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Formation Process and Fate of the Nuclear Chain After Injury in Regenerated Myofiber

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ABSTRACT

Although it is well known that regenerated myofibers contain nuclear chains (arrayed nuclear clusters), details of its process of formation and fate are still remained unclear. In the present study, we isolated single myofibers from injured ICR mouse tibialis anterior muscles by the alkali maceration-based method, and carried out histological observation and bromodeoxyuridine (BrdU) pulse-chase analysis on the nuclear chains. The nuclear chains were formed after injury and remained stable for at least 6 months after injury. When BrdU was administered during the first 4 days after injury, up to 56% of nuclei in the nuclear chains were labeled with BrdU, whereas when BrdU was administered 5 days or later after injury, less than 3% of myonuclei were labeled with BrdU. Among BrdU-positive nuclei in the nuclear chains, the nuclei showing attenuated and strong BrdU signal were dominant when BrdU was administered at the time points of 0–2 and 3–4 days after injury, respectively. These results suggest that successive nuclear divisions occur during the first 4 days after injury and might be involved in the appearance of the stable nuclear chains in regenerated myofibers. Anat Rec, 291:122–128, 2007.

Key words: nuclear chain; nuclear division; BrdU pulse-chase; single myofiber; muscle injury

It is well know that the regenerated myofibers show unique myonuclear distribution compared with normal ones (Landing et al., 1974; Newlands et al., 1998). In normal myofibers, myonuclei are scattered in the peripheral region of the myofibers, whereas in regenerated myofibers, myonuclei form arrayed clusters along the long direction of the myofibers (i.e., nuclear chains). Although many studies have investigated injured muscles, there have been few investigations on nuclear chains throughout the entire length of the myofiber due to the difficulty in observing all myonuclei in the myofiber, and the details in the formation process and fate of the nuclear chains still remained unclear.

Isolation of single myofibers is an excellent method of observing all nuclei in the myofiber. Single myofibers have been isolated by various methods, including extraction by glycerol (Natori, 1975; Allen et al., 1995), mechanical isolation (Landing et al., 1974), enzymatic digestion (Bischoff, 1990; Beauchamp et al., 2000; Newlands et al., 1998), nitric acid maceration (Gonyea et al., 1986; Tamaki et al., 1992), and alkali maceration (Nakada et al., 1997). In particular, the alkali maceration-based method has advantages to isolate fine single

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myofibers for morphological analysis; the alkali maceration-based method enables to prepare a large number of single myofibers without structural disruption from long-term denervated, newborn, and aged mice muscles, despite significant accumulation of connective tissues and/or structural weakness in these myofibers (Wada et al., 2002, 2003; Brack et al., 2005). These successes in isolating single myofibers suggest that the alkali maceration-based method can be used to isolate fine single myofibers from injured muscles, too.

In the present study, we first isolated fine single myofibers from injured muscles by the alkali maceration-based method, and then carried out histological and bromodeoxyuridine (BrdU) pulse-chase analysis on the nuclear chains in the regenerated myofibers throughout their entire length. Finally, we described the formation process and the fate of the nuclear chains in the regenerated myofibers after injury.

MATERIALS AND METHODS

Animals and Muscle Injury

All experiments were conducted in compliance with protocols approved by the University of Tsukuba's Animal Experimental Committee. Healthy 4- to 12-month-old and 2-week-old ICR mice were used in the present study. Injury to the tibialis anterior (TA) muscles was generated by either a mechanical or chemical method under pentobarbital sodium or diethyl ether anesthesia. For mechanical trauma, a sterile 28-gauge needle was percutaneously stabbed into the TA muscle 400 times. The 400-time needle stabbing was needed to injure the TA muscles with a similar and high degree. The needle-stabbed TA muscles were used for the investigation of the change in the frequency of the myofibers that have nuclear chains after injury. For chemical-induced injury, 50 μl of bupivacaine hydrochloride solution (BPVC; 0.1–0.5% with phosphate buffered saline [PBS]) was percutaneously injected into the TA muscles. The mice were housed and allowed to recover from the treatment. At the end of the experiment, the animals were killed by ether overdose and cervical dislocation.

Single-Myofiber Preparation for Histological Observation

Single myofibers were isolated as previously described (Wada et al., 2002). Briefly, portions of the hindlimb were fixed for 2 days with 4% paraformaldehyde in a relaxing solution (137 mM NaCl, 5.4 mM KCl, 5 mM $MgCl_2$, and 4 mM ethyleneglycoltetraacetic acid in 5 mM HEPES buffer, pH 7.0) to prevent myofiber contraction. After teasing the muscle samples, the resulting tissue bundles were macerated in 40% NaOH solution for 3 hr. After careful dissection of single myofibers under a binocular microscope, each myofiber was mounted on a gelatin-coated glass slide and stained with hematoxylin. When the isolated myofibers were mounted on glass slides, we confirmed that every myofiber was not bundle of small myofibers picking their end using fine tweezers.

BrdU Pulse-Chase Analysis

To analyze nuclear division events in the nuclear chains after injury, the mice were injected with 5-bromodeoxyuridine (BrdU; 50 mg/kg body weight) at the time points indicated in Figure 2A. BrdU was administered four times every 6 hr, with the first injection done by means of the tail vein followed by intra-abdominal routes. Ten days after injury, the animals were killed and individual myofibers were prepared as described above. The isolated myofibers were further treated with 20% NaOH solution for 2 hr at room temperature. This treatment was essential for the immunostaining of BrdU in the single myofibers. The myofibers were then rinsed with PBS 3 times, and immunoreacted with POD-conjugated anti-BrdU Fab fragment (1 U/ml; Roche Applied Science, Germany). The nuclei were counterstained with hematoxylin.

The effect of circulating levels of BrdU on its myonuclei incorporation was determined by a single intra-abdominal injection of different doses of BrdU (5, 25, and 50 mg/kg body weight) to noninjured 2-week-old mice. At 24 hr after BrdU injection, myofibers were isolated from the animals and were immunostained with anti-BrdU Fab fragment.

RESULTS

Properties of Isolated Single Myofiber

While large numbers of myofibers can be obtained from normal muscles without any treatments after alkali-maceration, muscle tissues inflicted with trauma still remained loosely bundled after the alkali-maceration step and thus required further dissection to separate the strands. With careful dissection with fine tweezers, a large number of single myofibers were obtained even from injured muscles. As shown in Figure 1, the resulting myofibers from both intact and injured muscles maintained structural integrity, and showed clear cross-striations and multiple nuclei, without any cytoplasmic contraction. Proliferating cell nuclear antigen, myogenin, and laminin were undetectable in the isolated single myofibers by immunochemical method (data not shown), perhaps, due to degeneration of the proteins accompanied with the alkali maceration, and thus, many other proteins also would be undetectable. These data indicate that, although it is difficult to detect specific cell cycle marker proteins by immunochemical method, the alkali-maceration–based method provides reliable samples for histological observation of the myofibers in injured muscles.

Nuclear Chains in Regenerated Myofibers

We observed all nuclei in the myofiber throughout its entire length using isolated single myofibers. In normal myofibers, less than 1.9% of the myofibers appeared to possess the nuclear chains composed of more than five nuclei at any ages of normal mice (Table 1). In contrast to normal myofibers, the injured muscles exhibited prominent nuclear chains (Fig. 1B). Figure 1F shows the time-course changes in the frequency of myofibers that have nuclear chain(s) with >7 nuclei after injury. In injured muscles, more than 60% myofibers contained nuclear chains, and the frequency of myofibers containing nuclear chain(s) did not decrease for at least 6 months after injury. These data indicate that muscle injury triggers the appearance of stable nuclear chains in the myofibers. In some instances, the nuclear chains were local-

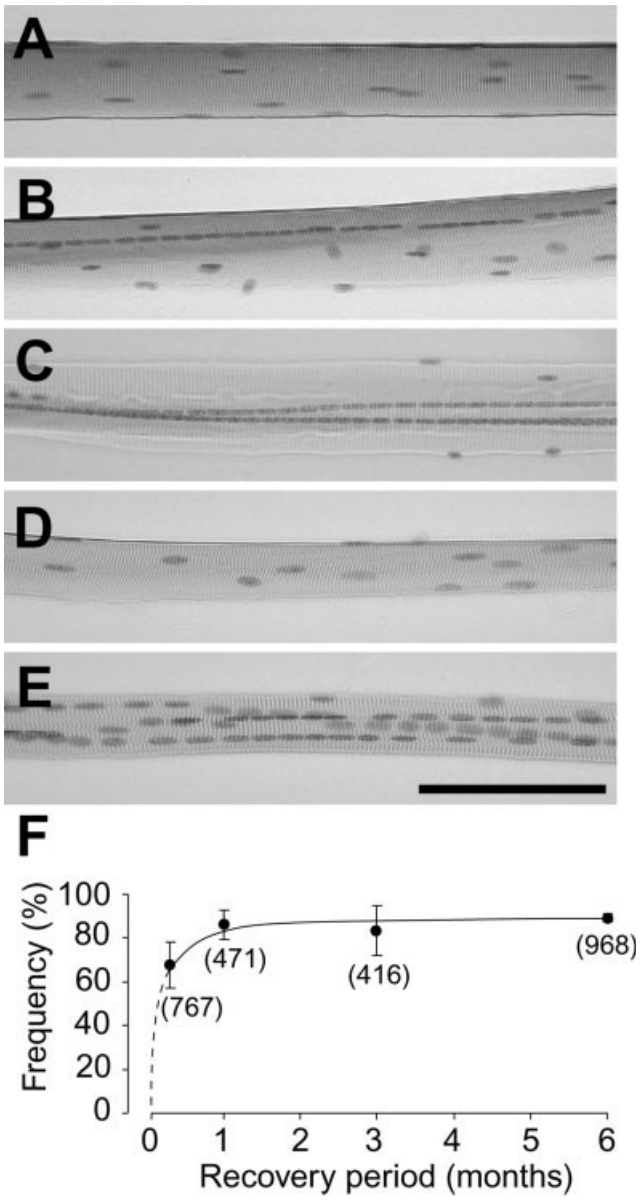

Fig. 1. Photomicrographs of single myofibers isolated from alkali-macerated tibialis anterior (TA) muscles. **A:** Single myofiber isolated from normal TA muscle. All myonuclei are scattered throughout the entire length of the myofiber. **B:** Single myofiber isolated from TA muscle 11 days after 0.1% bupivacaine hydrochloride solution (BPVC) injection. Distinct nuclear clustering is seen. **C:** Single myofiber isolated from TA muscle 6 months after injury. Even after the 6-month recovery period, arrayed nuclear clusters remain stable in the myofiber. **D,E:** Different regions of the same myofiber isolated from TA muscle 1 month after needle stubbing. The part shown in D has normal scattered nuclei; on the other hand, the other part shown in E contains arrayed nuclear clusters. **F:** Change in the frequency of the myofibers that contain arrayed nuclear cluster(s) during the 6-month recovery period. Four injured TA muscles were analyzed. Muscle injury was given by 400 times of needle stabbing, and analyzed at 1, 3, and 6 months after injury. Data was obtained from four injured muscles at each time point. The numbers in parentheses represent the total numbers of single myofibers examined. The values are means ± SD. Scale bar = 100 μm.

ized in a part of the myofiber with the distal parts of the myofiber still showing scattered nuclei (Fig. 1D,E).

BrdU Pulse-Chase Experiment in Regenerated Myofibers

To investigate the nuclear division event in the nuclear chains after injury, BrdU was administered at various time points and detected at 10 days after injury (Fig. 2A). The BrdU incorporated in the nuclei was clearly detected as brown chromogen by the immunochemical method. The intensity of BrdU signal level in each nucleus was classified as either type I (sparsely labeled, 1–6 spots), II (moderately labeled), or III (intensely labeled), and unlabeled nuclei were classified as type O (Fig. 2B). The frequencies of type I, II, and III nuclei in the nuclear chains are shown in Figure 2C. When BrdU was administered during the first 4 days after injury, up to 56% of nuclei in the nuclear chains were labeled with BrdU (i.e., showing either type I, II, or III), whereas when BrdU was administered 5 days or later after injury, less than 3% of myonuclei were labeled with BrdU. Among BrdU-positive nuclei in the nuclear chains, type I and II nuclei were dominant when BrdU was administered at the time points of 0–2 days after injury, whereas type III nuclei were dominant with BrdU administration at 3–4 days after injury. Taken together with our conclusion that the attenuated BrdU signals were result of successive nuclear division(s) after BrdU incorporation (see the Discussion section), these data suggest that the nuclei in the nuclear chains underwent multiple division(s) during the first 4 days after injury, but the nuclear divisions rarely occurred thereafter.

BrdU Pulse-Chase Experiment in Normal 2-Week-Old Mice Myofibers

To verify whether low dosage of BrdU results in the attenuated BrdU signal in the nuclei, a BrdU pulse-labeling experiment was performed on normal TA muscles of 2-week-old mice (see the Discussion section). One day after administration of BrdU with 5, 25, and 50 mg/kg body weight, all BrdU-positive nuclei in 2-week-old mice myofibers were type III regardless of the amount of BrdU dosage (Fig. 3).

Nuclear Fragmentation in Nuclear Chain

A few myofibers in the muscles 10 days after injury contained small or constricted shaped myonuclei. At 10 days after injury, the frequency of the myofibers containing the small nuclei was less than 1%, and no small or constricted shaped nuclei were found in the muscles 1 month or later after injury (data not shown). The small or constricted shaped nuclei were labeled with BrdU when BrdU was administrated during 0–2 days after injury (Fig. 4). These data suggest that a part of the nuclei underwent nuclear divisions before they fragmented to disappear in the myofiber regeneration process. The BrdU released from degenerating BrdU positive-nuclei might be reused in other nuclei for DNA replication (Grounds and McGeachie, 1987). However, due to the smallness in the quantity, the BrdU released from degenerating nuclei would rarely affect on the results of our BrdU pulse-chase experiments.

TABLE 1. Percentages of nuclear clusters in normal myofibers[a]

Cluster size (number of nuclei)	Age							
	0 days	3 days	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 months
3	3.7	13.5	18.7	27.7	19.1	6.6	7.0	11.0
4	0.0	2.1	6.2	5.2	4.0	4.6	2.8	2.1
5	0.0	0.0	0.0	1.9	0.8	0.3	1.4	0.2
6	0.0	0.0	0.0	0.6	0.3	0.0	0.0	0.7
>7	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.7

[a]Data were corrected from four or more tibialis anterior muscles at each age. The nuclear clusters that are composed of two nuclei were not counted because of the difficulty in estimating whether the two myonuclei formed a cluster or not.

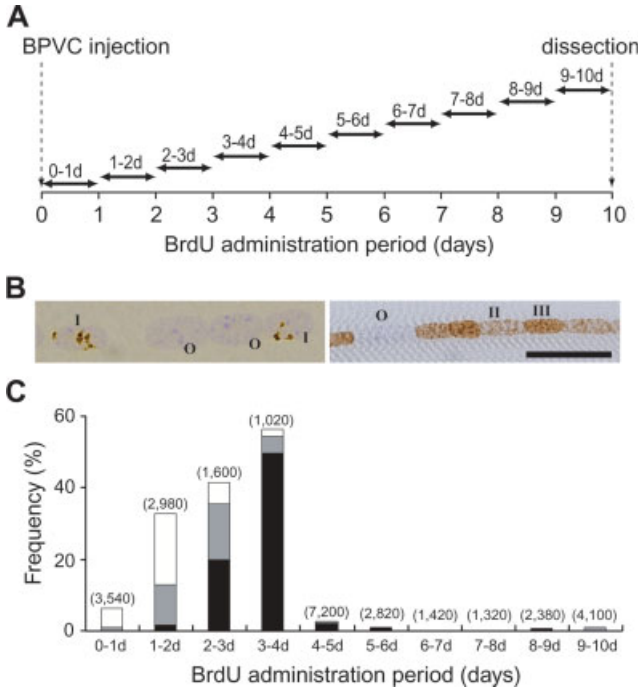

Fig. 2. The result of bromodeoxyuridine (BrdU) pulse analysis on the nuclear chains. **A:** The protocol of BrdU administration. Two mice were used for each group. Arrows represent the BrdU administration period with 4 times every 6 hr. **B:** Photomicrographs of the nuclear chains that were labeled with BrdU. The nuclei were labeled with BrdU at different levels classifiable into four types (types I, II, III, and O; see the Results section). **C:** The frequencies of type I, II, and III nuclei in the randomly selected nuclear chains. The white, gray, and black bars represent the frequencies of type I, II, and III nuclei in the nuclear chains, respectively. The BrdU administration periods are parallel to that shown in A. The numbers in parentheses represent the total numbers of myonuclei examined.

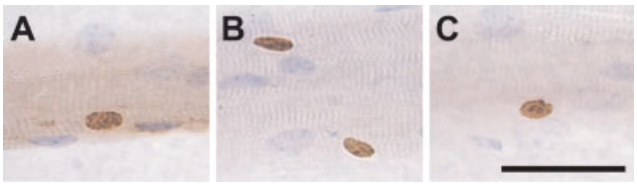

Fig. 3. The result of bromodeoxyuridine (BrdU) pulse-chase analysis in 2-week-old normal muscles. **A–C:** The myofibers were isolated from tibialis anterior (TA) muscles of 2-week-old mice 1 day after administration of BrdU with 50, 25, and 5 mg/kg body weight (A, B, and C, respectively). Note that all BrdU-positive nuclei are type III regardless of BrdU dosage. Scale bar = 100 μm.

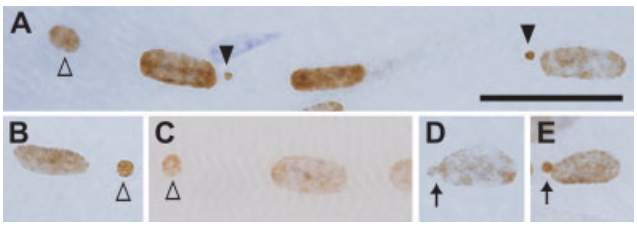

Fig. 4. Small nuclei in regenerated myofibers. **A:** Small (white triangle) and extremely small (black triangles) nuclei which were labeled with bromodeoxyuridine (BrdU). **B,C:** Other examples of BrdU-labeled small nuclei in regenerated myofibers (white triangles). **D,E:** Constricted shaped nuclei in the regenerated myofibers. Small nuclei seems to bud from the parent nucleus (arrows). BrdU was administrated over 3–4 days after injury, and the myofibers were isolated 10 days after injury. Scale bar = 30 μm.

DISCUSSION

Many previous studies have investigated the muscle regeneration process by [^{3}H]thymidine autoradiography (McGeachie and Grounds, 1987; Morlet et al., 1989; Grounds and McGeachie, 1990) or immunochemical detection of BrdU (Hurme and Kalimo, 1992; Saito and Nonaka, 1994), and clarified the time course of nuclear division event in regenerating muscle tissues. In the present study, we succeeded in a semiquantitative BrdU pulse-chase experiment in the single myofibers isolated from injured muscles (Fig. 2). This approach enabled the description of not only the time course of the nuclear division event after injury, but also nuclear distribution and history of nuclear divisions in each nucleus in the regenerated myofibers. The results of our observations proposed the hypothesis that successive nuclear divisions of myogenic cells after injury results in the appearance of the stable nuclear chains in the regenerated myofibers.

Because it is generally believed that true myonuclei have no mitotic activity (Stockdale and Holtzer, 1961; Konigsberg, 1963; Stockdale, 1971), the true myonuclei would not be the source of the nuclear chains. Although it has been reported that both the muscle interstitium (Polesskaya et al., 2003) and circulating bone marrow-derived cell types (LaBarge and Blau, 2002) can give rise to myogenic precursors, their quantitative contributions, compared with the satellite cells, are not signifi-

cant in terms of the overwhelmingly rapid regeneration of myonuclei after muscle injury (Sherwood et al., 2004). Therefore, the nuclear chains are conjectured to originate from satellite cells.

Some molecular markers for satellite cells such as M-cadherin (Irintchev et al., 1994), syndecan 3 and 4 (Cornelison et al., 2001), CD34 (Beauchamp et al., 2000), and Pax7 (Seale et al., 2000) have been identified, yet there are still no molecular markers for the nuclei originating from satellite cells that act as myonuclei in the regenerated myofibers. If the nuclear chains do indeed originate from satellite cells, their unique nuclear clustering would become a feasible morphological marker for the newly recruited nuclei originating from satellite cells in the regenerated myofibers as they remain stable in the myofibers for a long time (Fig. 1C,F).

More than 60% of myofibers isolated from needle-stabbed TA muscles contained the nuclear chains (Fig. 1), suggesting that the nuclear chains are major nuclear population in the regenerated myofibers. Thus, the gene expression profile in the nuclear chains (or satellite cells) would be consistent with the time course change previously reported. Roughly, the quiescent satellite cells, or Pax7-positive satellite cells are activated to start proliferation after injury, and Pax7 expression declines with the up-regulation of muscle determination genes such as MyoD, Myogenin, and Myf-5 (Seale et al., 2000; Yablonka-Reuveni and Rivera, 1994; Cornelison and Wold, 1997; Beauchamp et al., 2000). After then, satellite cells terminate proliferation and fuse to form myotubes expressing contractile proteins such as Myosins (Cantini et al., 1993). However, genes expression property of each nucleus in the same nuclear chain might be different. Newlands et al. (1998) have reported that troponin I mRNA expression level is different between each nucleus even in the same nuclear chain. Further investigations are needed to understand the gene expression properties in the nuclear chains.

We observed the attenuated BrdU signals in the nuclear chains (i.e., type I or II nuclei; Fig. 2B). The successive nuclear division(s) distributing incorporated BrdU into progeny nuclei would cause the attenuated BrdU signals in the nuclear chains. In this scenario, the number of successive nuclear divisions after incorporation of BrdU should be greater in type I nuclei than in type II, and that there should be no successive divisions in type III. The result of our BrdU pulse-chase experiment that the type III nuclei were dominant with BrdU administration at 3–4 days after injury, but BrdU administration at 5 days and later resulted in few BrdU positive nuclei (i.e., only a few nuclear divisions occurred after 5 days of injury), is consistent with this scenario. Taken together with a previous study showing the recovery in skeletal muscles within 4 days after injury (Whalen et al., 1990), the rapid increase in the nuclear number by successive nuclear divisions forming the nuclear chains might be related with the rapid recovery in the myofiber after injury.

The low levels of BrdU pulse also could cause the attenuated BrdU signals in the nuclei. To verify this, we administrated a much lower concentration of BrdU into 2-week-old mice, and the myofibers were analyzed after 1 day of BrdU administration. In this BrdU pulse-chase experiment, no successive myonuclear divisions would occur because the satellite cells divide in a 32-hr cell cycle

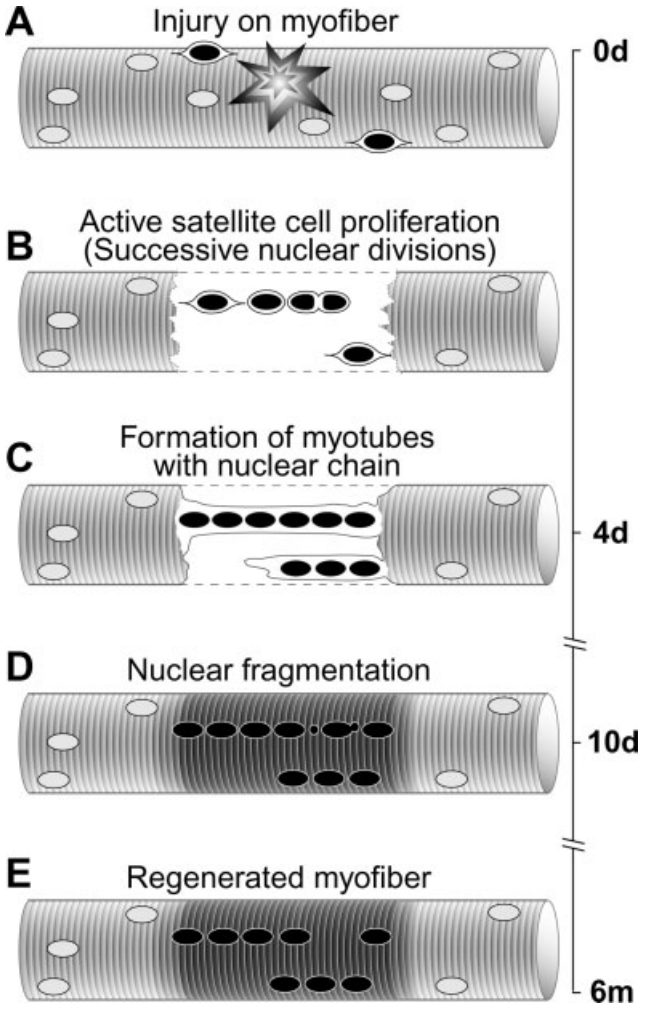

Fig. 5. Schematic illustrations of the nuclear chain formation. **A:** In normal myofiber, myonuclei (gray) and quiescent satellite cells (black) are scattered throughout the myofiber. **B:** When myofiber is injured, satellite cells are activated to start continuous cell division, which makes their nuclei array along the direction of the myofiber. The satellite cells proliferate (nuclei divide) dominantly during the first 4 days after injury but rarely do so thereafter. **C:** It is hypothesized that the satellite cells fuse with each other to form myotubes with the resultant nuclear chain(s). **D:** A part of nuclei in the nuclear chains fragment and would disappear thereafter. **E:** The necrotic region of the myofiber is recovered within 10 days after injury, and the nuclear chains remain stable in the regenerated myofiber for a long time (at least 6 months). The dark area represents the newly formed cytoplasm in the regenerated myofiber after injury.

in postnatal growing animal (Schultz, 1996). The result showed that much lower BrdU administration also resulted in strong BrdU signal (i.e., type III nuclei; Fig. 3). Although the situation in the postnatal growing muscles might be different from adult regenerating muscles, these data suggest that the low levels of BrdU pulse would not cause the attenuated BrdU signals in myofibers. Therefore, the hypothesis that low levels of BrdU pulse causes attenuated BrdU signals would be ruled out.

Although our data suggest that the nuclear chains are achieved by continuous nuclear divisions (perhaps, accompanied with satellite cells proliferation), that satel-

lite cells can migrate across the sarcolemma (Hughes and Blau, 1990; Watt et al., 1994), in contrast to the stationary true myonuclei (Bruusgaard et al., 2003), reminds us that nuclear arraying might be generated by migration of activated satellite cells. However, the structures that regulate the migration of activated satellite cells to align have not yet been reported.

In some regenerated myofibers, small or constricted shaped nuclei were found in the nuclear chains (Fig. 4). Because no small or constricted shaped nuclei were found in the regenerated myofibers 1 month or later after injury, the small nuclei would be a temporal structure to disappear. The myonuclear apoptosis have been reported under various situations such as unweighting (Allen et al., 1997), myofiber atrophy (Borisov and Carlson, 2000), and normal growth (Tranchtenberg, 1998). Taken together with these studies and our observations of small or constricted shaped nuclei, the nuclear apoptosis would occur in the regenerated myofibers, too. However, we have no evidence contradicting the possibility that the small nuclei survive and recover their size thereafter. A previous study has reported that the nuclear budding occurs in mammalian cells in the process of proliferation (Sundaram et al., 2004). We also found that the small or constricted-shape nuclei were labeled with BrdU. This finding suggests that some nuclei had undergone nuclear division(s) before they fragmented.

In summary, we successfully isolated fine single myofibers from injured muscles and carried out histological and BrdU pulse-chase analysis on the nuclear chains. In the present study, we showed that (1) muscle injury triggered the appearance of stable nuclear chains in the myofibers, (2) the nuclear chains were achieved by successive nuclear divisions during the first 4 days after injury, and (3) a part of nuclei fragmented after once they divide. These results lead us to propose a possible hypothesis about the formation process and fate of the nuclear chains after injury as summarized in Figure 5. The successive nuclear divisions start at least within 1 day after injury, which makes the nuclei array, and terminate within 4 days after injury. Perhaps, the successive nuclear divisions are accompanied with the satellite cells proliferation. After then, the arrayed satellite cells fuse with each other to form myotubes, which have nuclear chain(s). The newly formed myotubes with the nuclear chains would fuse to the parent myofiber and perhaps to the neighboring survived myofibers, too (Robertson et al., 1993; Irintchev et al., 1994). The necrotic region of the myofiber is recovered within 10 days after injury, and the nuclear chains remain stable in the regenerated myofiber for at least 6 months. Due to the numerical abundance and stability of the nuclear chains, the nuclear chains formation would be associated with the regenerated myofiber phenotypes. Further investigation on the nuclear chains will provide important information about muscle regeneration process.

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