

# Effect of heat stress soon after muscle injury on the expression of MyoD and myogenin during regeneration process

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## Abstract

Heat stress could promote skeletal muscle regeneration. But, in the regeneration process, effects of heat stress on myogenic cells and the regulating factors is unknown. Therefore, Influences of heat stress soon after injury on distribution of the myogenic cells and chronological changes in expression of MyoD and myogenin were examined. The first peak of MyoD expression was temporally correlated with the time when proliferating satellite cells began to appear, and the rapid decline of the MyoD expression from the first peak, with the appearance time of myoblasts, respectively in both the non-Heat and Heat groups. The first peak of myogenin expression was temporally correlated with the time when multinuclear cells began to form in the both groups. Due to the heat stress, proliferation and differentiation of myogenic cells and chronological changes in these factors were accelerated one day earlier than in the non-Heat group. As MyoD and myogenin are regulating factor of proliferation and differentiation, heat stress soon after the muscle injury could accelerate the proliferation and differentiation of myogenic cells and the expression of their regulating factors MyoD and myogenin.

**Keywords:** Heat Stress, MyoD, Myogenin, Regeneration, Skeletal Muscle

## Introduction

Skeletal muscle injury often occurs due to excessive exercise, laceration and contusion<sup>11,24</sup>. Especially, in muscle contusion, the sarcolemma is directly ruptured, and Ca<sup>2+</sup> would flow into the injured fiber. Consequently, Ca<sup>2+</sup>-dependent proteases can be activated to promote degeneration in necrotic muscle fibers<sup>1,17</sup>. With the disruption of sarcolemma and the degeneration progresses, satellite cells, remain quiescent in intact muscle, were activated<sup>21</sup> to regenerate injured muscle. Due

to the chemical reaction depending on the temperature, heat or cold stress to the muscle after the injury might be important in the degeneration and subsequent regeneration. Actually, we examined influences of heat stress applied soon after the crush injury on regeneration<sup>25</sup>, describing that heat stress applied soon after the injury could accelerate not only secondary degeneration but also distribution of activated satellite cells. As the activated satellite cells proliferate and differentiate due to newly generate skeletal muscle fiber<sup>9</sup>, the proliferation and differentiation of those cells is important in skeletal muscle regeneration. The heat stress soon after the injury, which can promote degeneration in necrotic fibers, also might effect on the proliferation of myogenic cells and subsequent differentiation. However, it is still unclear detailed effects of heat stress soon after the injury on the proliferation and differentiation of the myogenic cells and which factors regulating the myogenic cell could effected.

The activation, proliferation and differentiation of satellite cells are mainly regulated by skeletal muscle-specific transcription factors such as MyoD, myogenin and so on<sup>11,22</sup>. The MyoD and myogenin are proteins with a key role not only in myoge-

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nesis at embryonic and neonatal stages<sup>26</sup> but also during muscle regeneration<sup>14</sup>, because their deficiency impaired myogenesis including the development and regeneration<sup>14-16,19</sup>. As for the MyoD, it expresses in activated satellite cells<sup>7,30</sup> and its expression were required for going forward the cell cycle to S phase<sup>31</sup>. In terms of myogenin, myogenin associates with formation of muscle fibers in embryonic period<sup>12</sup> and might be involved with differentiation of myogenic cells, especially fusion from myoblast to myotube. From these reports, MyoD and myogenin has been reported to be involved with the proliferation and the differentiation of muscle precursor cells, respectively. Therefore, how expression of MyoD and myogenin were effected by heat stress soon after muscle injury were examined.

In the present study, therefore, influences of the heat stress applied soon after the injury on the proliferation and differentiation of myogenic cells and expression of MyoD and myogenin were examined in the crush injured rat skeletal muscle. Special references were paid on the temporal relationship between the chronological change in expression of MyoD and myogenin and those in proliferation and differentiation of the myogenic cells.

## Materials and methods

This study was approved by Institution Animal Care and Use Committee (IACUC) and carried out according to the Kobe University Animal Experimentation Regulations. All experiments were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No 123, Strasbourg 1985).

### Animals

A hundred-forty four 8-week-old male wistar rats weighing 180-200 g (Japan SLC, Shizuoka, Japan) were used in the present study. They were allowed to access food and water freely throughout the experiments. Animals were housed independently in normal cage with woodchip floor. All animals were kept in a controlled environment with fixed 12: 12 h light: dark cycles and ambient temperature maintained at 25C.

### Experimental protocols

Methods to induce the muscle injury and applying the heat stress were performed according to our previous studies<sup>24,25</sup>. Briefly, animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg), anterolateral aspect of the left hindlimb was shaved. Then we made a longitudinal incision about 20 mm long in the skin and peripheral fascia of tibialis anterior (TA) muscle and exposed only the extensor digitorum longus (EDL) muscle without injuring TA muscle. The middle part of the EDL muscle belly was crushed for 30 sec by using forceps, to which a weight (500 g) was attached. Immediately after the injury, the skin was closed with a 4-0 suture. The animals were randomly divided into heat stress applied and non-applied groups (Heat and non-Heat groups, re-

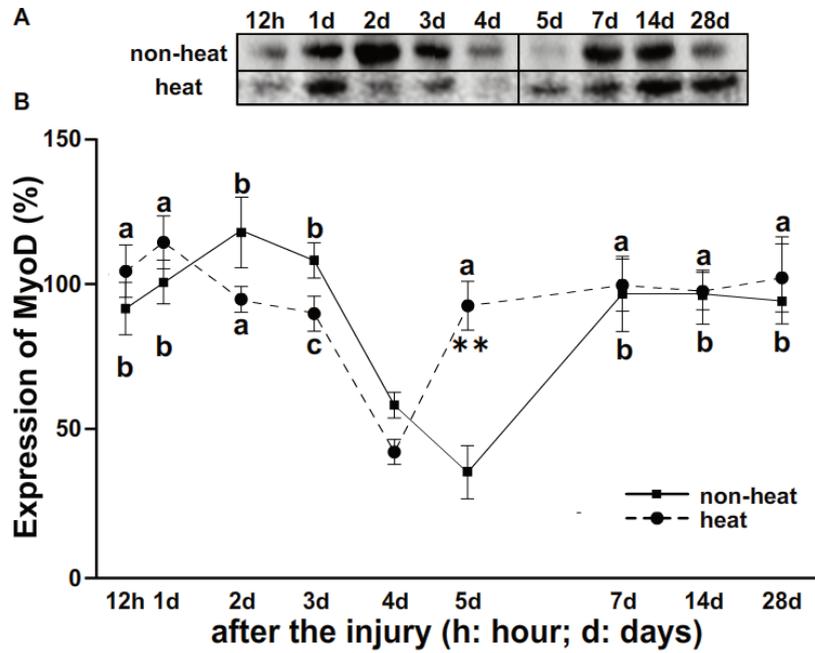
spectively; n=72 in each group). The heat stress was applied by the hot pack enclosing hot water (42C) in a polyethylene bag. Five minutes after the injury, animals in the Heat group received heat stress, but those in the non-Heat group did not. The hot pack was applied to the injured EDL muscle through the skin and TA muscle for 20 min. Applying time of the hot pack determined by preliminary experiment described below. We previously monitored surface temperature of the muscle by a thermometer (Tsuruga Electric Works, Osaka, Japan) remained in close contact with surface of the muscle and reported that surface temperature in the injured muscle after applying hot pack of our study increased, although that of the injured muscle without heat stress did not increase<sup>27</sup>. Applying time of the hot pack of this study (20 min) and temperature setting (42C) were enough to increase the surface temperature of the EDL, although the heat radiated from skin, through the TA muscle, to the EDL muscle, without inducing cell death by heat stress itself (see Takeuchi et al.<sup>27</sup>). In experimental procedures of this study, animals were monitored whether abnormal behavior occurred or not. These abnormal behaviors of the animals are hyperkinesia, bristling, crying loud and inadequate feeding etc. We observed all the animals in detail throughout the experimental procedure including the peri-operative analgesia, post-operative monitoring and crush injury to avoid any adverse effect. In this study, all the rats showed no overt signs of distress described above. Same as after the treatments, there were no rats exhibited such painful behavior. In addition, there were no adverse effects of the heat stress on the skin. At 12 h, 1, 2, 3, 4, 5, 7, 14 and 28 days (n=8/group at each time point) after the injury, animals were sacrificed by an overdose intraperitoneal injection of pentobarbital sodium. The injured EDL were harvested, and immediately frozen in dry ice-cooled acetone without any fixation, and stored at -80C until analyses. The harvested samples were used for morphological analysis (n=3/group at each time point) and western blot analysis (n=5/group at each time point, respectively).

### Morphological analysis

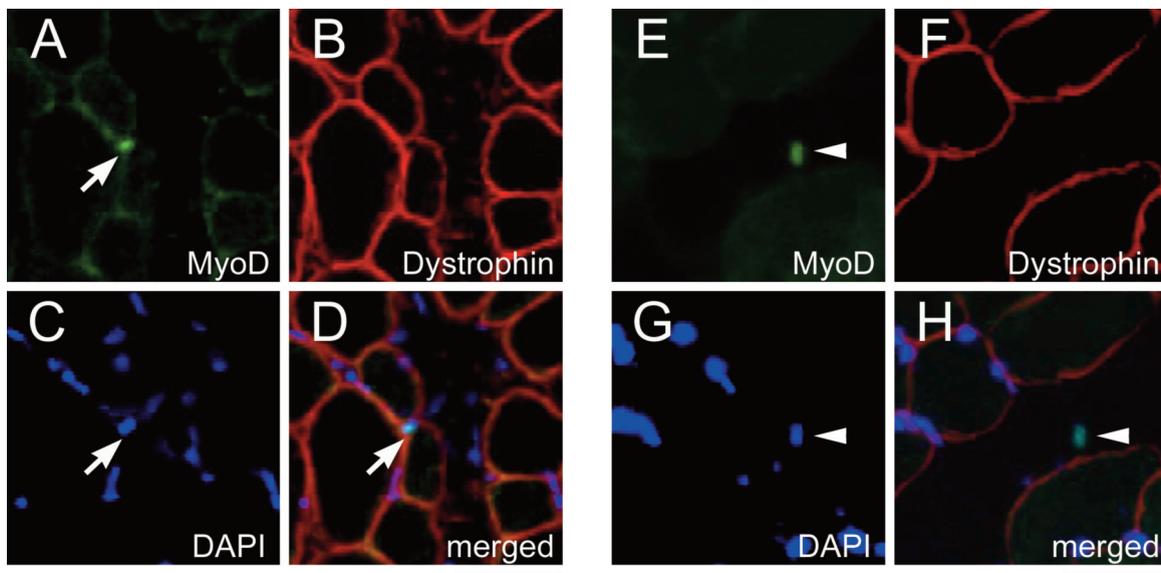
Cross sections of 10 µm in thickness were cut by a cryostat (CM-1510S, Leica Microsystems, Mannheim, Germany) and mounted on glass slides. These cross sections were cut in the middle part of the EDL muscle belly. This was just the crushed site. Therefore, these cross sections were just crushed site. To begin with, we confirmed these sections with hematoxylin and eosin (H-E) staining whether its site is injured site or not. Then these sections were cut repeatedly in injured sites with confirmation. Some of these sections were used for H-E staining for histological investigation, and others were used for immunofluorescence staining, as described below.

### Immunofluorescence staining

In the present study, anti-MyoD, anti-myogenin, anti-dystrophin anti-proliferating nuclear antigen (PCNA) and anti-Pax-7 were used as primary antibodies. These antibodies were obtained from Santacruz Biotech. Dystrophin, Pax-7 and PCNA were used for marker molecules of the plasma mem-



**Figure 1.** Chronological change of MyoD protein expression levels in regeneration. (A) Bands gained by Western blot. (B) Vertical axis shows the ratio of MyoD protein expression levels analyzed by Western blot. Horizontal axis shows the time course after the injury. This indicates that heat stress promote chronological change of MyoD protein by 1 day. Solid lines, non-Heat group; dashed line, Heat group. Data are expressed as the mean±s.e. a and b are significantly difference from 4 and 5 days in same groups, respectively, at  $p<0.01$  and c is significantly difference from 4 days, respectively, at  $p<0.05$ . \*\*and \* is significantly difference from non-Heat group at  $p<0.01$  and 0.05, respectively.



**Figure 2.** The spatial expression patterns of the MyoD and dystrophin immunoreactivities at 1 day after injury in Heat group (A-D). These findings are identical with the 2 days after injury in non-Heat groups (E-H). Triple immunostaining to show localization of MyoD (A, E), dystrophin (B, F) and nuclei (C, G) is performed and three images are merged (D, H). Arrows indicate a nuclei expressing MyoD inside dystrophin-positive membrane (MyoD-positive myonuclei). Arrowheads indicate nuclei expressing MyoD outside dystrophin-positive membrane (MyoD-positive nuclei of satellite cells). Scale bars: 50  $\mu$ m.

brane, satellite cells and proliferating cells, respectively<sup>9</sup>. Secondary antibodies, anti-mouse IgG (H+L) Alexa Fluor R 555 and anti-rabbit IgG (H+L) Alexa FluorR 448 were obtained from Cell Signaling Technology, and used for the primary mouse monoclonal antibodies and the primary rabbit polyclonal antibodies, respectively.

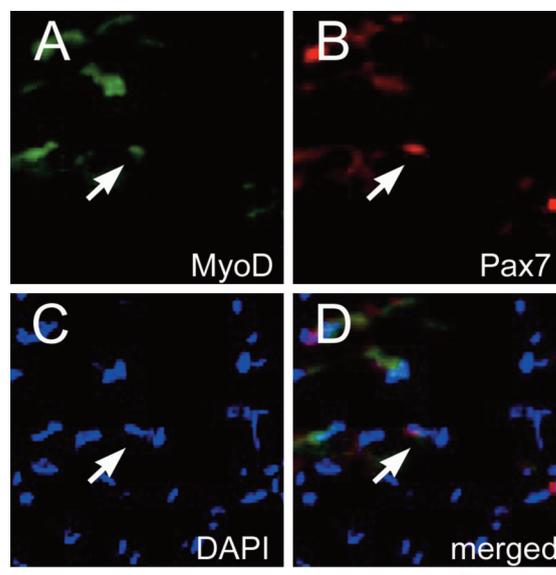
Frozen cross sections were mounted on 3-aminopropylethoxysilane-coated glass slides and air-dried for 30 minutes at room temperature. Sections were fixed in 4% paraformaldehyde diluted with 0.1M phosphate buffer (pH 7.4). These sections were then washed in 0.1M phosphate-buffered saline (PBS: pH 7.4). After the post-fixation, sections of Pax-7 and PCNA immunohistochemistries were treated with 10 mM sodium citrate (pH 6.5) for 10 minutes at 80C for epitope retrieval. To block a non-specific reaction, these sections were incubated in 0.1M PBS containing 10% normal serum and 1% triton X-100 for 1 hour. The primary antibodies were appropriately diluted with 0.1 M PBS containing 5% normal serum and 0.3% Triton X-100, and sections were incubated in them for 12-24 hours at 4C. The secondary antibodies were appropriately diluted with 0.1 M PBS containing 5% normal serum and 0.3% Triton X-100 from, and sections were incubated in them overnight at 4C. After washing in 0.1 M PBS, these sections were mounted in Vectashield mounting medium with 4'-d-diamino-2-phenylindole (DAPI; Vector Labs) to visualize nuclei and observed on a microscope (Olympus, co. Japan) with epifluorescence.

#### Gel electrophoresis and immunoblots

Primary antibodies used in this study were anti-MyoD and anti-myogenin, and secondary antibodies, anti-rabbit or anti-mouse IgG, conjugated to horseradish peroxidase (GE Healthcare, Waukesha, WI, USA). The frozen muscle samples were homogenized in homogenizing buffer containing 20 mM Tris-HCl (pH 7.4), 25 mM KCl and protease inhibitor cocktail (Nacalai tesque, Japan). The homogenates were centrifuged at 15,000 rpm for 15 minutes at 4C. Total protein concentration was determined by using a protein determination kit (Bio-Rad, Hercules, CA, USA). The homogenates were solubilized in sample loading buffer containing 50 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 5%  $\beta$ -mercaptoethanol and 0.005% bromophenol blue. Fifteen micrograms of sample protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to poly vinylidene di fluoride (PVDF) membrane. After the membranes were blocked in 10% skimmed milk in PBST for 2 hours, they were incubated with primary antibodies overnight at 4C. The membranes were incubated with secondary antibodies for 1 hour at room temperature. The signals were detected by using a chemiluminescent (ECL, GE Healthcare) and analyzed with an image reader (LAS-1000, Fujifilm, Tokyo, Japan).

#### Quantitative analysis

For the western blot analysis, a specific sample from the non-Heat group at 28 days after the injury was used as a stan-



**Figure 3.** Localizations of MyoD and Pax7 immunoactivities at 1 day after injury in Heat group. These findings are identical with the 2 days after injury in the non-Heat groups (data not shown). Triple immunostaining to show localization of MyoD (A), Pax7 (B) and nuclei (C) is performed and the three images are merged (D). Arrows indicate the Pax7-positive nuclei expressing MyoD (MyoD-positive satellite cells). These results show that MyoD also expressed in nuclei of satellite cells. Scale bar: 50  $\mu$ m.

dard sample. The samples were subjected to SDS-PAGE and the expression ratio to that of the standard sample was calculated<sup>5,20</sup>. For morphological analysis, H-E sections were used. As nuclei migrate from the center to periphery of the fibers with maturation<sup>8,23</sup>, we used muscle fiber exhibiting central nuclei as regenerating immature muscle fibers. In the H-E sections, the ratio of the fibers exhibiting central nuclei to total muscle fibers were calculated in 10 areas (1 area=0.0625 mm<sup>2</sup>) at 14 days after the injury.

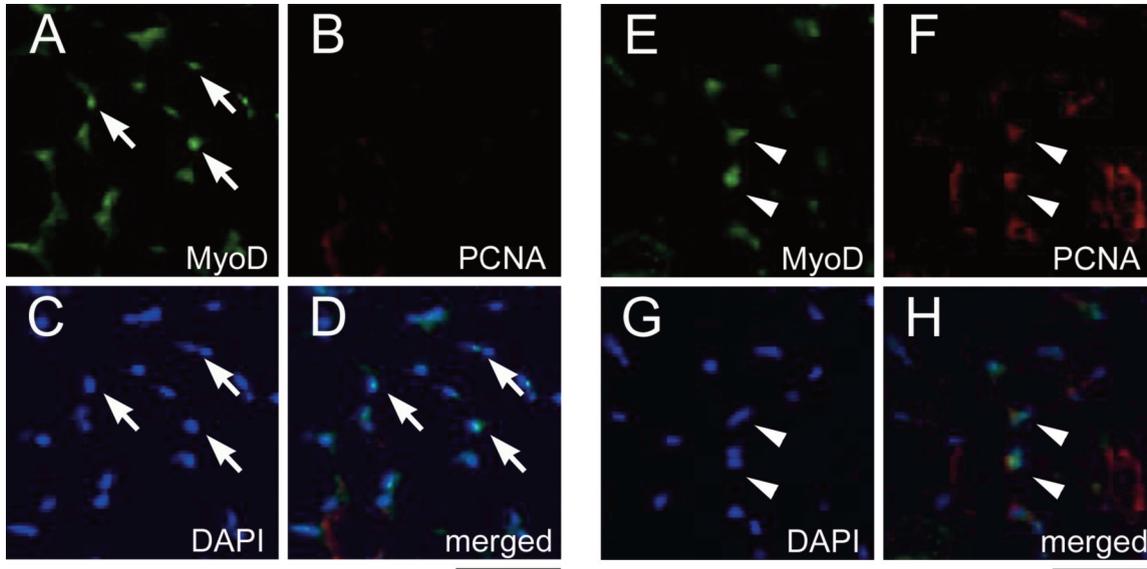
#### Statistical analysis

All values are represented as mean $\pm$ standard error. The effect of group (Injury vs Injury+Heat) and time (12 h, 1, 2, 3, 4, 5, 7, 14 and 28 days after the injury) were tested for significance with an analysis of variance (ANOVA). When the ANOVA revealed a significant interaction, Scheffe test were performed as post hoc tests. Statistical significance level was set at P<0.05. Two-way (group  $\times$  time) analysis of variance (ANOVA) was used and Scheffe test were performed as post hoc tests. Statistical significance level was set at P<0.05.

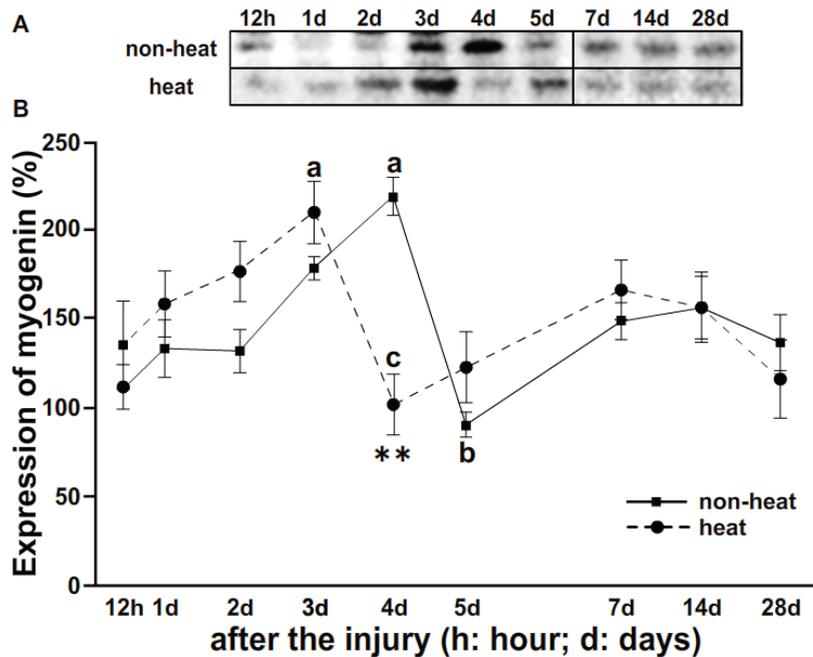
## Results

#### Expression of MyoD at early stages of muscle regeneration

Figure 1 showed the western blot analysis of MyoD. According to this analysis, these were a significant main effect for group (p<0.01) and time (p<0.01) and a significant group  $\times$



**Figure 4.** Photomicrographs showing triple immunostaining to show localization of MyoD (A, E), PCNA (B, F) and nuclei (C, G) is performed and three images are merged (D, H). (A-D) 12h after injury in Heat group (Figure 4D). Arrows indicate a nucleus expressing only MyoD. (E-H) 1 day after injury in Heat group. These findings are identical with the 2 days after injury in non-Heat group (A-D). Arrowheads indicate that a nuclei expressing both MyoD and PCNA. These results show that the timing of the MyoD expression was ahead of the PCNA by 12 h - 1 day. Scale bars: 50  $\mu$ m.



**Figure 5.** Chronological change of myogenin protein expression levels in regeneration. (A) Bands gained by Western blot. (B) Vertical axis shows the ratio of myogenin protein expression levels. Horizontal axis shows the time course after the injury. This indicates that heat stress promote chronological change of myogenin protein by 1 day. Solid lines, non-Heat group; dashed line, Heat group. Data are expressed as the mean $\pm$ s.e. a, b and c are significantly difference from 12 hours, 3 and 4 days, respectively, at  $p < 0.01$  in same groups. \*\* is significantly difference from non-Heat group at  $p < 0.01$ .

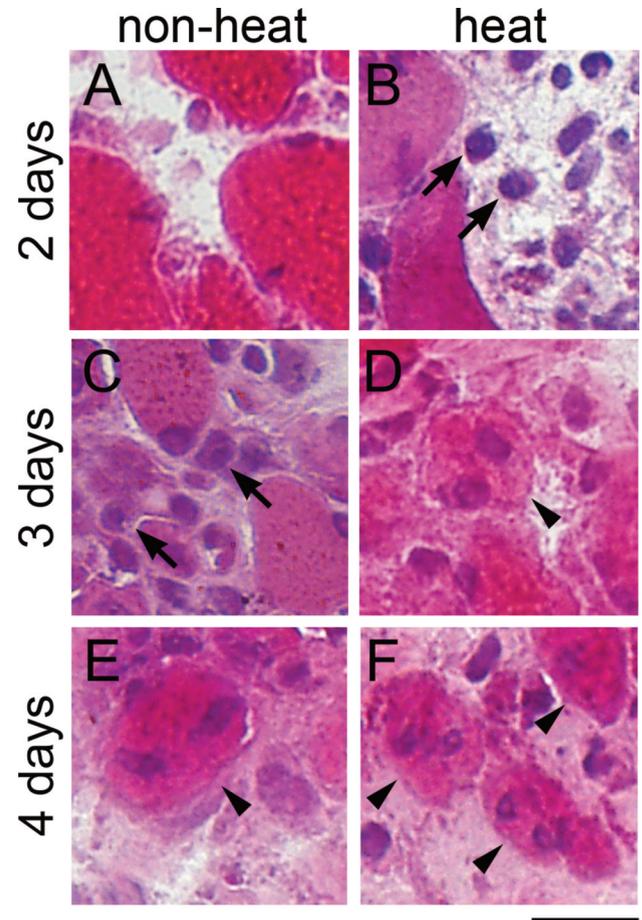
time interaction ( $p < 0.01$ ). Although not significant, the expression levels of MyoD in non-Heat group increased 24.1 % from 12 hours to 2 days after the injury, when that level reached first peak. Then that level significantly decreased until 5 days ( $p < 0.01$ , 66.8 % decrease) and significantly re-increased until 7 days after the injury ( $p < 0.01$ , 168.8% increase). In Heat group, the expression levels of MyoD increased 9.2% from 12 hours to 1 days after the injury ( $p > 0.05$ ), when that level reached first peak. Also, this expression level was higher than that in non-Heat group at the same time ( $p < 0.05$ ). Then, their levels significantly decreased until 4 days ( $p < 0.01$ , 63.5% decrease) and significantly re-increased until 5 days ( $p < 0.01$ , 159.4% increase). Also, this expression level was higher than that in non-Heat group at the same time ( $p < 0.01$ ).

Immunoreaction for MyoD was detected on the nuclei being stained with DAPI at 2 days after the injury in the non-Heat group, and at 1 day, in the Heat group (Figure 2). At these stages, some of the MyoD-positive cells were noted along the dystrophin- positive plasma membrane (Figure 2). Other MyoD-positive cells were considered to be myogenic cells or inflammatory cells migrating into the injured muscle tissues. Triple immunostaining for MyoD, Pax-7 and DAPI showed that immunoreactions for MyoD and Pax-7 were co-localized on the nuclei stained with DAPI. These cells were found at 2 days after the injury in the non-Heat group, and at 1 day in the Heat group (Figure 3).

Triple immunostaining for MyoD, PCNA and DAPI was shown in Figure 4. At 1 day after the injury in the non-Heat group and at 12 hours in the Heat group, MyoD-positive nuclei were found, but PCNA-positive nuclei were not yet detected (Figure 4 A-D). At 2 days after the injury in the non-Heat group and at 1 day in the Heat group, immunoreaction for DAPI was co-localized on the MyoD-positive nuclei (Figure 4 E-H). These findings indicate that 12 hours - 1 day prior to proliferation, MyoD began to be expressed in the myogenic nuclei.

#### Expression of myogenin at early stages of muscle regeneration

Chronological changes in the expression of myogenin were presented in Figure 5. According to this analysis, these were a significant main effect for time ( $p < 0.01$ ) and a significant group  $\times$  time interaction ( $p < 0.01$ ). In the non-Heat group, although not significant ( $p = 0.1597$ ), the expression level of the myogenin increased 62.3% from 12 hours to 4 days after the injury, when that level reached first peak. Also, the expression level was higher than that in Heat group at the same time ( $p < 0.01$ ). Then, the expression level significantly decreased until 5 days after the injury ( $p < 0.01$ , 58.9% decrease). Although not significant, the expression levels re-increased 73.7% from 5 to 14 days, and then it tended to decrease until 28 days after the injury. In Heat group, the expression significantly increased 87.6% from 12 hours to 3 days after the injury ( $p < 0.05$ ), when that level already reached first peak unlike non-Heat group. Then, the expression level significantly decreased until 4 days after the injury ( $p < 0.05$ , 51.5% decrease). Although the expression levels re-increased 63.4% from 4 to 7 days, and then it tended to decrease until 28 days after the injury.



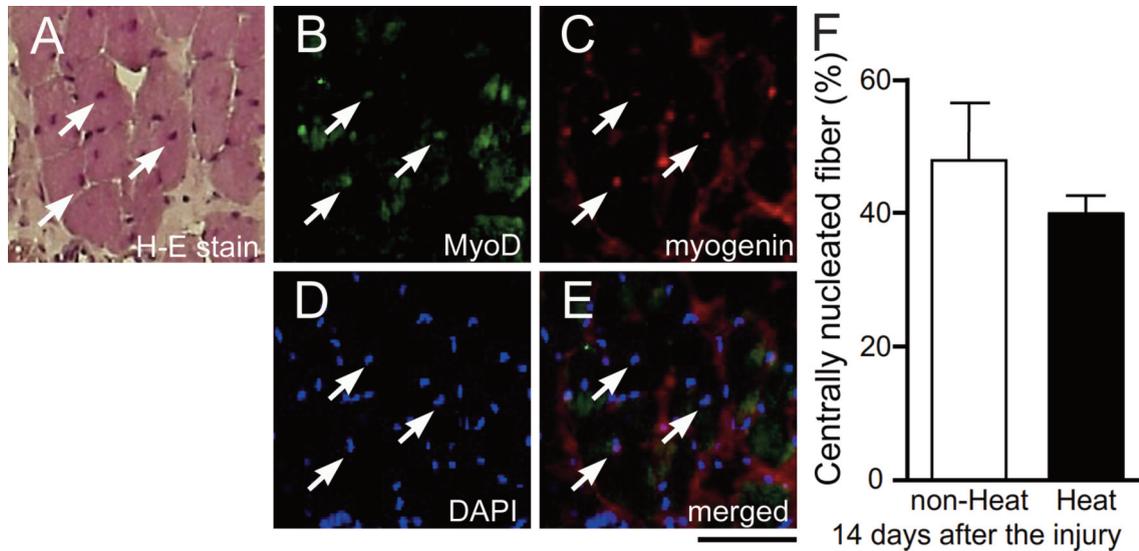
**Figure 6.** Transverse sections of the extensor digitorum longus muscle stained with hematoxylin and eosin in the non-Heat and Heat groups at 2, 3 and 4 days after the injury. Arrows and arrowheads indicate myoblasts and myotube, respectively. These results indicate that heat stress can accelerate regeneration process of the skeletal muscle including the myoblasts and myotube appearance. Scale bar: 100  $\mu\text{m}$ .

#### Morphological features at early stages of muscle regeneration

According to the observation in H-E sections, nascent regenerating myogenic cells (myoblasts) exhibiting a basophilic cytoplasm were first encountered at 3 days after the injury (Figure 6C) in the non-Heat group, and in the Heat group, at 2 days (Figure 6B). Regenerating muscle cells containing 2 or more nuclei (myotube) were first found at 4 days in the non-Heat group (Figure 6E), and at 3 days after the injury, in the Heat group (Figure 6D).

#### Expression of MyoD and myogenin at 14 days after the injury

Morphologically, regenerating muscle fibers exhibiting centrally located nuclei (central nuclei) were observed in both the non-Heat and Heat groups (Figure 7A). The ratio of these fibers to total fibers (Figure 7F) in the Heat group ( $39.7 \pm 2.3\%$ ) was lower than that in the non-Heat group ( $47.9 \pm 8.5\%$ ). Furthermore, by triple immunohistochemistry, MyoD and myo-



**Figure 7.** These serial cross sections indicate the MyoD and myogenin immunoactivities at the 14 days after injury. (A) Hematoxylin and eosin staining to show morphological characteristic at this later stage of regeneration. Triple immunostaining to show localization of MyoD (B), myogenin (C) and nuclei (D) is performed and the three images are merged (E). Arrows indicate central nuclei. (B-E) These figures show that the MyoD and myogenin co-express in the same nuclei as (A). Scale bar: 50  $\mu$ m. (F) Vertical axis shows the ratio of the fibers exhibiting central nuclei to total muscle fibers. Horizontal axis shows groups, non-Heat and Heat groups at 14 days after the injury. These results indicate that the ratio in Heat group is lower than that in non-Heat group at 14 days after the injury.

genin was co-expressed not only in the central nuclei but also on the nuclei along the margin of the regenerating fibers.

## Discussion

### *Heat stress accelerated proliferation of satellite cells and the regulating factor MyoD*

It was reported that up-regulation of MyoD m-RNA and synthesis of the protein were rapidly induced in injured muscle, namely muscle precursor cells began to express MyoD soon after the activation induced by the injury<sup>29</sup>. In the present study, the level of MyoD expression began to increase within 12 hours, and reached the first peak at 2 days after the injury in the non-Heat group, and at 1 day in the Heat group, respectively. Our immunohistochemical studies revealed that though MyoD-positive nuclei were noted at 1 day after the injury in the non-Heat group, and at 12 hours, in the Heat group. In addition, PCNA-positive proliferating nuclei were first found at 2 days after the injury in the non-Heat group, and at 1 day, in the Heat group. In the present study, myogenic cells in which labeled both with MyoD and Pax-7 were detected at 2 days after the injury in the non-Heat group, and at 1 day, in the Heat group. These findings indicate that MyoD begins to be expressed in myogenic cells before they begin to proliferate, and the timing of the peak in the expression of MyoD is well corresponded with the appearance time of their nuclei exhibiting co-expression of MyoD and PCNA in both groups. Megeney et al.<sup>15</sup> reported that in the MyoD knockout mouse, proliferating ability of myogenic precursor cells was weakened, thus the

muscle regeneration did not adequately occur. From these previous reports, MyoD have been reported to be involved with proliferation of myogenic cells during muscle regeneration. Therefore, these findings taken together suggest that heat stress soon after the injury could accelerate the proliferation of myogenic cells and the expression of the MyoD.

It was reported that withdrawal from the cell cycle is required for the proliferating satellite cells to differentiate into myoblasts<sup>9</sup>. Zammit et al.<sup>30</sup> reported that as the cell cycle withdrawal progressed, MyoD disappeared. These findings suggest that the decline of MyoD might be necessary for the differentiation of the proliferating satellite cells to differentiate into myoblasts. In the present study, after the first peak, MyoD expression began to decline until 5 days after the injury in the non-Heat group, and until 4 days, in the Heat group, respectively. Morphologically, young regenerating muscle cells (myoblasts) are characterized by basophilic cytoplasm containing a nucleus<sup>18,24</sup>. These cells were first found at 3 days after the injury in the non-Heat group, and at 2 days, in the Heat group, respectively. These findings suggest that heat stress could accelerate differentiation of myoblasts from proliferating satellite cells.

### *Heat stress accelerated differentiation to myotube and the regulating factor myogenin*

It has been reported that myogenin involves with differentiation of satellite cells, especially fusion from myoblast to myotube<sup>12</sup>. Wright et al.<sup>28</sup> reported that *in vitro*, at the time when myogenin transcripts reached the peak, myotube formation and myosin message began, and thereafter, it declined. In the pres-

ent study, according to our western blot analysis, the expression level of myogenin reached the first peak at 4 days after the injury in the non-Heat group, and at 3 days, in the Heat group. Thereafter, it rapidly decreased until 5 days in the non-Heat group, and until 4 days, in the Heat group. Regenerating muscle cells containing two or more nuclei (myotubes) were first found at 4 days in the non-Heat group, and at 3 days, in the Heat group. These findings indicated that the stage when the myotube began to be formed was temporally corresponded with the first peak of the myogenin expression in both non-Heat and Heat groups, respectively. Furthermore, the chronological change of myogenin expression and the appearance of myotube were accelerated by applying the heat stress. Therefore, these findings suggest that heat stress soon after the injury could accelerate the differentiation of myogenic cells and the expression of the myogenin.

#### *Effect of heat stress on expression of MyoD and myogenin at re-increasing stages*

In the present study, the rapid decline of MyoD and myogenin expressions finished at 5 days after the injury in the non-Heat group, and at 4 days, in the Heat group, and thereafter, the expression of these molecules began to increase again until 7 days after the injury, respectively. After 7 days, levels of the expression of MyoD in both groups did not change until 28 days, but levels of the myogenin appeared gradually to decrease until 28 days in both groups. The rapid decline and re-increase of MyoD and myogenin were also reported *in vivo*<sup>20</sup> and *in vitro*<sup>28</sup>. Since the production of myoblasts and formation of myotubes might finish at the early stages of muscle regeneration, roles of MyoD and myogenin expressed at the re-increasing stages should be different from those at the early stages described above.

Fuchtbauer and Westphal<sup>6</sup> reported that at 2 weeks after the injury, MyoD and myogenin were expressed in nuclei of the regenerating muscle fibers. In our materials at 14 days after the injury, expressions of these factors were co-localized mainly on the centrally located nuclei in the regenerating muscle fibers. At this stage, regenerating muscle fibers containing centrally located nuclei (central nucleus) were often observed, and morphologically, they were considered to be immature regenerating muscle fibers<sup>18</sup>. As to the possible roles of MyoD and myogenin expressed at the re-increasing stages, several researchers showed this phenomenon. It was reported that up-regulation of MyoD and myogenin occurred in denervated skeletal muscles<sup>27</sup> and intact senile muscles<sup>5</sup>. Launay et al.<sup>13</sup> reported that expression of these factors re-increased from 8 days after injury to denervated skeletal muscle, and suggested that, at this stage, MyoD and myogenin might have roles in formation of neuro-muscular junctions. These findings suggest that considerably high expression of these factors at re-increasing stages might be involved in further maturation of regenerating muscle fibers or functional recovery of neuro-muscular junctions, although their precise roles at late stages are still unknown. In the present study, heat stress soon after the injury could accelerate re-increase of MyoD and myogenin at later

stage, suggesting that heat stress could effect further maturation of regenerating muscle fibers or neuro-muscular junctions.

## Conclusion

Due to the heat stress applied soon after the crush injury, chronological changes in expression of MyoD and myogenin progressed about 1 day earlier than those in the non-Heat group, and proliferation of satellite cells, differentiation into myoblasts and formation of multinucleated regenerating muscle fibers were also accelerated. Therefore, this finding suggests that heat stress soon after the injury promotes the proliferation and the differentiation of myogenic cells and the expression of the MyoD and myogenin.

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