

Skeletal Muscle Satellite Cells: Timelapse Videomicroscopic Evidence that Renewal is Stochastic

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Abstract

When skeletal muscle satellite cells replicate in response to tissue trauma, most of the progeny terminally differentiate, but a few cells resume the role of muscle precursors. The mechanisms that allow some cells to escape commitment while the majority of the cells differentiate are unknown. A murine cell line, MM14, derived from adult skeletal muscle satellite cells, was used to investigate the process of satellite cell renewal. Retrospective analysis of timelapse video data demonstrated that clonally-related daughter cells do not all respond similarly to low mitogen conditions. While most of the cells did commit to terminal differentiation when bFGF was withdrawn, a small subpopulation, comprising about 5% of the total cell population, continued to divide, albeit more slowly. These cells could be re-stimulated into rapid proliferation by bFGF, and, when bFGF was subsequently withdrawn, their progeny recapitulated both the majority *terminal differentiation* and the minority *self-renewal* phenotypes of the original clonal population. We interpret these results as evidence that stochastic mitotic asymmetries underlie the process of satellite cell renewal.

Key words: satellite cell, MM14, bFGF, timelapse, skeletal muscle, cell cycle, in vitro.

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Skeletal muscle tissue harbors an efficient, yet somewhat mysterious system of repair, the basis of which is a population of latent "precursor" cells which constitute a reserve of regenerative myogenic potential. These cells, termed "satellite" cells for their anatomical location adjacent to multinucleated myofibers [37] can, at propitious times, be stimulated to replicate, yielding not only progeny which terminally differentiate so as to abate the consequences of trauma [49-51], but also progeny which re-establish the satellite cell population [39]. While regulation of this process is undoubtedly multi-faceted, *in vitro* studies suggest that a key element is growth factor availability [1-3, 8-11, 13, 16-18, 21, 22, 27, 34, 43, 55, 56]. The general hypothesis is that quiescent satellite cells are stimulated to replicate when they are exposed to critical factors, and when one or more of these factors becomes limiting, replicating satellite cell progeny cease proliferation.

Anatomical evidence suggests that the satellite cell population, *in toto*, decreases during the lifespan of a species [44]. Consistent with this observation, clonal analysis has shown that the replicative capacity of satellite cells from older donors [48], and from tissue which has undergone repeated regeneration [47], is attenuated. Nevertheless, these reserve cells normally are never entirely depleted,

although, under conditions of chronic repetitive muscle damage such as in the muscular dystrophies, satellite cells may be in extremely low abundance [25, 54].

The mechanism(s) whereby new satellite cells are produced from a pool of replicating and differentiating daughter cells is not understood. For strategic reasons, including the difficulties of using an *in vivo* approach, we have studied this and related questions in a model *in vitro* system using the MM14 cell line which was clonally derived from an adult mouse skeletal muscle satellite cell. Preliminary results have appeared in abstract form [5].

Previous publications from this laboratory [12, 32] have shown that MM14 cells are absolutely dependent on exogenous growth factors, notably FGF, for continued replication. Upon removal of FGF from the culture medium, cells normally complete their current cell cycle and commit to terminal differentiation during the subsequent G1-phase. Commitment entails the activation of muscle specific gene expression as well as an irreversible withdrawal from the cell cycle with loss of the ability to proliferate in response to added FGF. Subsequent studies showed that committed cells lose cell surface receptors for EGF and FGF [30, 41], and that the loss is transcriptionally regulated [53]. These studies concentrated on the behavior of the

majority myoblast population and would not have identified a minority subpopulation which might "break the rules" by *not* terminally differentiating in response to growth factor deprivation.

In this report, we show by time lapse videomicroscopy of individual clones that, upon mitogen withdrawal, the majority of MM14 cells mimic the process of muscle repair by terminally differentiating. However, a small percentage of MM14 cells do *not* exit the cell cycle and differentiate; rather, this subpopulation continues to divide, albeit much more slowly. Even after multiple slow cycles in differentiation conditions, these cells can be stimulated by bFGF to resume rapid cycling and, upon mitogen deprivation, their progeny recapitulate the terminal differentiation response of the original clonal cohort. The slowly replicating phenotype, which is reminiscent of an *in vivo* satellite cell phenotype recently reported by Schultz [45], appears to maintain the replicative and myogenic potential of the original clonal population. We interpret the occurrence of these different cell behaviors to suggest that maintenance of the satellite cell phenotype is a stochastic process based on mitotic asymmetries.

Materials and Methods

Cells

The cell line, MM14, was derived from a clonally isolated satellite cell obtained from leg muscle of a 60-day-old BALB/c male mouse [25]. Primary myoblasts were obtained by dissociation of leg muscle tissue from young adult mice, as described by Ontell, *et al* [42].

Culture conditions

Both MM14 and primary myoblast cultures were grown on gelatin-coated surfaces. High density cultures were set up at 50,000 cells per 100 mm dish in 10 ml of Ham's F10 plus 0.8 mM extra CaCl₂ (F10C), supplemented with 15% horse serum (HS), 2 ng/ml bFGF (human recombinant bFGF; Zymogenetics, Inc., Seattle, WA) and 50 mg/ml Gentamicin Sulfate (Gemini Bio-Products, Inc., Calabasas, CA). Cultures were reseeded daily and bFGF was replenished twice per day. In this regime, two nanograms per ml bFGF was determined by dose-response experiments to support continuous mitogenic stimulation of the cells. Differentiation was initiated by rinsing the cells twice with Saline G and then re-feeding with "differentiation medium" containing 1.5% HS, 1 mM Insulin and no added bFGF.

High density cultures were passaged with 0.25% Trypsin and 2.7 mM EDTA (Gibco BRL, Gaithersburg, MD) and diluted appropriately for clonal studies. Clonal cultures for videotaping were set up at 50 cells per T-25 screw-cap flask (No. 25100; Corning Glass Works, Corning, NY) in 7.5 mls of F10C medium supplemented with bFGF (2 ng/ml) and 15% HS. Flasks were flushed with CO₂, capped and then placed in the 37°C microscope chamber (see below). Following medium changes and/or bFGF replenishments, the pH was quickly re-established by flushing the cultures with 5% CO₂ before resuming videotaping.

Timelapse video microscopy

Within an hour of plating cells onto a T-25 flask, the flask was scanned to find a single cell which had attached and which was isolated from other cells. Cells were magnified through a 10x objective attached to a Nikon Diaphot inverted microscope equipped with an extra-long working phase condenser (Nikon, Inc., Garden City, NY). The cells were imaged through a video camera (Series 65; Dage-MTI, Inc., Wabash, MI) equipped with a high resolution vidicon tube, and were videotaped with a timelapse recorder (model TLC2051R; Gyr Products, Anaheim, CA) for playback at 180x normal speed. During the videotaping, the cells were maintained in an incubator (Nikon, Inc.) attached directly to the microscope, and the temperature was regulated at 37°C by an automatic temperature control unit (Nikon, Inc.). The videotape was subsequently viewed on a monochrome monitor (model 925; Lenco, Jackson, MI). Although the data presented in this paper concentrate on a single clone, similar cell behaviors were recorded in many clones.

Retrospective analysis of the videotape was used to measure cell cycle durations by calculating the time between successive telophases. Terminal differentiation following a cell division was manifest either as fusion of the cell, detected during subsequent portions of the videotape, or, in the case of unfused cells, by myosin-positive immunostaining with the MF20 anti-myosin monoclonal antibody [6] following fixation of the culture or videotaped clone.

In order to relocate the clone after the medium conditions were adjusted, a sketch of the clone was first made on a plastic sheet taped to the monitor screen. Then a circle was drawn around the microscope light path where it intersected the top of the flask. Noting the orientation of the flask on the microscope stage, the flask was then removed. Using these landmarks, we could change the medium, and precisely relocate and reorient the clone within 5 min.

Results

A. A subpopulation of satellite cell progeny escapes terminal differentiation following growth factor deprivation.

Skeletal muscle satellite cells *in vivo* undergo proliferation cycles that lead to both tissue repair and self-renewal of the initial satellite cell population. Muscle repair requires extensive proliferation of small numbers of satellite cells followed by their terminal differentiation to replace the injured muscle fibers. Self-renewal requires that a small percentage of the expanded satellite cell-derived myoblasts re-enter a quiescent or slowly-dividing state under the same environmental conditions in which the majority of their clonal relatives are differentiating. Previous studies from our group have focused on understanding the proliferation and terminal differentiation process of a satellite cell-derived mouse cell line, MM14, but we have not determined whether these cells also exhibit a self-renewal process akin to that of satellite cells *in vivo*.

Analysis of the satellite cell self-renewal process *in vitro* requires the ability to detect a small fraction of cells that either become quiescent or continue cycling slowly under environmental conditions that cause the majority of cells to initiate terminal differentiation. In order to increase the opportunity of detecting such a rare response, and to facilitate quantifying its frequency, our initial studies were carried out with high density cultures. Replicating cultures of MM14 cells were induced to commit to terminal differentiation by a switch to "reduced mitogen" medium (see Materials & Methods). After up to 6 days (i.e., a much longer time than the approximately 15 hrs that is sufficient for inducing commitment in greater than 95% of the population [12, 33]), the cultures were passaged and replated at clonal density in complete growth medium. Any remaining replicative cells were thus free to divide, yielding clonal progeny which could be analyzed for their myogenic potential. Clones were detected, and when they had expanded to about 6 population doublings, their myogenic potential was assessed. The results indicated that, after as long as 6 days in reduced mitogen medium, 0.1 - 1% of the total cell population (prior to induction of commitment) were indeed capable of giving rise to myogenic clones (Table 1). Furthermore, while most of the myogenic clones contained *only* myosin (+) cells, most of which had also undergone fusion, approximately 15% of the myogenic clones also contained a small percentage of unfused, myosin (-) cells (Figure 1), an observation whose relevance to the satellite cell renewal process became more apparent after further experiments (see below).

Because MM14 cells are a permanent myoblast cell line which is being used as a model of how satellite cell progeny may respond to reduced mitogen conditions, it was important to establish whether *primary* adult muscle satellite cells behaved similarly. When mass cultures of replicating primary mouse skeletal muscle cells were switched to reduced mitogen medium for up to 6 days, a small percentage of this population likewise escaped commitment, were cloneable and were myogenic. Qualitatively, primary muscle cells responded similarly to MM14 cells (Table 1).

B. Clonal analysis of MM14 cells which exhibit the satellite cell renewal phenotype.

Because studies of mass cultures could not provide important details of individual cell behavior, such as cell cycle times and the frequency of asymmetric cell divisions, we utilized timelapse videomicroscopy to study individual clones. By retrospective analysis of the videotape, cell-cell relationships could be documented through a series of divisions, as could the behavior of each cell at precise times following changes of mitogen concentration. The experimental protocol shown in Figure 2 was used in generating the data presented in Figure 3. This protocol was designed to provide enough time between successive manipulations of the mitogenic environment for the cells to differentiate and/or generate small subpopulations of slowly-replicating cells, while at the same time preventing

Table 1. Cloneable Myogenic Cells Remaining after Mitogen Withdrawal. Replicating mass cultures of MM14 cells or primary muscle cells were induced to commit to terminal differentiation by switching them to @147reduced mitogen@148 conditions (see Materials & Methods). After 4 or 6 days, the cultures were passaged and the cells replated at clonal density in complete growth medium. When clone sizes reached about 6 population doublings, their myogenic potential was assessed by switching them to reduced mitogen conditions for 2 days, and then scoring them for myosin-positive cells (Materials & Methods). Between 0.1 and 1% of the total MM14 cell population and about 0.01% of the primary cell population were cloneable muscle cells (based on cell counts prior to mitogen reduction, and an estimate of 1 subsequent population doubling). In all myogenic clones, at least 50% of the cells were myosin positive. a) Previous studies have shown that reduced mitogen conditions lead to a proportional increase in on-defective@148 (DD) variants which are able to continue proliferating while most MM14 cells undergo terminal differentiation. retain low-level myogenic potential as indicated by the observation that only a small percentage (< 0.4%) of cells in such clones undergo muscle differentiation, and these appear about a week after being switched to low mitogen conditions [31]. Thus, the finding that 100% of the cloneable cells did not give rise to myogenic colonies is attributable to the probability that 20-40% of the cells were DDs. b) Primary muscle cultures had been enriched for skeletal muscle cells but still contained some fibroblasts. When subjected to low mitogen conditions, most of the myoblasts underwent terminal differentiation while the fibroblasts became quiescent. When passaged and subjected to the clonal assay, the fibroblasts gave rise to non-muscle clones.

Cell Culture	Days after Withdrawal of bFGF	% of the Cloneable Cells that are Myogenic
MM14		
Experiment 1	4	57 ^a
2	6	79
Primary Muscle Cells		
Experiment 1	4	55 ^b
2	6	60 ^a

the clone from becoming too dense to track individual cells.

The lineage of a clone whose growth, commitment and terminal differentiation were documented over a period of about 10 days is shown in Figure 3. This is one of more than 30 clones which we have analyzed by timelapse videomicroscopy. Its development is similar to that of the other clones we have videotaped, except that we have managed to follow most of the cells in this clone for an

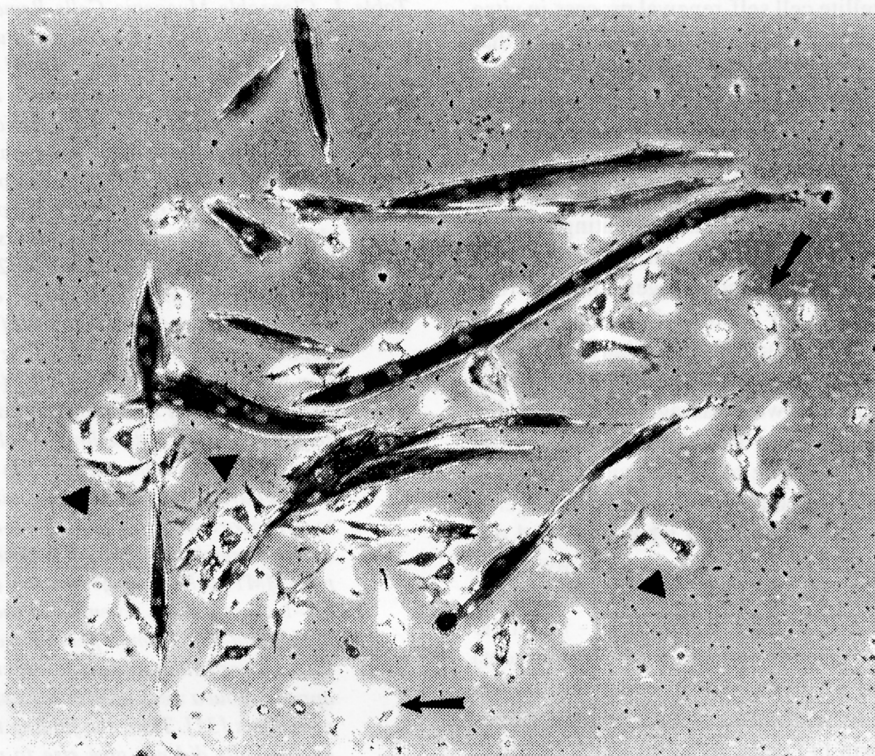


Figure 1. Some clonally-related MM14 cells do not terminally differentiate in low mitogen conditions. Clonal cultures were refed daily until the clones reached ~6 population doublings, and were then switched to differentiation medium. After 2 days, the cultures were stained with antibody to myosin heavy chain in order to evaluate the myogenic potential of each clone. Some clones contained only myosin-positive cells, while others (like the one presented in this Figure) contained both myosin-positive (darker staining cytoplasm) and myosin-negative cells. This particular clone was selected to illustrate a rare occurrence in which the myosin-negative cells were of two types. The small phase-bright cells (arrows) have the typical morphology of slowly dividing myoblasts while the larger, flattened stellate cells (arrow heads) have the typical morphology of @147differentiation defective@148 muscle cells (see Table 1 legend). Differentiation defective cells occur very infrequently, but the potential for their occurrence complicates the analysis of replicative cells within differentiated muscle cultures which are maintained under low mitogen conditions. Morphological differences between differentiation defective cells and slowly replicating myoblasts permit fairly reliable distinctions between the two phenotypes. However, slowly replicating myoblasts can be definitively distinguished based on their ability to form differentiated muscle cells when they are switched to FGF-containing medium for several cell doublings and then switched back to low mitogen conditions.

exceptionally long time (216 hrs), through 2 cycles of mitogen stimulation and deprivation.

As the clone expanded through 4 doublings in high-mitogen growth conditions, all of the cells remained in a growth cycle, as expected. The cells divided rapidly at a mean of 9.7 hrs per cycle (Figure 3, panel A & Table 2). Pairs of replicating sister cells exhibited remarkably similar cycle times - a mean percent deviation of only 4.6% was calculated from the combined data of 3 clones (24 pairs of sisters).

When the clone size reached 16 cells (35 hrs after the first cell division; see Figure 3, panel A), the clone was switched to differentiation medium (see Materials & Methods) in order to induce terminal differentiation. All 16 cells completed the cycle they were in, with no evidence of delay (mean cycle time = 10.4 hrs; Table 2). However, following

completion of this cycle, there were striking alternative behaviors among the 32 clonal progeny (Figure 3, panel B). Forty-four percent (44%) of the cells (14 of 32) terminally differentiated without further cell division (see example I in Figure 3, panel B). Of the remaining 18 cells, 9 (28% of the total) divided once more, producing 2 terminally differentiated daughters (see example II), and 2 cells (6% of the total) went through two additional doublings before *all* of their progeny were terminally differentiated (example V). In 5 cases, the fate of at least one cell per sublineage could not be assessed because the cell was lost from the microscope field (see examples IV and VI in panel B of Figure 3). However, even these sublineages contained myogenic cells. The most notable of the 32 cell cohort were the 2 cells (6%) which yielded progeny that continued to divide for 4 additional cycles (sublineages 1

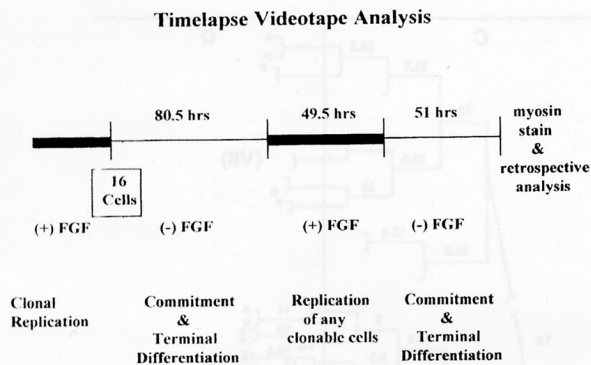


Figure 2. Protocol for a timelapse videomicroscopic experiment, the results of which are presented in Figure 3. The protocol was designed to permit clonal replication to 16 cells under conditions of optimal mitogen stimulation, followed by sufficient time in differentiation medium (80.5 hrs) for cells to either terminally differentiate or give an indication of their cycling behavior. Subsequently, any non-committed cells were re-stimulated with bFGF for enough time (49.5 hrs) to permit measurement of at least 2 cycle times, and these cells were then tested for their response to mitogen deprivation, as was done earlier in the experiment. At the end of the experiment, the clone was fixed and stained with antibody to myosin heavy chain in order to distinguish unfused cells which had terminally differentiated from cells which had not.

& 2; Figure 3) over a span of more than 3 days (80.5 hrs). Upon growth factor withdrawal, the cycle time of cells in these sublineages increased by about 85%, from a mean of 9.7 "Symbol" $\pm$ 0.5 hrs in growth medium containing bFGF to a mean of 18 "Symbol" $\pm$ 3.0 hrs in reduced mitogen medium (Table 2). This slow cycle time was not significantly different from that observed among all of the other 16 sublineages (17.2 "Symbol" $\pm$ 3.4 hrs) whose replicating progeny differentiated during the low mitogen period.

The few cells in sublineages 1 and 2 which continued to divide throughout the 3 day period of FGF deprivation appeared to be breaking the "rules" of terminal differentiation that had been inferred from previous end-point analyses of hundreds of MM14 clones [12]. However, the pooled clonal data from such studies would not have resolved such minor discrepancies from the norm. Additionally, since MM14 cells can give rise to differentiation-defective variants which have greatly reduced myogenic potential (see Figure 1 and the legend to Table 1), the small fraction of undifferentiated cells could easily be confused with such variants. It thus was necessary to establish whether these slowly-cycling cells in videotaped cultures were in fact myogenic.

To determine the myogenic potential of the cells which continued cycling under low mitogen conditions, the clone

was first switched back to high mitogen medium (15% serum; (+)bFGF; see Figure 3, panel C) for 2 days (from 115.5 - 165 hrs, total elapsed time) to stimulate rapid proliferation in the pool of putative satellite cell progeny (much as they might be stimulated *in vivo* during a regeneration response). At this time within the 2 sublineages of interest, there were 6 replicating cells whose complete clonal history would be known (see 1a-c and 2a-c; Figure 3; panel C). Other cells had either migrated from the field of view, or their exact relationship to the other cells in the clone became confused during cell movement. Upon restoration of high mitogen conditions, all 6 cells completed their cell cycles within the next 18 hrs. The mean time for the completion of each cell's current cycle after mitogen restoration (13.9 hrs; Table 2) was 3 hrs longer than the total mean cycle time of all subsequent cell divisions (10.8 hrs; Figure 3, panel C; & Table 2), suggesting that an initial lag time is required for readjusting to high mitogen conditions.

After 49.5 hrs (165 hrs total elapsed time), mitogens were again removed in order to assess the myogenic potential of the rapidly dividing progeny. During this second exposure to low mitogen medium (Figure 3, panel D), considerably more cells were lost from the microscope field than were lost during the first exposure to low mitogen medium (see Figure 3, panels B vs D) because the clone was so much larger. Twenty-four cells were documented as they completed division following the medium switch. The mean cell cycle time during the transition period was 11.9 hrs. (Figure 3, panel D; Table 2). Of the 48 progeny, 13 were lost from the field and their fate is unknown (see example VII, Figure 3, panel D). Of the other 35 cells, 17 (49%) terminally differentiated without further cell division; 7 (20%) divided one additional time prior to differentiation, yielding 2 terminally differentiated daughters; and 1 cell (3%) produced all terminally differentiated progeny after two additional divisions. These behaviors are comparable to the response of the earlier progenitor cell cohort (Figure 3, panel B) when they were subjected to low mitogen conditions (between 35 and 115.5 hrs). Likewise, one cell (about 3% of the sublineage 1b cohort) completed two additional doublings in low mitogen medium, generating a sister pair which did not stain for myosin ["M(-)"] when the experiment was terminated at 216 hrs (Figure 3, sublineage 1b, upper section). These cells had divided 17 hrs earlier and hence appeared to be expressing the same slowly-cycling, renewal phenotype which was illustrated previously in panel B for sublineages 1 and 2.

The cells which continue to cycle in low mitogen medium often produce daughters with non-identical behaviors: one daughter commits to terminal differentiation while the other continues to cycle. Following the switch to reduced mitogen medium at 35 hrs (Figure 3; panel B), 25% (4 of 16) of the divisions were "asymmetric" (see example VIII, Figure 3). Those cells which cycled again produced asymmetric progeny 35% of the time (6 of 17; see example V); and cells completing yet another cycle

In vitro model of satellite cell renewal

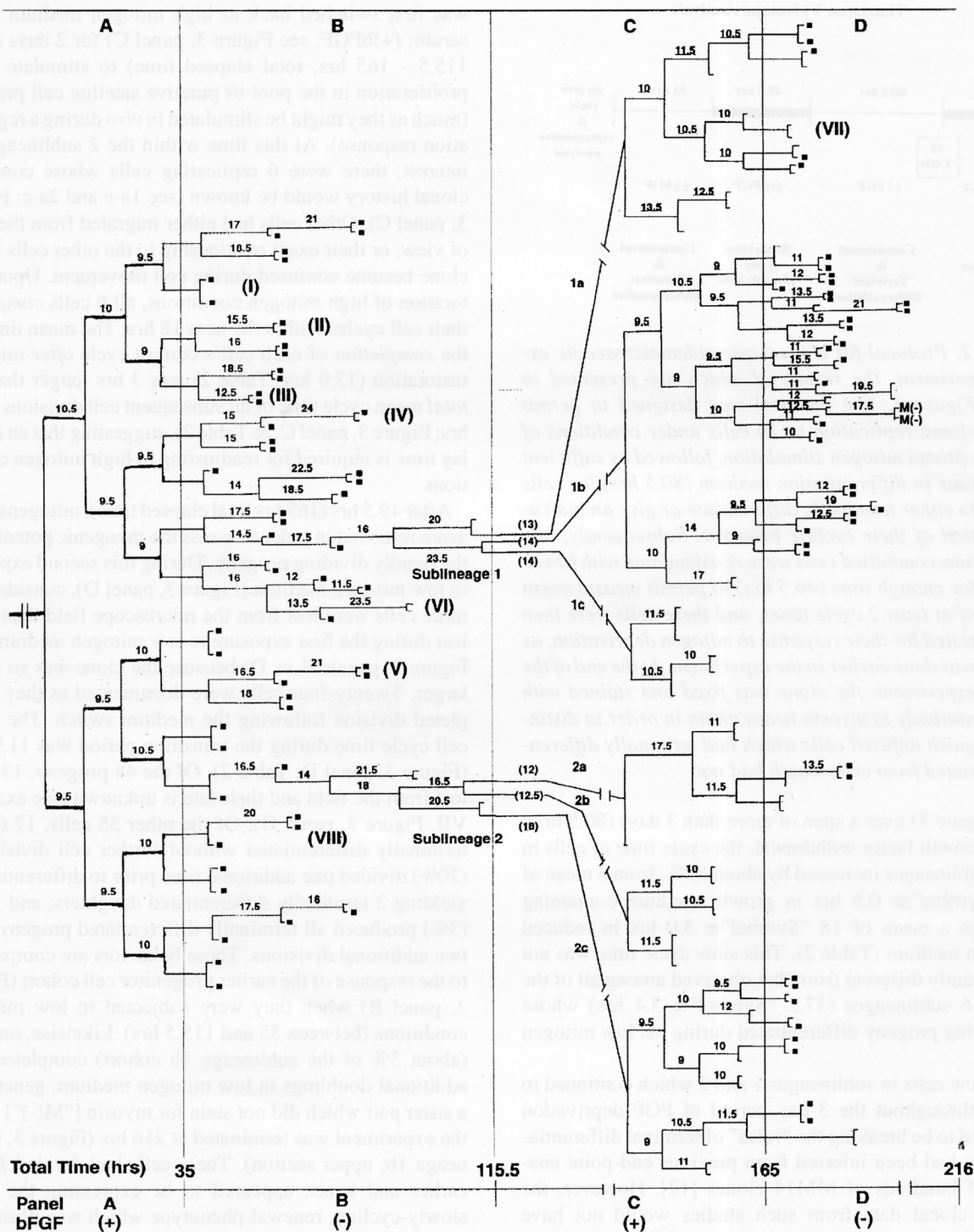


Figure 3. Clonal lineage of an MM14 cell. This clone was grown and subjected to two cycles of mitogen deprivation, as detailed in Figure 2. The videotape was retrospectively analyzed to determine cell cycle times, sister cell relations, and cell fusion. Cells which terminally differentiate, as determined either by their fusing or by their being myosin-positive at the end of the experiment, are indicated by filled squares (■); cells which were myosin-negative are designated as "M(-)"; cells whose subsequent lineage was indeterminate are designated by brackets which end without additional symbols. This designation was used for cells that either wandered out of the microscopic field or which crawled above or below another cell in a manner that made it impossible to distinguish which cell was which.

In vitro model of satellite cell renewal

Table 2. Intraclonal cycling behavior of MM14 cells through 2 rounds of mitogen modulation. These data are compilations of cell behaviors shown in Figure 3. Means $\pm$ standard deviations are shown. Numbers in parentheses are the number of documented intervals (i.e., cell cycle times or the times for completions of cell cycles). "Transition" cycles are those which span two media conditions. "Subsequent" cycles are those completed within one medium condition.

Culture Period	Cell Cycle Time
Initial clonal expansion (+) FGF	9.7 $\pm$ 0.5 hrs [14]
First mitogen restriction (-) FGF	
"transition" cycle	10.4 $\pm$ 0.7 hrs [16]
subsequent cycles	
sublineages 1 & 2	18.0 $\pm$ 3.0 hrs [11]
all other sublineages	17.2 $\pm$ 3.4 hrs [26]
Second mitogen stimulation (+) FGF	
completion time of	
"transition" cycle*	13.9 $\pm$ 2.2 hrs [6]
subsequent cycles	10.8 $\pm$ 1.9 hrs [41]
Second mitogen restriction (-) FGF	
"transition" cycle	11.9 $\pm$ 3.6 hrs [24]
subsequent cycles	13.4 $\pm$ 3.2 hrs [21]

*: This is *not* the total cycle time of these cells; it is the mean time for these cells to *complete* their current cycle after re-introduction of bFGF.

produce asymmetric daughters 43% of the time (3 of 7; see example VI). Subsequent cell divisions were exceedingly rare, and were mainly confined to sublineages 1 and 2. There were 5 documented instances of four successive divisions. In 3 of these cases, at least one daughter was lost from the field. Hence, it could not be determined if these divisions were asymmetric (see example VI and sublineage 2). Still, one daughter was terminally differentiated (see example VI), suggesting that some cells, if only a small percentage, can still commit after four successive cycles in reduced mitogen conditions. The two divisions whose progeny *could* be followed (1 each in sublineage 1 & 2) were both symmetric, producing only cycling daughters. Subsequent documented cell divisions were exclusively in sublineages 1 and 2, and none of the 6 progeny whose behavior could be documented (see: 1a-c & 2a-c; Figure 3; panel C) were terminally differentiated (see above).

To obtain additional data on the frequency of commitment by cells which cycle multiple times in low mitogen medium, we again turned to high density cultures. Mass cultures of replicating MM14 cells were switched to reduced mitogen medium according to the protocol in Figure 4. After 46 hrs, the cultures were pulsed for 2 hrs with 3H-TdR to label cells that continued to replicate under low mitogen conditions. The cultures were then incubated for an additional 22 hrs in reduced mitogen medium to allow time for labeled cells to divide and, if they had become committed, to terminally differentiate. When the cultures were myosin stained and then subjected to autoradiography, only 20% of the labeled nuclei were found in myosin (+) cells. These data are consistent with the clonal data, suggesting that cells which escape commitment but continue cycling in low mitogen conditions have a relatively low probability of terminal differentiation as long as low mitogen conditions persist.

Slowly-Cycling Cells: Frequency of Commitment

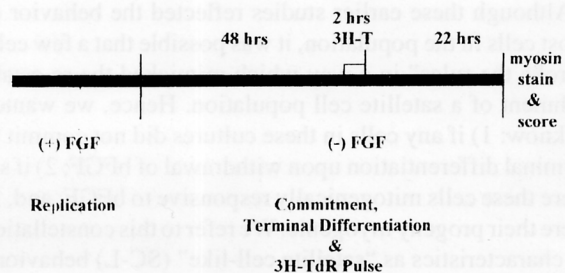


Figure 4. Protocol for analyzing the myogenic differentiation of slowly proliferating MM14 cells in high density cultures. MM14 cells were cultured on 100 mm plates in complete growth medium until the cell density reached 100,000. The cells were rinsed and refed with differentiation medium (see Materials and Methods). Forty-six hours later, 1 μ C/ml 3H-TdR was added for a 2 hr pulse. Upon removing the label and rinsing the cultures, differentiation medium was re-added and the cultures incubated for another 22 hrs. Depending on when mitosis occurred relative to the 3H-TdR pulse, the daughters of labeled cells had between 11 and 20 hrs to terminally differentiate before the end of the experiment. At the end of the experiment, the cultures were fixed and stained for myosin, and then autoradiographed. The probability that daughters from cells which were still proliferating (3H-TdR-positive) after 46 hrs in reduced mitogen medium would terminally differentiate was estimated by calculating the percentage of 3H-labeled nuclei which were in terminally differentiated (myosin-positive) cells.

Discussion

Skeletal muscle satellite cells [37] are reserve cells with myogenic potential which is periodically called upon to repair damaged tissue [49-51]. In the process, satellite cells are stimulated to replicate, producing a cohort of progeny which subsequently commit to terminal differentiation. Amazingly, and of great importance to the animal, not all of the progeny terminally differentiate. Rather, some cells are set aside, resuming the role of latent precursors in order to preserve replicative and myogenic potential in the event of subsequent muscle injury [39].

In order to study the processes underlying re-establishment of the skeletal muscle satellite cell phenotype, we developed an *in vitro* system using the well-studied satellite cell-derived line, MM14 [12]. These cells are myogenic both *in vitro* and *in vivo* [52]. Like satellite cell progeny from many sources [2, 8, 15, 18, 20, 21, 27, 34, 38, 55, 56], MM14 cells are stimulated to divide by bFGF. Previous reports from this lab have shown that, upon withdrawal of bFGF, MM14 cells cease replication and commit to a program of terminal differentiation [12, 32, 33]. In the process, the cells irreversibly lose their FGF receptors and are thus refractory to any subsequent exposure to this mitogen [41].

Although these earlier studies reflected the behavior of most cells in the population, it was possible that a few cells "broke the rules" in a way which mimicked the re-establishment of a satellite cell population. Hence, we wanted to know: 1) if any cells in these cultures did not commit to terminal differentiation upon withdrawal of bFGF; 2) if so, were these cells mitogenically responsive to bFGF; and, 3) were their progeny myogenic. We refer to this constellation of characteristics as "satellite cell-like" (SC-L) behavior.

Evidence for cells with SC-L properties was first obtained with high density cultures. Following an extended period of time in reduced mitogen medium (up to 6 days), 0.1-1% of the cells were still both cloneable and myogenic (Table 1). Similar results were obtained with primary muscle cells suggesting that the option to avert commitment under differentiation-promoting conditions may indeed be an opportunity available to satellite cell progeny *in vivo* [40]. The apparent quantitative difference between MM14 cultures and primary cell cultures in the percent non-committed muscle cells is probably largely attributable to contaminating fibroblasts in the latter. The presence of SC-L cells in MM14 and primary mouse muscle cultures is consistent with recent studies of human muscle cells in which self-renewing myoblasts could be identified within primary clones [7].

To obtain information regarding the genesis and subsequent behavior of individual cells displaying a SC-L phenotype, we turned to timelapse videomicroscopy of individual MM14 clones. By retrospective analysis of the videotape, cell-lineage relationships were documented through a series of divisions, as were the behaviors of cells at precise times following changes in mitogen concentration. In this report, we focus on an especially informative

clone, one which is atypical only in that we could document the behavior of many cells, over a 10 day period, through two rounds of mitogen stimulation and deprivation.

By analysis of the clone shown in Figure 3, it was determined that, as expected, most of the cells terminally differentiated when bFGF was removed. Also, there were *no* cells which stopped cycling when bFGF was removed and subsequently resumed proliferation when bFGF was restored. However, it was clear, even during the course of the experiment, that some cells continued to cycle slowly after mitogen reduction. Hence, while MM14 cells, under the culture conditions employed in this experiment, do not appear to model a satellite cell phenotype of quiescence [9, 46], they do appear to model the slowly-cycling satellite cell phenotype described by Schultz [45]. However it is not known whether satellite cells which seem to exhibit long-term quiescence *in vivo* actually enter this state after a series of longer cell cycles.

It was determined that the slowly-cycling MM14 cells were in fact myogenic (see below), but that they had only a low probability of committing to terminal differentiation (perhaps about 20%) unless first restimulated with bFGF. (Figure 3, panels C-D). Further insight into SC-L cell behavior was obtained by videomicroscopy.

Analysis of a representative clone growing in high mitogen medium (15% serum (+)bFGF) indicated rapid and near-synchronous cell cycles with a mean time of 9.7 hrs (Fig. 3, panel A; & Table 2). This rapid doubling time may at first appear inconsistent with the 12.5 hr mean doubling time previously reported for MM14 cells [12]. However, those data reflect the mean cycling time of many clones, and the calculated growth rate was subject to artifactual inflation due to cell loss via cell death and/or fixation and staining. Moreover, we have observed a range of mean cycling times among the various clones we have videotaped (range = 9.7 - 15.1 hrs; with a mean time of 11.8 ± 1.6 hrs calculated from the data of 17 such clones). To shorten the experiment time as much as possible, we endeavored to select for more rapidly dividing clones by immediately measuring the length of the first few divisions.

When this particular clone was challenged with low mitogen conditions at the 16 cell stage, all the cells completed their current cycle, but the behaviors among the ensuing 32-cell cohort differed markedly. Among these cells (Figure 3, panel B), about 95% either terminally differentiated or gave rise to daughter cells, granddaughter cells, or great-granddaughter cells which differentiated, while two cells (6% of the population) that did not commit went on to exhibit a slowly-cycling SC-L phenotype (Fig. 3; sublineages 1 & 2). Six percent may in fact be a lower estimate of the probability that MM14 cells can exhibit an SC-L phenotype. Five (of 32) cells, other than those giving rise to sublineages 1 & 2, were potential founders of SC-L lineages. These 5 cells begot lineages in which at least 1 cell, which may have been expressing a SC-L phenotype, was lost from the field. Example VI in Figure 3 perhaps

best represents this possibility: this sublineage contains cells which transited 4 successive cell divisions, some of them asymmetric, subsequent to mitogen reduction. Any of 4 "lost" cells in this sublineage might have continued cycling, thus establishing SC-L sublineage.

The 18 hr mean cell cycle time of the SC-L sublineages was significantly different ($P < 0.001$) from the 9.7 hr mean cycle time of the clonal progenitors (Table 2). Qualitatively, the behavior of the SC-L cells is consistent with strong *in vivo* evidence recently presented by Schultz [45] showing that at least some satellite cells, termed "reserve" cells, continue to divide slowly rather than become quiescent. Reserve satellite cells are hypothetical precursors of a more rapidly cycling population of "producer" cells which are competent to terminally differentiate after only a few divisions.

Schultz has proposed that producer cells are generated by asymmetric division of reserve cells, but the nature of such asymmetry is unknown. Our data indicate that when MM14 cells are switched to low mitogen conditions, there are many subsequent divisions (about 30% - 13 of 40; Figure 3, panel B) in which one daughter terminally differentiates while the other remains in cycle. In sublineages 1 and 2, there were 4 such asymmetric cell divisions documented among the first 4 doublings following the medium switch (Fig. 3, panel B). 3H-TdR labeling of high density cultures suggested that approximately 20% of the cells could commit after a series of slow cycles in reduced mitogen medium. From these data, we infer that MM14 cells in low mitogen conditions may invoke a specific program for slow cycling rather than commit to terminal differentiation. Once such slowly dividing myogenic sublineages become established, their relatively low frequency of differentiation leads to their gradual expansion. Consistent with this hypothesis, we have recently established conditions under which MM14 cells, as well as cells of another, independently-derived mouse satellite cell line, continue to proliferate for as long as 6 weeks following removal of exogenous bFGF. Yet, these cells retain the ability to terminally differentiate when passaged, regrown in FGF-containing medium and then switched to low mitogen conditions.

Following mitogen restoration, the mean cycle time of the SC-L MM14 cells decreases to that of the original clonal cohort. However, like their putative *in vivo* counterparts [9], SC-L cells exhibit a lag time to the resumption of rapid cycling following mitogen restoration (see the cells of sublineages 1 and 2, Figure 3, panels B-C; and Table 2). This results in the rapid accumulation of a second generation of progeny which are fully competent to commit to terminal differentiation (compare Fig. 3; panels B & D). Quantitatively, the second wave of commitment appears similar to the first. Following mitogen withdrawal, almost all of the cells which could be analyzed became terminally differentiated within 3 divisions. However, two cells ["M(-)" in Fig. 3; panel D; upper section of sublineage 1b] were still myosin-negative at the end of the experiment,

i.e., they were not terminally differentiated, even 17 hrs after cell division. We hypothesize that these cells were exhibiting a SC-L phenotype similar to that of the ancestral cells which gave rise to sublineages 1 and 2.

Our working hypothesis is that expression of the SC-L phenotype is triggered by mitotic asymmetries. We propose that mitotic asymmetries bearing on mitogenic responsiveness are common among satellite cell progeny dividing in mitogen-rich conditions; however, expression of a SC-L phenotype is "cryptic" unless mitogen concentration is limiting, such as when muscle clones are switched to "differentiation conditions".

The molecular basis of the asymmetries which allow mitotic sisters to respond differently within what would seem to be identical microenvironments is not known, but biochemical asymmetries have been reported in other developmental contexts [14, 26]. One likely class of candidates is growth factor receptors and their intracellular signal transduction pathways. According to this hypothesis, in most cell divisions there are only small asymmetries in the distribution of these components, leading to minor effects such as those responsible for the slight intermitotic time differences between most sister pairs. However, on a purely stochastic basis, cytokinesis may occasionally lead to even more disproportionate distributions of mitogen receptor / signal transduction components. Hypothetically, sister cells with functionally significant increases or decreases in at least one such component then initiate the SC-L slow-cycling response to mitogen deprivation. Once triggered by the appropriate mitotic asymmetry, the SC-L phenotype could include upregulation of FGF receptors [24, 28, 35, 36] and/or FGF synthesis [4, 19, 23, 29], as inferred from other satellite cell studies. These changes would, in turn, make it less likely that the slowly cycling cells within such renewal subpopulations terminally differentiate until their phenotypes were subsequently altered via re-exposure to high mitogen conditions. The molecular mechanisms underlying these alternative satellite cell phenotypes remain to be identified.

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