adams, 1956; Roth and Oron, 1985). Gay and Hunt (1954) dissected individual sprouted fibers from partially transected rat muscles to demonstrate the continuity between sprouts and mature fibers, later confirmed by electron microscopy (Shafig, 1970; Ali, 1979; Roth and Oron, 1985). Based on examination of fibers at various times after injury, Gay and Hunt (1954) concluded that sprouts from fibers on both sides of a small cut eventually fuse together, completely bridging the site of the lesion.

The means by which muscle fibers sprout has not been determined. A sprout could be the result of one or more satellite-cell myoblasts fusing onto the end of a mature fiber. Myotubes arising from myoblast fusion secondarily fuse with mature fibers in injured bat web muscle (Church, 1970). Sprouting was not seen at cut injuries in rats following vinblastine injection, indicating that sprouting may involve cell division (Roth and Oron, 1985).

Another possibility is that some sprouts originate entirely from mature fibers by 'budding' (Shafig, 1970; Ali, 1979). This phenomenon would be difficult to distinguish from myoblast fusion in histological sections of injured whole muscles, but it was clearly demon-
strated by individual adult rat muscle fibers maintained in cell culture. Bischoff (1980, 1986, 1988) used the flexor digitorum brevis muscle, a small, multipennate muscle particularly well suited for dissociation and culturing due to the shortness of its fibers (0.4–1 mm). Collagenase digestion made it possible to separate the fibers from the connective tissue but left the fiber basal lamina and underlying satellite cells attached (Bischoff, 1979). Cultured mature fibers actively participated in sprouting, with small pseudopodial outgrowths appearing after about a week and filling with myonuclei. Many fibers eventually underwent complete dedifferentiation into structures resembling embryonic myotubes (Bischoff, 1980). When Bischoff (1980, 1986) treated some cultures with the antimitotic drug cytosine arabinoside (araC) to interfere with satellite cell proliferation, he indicated that araC had no effect on the behavior of mature fibers but presented no specific data.

Of course, fibers might sprout chiefly by one means in vivo and by another in vitro. However, the potential of both phenomena to occur together has been shown in culture, where myoblasts can fuse with a fiber already undergoing dedifferentiation (Bischoff, 1988). The nuclei within this muscle fiber would then originate from two different sources, preexisting myonuclei and satellite cell myoblasts.

Leaving aside the question of the relationship between muscle fiber behavior in vitro and muscle regeneration in vivo, the present cell culture study addresses the role of satellite cells in muscle fiber sprouting and dedifferentiation. We used araC to eliminate proliferating cells (including fibroblasts and any other residual cell types as well as satellite-cell myoblasts) from some of the cultures. Since myonuclei do not replicate (Stockdale and Holtzer, 1961; Shafiq et al. 1968; Bischoff and Holtzer, 1969; Pullman and Yeoh, 1978), myotubes and mature fibers are unaffected by araC treatment. We found that essentially all fibers in untreated cultures lost their adult morphology and acquired characteristics of embryonic myotubes. 

Preparation of rat muscle fiber cultures

Fibers were isolated from flexor digitorum brevis muscles of Wistar rats 4–12 months old through a modification of the procedures of Bekoff and Betz (1977), Jay and Barald (1985), and Bischoff (1986). After the animals were killed by CO₂ asphyxiation, the muscles were aseptically removed and incubated with 0.3 % collagenase (Sigma, type I, 1,830 collagene units mg⁻¹) in 5 ml of Dulbecco's PBS (Gibco) in a tube mounted on a tilted rotor at 37°C for 3 h. They were then transferred to D-PBS plus 5 % horse serum (M.A. Bioproducts), and bundles of fibers were carefully loosened from their tendons with fine forceps. To do this, the tips of closed forceps were inserted and spread between the softened tendons and the rows of muscle fibers. As much of the tendons and nerves as possible were removed, and the fiber bundles were triturated through a wide-mouthed pipet followed by a Pasteur pipet to yield individual fibers. These were collected by sedimentation through a standing tube of D-PBS plus 5 % horse serum. Remaining fragments of tendons and nerves as well as undissociated fiber bundles were picked out of the fibers under a dissecting microscope, and sedimentation was repeated. Typically ten to twenty thousand viable muscle fibers were obtained from each animal.

Fibers were plated on 35-mm tissue culture dishes (Falcon) that had been coated with polylysine and laminin (Sigma) as described by Foster et al. (1987) but with the laminin solution left on the dishes for 5–10 days at 4°C before rinsing with medium. Approximately 500–1000 fibers were placed in 1–1.5 ml of culture medium per dish. The medium consisted of Dulbecco's modified Eagle's medium (D-MEM, Gibco), 10 % horse serum (M.A. Bioproducts), 5 % chick embryo extract (Nishi and Berg, 1979), 2 mM glutamine, 1 % antibiotic–antimycotic solution, and 12 μg ml⁻¹ gentamycin (Gibco or Sigma). AraC (Sigma) was added to individual dishes from 100 x stock solution to give 10 μM. Cultures were maintained at 37°C in 5 % CO₂. Medium was changed on the cultures 4 days by careful pipeting under a dissecting microscope, in order to avoid detaching the fibers. For fiber counting, cultures were stained with 0.1 % trypan blue (Gibco) in D-PBS, rinsed in D-PBS, and observed with phase contrast at 100 x. Only fibers excluding the dye were counted.

Materials and methods

Cocultures of mouse myoblasts with rat muscle fibers

C2C12 myoblasts, a subclone (Blau et al. 1983) of cells originally derived from adult mouse muscle satellite cells (Yaffe and Saxel, 1977), were from a stock provided by Dr H. Blau, Stanford University. Non-fusing, ras-transformed C2C12 cells (Olson et al. 1987), designated C41, were provided by Dr E. Olson, University of Texas, Houston. Both of these cell lines were grown in D-MEM with 20 % fetal calf serum (Flow), 0.5 % chick embryo extract, and 0.1 mg ml⁻¹ kanamyacin (Sigma). Balb/c 3T3 fibroblasts, obtained from Dr M. Imperiale, University of Michigan, were grown in D-MEM plus 5–10 % horse serum and 1 % antibiotic–antimycotic solution. After aliquots of these cells were added to 4 day muscle fiber cultures, the cocultures were maintained in culture medium, which was replaced after 2 days. For identification of mouse cell nuclei, cocultures were stained with bisbenzimide (Hoechst 33258, Sigma) according to the procedure described by Blau et al. (1983). Bisbenzimide-stained cultures were observed with Leitz filter combination D (excitation 355–425 nm, transmission >460 nm). Mouse nuclei appear speckled due to the presence of A-T rich satellite
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DNA, while rat nuclei appear uniformly pale (Hardeman et al. 1988).

$[^3H]$thymidine labeling

$[^3H]$thymidine was added to individual culture dishes to a final concentration of 10 nCi ml$^{-1}$. To demonstrate fusion between satellite-cell myoblasts and muscle fibers in cultures not treated with araC, $[^3H]$thymidine was present continuously from the start of culturing. To determine whether any cells proliferated and fused with fibers beginning after the end of araC treatment, the label was added only after removal of the drug on day 4. For autoradiography, cultures were fixed in methanol:formalin 9:1, rinsed in distilled water, and air dried. They were coated with Kodak NTB2 emulsion by pipeting several drops onto the dishes, tilting them to cover the surface, and pipeting out the excess. After drying, they were exposed at 4°C for 2 weeks.

Results

Muscle fiber remodeling in the presence of satellite cells

Immediately after dissociation and usually for the first 2 days in culture, the muscle fibers retained their mature appearance, displaying prominent striations, peripheral myonuclei, and jagged myotendinous junctions. Mononucleated cells, presumably satellite cells, were attached to the surface of most fibers (Fig. 1). The satellite cells, as well as fibroblasts from remaining connective tissue fragments, began to migrate onto the laminin substrate after 1 day. These cells proliferated rapidly, and by 4 days, satellite-cell myoblasts had begun to fuse to form small myotubes (Figs 2, 3).

Also by 4 days, the appearance of the mature muscle fibers began to change to resemble embryonic myotubes. Vesicles appeared within the fibers, and portions of some fibers flattened onto the substrate yet remained striated. In many cases, mononucleated cells made contact with muscle fibers as though preparing to fuse with them (Fig. 3A). Myotubes that had formed from satellite cells fused with the fibers as well (Figs 2, 3B). Typically by 6-7 days, nearly all the muscle fibers were remodeled to various extents. Many entirely lost their mature appearance and fused into the network of myotubes forming from satellite cells. Myotubes derived from mature fibers were usually marked by clusters of large vesicles (Fig. 2) not stainable for lipid or for glycogen (data not shown). At the same time, some fibers had only a few vesicles or small myotube-like sprouts or small regions of flattening. Many fibers twitched extensively after 5-6 days in vitro. This appeared to contribute to adhesion failure, which led to detachment and rounding up of some remodeled fibers.

Muscle fiber remodeling thus generated a wide range of morphologies, from flattened sheets to thin tubes to rounded 'myoballs,' with many fibers being partly one shape and partly another (Fig. 2). By day 8, 97.5±0.75% (mean of 3 experiments±s.e.m.) of the viable muscle fibers in untreated, control cultures had become remodeled (Fig. 4). In all experiments, fibers were scored as remodeled if they were at least partially flattened onto the substrate, if they had sprouts containing one or more nuclei, or if they had rounded up and accumulated vesicles. Fiber contraction without vesicle formation, or the appearance of vesicles in an otherwise unaltered fiber were not considered sufficient evidence of remodeling.

In order to substantiate the assumption, based on observations of living cultures, that myoblasts fused with mature fibers, $[^3H]$thymidine labeling was used to track the nuclei of proliferating cells. In cultures exposed continuously to 10 nCi ml$^{-1}$ $[^3H]$thymidine for 8–10 days, muscle fibers remodeled as usual; following autoradiographic processing, these were all found to contain labeled nuclei (Fig. 5). At higher concentrations of $[^3H]$thymidine, cell proliferation and myogenesis were inhibited (data not shown), in agreement with the results of Levis et al. (1970) with embryonic rat myoblasts.

Fig. 1. Muscle fibers 1 day in vitro with apparent satellite cells (arrows) still attached. Phase contrast. Scale line=0.1 mm.
Fig. 2. Four representative adult muscle fibers (a–d) photographed at daily intervals from 4 or 5 days to 8 days in vitro. Arrowheads of graduated sizes follow single fibers throughout the time course (in a,b,d). Transition of arrowheads from small to large indicate that the fiber has remodeled. Note small myotubes (m), derived from satellite cells [see examples in a days 6 and 7], and vesicles (*s in c and d days 7 and 8] in remodeling fibers. Stars [see d days 7 and 8] indicate partially detached fibers forming 'myoballs.' The dark corners seen on some frames are from orientation marks drawn on the dishes. Phase contrast. Scale line=0.5 mm.

Effect of araC treatment
Proliferating cells were eliminated by exposure to 10 μM araC during the first 4 days in vitro. In these cultures, the extent of muscle fiber remodeling observed at 8 days was drastically reduced; only 2.5±0.45% (3 experiments) of the viable fibers in these cultures became remodeled to resemble myotubes (Figs 4, 6). Satellite cells were not found on the surface of araC-treated fibers that retained their mature appearance, although retention of satellite cells on a small fraction of such fibers cannot be ruled out. Some myoblasts and fibroblasts initiated mitosis after the removal of araC. To test whether myoblast proliferation and fusion were responsible for the remodeling of those araC-treated fibers that did so, araC was maintained in some cultures for the entire 8-day culture period and replenished after 4 days. With this protocol, 1.5±0.40% (2 experiments) of the fibers still remodeled. To examine further whether satellite cell proliferation following the removal of araC and subsequent myoblast fusion with muscle fibers might lead to fiber remodeling, 4 cultures that had been treated with araC for 4 days were exposed to [3H]thymidine for 4 days in the absence of the drug. None of the 53 fibers that remodeled in these cultures contained any labeled nuclei (Fig. 7), indicating that myoblasts had not divided and then fused with them during the labeling period.

Effects of myoblast addition to araC-treated cultures
As a test of the hypothesis that myoblast fusion can promote muscle fiber remodeling, exogenous myoblasts were added to fibers whose native, satellite-cell myoblasts had been largely eliminated by araC treatment. In cultures that received 10⁵ mouse C2C12 cells after araC medium was replaced with regular medium on day 4, muscle fiber remodeling was largely restored (Fig. 4). Within 1 day after their addition, the myoblasts were fusing with muscle fibers, and a few of the fibers began
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Fig. 3. Details of contacts between adult muscle fibers (af) grown without araC and other cells at 4 days in vitro. Note small myotubes (mt), points of contact or fusion between fibers and other cells (arrows), and a dead fiber fragment (*). Phase contrast. Scale line=0.1 mm.

spreading onto the laminin substrate. Often by 2–3 days of coculturing, many C2C12 myotubes had formed and were fusing with the rapidly remodeling fibers. In 4–5 day cocultures (i.e., 8–9 days in vitro for the fibers), a network of myotubes, consisting of both remodeled rat muscle fibers and C2C12 mouse myotubes, covered the dishes. Distinguishing between remodeled rat muscle fibers and newly formed C2C12 myotubes was difficult with phase contrast microscopy, but we estimate that at least 95% of the fibers remodeled. Fusion between the C2C12 cells and the muscle fibers was confirmed with bisbenzimide staining, which showed nuclei from both species mingled in the same myotubes (Fig. 8).

To control for the possibility that simply the presence of many proliferating cells, rather than fusion between the myoblasts and muscle fibers, was in fact responsible for fiber remodeling, the same number of non-fusing C41 mouse myoblasts was added to some araC-treated cultures in place of C2C12 myoblasts following removal of araC on day 4. Precise fiber counts were impossible due to the overgrowth by C41 cells, but clearly fewer than 5% of the fibers flattened onto the substrate, formed sprouts, or acquired large vacuoles as fibers did in untreated cultures (Fig. 4). However, approximately half of the viable fibers in cocultures with C41 cells became highly contracted, far more than in untreated or araC-treated cultures (data not shown). In another control experiment, 10^5 Balb/c 3T3 mouse fibroblasts were added to araC-treated rat muscle fibers on day 4. This too resulted in fewer than 5% of fibers remodeling after 8–9 days in vitro (Fig. 4), although, again, many more fibers were highly contracted than in untreated or in araC-treated cultures.

Another concern was that some diffusible factor from the C2C12 myoblasts, rather than their demonstrated
fusion with the muscle fibers, could be promoting remodeling. Therefore conditioned medium from C2C12 cultures, filtered and mixed 2:1 with fresh muscle fiber medium, was placed on muscle fibers after 4 days araC treatment. The number of fibers that remodeled in these cultures (4.05±0.60%, 2 experiments) was not significantly different (P>0.05, two-tailed t-test) from the number that did so in araC-treated cultures maintained in unconditioned C2C12 medium (2.15±0.15%, 2 experiments).

Discussion

Satellite cells from adult rat muscle promoted a striking remodeling of mature muscle fibers in this in vitro study. Over a period of about 1 week, most of the fibers maintained on laminin substrates under conditions that permitted proliferation and fusion of their satellite cells came to resemble embryonic myotubes. Identification of preexisting myonuclei within the remodeled fibers supports the idea that sprouted or remodeled regions of fibers can form by 'budding' from mature regions (Shafiq, 1970; Ali, 1979; Bischoff, 1980, 1986). At the same time, our results indicate that remodeling of muscle fibers may depend on satellite cell or myoblast fusion with the fibers.

Eliminating most of the mononucleated cells from the cultures by araC treatment greatly decreased the number of muscle fibers that remodeled into myotubes by 8 days in vitro. In this respect, our results appear to differ from those of Bischoff (1980, 1986), who reported that virtually all fibers eventually converted into myotube-like structures in both araC-treated and untreated cultures. However, this may be a function of the time in culture, since Bischoff maintained fibers in vitro for 3 weeks or longer. When the culture period for our araC-treated muscle fibers was extended beyond 2 weeks, we also noted changes in the morphology of many more fibers, including shrinkage and constriction, centralization and clumping of nuclei, and loss of striations (unpublished observations). Since these events were different from those observed in untreated cultures and their onset was substantially delayed, we consider them to represent a separate phenomenon, possibly denervation atrophy (Gutman and Zelena, 1962). Also, Bischoff (1980) exposed fibers to araC for only 2–3 days, possibly allowing more satellite cells to survive and then fuse with the fibers. Based on our results, we would expect that changes in muscle fiber morphology
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should be significantly delayed and protracted, as well as qualitatively different, in any thoroughly araC-treated cultures compared with untreated ones.

The finding that addition of exogenous myoblasts restored normal remodeling ability to araC-treated muscle fibers demonstrated that the fibers themselves were unaffected by araC. More importantly, it supports the hypothesis that satellite-cell myoblasts somehow promoted fiber remodeling in the untreated cultures. In the presence of either the native satellite cells or the added C2C12 myoblasts, fiber remodeling was associated with fusion between myoblasts and fibers. To control for the possibility that it was simply the presence of proliferating cells rather than myoblast fusion with fibers that was responsible for promoting fiber remodeling, either non-fusing C41 myoblasts or 3T3 fibroblasts were added to araC-treated muscle fibers instead. Neither of these cell types significantly promoted fiber remodeling, although static contraction of the muscle fibers was more prevalent in their presence. Another control procedure, the addition of conditioned medium from growing C2C12 cultures to araC-treated muscle fibers, also failed to restore remodeling ability to a statistically significant extent. Yet C2C12 conditioned medium enhanced fiber remodeling slightly, suggesting that diffusible factors from myoblasts may play some role. The fiber contraction seen in the presence of C41 or 3T3 cells also might well be caused by some factor released from those cells.

Our results raise fundamental questions about the maintenance of the differentiated state by adult muscle fibers, both in vitro and in vivo. Why did muscle fiber dissociation and culturing lead to their fusion with myoblasts, and why did fusion in vitro lead to such complete fiber remodeling? Fusion between satellite-cell myoblasts and mature muscle fibers in a variety of situations in vivo is not associated with fiber remodeling. Satellite cells proliferate and fuse with fibers during normal growth (Moss and Leblond, 1971), and fusion is accelerated during muscle hypertrophy (Schiaffino et al., 1976) or when satellite cells are propagated in vitro and then grafted back into a muscle (Lipton and Schultz, 1979), without noted loss of adult fiber morphology. The sprouting of fibers, accumulation of vesicles, fusion between myoblasts and fibers, and fusion between remodeled fibers that we observed in vitro is characteristically seen in vivo only following muscle injury (Clark, 1946; Gay and Hunt, 1954; Walton and Adams, 1956; Roth and Oron, 1985). Myoblast fusion may turn out to be a necessary but insufficient condition for the

Fig. 8. Portion of a muscle fiber cocultured with $10^5$ C2C12 mouse myoblasts for 4 days following araC treatment for 4 days. Rat myonuclei (arrows) are mixed with mouse myonuclei in the remodeled fiber. Bisbenzimide stained. Scale line=50mm.
remodeling of mature fibers, with denervation or disruption of normal tissue architecture also being required. Growth factors present in the serum and embryo extract that were used in vitro, or factors released from proliferating myoblasts and fibroblasts in vivo, may help to trigger remodeling. Our conclusion that myoblast fusion promotes cultured muscle fiber remodeling still remains to be explicitly tested in injured muscle in vivo, although the observation that vinblastine injection reduced fiber sprouting in rats (Roth and Oron, 1985) is supportive.

While these results demonstrate a major role for myoblast fusion in promoting morphological remodeling of muscle fibers in vitro, the mechanisms of remodeling and its control remain unknown. Myoblasts and myotubes arising from satellite cells certainly added nuclei and cytoplasm to the mature fibers. Yet the fibers also appeared to be active participants in the remodeling process, as they rapidly (sometimes less than 2 days) and often completely lost their normal morphology. This participation presumably involves structural gene products not normally expressed in mature fibers, and one possibility is that these genes might be expressed only by the newly added myoblast nuclei. We have found that the embryonic rat muscle cell surface antigen H36 (Kaufman et al. 1985) appears to become more abundant on remodeling fibers when native satellite-cell myoblasts are present than when C2C12 cells are added (unpublished observations), suggesting that the myoblast nuclei may be responsible for H36 expression. Alternatively, factors in the myoblast cytoplasm, or factors expressed by their nuclei after fusion, could influence gene expression by preexisting myonuclei. Such factors might be similar or identical to those that trigger muscle gene expression by non-muscle nuclei in mixed heterokaryons (review, Blau et al. 1989). Gene products characteristic of muscle fiber remodeling may of course be expressed both by preexisting and by newly added myonuclei. Studies using additional species-specific antibodies or nucleic acid probes for these gene products in cocultures of muscle fibers and myoblasts from different animals will distinguish among these possibilities. Even without reagents that are species-specific, it may be possible to visualize products expressed by individual newly added and preexisting myonuclei in the remodeled fibers, just as mRNAs (Harris et al. 1989; Fontaine and Changeux, 1989) and membrane proteins (Pavlath et al. 1989; Ralston and Hall, 1989) have been shown to be localized around individual nuclei in cultured myotubes. This is the first report of fusion between adult individual rat muscle fibers and a mouse satellite cell line. This novel culture system presents muscle biologists with a new model system for studies of gene expression, extinction, regeneration and possible recapitulation (or not) of molecular events in embryonic muscle development.

Although the present results provide strong evidence that myoblast fusion promoted rapid muscle fiber remodeling within the culture period examined, they do not address the question whether myoblast fusion is an absolute requirement for fiber remodeling. The observation that 2-3% of fibers in araC-treated cultures without added myoblasts underwent remodeling, and the observation that myonuclei of remodeled fibers exposed to [3H]thymidine following araC treatment were unlabeled, might suggest that some fibers remodeled in the absence of myoblast fusion. However, these results are not inconsistent with an absolute requirement for myoblast fusion in fiber remodeling, since satellite cells may have divided once during the several hours that elapsed between muscle dissection and addition of araC to the final cultures, thereby escape elimination, and subsequently fuse with the fibers. Satellite cell mitosis has been observed within 30 min after explantation of rat muscles into organ culture (Maltin, 1987), and many satellite cells can fuse following only one round of mitosis in injured muscles in vivo (Grounds and McGeeachie, 1987, 1989). Furthermore, it is entirely possible that some satellite cells fused with fibers in the presence of araC, not dividing even once. Approaches that facilitate identification of non-dividing satellite cells on araC-treated muscle fibers, and then correlate fusion between these cells and their fibers with subsequent fiber remodeling, will be needed to determine whether any mature fibers can in fact initiate remodeling independently of myoblast fusion.

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