A Novel Approach to Collecting Satellite Cells From Adult Skeletal Muscles on the Basis of Their Stress Tolerance

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INTRODUCTION

Adult tissue stem cells are unique cells that retain their ability to self-renew and differentiate, play an essential role in tissue maintenance and regeneration, and are generally in a quiescent state in vivo. When tissues are damaged or exposed to stress, functional differentiated cells degenerate, whereas tissue stem cells are activated to enter into the cell cycle, where they self-renew, generate differentiated cells, and contribute to regeneration. For example, mature neurons and glial cells degenerate or die after cerebral ischemia, but neural stem/progenitor cells are activated to proliferate and differentiate into neural cells [1–4]. Thus, tissue stem cells are generally stress-tolerant.

Flow cytometry is widely used to collect stem cells from tissues by detecting specific surface markers. For example, human hematopoietic stem cells, one of the most extensively studied adult stem cell types, can be collected using the anti-CD34 antibody [5–7]. Although flow cytometry is a powerful tool, it is not applicable to stem cells whose surface markers are not well determined.

Tissue stem cells of skeletal muscle, namely satellite cells, are located between the basal lamina and the plasma membrane of muscle fibers in vivo [8]. When muscle fibers are damaged, satellite cells are activated to proliferate and generate myocytes that fuse together to form myotubes and contribute to muscle regeneration [9–11]. Paired box protein 7 (Pax7) is considered a reliable mouse satellite cell-specific marker [12, 13]. Mice transgenic for Pax7 were developed for analysis of the origin and development of satellite cells [13–15]. Flow cytometry is not practical for separating human satellite cells, however, particularly for clinical application, because Pax7 is a transcription factor and is therefore not located on the cell surface.

In the past few years, several groups have attempted to identify surface markers for mouse satellite cells. CD34 [16], M-cadherin [17], and vascular cell adhesion molecule-1 [18], or combinations of different markers, such as α7 integrin and CD34 [19], CXCR4 and β1-integrin [20], or syndecan-3 and -4 [21], are reported to be useful for collecting satellite cells, although these markers are not specific for satellite cells. The SM/C-2.6 antibody was recently identified as a marker...
of mouse dormant satellite cells for use in flow cytometry [22]. The details of this marker (e.g., localization, species specificity, function, and expression in other tissues or cells), however, have not yet been clarified. In human studies, neural cell adhesion molecule (CD56) has been used to mark satellite cells [23], but it is not specific for satellite cells and is also expressed on Schwann cells and natural killer lymphocytes [24–26]. For this reason, flow cytometry methods are limited for collecting human satellite cells.

In the present study, we turned our attention from flow cytometry to the stress tolerance of adult stem cells. Among primary cultured cells harvested from mouse and human skeletal muscle tissues, Pax7(+) satellite cells are tolerant of stress, and their ratio is substantially increased by exposure to stress, such as long-term trypsin incubation (LTT). Heat shock protein 27 (Hsp27) and αB-crystallin, known antistress factors [27–29], are related to the stress tolerance of Pax7(+) satellite cells. Furthermore, we confirmed that these enriched satellite cells retained their myogenic stem cell function both in vitro and in vivo. In this report, we developed a very efficient and inexpensive method of collecting human satellite cells that retain stem cell function in vivo. Our approach may also be used for other tissue stem cells whose specific surface marker is unknown and for which flow cytometry is not directly applicable for collection.

**MATERIALS AND METHODS**

All animal experiments were approved by the Animal Care and Experimentation Committee of Tohoku University Graduate School of Medicine.

**Skeletal Muscle Cell Isolation**

Primary mouse skeletal muscle cells were isolated from the hind limb skeletal muscle of 7- to 8-week-old C57BL/6NCrSlc mice (Japan SLC, Inc., Shizuoka, Japan, http://jslc.co.jp), dissected free of fat and connective tissue and washed with 0.01 M phosphate-buffered saline (PBS). Normal human skeletal muscle samples were purchased from Asterand, Inc. (Detroit, MI, https://www.asterand.com) and obtained from two healthy donors (an 80-year-old woman and an 82-year-old man). Approximately 3–10 cm³ of human skeletal muscle was dissected free of fat and connective tissue, and washed with 0.01 M PBS. The sample was minced finely and digested with 0.1 mg/ml Collagenase/Dispase (collagenase activity >0.1 U/mg lyophilizate and dispase activity >0.8 U/mg lyophilizate; Roche Applied Science, Mannheim, Germany, https://www.roche-applied-science.com) for 1.5 hours at 37°C. After digestion, the cell suspension was filtered through a 0.8-μm-thick cryosections were cut. Skeletal muscle cells on type I collagen-coated cover glasses were fixed with 4% (vol/vol) paraformaldehyde in 0.01 M PBS before immunocytochemistry.

**Cell Culture**

Cells were cultured in 5% CO₂ at 37°C. Primary mouse culture cells were maintained in growth medium: Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, http://www.invitrogen.com) containing 20% (vol/vol) fetal bovine serum (FBS; HyClone; Thermo Fisher Scientific, Logan, UT, http://www.thermofisher.com), 0.1 mg/ml kanamycin sulfate (Gibco, Grand Island, NY, http://www.invitrogen.com), 10 ng/ml basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, http://www.peprotech.com), and 500 U/ml ESGRO (leukemia inhibitory factor; Millipore, Billerica, MA, http://www.millipore.com). Normal human skeletal muscle cells (SkMCs; Lonza, Walkersville, MD, http://www.lonza.com) and primary human skeletal muscle cells were cultured in growth medium, SkGM BulletKit (Lonza).

**Stress Condition Tests**

We tested four stress conditions: (a) culture in DMEM containing no serum for 2 days, (b) culture in Hanks’ balanced salt solution (HBSS) buffer (Invitrogen) for 2 days, (c) culture in 20% (vol/vol) FBS in DMEM combined with low O₂ (2%) for 2 days, and (d) LTT for 6 hours (described below). After the cells were exposed to stress conditions, trypan blue staining was used to count the number of live cells, from which the survival ratio was calculated. The surviving cells were resuspended in growth medium and plated in a collagen-coated dish. After 24 hours of plating, cells were subjected to Pax7 staining as described below. The experiments were repeated at least three times.

**LTT Incubation**

Skeletal muscle cells (5 × 10⁵) were suspended in 5 ml of trypsin solution (0.25% trypsin-HBSS; Invitrogen), transferred to a 6-cm diameter dish, and incubated at 37°C for 1, 2, 2.5, 3, 3.5, 4, 6, or 8 hours. After incubation, the cells were washed with 0.01 M PBS and suspended in 5 ml of PBS in a 15-ml Falcon tube. The tube was vortexed for 1 minute by MS1 Minishaker (IKA Works, Inc., Cincinnati, OH, http://www.ika.com) at 1,800 rpm and then centrifuged at 400g for 15 minutes. Finally, the supernatant containing the dead cells was removed and the surviving cells were counted on the basis of trypan blue staining. The surviving cells were resuspended in growth medium and plated in a collagen-coated dish. After 24 hours of plating, cells were subjected to immunocytochemistry as described below. The experiments were repeated at least three times.

**Immunocytochemistry**

Cells were fixed with 4% (vol/vol) paraformaldehyde in 0.01 M PBS. Primary human skeletal muscle cells just after trypsin incubation were collected by centrifugation and embedded in O.C.T. Compound (Sakura Finetek, Tokyo, Japan, http://www.sakura.com), and 8-μm-thick crossections were cut. Skeletal muscle cells on type I collagen-coated cover glasses were fixed with 4% (vol/vol) paraformaldehyde in 0.01 M PBS before immunocytochemistry.

Samples were incubated with block solution containing 20% (vol/vol) Block-Ace (DS Pharma Biomedical, Osaka, Japan, http://www.dspbio.co.jp), 5% (wt/vol) bovine serum albumin (BSA; Sigma-Aldrich Japan, Tokyo, Japan, http://www.sigmaaldrich.com), and 0.3% (vol/vol) Triton X-100 (Wako Pure Chemical, Osaka, Japan, http://www.wako-chem.co.jp/english) in 0.02 M PBS at room temperature for 1 hour. Samples were then incubated overnight at 4°C with primary antibodies diluted in antibody diluent solution (0.02 M PBS supplemented with 5% [vol/vol] Block-Ace, 1% [wt/vol] BSA, and 0.3% [vol/vol] Triton X-100). Primary antibodies used were Pax7 (1:6,000; Developmental Studies Hybridoma Bank [DSHB], University of Iowa, Iowa City, IA), MyoD (1:200; BD Pharmingen, San Diego, CA, http://wwwbdbiosciences.com/index_us.shtml), myogenin (1:1,000; BD Pharmingen), von Willebrand factor (1:1,200; Dako Cytomation,
Glostrup, Denmark, http://www.dako.com), type I collagen (1:1,000; SouthernBiotech, Birmingham, AL, http://www.southernbiotech.com), S100A4 (1:2,000; Dako Cytomation), glial fibrillary acidic protein (GFAP; 1:1; Dako Cytomation), P0 (1:100; kindly provided by Dr. J.J. Archelos, Karl-Franzens Universität, Graz, Austria), Ki67 (1:100; Thermo Fisher Scientific), heat shock protein 72 (Hsp72; 1:100; Enzo Life Sciences, Plymouth, PA, http://www.enzolifesciences.com), and αβ-crystallin (1:100; Enzo Life Sciences). After being washed three times with PBS, samples were incubated with secondary antibodies (Alexa 488- or Alexa 568-conjugated anti-mouse IgG, anti-rabbit IgG, anti-goat IgG antibodies [Invitrogen]) for 1 hour at room temperature. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). All images were taken with a C1si Nikon confocal microscope system (Nikon, Tokyo, Japan, http://www.nikon.com). We counted Pax7- and MyoD-positive cells among more than 1,000 mouse cells and 500 human cells.

**Cell Transplantation**

Primary human skeletal muscle cells were labeled with green fluorescent protein (GFP)-lentivirus, as previously reported [30]. To induce muscle degeneration, cardiotoxin was injected into the gastrocnemius muscles of NOG mice (NOD/Shi-SCID [31], IL-2RγKO Jic, 7–8 weeks old; ICLAS Monitoring Center, Kawasaki, Japan, http://www.iclasmonic.jp) or SCID mice (7–8 weeks old; CLEA Japan, Inc., Tokyo, Japan, http://www.clea-japan.com) under anesthesia with avertin as described previously [32]. Nontreated cells and the cells obtained after trypsin incubation (1 × 10^6) were transplanted by local injection 2 days after inducing the damage. At 4 weeks after transplantation, cardiotoxin was administered again. At 2, 4, and 6 weeks after transplantation, mice were killed by an overdose of diethyl ether, fixed, and then subjected to immunohistochemistry.

In this study, we defined a damaged area as an area that satisfies both of the following conditions: (a) an area containing skeletal muscle fibers with centrally located nuclei, and (b) an area with a total number of nuclei >1,200 per mm^2. Generally, at an earlier time point of regeneration, such as 2 weeks after damage, regenerating myofibers exhibit centrally located nuclei, but later the nuclei change their location to the periphery of the myofibers [32]. In the present study, we collected samples not only at 2 weeks but also at 6 weeks, 2 weeks after cardiotoxin treatment, and thus regenerating myofibers could be counted as myofibers with centrally located nuclei in both samples. Furthermore, we found that the number of nuclei per mm^2 increased after damage because of a decrease in the size of the myofibers and the infiltration of inflammatory cells. For a pilot study, we counted the number of nuclei in both normal muscles and damaged tissues (2 weeks after cardiotoxin treatment) and found that a damaged area is represented by a total number of nuclei >1,200 per mm^2. In this experiment, we calculated the ratio of regenerating myofibers in damaged areas as number of GFP(+) myofibers with centrally located nuclei per number of all myofibers with centrally located nuclei. We counted at least three sections for each sample.

**Immunohistochemistry**

Mouse muscles were fixed with freshly prepared periodate-lysine-paraformaldehyde for 6 hours at 4°C, and a cryostat was used to cut 10-μm-thick sections. Samples were washed with 0.01 M PBS and incubated with blocking solution (5% [vol/vol] normal goat serum [Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com], 0.1% Triton X-100, and 0.3% BSA in 0.01 M PBS) for 1 hour at room temperature. After the blocking step, the slides were incubated overnight at 4°C with primary antibodies diluted in blocking solution. The primary antibodies used were Pax7 (1:1,000; DSHB) and human dystrophin (1:100; Vector Laboratories). After being washed three times with PBS, the slides were incubated with anti-mouse IgG antibody conjugated with Alexa 568 (1:500; Invitrogen) in 0.01 M PBS containing 0.1% Triton X-100 for 2 hours at room temperature. The cell nuclei were stained with DAPI. Samples were inspected with a C1si Nikon confocal microscope system.

**Statistical Analysis**

Statistical significance was assessed using Student’s t test. For comparisons of more than two groups, one-way analysis of variance was used, followed by the Bonferroni multiple comparisons test. A p value of less than .05 was considered statistically significant. All data are presented as means ± SEM.

**RESULTS**

**Enrichment of Mouse Satellite Cells**

Primary cultured cells were collected from adult mouse skeletal muscles by digesting them with collagenase/dispase, and analyzed by immunocytochemistry. The cultured cells comprised Pax7(+) satellite cells (9.3 ± 3.4%), MyoD(+) myocytes (10.0 ± 5.4%), and Pax7(+) /MyoD(+) cells (2.5 ± 0.6%), as well as non-muscular cells, such as fibroblasts (S100A4(+) /Pax7(−)/MyoD(−) cells; 78.4 ± 2.6%), vascular endothelial cells (von Willebrand factor(+) cells; 2.6 ± 0.6%), and Schwann cells (P0(+) cells; 3.2 ± 0.5%; Fig. 1A–1F, 1K, Before LTT).

To determine the optimal stress condition for the enrichment of satellite cells, we subjected primary cultured cells to four different stress conditions: low serum, poor nutrition, low O2, and LTT for 6 hours. Among the stress conditions tested, LTT was the most effective condition for the enrichment of Pax7(+) satellite cells (Table 1).

Primary cultured cells were then incubated with LTT for either 1, 3, 6, or 8 hours to determine the best condition for enrichment of satellite cells. The survival ratio was calculated, and surviving cells were further subjected to Pax7 immunocytochemistry. The enrichment efficiency, calculated by multiplying the survival cell ratio by the ratio of Pax7(+) satellite cells, was 611.3 ± 108.3 for 1-hour LTT, 1,002.8 ± 282.1 for 3-hour LTT, 3,671.9 ± 487.0 for 6-hour LTT, and 3,091.7 ± 334.8 for 8-hour LTT (Fig. 1G). The enrichment efficiency score was highest in the 6-hour LTT condition, and the ratio of Pax7(+) cells increased up to 28.6 ± 1.9% following 6-hour LTT, an approximately threefold increase compared with before LTT (Fig. 1H). These findings indicated that 6-hour LTT was the most effective of the four tested conditions for the enrichment of mouse satellite cells. We also examined the ratio of MyoD(+) myocytes. The enrichment efficiency score of MyoD(+) cells was 452.3 ± 196.6 for 1-hour LTT, 596.6 ± 62.9 for 3-hour LTT, 4,899.6 ± 612.6 for 6-hour LTT, and 4,125.2 ± 460.1 for 8-hour LTT (Fig. 1I). As for Pax7(+) cells, MyoD(+) myocytes had the highest enrichment efficiency score after 6-hour LTT, and the ratio of MyoD(+) cells increased up to 34.3 ± 0.6% following 6-hour LTT, which was approximately threefold higher than that before LTT (Fig. 1J). The ratio of...
Figure 1. LTT enrichment of mouse Pax7(+) satellite cells in mouse primary cultured cells. (A): Phase contrast image of primary mouse cultured cells. (B–F): Immunocytochemistry of Pax7 (B), MyoD (C), S100A4 (D), vWF (E), and P0 (F) in the cells at 4 days of culture. Nuclei were counterstained with 4’,6-diamidino-2-phenylindole (blue). Scale bars = 100 μm (A) and 50 μm (B–F). (G): Enrichment efficiency score of mouse Pax7(+) cells. LTT incubation time was 1, 3, 6, or 8 hours. Enrichment efficiency was calculated as follows: Survival cell ratio × Ratio of Pax7(+) cells × 1,000. Error bars indicate the mean ± SEM, ***, p < .001. (H): Ratio of mouse Pax7(+) cells before and after 6-hour LTT; *, p < .05. (I): Enrichment efficiency score of mouse MyoD(+) cells. ***, p < .001. (J): Ratio of mouse MyoD(+) cells before and after 6-hour LTT; *, p < .05. (K): Cell population before and after 6-hour LTT. The cell population included muscle-lineage cells (Pax7, Pax7+MyoD, and MyoD; red groups) and nonmuscular cells (S100A4, vWF, and P0; blue groups). Abbreviations: LTT, long-term trypsin incubation; Pax7, paired box protein 7; vWF, von Willebrand factor.
of enriching mouse Pax7(+). The above results demonstrated that LTT is an effective method to enrich satellite cells in mouse skeletal muscles.

Satellite cells change their morphology during differentiation. Upon differentiation, satellite cells proliferate, develop into mononuclear myocytes, and generate multinuclear myotubes by fusing together. To confirm the effect of LTT on the myogenic stem cell function of surviving cells, we verified the proliferation and spontaneous formation of multinuclear myotubes in vitro. The enriched Pax7(+)/satellite cells expressed proliferation marker Ki67 (Fig. 2A–2C). At 4 days after 6-hour LTT, the formation of multinuclear myotubes from surviving cells was confirmed under a phase-contrast microscope (Fig. 2D). The formed myotubes were positive for myogenin, a myogenic differentiation marker (Fig. 2E, 2F). These results suggest that treatment with LTT does not largely affect in vitro myogenic stem cell function.

Table 1. Stress condition tests in primary mouse cultured cells

| Stress condition | Start cell number | Survival after stress (%) | Pax7-positive (%) |
|------------------|-------------------|---------------------------|-------------------|
| Control (normal conditions) | 200,000 | ≥99.9 | 4.0 ± 0.2 |
| Nonserum | 500,000 | 86.7 ± 2.7 | 1.6 ± 0.2 |
| HBSS | 500,000 | 80.2 ± 3.2 | 3.7 ± 0.2 |
| 20% FBS + low O2 | 500,000 | ≥99.9 | 0.9 ± 0.1 |
| LTT, 6 hours | 500,000 | 16.8 ± 1.1 | 27.3 ± 1.9 |

Abbreviations: FBS, fetal bovine serum; HBSS, Hanks’ balanced salt solution; LTT, long-term trypsin incubation; Pax7, paired box protein 7.

Pax7(+) cells also increased up to 19.5 ± 2.6% after LTT, and the total ratio of the cells committed to the myocyte lineage (Pax7(+) and/or MyoD(+) cells) was 43.3 ± 1.0% (Fig. 1K). We further examined the ratio of nonmuscular cells before and after LTT. The ratio of nonmuscular cell markers was substantially reduced after 6-hour LTT: 61.7 ± 1.5% for fibroblasts, 0.4 ± 0.2% for vascular endothelial cells, and 0.3 ± 0.1% for Schwann cells (Fig. 1K). These findings indicate that LTT decreases the number of nonmuscular cells and that LTT is an effective method to enrich satellite cells in mouse skeletal muscles.

Enrichment of Human Satellite Cells by LTT

The above results demonstrated that LTT is an effective method of enriching mouse Pax7(+) satellite cells. We next verified the efficacy of LTT for human skeletal muscle cells. Commercially obtained normal cultured human SkMCs containing Pax7(+) cells (11.8 ± 1.1%) were treated with LTT for 1, 3, 6, or 8 hours (Fig. 3A–3C). The enrichment efficiency was calculated as described above for mouse primary cultured cells, and the ratios were 1,408.9 ± 182.7 for 1-hour LTT, 2,546.2 ± 252.6 for 3-hour LTT, 1,473.6 ± 191.2 for 6-hour LTT, and 1,259.1 ± 33.4 for 8-hour LTT (Fig. 3D). For human SkMCs, the enrichment efficiency score peaked at 3-hour LTT. In human T-SkMCs, we further examined the enrichment efficiency every 30 minutes between 2 and 4 hours to determine the highest enrichment efficiency. The enrichment efficiency scores were 6,706.0 ± 174.5 for 2-hour LTT, 9,275.5 ± 322.6 for 2.5-hour LTT, 10,470.9 ± 754.3 for 3-hour LTT, 7,697.2 ± 237.6 for 3.5-hour LTT, and 7,093.3 ± 180.2 for 4-hour LTT (Fig. 4K). In this examination, the enrichment efficiency score also peaked at 3-hour LTT. We additionally found statistically significant differences (p < .05) between 2 and 2.5 hours and between 3 and 3.5 hours, but no statistical difference between 2.5 and 3 hours. These findings suggested that the LTT treatment time from 2.5 to 3 hours is optimal for human muscles. The ratio of Pax7(+) cells increased up to 79.9 ± 2.2% in 3-hour LTT (Fig. 4L). Like that of Pax7(+) cells, the ratio of MyoD(+) cells increased up to 63.9 ± 1.3% after LTT, and that of Pax7(+) / MyoD(+) cells increased up to 48.9 ± 3.0%. In T-SkMCs, the cells surviving incubation with LTT comprised 94.9 ± 2.2% of Pax7(+) and/or MyoD(+) cells (Fig. 4M). Furthermore, we checked the expression of nonmuscular cell markers in human LTT-treated cells after 3-hour LTT and found that the ratios of fibroblasts (1.9 ± 0.4%), vascular endothelial cells (0.9 ± 0.3%), and Schwann cells (0.7 ± 0.1%) were substantially decreased (Fig. 4M). These results demonstrate that although the period of treatment differs, LTT is effective for the enrichment of both mouse and human Pax7(+) satellite cells. We refer to the LTT-treated cells as the satellite cell-enriched cell (SEC) population in the following sections.

Contribution of the SEC Population to Muscle Regeneration in Vivo

We next isolated cells from fresh human skeletal muscle according to the same protocol used for the mouse muscle tissue, and analyzed the collected cells, namely T-SkMCs (Fig. 4A, 4B). T-SkMCs contained Pax7(+) cells (31.2 ± 4.7%), MyoD(+) cells (53.9 ± 2.3%), and Pax7(+) / MyoD(+) cells (6.6 ± 1.3%), as well as nonmuscular cells, such as fibroblasts (type I collagen (+) / Pax7(−) / MyoD(−) cells; 10.1 ± 0.4%), vascular endothelial cells (3.9 ± 0.5%), and Schwann cells (GFAP (+) cells; 3.1 ± 0.2%); Fig. 4C–4M, Before LTT).

We next applied LTT to the T-SkMCs, and the enrichment efficiency scores were as follows: 4,172.4 ± 168.4 for 1-hour LTT, 10,674.3 ± 516.2 for 3-hour LTT, 4,088.2 ± 261.7 for 6-hour LTT, and 3,358.9 ± 261.1 for 8-hour LTT (Fig. 4J). As was the case with SkMCs, the enrichment efficiency score in T-SkMCs peaked at 3-hour LTT. In human T-SkMCs, we further examined the enrichment efficiency every 30 minutes between 2 and 4 hours to determine the highest enrichment efficiency. The enrichment efficiency scores were 6,706.0 ± 174.5 for 2-hour LTT, 9,275.5 ± 322.6 for 2.5-hour LTT, 10,470.9 ± 754.3 for 3-hour LTT, 7,697.2 ± 237.6 for 3.5-hour LTT, and 7,093.3 ± 180.2 for 4-hour LTT (Fig. 4K). In this examination, the enrichment efficiency score also peaked at 3-hour LTT. We additionally found statistically significant differences (p < .05) between 2 and 2.5 hours and between 3 and 3.5 hours, but no statistical difference between 2.5 and 3 hours. These findings suggested that the LTT treatment time from 2.5 to 3 hours is optimal for human muscles. The ratio of Pax7(+) cells increased up to 79.9 ± 2.2% in 3-hour LTT (Fig. 4L). Like that of Pax7(+) cells, the ratio of MyoD(+) cells increased up to 63.9 ± 1.3% after LTT, and that of Pax7(+) / MyoD(+) cells increased up to 48.9 ± 3.0%. In T-SkMCs, the cells surviving incubation with LTT comprised 94.9 ± 2.2% of Pax7(+) and/or MyoD(+) cells (Fig. 4M). Furthermore, we checked the expression of nonmuscular cell markers in human LTT-treated cells after 3-hour LTT and found that the ratios of fibroblasts (1.9 ± 0.4%), vascular endothelial cells (0.9 ± 0.3%), and Schwann cells (0.7 ± 0.1%) were substantially decreased (Fig. 4M). These results demonstrate that although the period of treatment differs, LTT is effective for the enrichment of both mouse and human Pax7(+) satellite cells. We refer to the LTT-treated cells as the satellite cell-enriched cell (SEC) population in the following sections.
the damaged area (Fig. 5H, left, white bar). In this result, we found that the grafting efficiency of the SEC population at 2 weeks was significantly higher than that of nontreated groups ($p < .05$). After 4 weeks, we confirmed that GFP(+) myofibers expressed human dystrophin (Fig. 5C, 5D). Furthermore, some of the GFP(+) transplanted cells expressed Pax7 and located in the satellite cell position among the regenerating muscles (Fig. 5E, 5F). These findings suggest that the human SEC population can be integrated into damaged muscles and contribute to regeneration.

We further examined whether the transplanted satellite cells contained in the SEC population could function normally as muscle stem cells in vivo. Four weeks after the initial transplantation, the same muscles were damaged again with cardiotoxin but without additional transplantation of the human SEC population. Two weeks after the second cardiotoxin treatment (i.e., 6 weeks after the initial transplantation), we observed regenerating GFP(+) myofibers with centrally located nuclei, and detected 19.9 ± 1.2% of GFP(+) myofibers in the SEC population samples (Fig. 5G, 5H, right, black bar). On the other hand, 3.1 ± 0.4% of regenerating GFP(+) myofibers were detected in the samples with nontreated cells (Fig. 5H, right, white bar), and we found that the regeneration efficiency of the SEC population at 6 weeks was significantly higher than that of nontreated cells at 6 weeks ($p < .01$). This finding indicates that, once transplanted, satellite cells of the human SEC population can integrate into the damaged tissue, where they can differentiate into muscle fibers and continue to contribute to muscle regeneration following repetitive damage in vivo.

Factors Related to Stress Tolerance in Satellite Cells

These results suggest that LTT is an effective method of enriching mouse and human satellite cells derived both from muscle tissues and cultured muscle cells that retain their ability to regenerate muscles. We next examined the responsiveness of Pax7(+) and Pax7(−) cells to stress stimulation by LTT.

Various factors are related to stress tolerance, and among them, Hsp27 and αB-crystallin, members of the small heat shock protein family, are upregulated in response to various types of stress stimuli in skeletal muscles to protect against stress-induced apoptosis [33, 34]. We investigated the expression of Hsp27 and αB-crystallin in T-SkMCs (Fig. 6A). Many Pax7(+) cells expressing Hsp27 and/or αB-crystallin survived treatment with LTT, whereas the majority of Pax7(+) cells negative for these markers or Pax7(−) cells with or without the expression of these two factors did not survive after LTT (Fig. 6B, 6C). These findings suggest that Hsp27 and αB-crystallin are involved in the survival of Pax7(+) satellite cells against stress-induced apoptosis under LTT.

DISCUSSION

Previous studies demonstrated that tissue stem cells (e.g., hematopoietic stem cells, neuronal stem cells, and satellite cells) are tolerant of various types of stress, such as hypoxia, heat, and mechanical stress [35–37]. We previously reported that, among other stressors, such as low serum, poor nutrition, and low $O_2$, LTT was the most potent condition to enrich a specific type of mesenchymal stem cell, namely multilineage-differentiating
stress enduring cells, in adult human bone marrow stromal cells and skin fibroblasts [38]. In the present study, LTT was also the most effective condition for the enrichment of satellite cells. Treatment with LTT increased the ratio of mouse satellite cells threefold and substantially decreased the ratios of nonmuscular cells such as fibroblasts, vascular endothelial cells, and Schwann cells among primary cultured cells harvested from fresh muscles. In addition, LTT was effective not only for mouse satellite cells but also for human satellite cells. The LTT incubation time most effective for increasing the ratio of Pax7(+) cells was 6 hours for mouse cells and 3 hours for human cells. This difference might be due to species-specific differences; although human and mouse

**Figure 3.** LTT enrichment of human Pax7(+) satellite cells in skeletal muscle cells (SkMCs). (A–C): Characterization of SkMCs. (A): Phase contrast image of SkMCs. (B, C): Expression of Pax7 in SkMCs. (B): Merged image of Pax7/DAPI. (C): Pax7 signal. Nuclei were counterstained with DAPI (blue). (D): The enrichment efficiency score of human Pax7(+) cells in SkMCs by LTT. Each LTT incubation time was 1, 3, 6, or 8 hours. Error bars indicate the mean ± SEM. *, p < .05. (E): Ratios of human Pax7(+) cells before and after 3-hour LTT. *, p < .05. (F–H): Expression of Ki67 in Pax7(+) cells. (F): Merged image (Pax7/Ki67/DAPI). (G): Pax7 signal. (H): Ki67 signal. (I–K): Formation of multinuclear myotubes with expression of myogenin. (I): Merged image of myogenin/DAPI. (J): DAPI. (K): Myogenin signal. Nuclei were counterstained with DAPI (blue). Scale bars = 100 μm (A) and 50 μm (B–I). Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; LTT, long-term trypsin incubation; Pax7, paired box protein 7.
satellite cells express similar markers, it is unclear whether their properties and functions are equivalent.

Hsp27 and B-crystallin, small heat shock proteins, are expressed in many cell types and tissues under normal conditions [39–41] and tend to form variable size oligomers [42]. Many groups have reported that these factors might be important for regulating cell survival and apoptosis. For example, when tissues are damaged or exposed to stress, the oligomers rapidly dissociate by phosphorylation and are thought to form a dimer that suppresses apoptotic cell death [43]. Recently, Bruey et al. proposed a model in which Hsp27 interacts with cytochrome c, an apotosome, and then negatively regulates cell death by preventing cytochrome c-mediated interaction of Apaf-1 with procaspase-9 [44]. Our results suggest that Hsp27 and B-crystallin are, at least in part, correlated with the stress tolerance and survival of Pax7(+) cells. Apoptotic cell death is controlled by an exquisite balance between apoptotic factors and anti-apoptotic factors. We speculate that some other factors may critically regulate the survival of Pax7(+) cells through a pathway that involves Hsp27/B-crystallin, and such a mechanism will be

Figure 4. LTT enrichment of human Pax7(+) satellite cells in primary T-skeletal muscle cells (SkMCs). (A): Fresh human skeletal muscle tissue. (Ruler is marked in centimeters.) (B–I): Primary cultured cells collected from the adult human skeletal muscles. (B): Phase contrast image of T-SkMCs. (C–F): Immunocytochemistry of Pax7 (C, D) and MyoD (E, F). (C): Merged image (Pax7/4',6-diamidino-2-phenylindole [DAPI]). (D): Pax7 signal. (E): Merged image (MyoD/DAPI). (F): MyoD signal. Arrowheads indicate each positive cell. (G–I): Expression of type I collagen (G), vWF (H), and GFAP (I) in T-SkMCs. Nuclei were counterstained with DAPI (blue). Scale bars = 100 μm (B) and 50 μm (C–I). (J, K): The enrichment efficiency score of human Pax7(+) cells in T-SkMCs by LTT. LTT incubation times were 1, 3, 6, or 8 hours (J) and 2, 2.5, 3, 3.5, or 4 hours (K). **, p < .05; ***, p < .001. (L): Ratios of Pax7(+) cells in T-SkMCs before and after 3-hour LTT. **, p < .05. (M): Cell population of T-SkMCs before and after 3-hour LTT. Red groups show muscle-lineage cells expressing Pax7, Pax7+MyoD, or MyoD, and blue groups show nonmuscular cells expressing type I collagen, vWF, or GFAP. Abbreviations: GFAP, glial fibrillary acidic protein; LTT, long-term trypsin incubation; Pax7, paired box protein 7; vWF, von Willebrand factor.
Figure 5. Transplantation of the human satellite cell-enriched cell (SEC) population into degenerated muscles of immunodeficient mice. (A, B): Incorporation of the GFP-labeled human SEC population into regenerating muscles at 2 weeks after transplantation. (B): Higher-magnification view of the boxed area in (A). (C, D): At 4 weeks after transplantation, GFP(+) myofibers (C) expressed human dystrophin (D). (E, F): Arrowhead indicates the transplanted GFP-labeled cells (E) positive for Pax7 (F). (G): Specimen obtained from biopsy at 2 weeks after the second cardiotoxin treatment. GFP-labeled regenerating muscle fibers exhibited centrally located nuclei. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). Scale bars = 50 μm. (H): The ratio of regenerating GFP(+) myofibers at 2 weeks after the first cardiotoxin treatment and transplantation, and at 6 weeks (2 weeks after the second cardiotoxin treatment without additional transplantation). Black bars represent the SEC population (LTT+) and white bars represent nontreated cell population (LTT–). *p < .05; **p < .01. Abbreviations: GFP, green fluorescent protein; LTT, long-term trypsin incubation; Pax7, paired box protein 7.

Figure 6. Expression of Hsp27 and αB-crystallin in human Pax7(+) satellite cells. (A): Immunocytochemistry of Pax7 (green) and Hsp27 or αB-crystallin (red) in the human satellite cell-enriched cell (SEC) population. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). Scale bars = 25 μm. (B): Change in the cell number expressing Hsp27 in Pax7(+) and Pax7(–) cell populations before and after 3-hour LTT incubation. Black bars represent Hsp27(+) cells, and white bars represent Hsp27(–) cells. (C): The number of cells expressing αB-crystallin included in Pax7(+) and Pax7(–) cell populations before and after 3-hour LTT incubation. Black bars represent αB-crystallin(+) cells, and white bars represent αB-crystallin(–) cells. Abbreviations: Hsp27, heat shock protein 27; Pax7, paired box protein 7.

clarified by gain- and loss-of-function experiments in future studies.

Pax7(+) satellite cells contain quiescent satellite cells (Pax7(+) /MyoD(–) cells) and activated satellite cells, including myogenic precursor cells (Pax7(–) /MyoD(+) cells) [45]. After incubation with LTT, the ratio of both Pax7(+) /MyoD(–) and Pax7(+) /MyoD(+) cells increased among T-5kMcs, and both made up ~95% of the SEC population, suggesting that LTT enriches both quiescent and activated satellite cells. Interestingly, the ratio of Pax7(+) /MyoD(+) cells after LTT was higher than that...
of Pax7(+)/MyoD(−) in both mouse and human cells. Therefore, activated satellite cells might be more tolerant than quiescent satellite cells to the stress of LTT.

LTT uses trypsin, which is naturally produced in the body, so LTT seems to be less hazardous to cells. Treatment of the cells with trypsin for 3 or 6 hours, however, is a strong stress and thus might affect the myogenic stem cell function of enriched Pax7(+) cells. Nevertheless, in our study, we confirmed the expression of Ki67, which indicates proliferation activity of Pax7(+) cells and the formation of myogenin(+) multinuclei myotubes in the SEC population in vitro. Furthermore, in the transplantation experiment, we confirmed the incorporation of GFP-labeled myoblasts expressing human dystrophin and of GFP/Pax7 double-positive cells in the satellite cell location in immunodeficient mouse regenerating muscle. In addition, these transplanted cells contributed to subsequent muscle regeneration following repetitive muscle damage without additional cell transplantation. These findings indicate that LTT does not affect the differentiation and regeneration ability of the SEC population and that the toxicity of trypsin is low.

Duchenne muscular dystrophy (DMD), the most common subtype of muscular dystrophy, is an X-linked muscle disorder caused by mutations in the dystrophin gene [46]. DMD is a serious disease that ultimately leads to death due to progressive muscle weakness [47–49]. Although various approaches to treat DMD have been investigated, there is still no curative treatment for DMD. Clinical trials of myoblast transplantation to DMD patients lead to transient functional recovery, but poor cell survival and limited proliferation after implantation remain serious limitations [50]. Thus, treatments using human leukocyte antigen-matched healthy donor- or relative-derived satellite cells that can self-renew and continuously provide new myocytes upon transplantation would be an ideal treatment. Our method can be used for transplantation of satellite cells for muscle degenerative diseases without genetic modification and thus might be of practical benefit for clinical application.

Several groups have reported methods to enrich myogenic cells. For example, collecting skeletal muscle cells using a preplating technique reduces the contamination of nonmuscular cells [51–54]. Although this method takes advantage of the fibroblast property to adhere faster to the uncoated dish than skeletal muscle cells, this system is not specialized for satellite cells. In the present study, however, we focused only on satellite cells, and we propose LTT as a novel approach specialized for enrichment of satellite cells and not for general myogenic cells. In addition, preplating takes at least 4 days to obtain myogenic cells at the highest enrichment efficiency and must be repeated several times. In contrast, LTT can be performed in a few hours. Culturing cells under hypoxic conditions is also reported to promote the proliferation of satellite cells [55], whereas this method requires devices to maintain the hypoxic condition. LTT requires no long-term processes, no expensive devices, and little labor, and thus it can be easily performed in regular laboratories. Furthermore, LTT is the enrichment method based on the stress resistance of tissue stem cells. Therefore, another important advantage of LTT is that this system may also be used for collecting other tissue stem cells whose surface marker is unknown.

**Conclusion**

Treatment with LTT to induce stress tolerance in tissue stem cells is an effective method of enriching satellite cells that retain their stem cell functions. LTT has several benefits. (a) LTT is a novel approach for enrichment of satellite cells. (b) LTT can be easily performed in any laboratory without the need for expensive devices, special agents, long-term processes, or excessive labor. (c) The toxicity of trypsin is expected to be low, because trypsin is a natural product and can be inactivated by the addition of serum. (d) LTT may be applicable to other tissue stem cells for which flow cytometry is not directly applicable. Our method will be beneficial for studies of stem cells whose collection method has not been specified.

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**Author Contributions**

T.S.: conception and design, financial support, provision of study materials, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; Y.K.: conception and design, data analysis and interpretation; S.W.: provision of study materials; M.D.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

**Disclosure of Potential Conflicts of Interest**

The authors indicate no potential conflicts of interest.

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