

Proliferative Dynamics and the Role of FGF2 During Myogenesis of Rat Satellite Cells on Isolated Fibers

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Abstract

Myogenic precursors in adult skeletal muscle (satellite cells) are mitotically quiescent but can proliferate in response to a variety of stresses including muscle injury. To gain further understanding of adult myoblasts, we are analyzing myogenesis of satellite cells on fibers isolated from adult rat muscle. In this culture model, satellite cells are maintained in their *in situ* position underneath the fiber basement membrane. Employing two different approaches to monitor proliferation of satellite cells on isolated fibers (autoradiography following ^3H -thymidine incorporation and immunofluorescence of cells positive for proliferating cell nuclear antigen (PCNA)), we show in the present study that satellite cells initiate cell proliferation at 12 to 24 hours following fiber culture establishment and that cell proliferation is reduced to minimal levels by 60 to 72 hours in culture. Maximal number of proliferating cells is seen at 36 to 48 hours in culture. These PCNA+ satellite cells transit into the differentiated, myogenin+ state following about 24 hours in the proliferative state. Continuous exposure of the fiber culture to FGF2 (basic FGF; added at the time of culture establishment) leads to a 2 fold increase in the number of PCNA+ cells by 48 hours in culture but the overall schedule of proliferation and transition into the myogenin+ state is not affected. Delaying the addition of FGF2 until 15 to 18 hours following the initiation of the fiber culture does not reduce its effect. However, the addition of FGF2 at 24 hours or later results in a progressive reduction in the number of proliferating satellite cells. Exposure of fiber cultures to transforming growth factor β (TGF β 1) leads to a reduction in the number of proliferating cells in both the absence or presence of FGF2. We propose that FGF2 enhances the number of proliferating cells by facilitating the recruitment of additional satellite cells from the quiescent state. However, satellite cells on isolated fibers conform to a highly coordinated program and rapidly transit from proliferation to differentiation regardless of the presence of FGF. The identification of agents that can prolong the proliferative state of satellite cells when the cells undergo myogenesis in their native position by the intact myofiber might be useful in improving myoblast transplantation into skeletal muscle for cell-mediated gene therapy.

Key words: myogenesis, satellite cells, proliferation, fibroblast growth factor, transforming growth factor β , PCNA, myogenin, myoblast transplantation.

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Satellite cells are situated between the basement membrane and the plasma membrane of the myofiber and are thought to be the primary source of myogenic cells in postnatal and adult skeletal muscle [8, 36; reviewed in 14, 27, 52]. Individual myofibers become encased by a basement membrane during late embryogenesis and it is at that stage that the distinction of satellite cells by their morphology and location is first possible [reviewed in 27, 63]. *In vivo* studies analyzing the incorporation of ^3H -thymidine into satellite cell nuclei and, subsequently, into fiber nuclei, indicated that at least some of the satellite cells are

proliferative in young animals, contributing additional nuclei to the fibers [40]. In the adult, satellite cells are mitotically quiescent but can reinitiate proliferative activity in response to injury, and their progeny eventually fuse into preexisting or new myofibers [15, 33, 50, 51, 54]. Muscle trauma involving fiber death and wound-related processes is not the only condition that leads to satellite cell proliferation; activation of these precursors also occurs in response to stresses such as stretch and compensatory hypertrophy, exercise, and denervation [4, 19, 55, 56, 61; summary in 3, 10, 52].

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Myogenic cultures of cells isolated from normal juvenile or adult postnatal muscle are commonly presumed to be cultures of satellite cells [1, 7, 67]. In such *in vitro* models the myogenic precursors become mitotically active upon culturing and their progeny eventually differentiate and fuse into multinucleated fibers [23, 30, 64, 66]. A number of growth factor families including fibroblast growth factor (FGF), insulin-like growth factor (IGF), transforming growth factor (3 (TGF β)) and platelet-derived growth factor (PDGF) have been shown to be involved in the proliferation and differentiation of myogenic cells in such cultures of tissue dissociated satellite cells [1, 2, 17, 18, 22, 28, 31, 60, 70; reviewed in 27, 63]. These cell culture studies contribute immensely to our current understanding of the regulation of satellite cells. However, the association between the satellite cells and their neighboring fibers is not maintained in these cultures. Such interactions between the satellite cells and the myofibers could be important for maintaining the satellite cell in a quiescent or proliferative state [11, 12]. Gaining further understanding regarding the regulation of satellite cell proliferation *in vivo* can provide further insight for improving the transfer of donor myoblasts into skeletal muscle for cell-mediated gene therapy.

We have adopted the rat single fiber system pioneered by Bekoff and Betz [6] and further developed by Bischoff [9, 10] in order to more critically evaluate myogenesis of adult satellite cells in their native position without the complexity of the intact tissue. In this model the fibers are isolated intact, retaining their few satellite cells in the original site underneath the basement membrane. These fiber-associated satellite cells can be induced to proliferate on the isolated fibers as was initially demonstrated by incorporation of ³H-thymidine and autoradiography [9, 10, 12]. More recently, we reported on the utilization of indirect immunofluorescence to trace myogenesis of satellite cells in the isolated fiber model [68]. We demonstrated that myogenesis of satellite cells in their native position follows a highly coordinated, multistep program of regulatory and structural protein expression. As the satellite cells enter the cell cycle their nuclei become positive for proliferating cell nuclear antigen (PCNA) [68]. PCNA is an auxiliary protein to DNA polymerase δ whose levels correlate with DNA synthesis during the cell cycle, becoming maximal during the S phase [5, 13]. We also analyzed the expression of the myogenic regulatory factor proteins MyoD and myogenin by the fiber associated satellite cells. MyoD and myogenin are members of the basic-helix-loop-helix family of myogenic transcription factors [20, 24, 62]. The four known members of this family are thought to be important in myogenic determination and in the progression from proliferation to differentiation during myogenesis [reviewed in 37, 41, 42, 59, 71]. We showed that nuclei of quiescent satellite cells and of the myofiber itself do not express detectable levels of MyoD or myogenin proteins but that activated satellite cells transit through a MyoD-myogenin expression program. In brief, our study demonstrated that

the PCNA+ satellite cells coexpress the myogenic regulatory factor protein MyoD and that this dual expression is transient - following about 24 hours in the PCNA+/MyoD+ state the cells become negative for both PCNA and MyoD but are found positive for myogenin. The myogenin+ state is also transient, diminishing after 24 hours. Upon transitioning into the myogenin+ state the satellite cells additionally become positive for smooth muscle α -actin and about 50% of these cells are detectable with an antibody against a developmental form of sarcomeric myosin. The expression of smooth muscle α -actin is transient and follows the same kinetics of myogenin expression but the expression of sarcomeric myosin is stably maintained [68].

We additionally demonstrated that FGF2 (basic FGF) can enhance the number of proliferating satellite cells (PCNA+/MyoD+ cells) without suppressing the subsequent transition of the cells into the differentiating, myogenin+ state [68]. Collectively, our study suggested that FGF2 may be involved in cell cycle entry and/or proliferation of satellite cells but that regardless of the presence of the mitogen, the cells rapidly withdraw from the cell cycle and differentiate into the myogenin+ phenotype. The present study was conducted to analyze in finer details the influence of FGF2 on proliferation of adult rat satellite cells in the isolated fiber model. The study was especially designed to analyze whether the response of satellite cells to FGF2 is limited to specific time in culture. We concluded that maximal response to FGF (as determined by the number of PCNA+ cells) can be reached if FGF2 is added no later than 15-18 hours following culture establishment. Also, a subpopulation of satellite cells remains (or just becomes positive for) PCNA+ at 72 hours in culture only if the cultures are exposed constantly (i.e., up to 72 hours) to FGF2. Taken together, the study supports the proposal that FGF enhances the number of satellite cells capable of undergoing proliferation. However, a prolonged maintenance of the proliferative state may require the addition (or removal) of yet unknown agents.

Methods

Animals

Adult rats (males, 8- to 10-week old, Sprague-Dawley) were used throughout the study.

Isolation and culture of rat muscle fibers

Single muscle fibers with associated satellite cells were prepared from the flexor digitorum brevis (FDB) muscle of the rat hind feet as we previously described [68]. Typically, muscles from both hind feet of two rats were used for each preparation. The outer connective tissue was removed and the muscles were immersed in 10ml solution of 0.2% collagenase type 1 (Sigma, St. Louis, MO; resuspended in Eagle's minimal essential medium (MEM)). Digestion was for 2 to 3 hours at 37°C without agitation. The collagenase treated muscle was transferred into MEM containing 10% horse serum, teased, and further triturated with a wide-mouth Pasteur pipet in a similar way to that

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described by Bischoff [9, 10]. Fibers were then allowed to settle at 1 xg for 15 min at room temperature through 10 ml of MEM containing 10% horse serum in a 15 ml Sorvall polycarbonate tubes. Fiber precipitation was repeated a total of three times to free the fibers from debris and connective tissue cells liberated by the digestion and teasing. Final fiber sediment, in residual medium (about 3 to 3.5 ml), was aliquoted into fifty five to sixty five 35-mm tissue culture plates coated with 0.1 ml of Vitrogen solution made isotonic (pH 7.0) by the addition of 1 vol of 7X DMEM to 6 vol of stock Vitrogen 100 (Celtrix Laboratories, Palo Alto, CA). About 50 μ l of fiber suspension, dispensed using a trimmed, wide mouth micropipet tip to prevent fiber breakage, was added to the center of the plates immediately following Vitrogen coating. Plates were gently swirled to allow even spreading of fiber aliquots throughout the plates and incubated for 20 min at 37°C to allow formation of Vitrogen gel and adherence of fibers to the matrix. Cultures then received 1.0 ml of basal medium which consists of MEM containing 20% Controlled Process Serum Replacement (CPSR2, Sigma) and 1% horse serum (Sigma; preselected for supporting clonal growth of chicken myoblasts, for details see ref. 70). Medium (\pm FGF2) was replenished daily to allow adequate supply of the reagents. Human recombinant, yeast-produced FGF2 was kindly provided by Dr. S. Hauschka (Dept. of Biochemistry, University of Washington, Seattle, WA) and was added to the medium of the fiber cultures at 2 ng/ml. Higher FGF2 concentrations in the range of 5 to 10 ng/ml had similar mitogenic effect on satellite cells in the intact fiber system as those obtained with 2 ng/ml FGF (data not shown). In some experiments we examined the effect of TGF β 1 on proliferation of satellite cells in fiber cultures. TGF β 1 was from Genzyme Corporation (Cambridge, MA) and was added to the basal medium (\pm FGF2) at 10 ng/ml. As for the FGF studies, medium and growth factors were replenished daily.

Immunolabeling

Immunolabeling of fiber cultures was performed using indirect immunofluorescence as previously described [68]. Cultures were rinsed with MEM at room temperature, fixed for 10 min at 4°C with ice-cold, 100% methanol, and air dried at room temperature for 10 min. Cultures were then kept at 4°C, in sterile Tris-buffered saline containing normal goat serum (TBS-NGS; 0.05 M Tris, 0.15 M NaCl, 1% normal goat serum, pH 7.4) to block non-specific antibody binding. Following a minimum of 24 hr in TBS-NGS, fiber cultures were rinsed (3X) with Tris-buffered saline containing Tween 20 (TBS-T20; 0.05 M Tris, 0.15 M NaCl, 0.05% Tween 20, pH 7.4). Cultures were then incubated with the primary antibody (listed below) for 1 hr at room temperature and the incubation was continued overnight at 4°C. Cultures were then rinsed (3X) with TBS-T20 and incubated at room temperature for 1-2 hr with the secondary antibody. Secondary antibody was fluorescein-conjugated rabbit anti-mouse IgG (obtained from Or-

ganon-Technika Cappel, Downington, PA) and was diluted 1:100 with the blocking buffer TBS-NGS. Following staining with the secondary antibody cultures were rinsed with TBS-T20 and nuclei were counterstained with DAPI as previously described by us [68]. Cultures were finally rinsed again with TBS-T20 and mounted in VECTASHIELD mounting medium from Vector Laboratories (Burlingame, CA).

Primary antibodies

The following primary antibodies were used to study fiber cultures:

(a) A mouse monoclonal antibody against PCNA (mAb 19F4; from Boehringer Mannheim, Indianapolis, IN). The antibody was diluted 1:100 in TBS-NGS.

(b) A mouse monoclonal antibody against rodent myogenin (mAb F5D, hybridoma supernatant form) which was developed and provided by Dr. W. Wright, University of Texas.

Further discussion regarding the characteristics of the two antibodies, demonstration that the antibodies stain nuclei of satellite cells but not of the myofiber itself, and immunofluorescent photographs of fibers stained with the antibodies are included in our previous study [68]. Figure 1 in this paper shows examples of immunostaining with the two antibodies.

Autoradiography

Detection of proliferating satellite cells on isolated fibers by their incorporation of ³H-thymidine and subsequent autoradiography was done in a similar fashion to what was previously described [9, 66]. Cultures were exposed to ³H-thymidine (0.5 μ Ci/ml final concentration, 6.7 Ci/mmol, NEN, Boston, MA) during the last 12 hours prior to fixation. At the end of the radiolabeling period cultures were rinsed with MEM and fixed with methanol as for immunofluorescence. Cultures were then coated with NTB2 emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with water. Cultures were stored in the dark for five days at 4°C and developed with Dektol (Eastman Kodak). Figure 2 shows examples of fiber cultures visualized via autoradiography for ³H-thymidine-labeled cells.

Counting positive cells on isolated fibers

Fibers were monitored for the number of nuclei positive for the different antibodies or for nuclei which incorporated ³H-thymidine as seen by autoradiography. Counting of fiber cultures was done using a 25x objective for immunostaining and a 40x objective for autoradiography. Two parallel 35-mm plates were used for each time point analyzing 30 fibers per plate. Total positive cells for 30 fibers was then averaged for the duplicate plates. This value is eventually expressed per 10 fibers as shown in the Figures and Tables. Observations were made with a Zeiss microscope equipped for epifluorescence, and Kodak EL 135 film (400 ASA) was used for photography.

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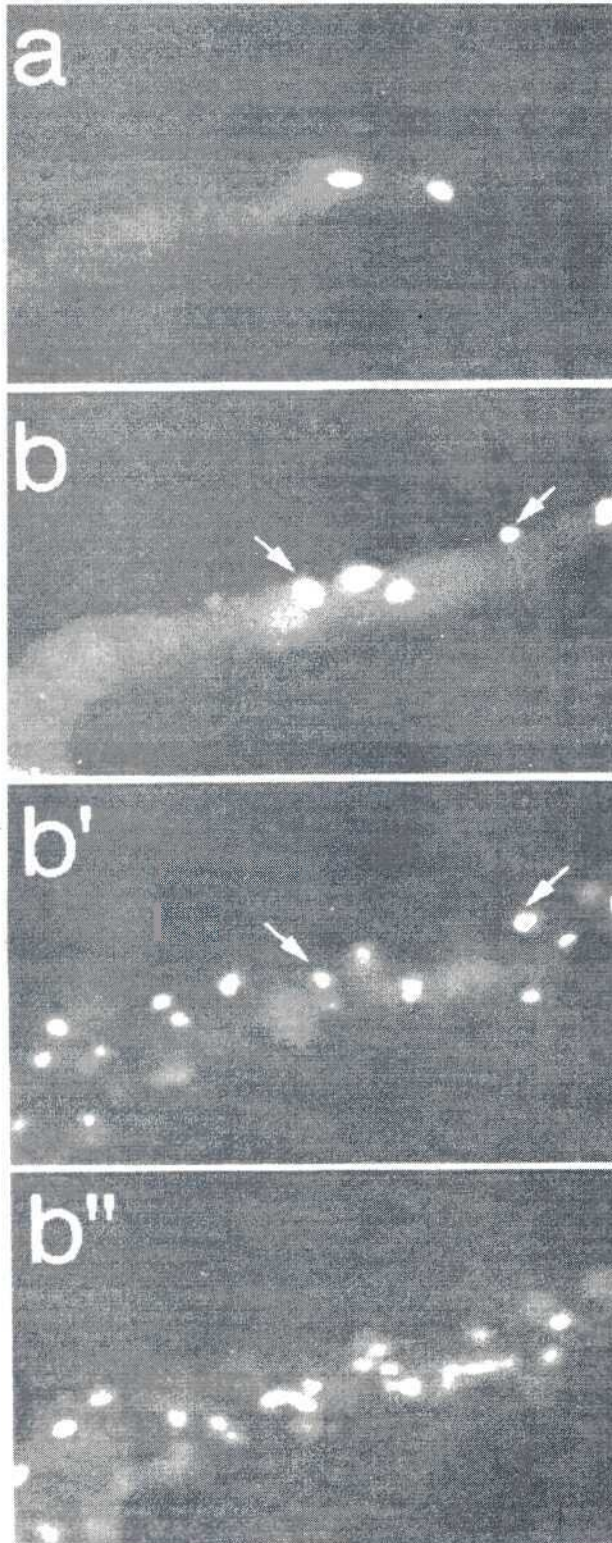


Figure 1. Representative micrographs of cultured fibers isolated from 8- to 10-week-old rats and reacted via indirect immunofluorescence with antibodies against PCNA (a) or myogenin (b). Micrographs in panels b' and b'' show the same fiber as in panel b counterstained with DAPI to visualize myofiber nuclei. Micrograph in panel b' was taken at the same focal level as for panel b while micrograph in panel b'' was taken at a different focal level to show additional myonuclei. Arrows in panels b and b' identify the same nuclei in the two different micrographs. Cultures used for the micrographs vary from 2-day old cultures (panel a, anti-PCNA) to 3-day-old cultures (panel b, anti-myogenin). A magnification of 40x was used for all micrographs.

Results

Monitoring proliferation of satellite cells on isolated fibers

Two methods were used to detect proliferation of satellite cells on fibers maintained in basal medium. One approach utilizes autoradiography of satellite cells following incorporation of ^3H -thymidine (see Figure 2 for micrographs of radiolabeled cells). Figure 3 summarizes our quantification of the ^3H -thymidine-labeled cells when fiber cultures were exposed during different times in culture to ^3H -thymidine for 12 hours and subsequently fixed at the end of 12-hour pulse and processed for autoradiography. Labeling periods (hours in culture following culture establishment) were: 0-12, 12-24, 24-36, 36-48, 48-60 and 60-72 and are shown in Figure 3. The increase in proliferating cells begins within the 12-24 hour time point. Maximal proliferation is detected in the 24-36 and the 36-48 hour time points. A decline in proliferation is observed in the 48-60 hour label period and further decrease is seen in the 60-72 hour label period. A second approach for monitoring proliferating cells utilizes immunodetection with an antibody against PCNA (see Figure 1 for a micrograph of PCNA+ satellite cells associated with the myofiber). In this approach all fiber-associated nuclei positive for the antibody at the different time points are recorded. Results of such an analysis when fibers are maintained in basal medium are summarized in Figure 4. This immunodetection of proliferating cells demonstrates that the increase in cell proliferation begins by 24 hours in culture and that maximal number of PCNA+ cells is detected between 36 and 48 hours in culture. Subsequently, by 72 hours in culture there is a sharp decline in the number of proliferating (PCNA+) cells. Both approaches for detection of proliferating satellite cells yielded similar conclusions regarding the kinetics of satellite cell proliferation in basal medium. Given the ease of the immunoanalysis, we further used this method in our experiments on the role of FGF2 during proliferation of satellite cells on isolated fibers.

FGF2 enhances proliferation of satellite cells

Figure 5 summarizes our analysis of satellite cell proliferation on fibers maintained in basal medium in the absence and presence of FGF2 (open and shaded symbols, respectively). The results in Figure 5 are based on an individual experiment and have been reproduced by us in multiple experiments (some of which are summarized in Tables 1 and 3). The continuous exposure of the fiber cultures to FGF2 (2 ng/ml) results in about a 2-fold increase in the number of PCNA+ cells by 48 hours in

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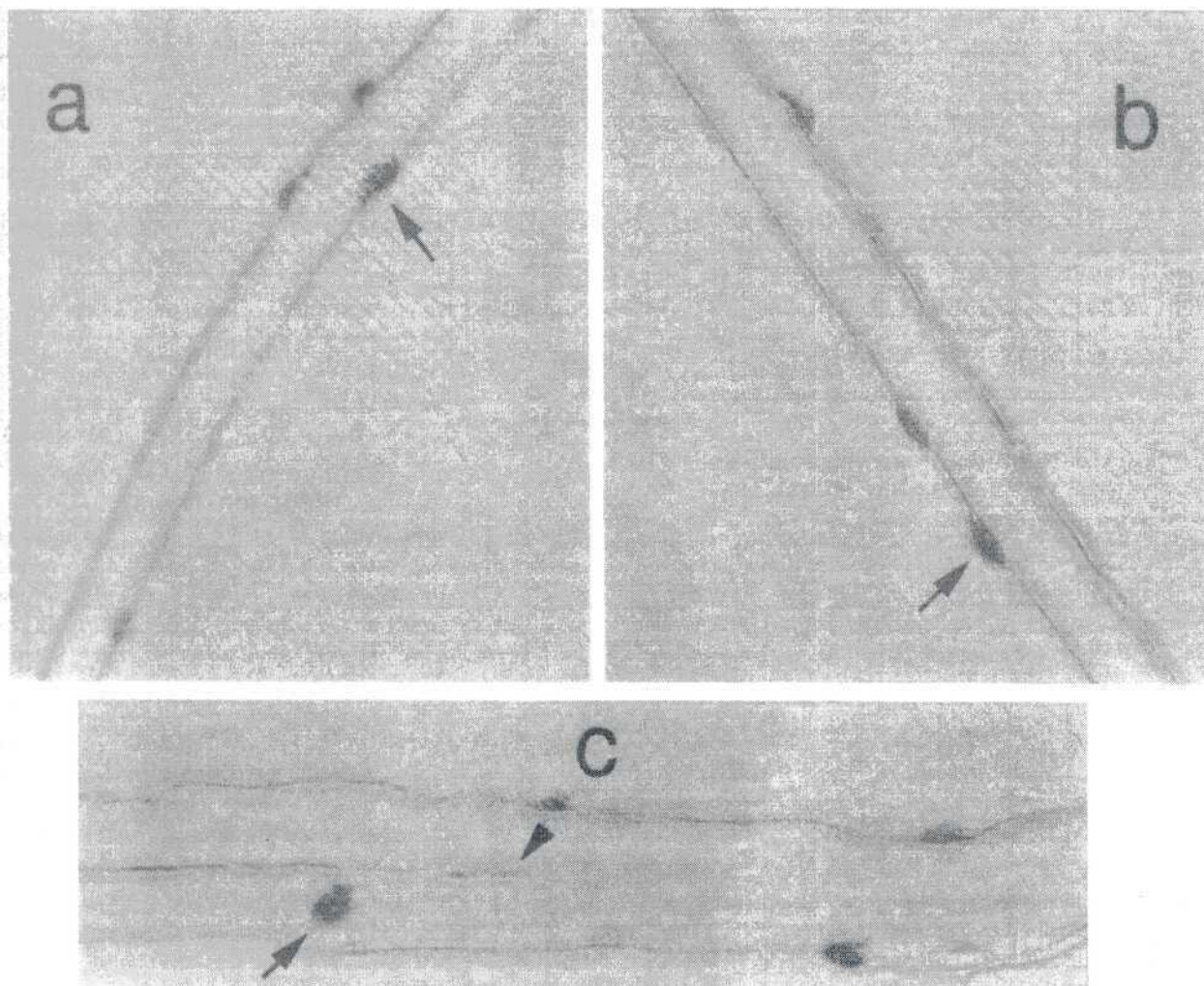


Figure 2. Autoradiographs of single fibers in cultures fixed at 48 hours. Cultures were maintained in basal medium and received ^3H -thymidine during the last 12 hours prior to fixation. Arrows point to various positions of ^3H -thymidine-labeled nuclei. Note the split fiber shown in panel c; the branching point is indicated with an arrowhead. Such split fibers are infrequently detected in the cultures. A magnification of 40x was used for all panels.

culture. By 72 hours in culture there is a decline in the number of PCNA+ cells regardless the absence or presence of FGF2. However, at the 72 hour time point there is still a far higher number of PCNA+ cells in the FGF-treated cultures compared to the cultures maintained in the absence of FGF. This reproducible, 2-fold increase in the number of PCNA+ cells induced by FGF2 might indicate that FGF2 allows the satellite cells to transverse through one additional round of cell cycle. Alternatively, FGF2 might increase the number of satellite cells which enter the cell cycle.

Influence of delayed exposure to FGF2 on satellite cell proliferation

We were interested in determining whether there is a critical time point by which FGF2 needs to be presented to the fiber cultures in order to facilitate the increase in PCNA+ cells shown in Figure 5. Routinely we add FGF to the cultures upon the initial establishment of the fiber cultures (i.e., the time when the fiber cultures receive the

basal medium which follows the initial 20 minutes of fiber adherence to the Vitrogen). However, to resolve whether there is such a critical time, the addition of FGF2 to the basal medium was delayed to progressively later time points as summarized in Table 1. The three experiments included in Table 1 indicate that the addition of FGF 24 hours following culture establishment results in a lower number of PCNA+ cells at 48 hours in culture as compared to parallel cultures that were maintained in FGF from 0-time. The addition of FGF 12 hours following culture establishment results in a kinetic which is nearly identical to that obtained when FGF was added at the time of culture establishment (see Experiments 2 and 3 in Table 1). The addition of FGF 15 hours or 18 hours following culture establishment also resulted in subsequent kinetics similar to that obtained when FGF was added at culture establishment. However, the number of PCNA+ cells at 36 hours in cultures is slightly reduced in cultures receiving FGF at 15 or 18 hours compared to cultures receiving FGF at 0-time. We additionally show in Table 2 that the number

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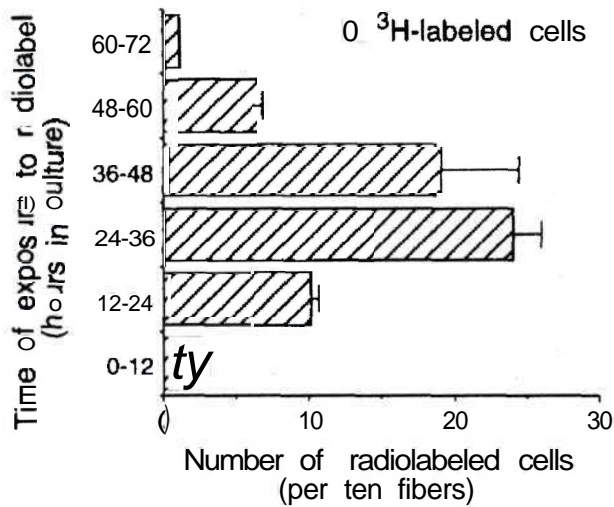


Figure 3. Quantification of ³H-thymidine-labeled nuclei on isolated fibers maintained in basal medium. Cultures received ³H-thymidine for 12 hours at different time intervals as indicated on the Y-axis. At the end of the labeling period cultures were fixed and processed for autoradiography. Duplicate plates were analyzed for each time point monitoring 30 fibers per plate. Results are eventually averaged for the two plates and are expressed per 10 fibers. Standard deviation reflects the difference between the duplicate plates.

of myogenin+ cells peaks 24 hours following the peak in PCNA+ (i.e., myogenin+ cells peak at 72 hours in culture) [previously described by us in ref. 68]. Results in Table 2 indicate that the number of myogenin+ cells at 72 hours is reduced by about 30% if FGF addition is delayed until 24 hours following culture establishment and that there is a further decline in the number of myogenin+ cells if FGF is added at 48 hours or later. Hence, a delayed addition of FGF2 to fiber cultures leads to similar effects on the reduction of first proliferating and subsequently differentiating cells. This indicates that FGF2 modulates the number of proliferating cells but does not influence the transition of the proliferating cells to the myogenin+ state.

Collectively, the experiments with the delayed exposures to FGF suggest that the satellite cells respond to exogenous FGF during earlier time points in culture as the cells begin

Figure 5. Quantification of PCNA+ nuclei on isolated fibers maintained in basal medium in the absence and presence of FGF2. Cultures were fixed at various time points and reacted with the antibody against PCNA employing indirect immunofluorescence. FGF2 was added at 2 ng/ml at the time of culture establishment and medium (\pm FGF2) was replaced daily. Duplicate plates were analyzed for each time point monitoring 30 fibers per plate. Results are eventually averaged for the two plates and are expressed per 10 fibers. Standard deviation reflects the difference between the duplicate plates.

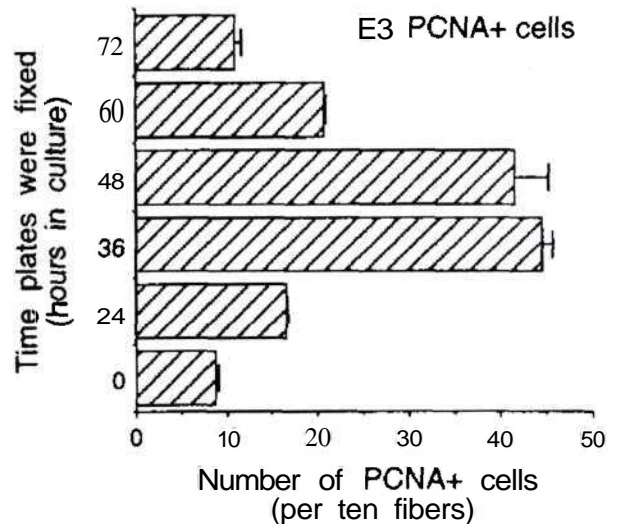
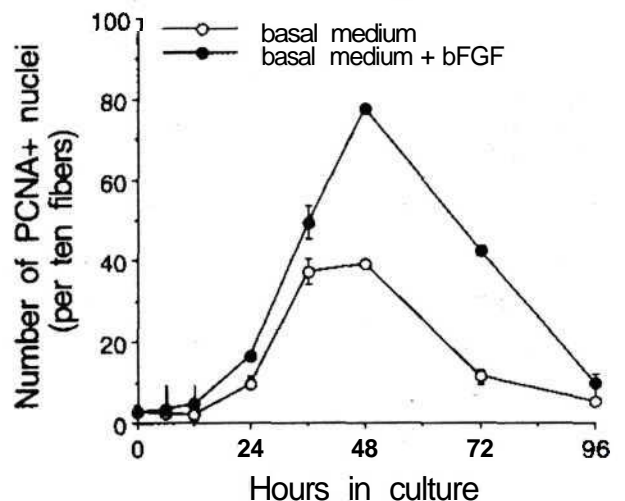


Figure 4. Quantification of PCNA+ nuclei on isolated fibers maintained in basal medium. Cultures were fixed at various time points as indicated on the Y-axis and reacted with an antibody against PCNA employing indirect immunofluorescence. Duplicate plates were analyzed for each time point monitoring 30 fibers per plate. Results are eventually averaged for the two plates and are expressed per 10 fibers. Standard deviation reflects the difference between the duplicate plates. The figure is based on the data shown in the first line of Table 3. Similar data to those shown in this figure have been reproduced in independent experiments as shown for example in Figure 5 and in the various studies summarized in Tables 1 and 3.

proliferating. The cells, however, lose their ability to respond mitotically to FGF past a critical time window. Also, the effect of FGF does not prolong the proliferative state, and the cells rapidly leave the cell cycle and transit into the myogenin+ state.



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Table 1. Influence of delayed exposures to FGF on the number of PCNA+ cells.

FGF addition conditions ^{a,b}	Time in culture (hours)						
	24	36	48	72	96	120	144
Exp 1 no FGF	15.7 ± 1	35.7 ± 1.7	49.3 ± 2.7	11.5 ± 0.8	7.7 ± 0.7	6.0 ± 2.0	ND ^d
at 0 time	9.7 ± 1	57.7 ± 5	89.5 ± 7.5	36.8 ± 8.5	11.3 ± 0.3	14.3 ± 2.0	ND
added at 24 hours	- ^c	34.8 ± 0.5	78.3 ± 7.7	41.3 ± 6.3	17.2 ± 3.2	3.3 ± 0.3	ND
added at 30 hours	-	30.6 ± 2.7	67.0 ± 1.0	36.5 ± 0.8	20.7 ± 0.3	6.0 ± 2.0	ND
added at 36 hours	-	-	59.7 ± 2.7	47.6 ± 6.7	9.5 ± 1.2	6.5 ± 2.8	ND
added at 42 hours	-	-	49.8 ± 6.5	38.6 ± 2.7	14.7 ± 1.3	9.5 ± 1.5	ND
added at 48 hours	-	-	-	20.2 ± 1.2	11.5 ± 2.5	6.5 ± 3.5	ND
Exp 2 no FGF	9.8 ± 0.8	ND	51.8 ± 2.5	18.8 ± 2.8	6.0 ± 0.3	4.2 ± 0.2	5.5 ± 1.8
at 0 time	27.0 ± 1.3	ND	95.0 ± 4.7	44.5 ± 8.8	7.8 ± 0.8	6.5 ± 3.2	4 ± 0.7
added at 12 hours	16.7 ± 1.3	ND	100.3 ± 0.3	48.5 ± 2.8	10.5 ± 2.2	ND	ND
added at 24 hours	-	ND	80.3 ± 2.0	66.2 ± 1.2	16.8 ± 0.8	5.2 ± 0.5	ND
added at 48 hours	-	ND	-	43.5 ± 2.8	22.3 ± 0.7	2.7 ± 0.3	11.8 ± 0.2
added at 72 hours	-	ND	-	-	15.2 ± 2.8	1.18 ± 0.2	12.2 ± 0.8
Exp 3 no FGF	10.0 ± 5.3	43.8 ± 0.5	58.0 ± 6.3	15.8 ± 2.2	ND	ND	ND
added at 0 time	18.3 ± 0.0	63.8 ± 0.2	93.3 ± 1.0	50.0 ± 3.3	16.8 ± 5.2	ND	ND
added at 12 hours	15.3 ± 0.3	57.8 ± 8.2	95.8 ± 7.2	61.7 ± 1.3	13.7 ± 0.3	ND	ND
added at 15 hours	11.7 ± 2.3	54.0 ± 0.3	93.3 ± 2.3	52.5 ± 1.5	13.3 ± 1.0	ND	ND
added at 18 hours	9.5 ± 1.2	52.5 ± 1.5	87.1 ± 1.0	59.2 ± 0.5	14.0 ± 0.7	ND	ND
added at 24 hours	-	50.8 ± 1.8	76.7 ± 3.3	58.3 ± 0.7	15.5 ± 3.5	ND	ND

^a Analyses were conducted via indirect immunofluorescence using a monoclonal antibody against PCNA. For each time point results reflect the average of duplicate plates. The total number of PCNA+ cells was first determined for 30 fibers per plate. These values were then averaged for the duplicate plates and expressed per 10 fibers. standard deviation (±) reflects the difference between the duplicate plates.

^b Basal medium ± FGF was replaced every 24 hours to avoid possible depletion of the growth factor. FGF was added directly to the culture medium at the indicated time.

^c "-" indicates that the value determined at the same timepoint for the "no FGF" treatment can be inserted.

^d ND = not determined.

Table 2. Influence of delayed exposures to FGF on the number of myogenin+ cells.

FGF addition conditions ^{a,b,c,d}	Time in culture (hours)						
	24	48	72	96	120	144	168
no FGF	1.5 ± 0.2	20.2 ± 1.2	45.2 ± 0.8	12.8 ± 1.8	5.7 ± 0.3	4.2 ± 0.5	3.8 ± 1.2
added at 0 time	ND	28.3 ± 3.7	97.5 ± 1.8	53.7 ± 2.3	43.2 ± 1.2	20.2 ± 1.5	8.8 ± 0.2
added at 24 hours	-	9.8 ± 0.8	67.7 ± 2.3	65.3 ± 2.7	ND	ND	ND
added at 48 hours	-	-	22.5 ± 4.2	37.2 ± 2.5	28.3 ± 3	ND	ND
added at 72 hours	-	-	-	15.3 ± 0.7	14.7 ± 3.3	18.6 ± 2.3	ND
added at 96 hours	-	-	-	-	5.2 ± 1.8	6.5 ± 0.2	8.7 ± 0.3

^{a, c, d} Analysis was conducted via indirect immunofluorescence using a monoclonal antibody against myogenin. Data gathering and all other details are as in Table 1.

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Table 3. Influence of pulse exposure to FGF on the number of PCNA+ cells.

FGF pulse conditions ^{a,b}	Time in culture (hours)						
	0	24	36	48	60	72	96
Exp 1 no FGF	8.8 ± 0.2	16.5 ± 0.2	44.5 ± 1.2	41.5 ± 3.8	20.7 ± 0.3	11 ± 0.7	4.5 ± 0.2
continuous FGF		22.8 ± 2.8	60.7 ± 0.7	78.8 ± 1.5	45.0 ± 5.0	21.5 ± 0.5	8.2 ± 0.2
initial 24 hours		- ^c	62.7 ± 1.7	77.0 ± 1.7	37.3 ± 2.0	11.2 ± 0.2	4.3 ± 1.3
initial 36 hours		-	-	80.7 ± 2.7	41.6 ± 0.0	11.2 ± 0.5	5.8 ± 2.5
initial 48 hours		-	-	-	54.8 ± 1.8	13.5 ± 0.2	6.2 ± 2.5
initial 60 hours		-	-	-	-	10.5 ± 3.2	8.2 ± 3.2
initial 72		-	-	-	-	-	6.7 ± 0.0
Exp 2 no FGF	ND ^d	19.7 ± 2.7	41.7 ± 5.0	54.0 ± 0.3	ND	12.5 ± 0.5	ND
continuous FGF		31.2 ± 5.8	66.8 ± 1.8	84.5 ± 4.2	ND	25.2 ± 2.8	ND
initial 1 hour		21.3 ± 1.3	49.5 ± 2.5	63.5 ± 2.5	ND	9.5 ± 4.5	ND
initial 3 hours		24.3 ± 2.7	50.0 ± 1.7	69.7 ± 5.0	ND	8.7 ± 0.3	ND
initial 6 hours		27.2 ± 2.2	57.2 ± 1.2	74.0 ± 4.3	ND	12.8 ± 0.8	ND
initial 12 hours		24.5 ± 2.5	58.3 ± 1.7	73.3 ± 1.0	ND	12.0 ± 0.7	ND
initial 24 hours		-	62.3 ± 1.7	75.7 ± 4.7	ND	8.5 ± 0.2	ND

^a Analyses were conducted via indirect immunofluorescence using a monoclonal antibody against PCNA. Results for each time point reflect the average of duplicate plates. The total number of PCNA+ cells was first determined for 30 fibers per plate. These values were then averaged for the duplicate plates and expressed per 10 fibers. Standard deviation (±) reflects the difference between the duplicate plates.

^b Basal medium ± FGF was replaced every 24 hours to avoid possible depletion of the growth factor. To terminate an FGF pulse the cultures were washed three times with MEM and received basal medium only.

^c "-" indicates that the value determined at the same timepoint for "continuous FGF" can be inserted.

^d ND = not determined.

Influence of early pulse exposure to FGF2 on satellite cell proliferation

In the experiments described in the previous section we examined the critical time by which FGF2 needs to be presented to support maximal proliferation of satellite cells. The experiments described in this section (summarized in Table 3) were conducted in order to determine how long the fibers need to be exposed to FGF2 to facilitate maximal proliferation of satellite cells (i.e., kinetics of PCNA+ cells which are similar to that seen when the cultures are treated with FGF2 continuously upon their establishment). In the first experiment the removal of FGF2 at 24 or 36 hours following culture establishment resulted in a peak of PCNA+ cells at 48 hours in culture that was similar to that seen when the cultures were continuously exposed to FGF (Exp 1, Table 3). In the second experiment we concluded that the addition of FGF for just the first hour following culture establishment resulted in some increase in the number of PCNA+ cells at the 48 hour time point (Exp 2, Table 3). The ability of such a brief pulse exposure to support proliferation of satellite cells can be attributed to internalization of FGF by the cells for later use or to the ability of the FGF to influence the cells even

during this early time in culture. Our pulse exposure experiments can potentially be criticised in that the washes of the cultures with MEM to terminate the FGF pulse might have not fully removed the FGF from the culture; remaining FGF could be sufficient to facilitate the increase in PCNA+ cells seen at 48 hours. This possibility is not favored by us in view of our additional findings with both of the experiments summarized in Table 3. The data from both experiments indicate that a continuous exposure to FGF is required to obtain the maximal number of PCNA+ cells at the 72 hour time point. This lower value of PCNA+ cells at 72 hours in culture has been seen even if cultures were maintained in FGF2 for the initial 60 hours (Exp 1, Table 3). This reduction in PCNA+ cells at 72 hours unless the cultures are continuously exposed to FGF may hint to the existence of a cell population whose proliferation is initiated later and further argues against the possibility that FGF is not eliminated by the MEM washes at the termination of the FGF pulse. It is also noteworthy that the medium removed from the cultures at the end of pulse exposures to FGF can support mitogenesis of various cells to a similar level as freshly added FGF2 (data not shown), indicating that the exogenously added FGF2 is not simply absorbed by the varying components of the fiber culture (e.g., Vitro-

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gen, tissue culture plastic). Previously, using an antibody which blocked the activity of FGF2, we were able to suppress the influence of FGF2 on enhancing the number of PCNA+ cells [68]. A similar approach can potentially be used to block the activity of FGF if traces of the growth factor indeed remain in the fiber cultures and promote proliferation following the removal of FGF from the culture dish by the MEM washes.

TGF β reduces proliferation of satellite cells on isolated fibers

The decline in proliferating satellite cells following 48 hours in culture regardless of the presence of FGF2 was unexpected as various studies of cultured mouse myoblasts suggested that several FGFs can support continuous proliferation and in some instances were also shown to inhibit differentiation [16, 29, 44, 45, 47, 53, 69]. The difference between our results with the rat fiber cultures and those reported by the other investigators with cultures of tissue dissociated mouse myoblasts and mouse-derived cell lines can not be simply explained by species difference. Our analysis of myogenesis of mouse satellite cells on isolated fibers has revealed a similar influence of FGF2 on proliferation (PCNA+/MyoD+ cells) as that seen in the rat model (unpublished data). It was therefore possible that satellite cells on isolated fibers follow a highly regulated program of cell proliferation controlled by enhancers and inhibitors which might be present within the fiber unit. With the view that TGF β has been detected in the skeletal muscle tissue [35] and was shown to depress proliferation of satellite cells from various species [2, 11, 31] we began analyzing the influence of TGF β on proliferation of rat satellite cells in the isolated fiber model. The analysis of TGF β effect was carried out in the absence and presence of exogenously added FGF2. The results of such a study are summarized in Figure 6. FGF2 (2 ng/ml) and/or TGF β 1 (10 ng/ml) were added at the time of culture establishment and replaced together with the basal medium daily to ensure ample supply of the different additives. The number of PCNA+ cells was subsequently examined for the 48 hour time point. The addition of TGF β 1 to cultures reduced satellite cell proliferation by about 50%. This reduction was observed for cultures maintained in the absence or presence of FGF2. TGF β is known to arrest proliferating cells at G1 [25, 32, 48] and it is thus possible that TGF β exerts a similar effect on satellite cell proliferation in the rat fiber model. More detailed work on the expression of TGF β in the fiber model may hint to as whether TGF β is indeed involved in the decline in proliferation of satellite cells in the fiber model by 72 hours in culture. Other agents, including the heparan sulfate proteoglycans which were implicated in the regulation of FGF actions during myogenesis [44] and are present in the fiber unit and its basement membrane [work in progress in our laboratory], might also play a role in regulating satellite cell proliferation.

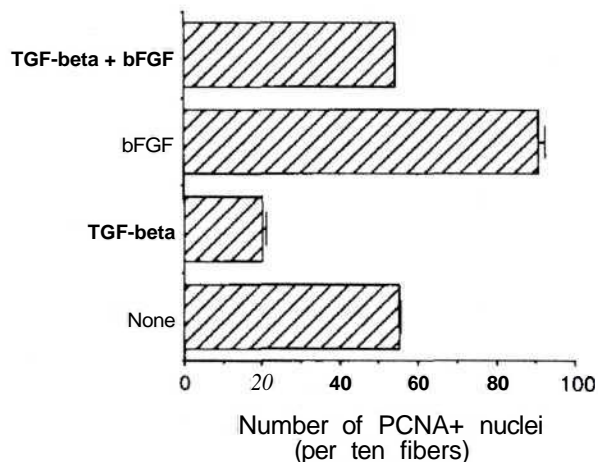


Figure 6. Effect of TGF β 1 on proliferation of satellite cells in fiber cultures. Fiber cultures were maintained in basal medium in the absence and presence of FGF2. TGF β 1 (10 ng/ml) and FGF2 (2 ng/ml) were added to the basal medium at the time of culture establishment and medium \pm additives was replaced at 24 hours in cultures. Cultures were fixed following 48 hours in culture and reacted with the antibody against PCNA employing indirect immunofluorescence. Duplicate plates were analyzed for each time points monitoring 30 fibers per plate. Results are eventually averaged for the two plates and are expressed per 10 fibers. Standard deviation reflects the difference between the duplicate plates.

Discussion

The present study was conducted as part of our ongoing investigation on the regulation of satellite cell proliferation in the postnatal muscle. Previously, our studies on the regulation of satellite cell proliferation utilized cultures of myogenic cells dissociated from adult chicken muscle [63, 64, 70] or cultures of the myogenic line C2, derived from adult mouse skeletal muscle [63, 65, 69]. To gain further insight regarding the regulation of satellite cell proliferation we began analyzing rat satellite cells in the isolated fiber model [68]. In this culture model the satellite cells remain in their native position situated by the myofiber and underneath the fiber basement membrane. We show in the present study that satellite cells on isolated fibers enter and exit the cell cycle within the first 3 days in culture, reaching maximal number of proliferating cells between 36 to 48 hours in culture. The maximal number of proliferating cells is enhanced by 2-folds in the presence of FGF2 but cells withdraw from the cell cycle and enter the differentiating, myogenin+ state regardless of the presence of FGF. Earlier studies of Bischoff [9, 10] concerning the proliferation of rat satellite cells on isolated fibers focused on proliferative kinetics within the initial days in culture and did not recognize that proliferation of satellite cells is limited to the early days and that satellite cells transit from proliferation to differentiation. Extending our investigation to later

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days in culture we were able to recognize a highly coordinated program of proliferation, withdrawal from the cell cycle and differentiation when satellite cells undergo myogenesis at their native position along the muscle fiber.

Our findings that FGF2 enhances the number of PCNA+ cells by 2 folds at 48 hours in culture could suggest that FGF2 facilitates an additional round of cell proliferation. However, the kinetics of myogenin+ cells in the absence and presence of FGF2 argue against this possibility as differentiation is not delayed in the presence of FGF. An alternative explanation which seems to be more in agreement with the results is that FGF2 allows more quiescent satellite cells to enter the cell cycle. The proliferation of these additional satellite cells might be differently regulated than that of the satellite cells which enter the cell cycle in the absence of exogenously added FGF2. These additional satellite cells might represent myogenic precursors which are recruited only following a more intense trauma to the muscle; perhaps, additional FGF (or other growth factors) is released to the environment under the conditions associated with such a more severe injury. Clarification as to how many times satellite cells proliferate during myogenesis on isolated fiber in the absence and presence of FGF2 will assist in determining the role of FGF during satellite cell proliferation in the fiber model. We are in the process of analyzing how many times the satellite cells can proliferate on the isolated fibers in the absence and presence of FGF by quantifying the number of ³H-thymidine-labeled cells that can subsequently enter an additional round of cell cycle. Cells that are going through a second round of S-phase are detected by exposure of the fiber cultures to 5-bromo-2'-deoxyuridine (BrdU) and subsequent immunolocalization of the BrdU+ cells in combination with autoradiography to determine whether the cells previously incorporated ³H-thymidine (Rivera and Yablonka-Reuveni, work in progress). A similar approach was recently taken by Schultz [49] for the *in vivo* analysis of satellite cell proliferation in growing (30-day-old) rats. The latter study concluded that the majority of the satellite cells go through only one round of cell proliferation and suggested that the purpose of these cells is chiefly to provide myonuclei to the growing myofibers. This observation of limited proliferative capacity of satellite cells *in vivo* fits well with our findings with satellite cells in the isolated fiber model. The current study was performed with 8- to 10-week-old rats; we also observed limited proliferation of satellite cells on fibers from young (21-day-old) rats [Yablonka-Reuveni and Rivera, manuscript in preparation]. Schultz [49] has further suggested that there is a second population of satellite cells which divide slowly and might be the 'real' reserved myogenic cells in the postnatal environment, generating the fast dividing satellite cells with the limited proliferative capacity. The possible existence of such slow dividing myogenic stem cells is presently being investigated by us in the isolated fiber model.

The finding of the limited proliferation of satellite cells in the fiber model regardless of the presence of FGF2 was unexpected. Various reports employing primary satellite cell cultures or cultures of myogenic cell lines isolated from adult muscle (presumably satellite cell derived lines), have shown that members of the FGF family of growth factors can support ongoing myoblast proliferation [16, 47, 63, 69] and in some instances various FGFs were implicated in delaying myogenic differentiation [16, 26, 29, 44, 45, 57]. Analyses of mRNA transcripts for FGF receptors in several myogenic cell lines have suggested that levels of FGF receptor transcripts are reduced in correlation with myogenic differentiation [21, 39, 43, 58]. We are currently investigating which types of FGF receptors are expressed by satellite cells during myogenesis on isolated fibers and whether the expression of these receptors is modulated as the fiber-associated satellite cells withdraw from the cell cycle and differentiate [Graves and Yablonka-Reuveni, work in progress]. At any rate, the limited proliferation of the satellite cells in the fiber culture suggests that the control of myoblast proliferation in the fiber system is different from that of the tissue dissociated myogenic cultures which demonstrate a long-term proliferative capacity. The possibility that other members of the FGF family or other growth factors including hepatocyte growth factor, IGF-1 and PDGF may facilitate continuous proliferation of satellite cells on isolated fibers has been examined by us. None of the factors tested increased proliferation of satellite cells on isolated fibers beyond what has been detected in the presence of FGF2 [Yablonka-Reuveni and Rivera, unpublished work]. We also demonstrated that BudR blocks differentiation of satellite cells on isolated fibers but that this interference did not eliminate the withdrawal from the cell cycle of the satellite cells as seen in the absence of BudR [68]. These various observations further support the notion that the satellite cells we visualized in the fiber model are 'programmed' or 'fated' to limited proliferation and their role, similar to what was suggested by Schultz [49], is to provide myoblasts that rapidly join the growing or regenerating myofiber.

It is interesting to note that the addition of exogenous FGF2 to regenerating mouse skeletal muscle did not enhance the process of regeneration [38]. This study was performed in three separate models of mouse muscle injury: crush-injury, denervated, and dystrophic (mdx) muscle [38]. However, the incorporation of donor non-dystrophic mouse myoblasts into myofibers of uninjured host mdx skeletal muscle was enhanced if the donor myoblasts (obtained by expansion in primary culture) were grown in culture with FGF2 [34]. Given the different observations regarding the influence of FGF2 in the different culture models (single fiber cultures versus tissue dissociated primary cells) and *in vivo* (regeneration versus transplantation of cultured myoblasts into noninjured mdx muscle), it is possible that the mitogenic influence of FGF2 might be regulated by various additional factors which can

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have positive/negative influences on myogenesis in general and on FGF2 action in specific and which might be present at different levels depending on the model studied.

Our studies with the delayed exposure of FGF have identified the latest time by which FGF2 needs to be added to the fiber culture to achieve maximal stimulation of cell proliferation. This time (12-18 hours following culture establishment) precedes the time by which an increase in the number of PCNA+ cells is first recognized. Although not analyzed by us yet in further details, we propose that this time represent the G₁ phase of the cell cycle. The results of the experiments with early pulses of FGF2 may further suggest that an exposure to FGF2 during the early phase of recruitment from G₀ to G₁ is sufficient for cell proliferation and that FGF is not required for the subsequent step of DNA synthesis. Mammalian cell studies showed that both FGF1 (acidic FGF) and FGF2 were important during the progression from G₀ to near S phase but were not needed anymore during the actual period of DNA synthesis [46, 72]. In a study of transformed fetal bovine aortic endothelial cells [46], it was suggested that an incubation of about 12 hours with FGF2 (from G₀ to near S) was required for long lasting activation of protein kinase C - a requirement for a subsequent cell proliferation. Similarly, in a study of BALB/c 3T3 cells, a longer term exposure to FGF1 (about 12 hours) was required to support certain protein phosphorylations during G₁ in addition to the initial FGF1 requirement during the G₀-G₁ transition [72, 73].

In summary, the present study describes some of our studies on the regulation of satellite cell proliferation in the isolated rat fiber model. In this model the satellite cells first enter the cell cycle, subsequently spend a limited time in a proliferative compartment, and eventually withdraw from the cell cycle and transit into the differentiated state. This isolated fiber model offers an excellent tool for the analysis of regulatory steps linked to the recruitment, proliferation and differentiation of satellite cells in their native position by the myofiber. Utilizing this model to dissect in finer details the agents involved in regulating myogenesis in the postnatal environment has the potential of contributing significant findings for improving donor myoblasts transplantation into skeletal muscle for cell-mediated gene therapy.

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