1. Introduction

Skeletal muscle is crucial for physical support and motion [1]. However, loss of skeletal muscle function may cause severe muscular disorders, such as muscular dystrophy and age-associated muscle atrophy [2]. Although several curative treatments for these severe muscular diseases are under development, screening of molecules that enhances muscle regeneration or cell transplantation approach has been expected to be the promising treatment as to promote the regeneration of muscle cells. Muscle regeneration is mediated by satellite cells, which are located in the sarcolemma and beneath the basement membrane of muscle fibers [3,4], composed of collagens, laminins, and proteoglycans [5]. The function and survival of satellite cells are maintained by multiple adhesion receptors, including integrins, which are responsible for cell adhesion to the extracellular matrix (ECM) [6–8]. Signals are transmitted into cells upon binding to the ECM, and integrins are important for regulation of physiological functions, such as survival, proliferation, and differentiation [9,10]. The paired box transcription factor
Pax7 is critical for maintenance of satellite cell function, and its inactivation causes severe depletion of satellite cells in skeletal muscle [4,11,12]. Pax7, together with the myogenic regulator MyoD, determines the fate of satellite cells [13–15]. In adult skeletal muscle, satellite cells remain in the Pax7+MyoD− quiescent phase [16]. However, once the muscle tissue is damaged, satellite cells convert into the Pax7+MyoD+ activated state and proliferate, followed by differentiation into mature myofibers [17]. After tissue repair, a small number of activated satellite cells self-renew and return to the quiescent state [3,7,18]. To date, methods have been established to isolate satellite cells from skeletal muscle tissue using fluorescence-activated cell sorting (FACS). In mice, isolated satellite cells were transplanted into the damaged muscle, and engraftment, regeneration, and self-renewal were observed [19,20]. During the process of isolating mononuclear cells from skeletal muscle, enzymatic digestion using collagenase is needed for efficient isolation [21,22]. Conventionally used collagenase II is prepared from *Clostridium histolyticum* extracts, which contains multiple enzymes such as collagenases, neutral proteases, and others in various ratios depending on the company and the product batch [23,24]. Since most of these enzymes are not defined and free of unknown derivatives, therefore, using conventional collagenase II does not necessarily fit to the biological raw material criteria. Also, isolating stem cells with intact surface antigens is another important point for analysis and clinical applications.

In this study, we compared the effects of purified recombinant collagenases (collagenase G, ColG and collagenase H, ColH) and conventional collagenase II to isolate skeletal muscle satellite cells. We showed an efficient method of satellite cell preparation using ColG and ColH with a high cell yield, viability of cells, and regeneration potency to fit the biological raw material criteria. This approach can be applicable to isolate somatic stem cells, such as mesenchymal stem cells and pancreatic islet cells.

2. Methods

2.1. Animals

C57BL/6 wild-type mice and C57BL/6-Tg (CAG-EGFP) mice were purchased from CLEA Japan, Inc and Japan SLC, Inc., respectively. Eight to twelve-week-old male mice were analyzed. All procedures for animal experiments were approved by the Tokyo Medical and Dental University Animal Care and Use Committee (Protocol number: 0170282C).

2.2. Satellite cell isolation

Mouse skeletal muscles from the fore- and hind-limbs were dissected and digested with collagenases. In terms of enzyme concentrations, we measured enzymatic activities of ColG (Meiji Seika Pharma) and collagenase type II (Warthong Biochemical) using a substrate, AzoColl (Sigma). Also, enzymatic activities of ColH (Meiji Seika Pharma) and collagenase type II using a substrate, N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala (Sigma), were measured as well. From the measurements, the appropriate concentrations of ColG (57.456 μg/ml) and ColH (12.125 μg/ml) that exert equal activities to that of collagenase type II (1.4 mg/ml) was determined and used for the experiments. Since collagenase type II is crude and possesses neutral protease activity, Dispase II (Godo shusei) was used as a supplementation of neutral protease into the ColG/ColH solution. The neutral protease activities of Dispase II and collagenase type II were measured using a substrate, FA-Gly-Leu-NH2 (Bachem). According to the measurement, 155.4 μg/ml of Dispase II was expected to have an equal activity to that of collagenase type II. As a result of an optimization for the satellite cell isolation, 2-fold the concentration (310.8 μg/ml) of Dispase II was suitable and used as a supplementation of neutral protease to ColG and ColH in this study. Collagenases were used for digestion at 37 °C for 1 h. Then, the digested tissue was filtered through 100 μm- and 40 μm-cell strianizers (BD Biosciences). The filtered mononuclear cells were stained with phycoerythrin (PE)-conjugated anti-CD31 (BD Biosciences), PE-conjugated anti-CD45 (BD Biosciences), PE-conjugated anti-Sca1 (BD Biosciences), and biotinylated anti-SM/C-2.6 antibodies [26], and streptavidin–allophycocyanin (Becton, Dickinson and Company), on ice for 30 min. To analyze expression of integrins, a fluorescein isothiocyanate-conjugated anti-integrin α7 antibody (3C12; Novus Biologicals) and a PE-conjugated hamster anti-rat CD29 antibody (BD Bioscience) were also added. All the cells were resuspended in HBSS and propidium iodide (PI). Cell sorting was performed using a MoFlo flow cytometer (Beckman), and CD31 , CD45 , Sca-1 , and SM/C-2.6+ cells were collected as mouse satellite cells.

2.3. Cell culture

Isolated mouse satellite cells were plated on glass chamber slides coated with Matrigel (BD Biosciences). For proliferative conditions, satellite cells were cultured in Dulbecco’s modified Eagle’s medium with GlutaMAX (Life Technologies) containing 20% fetal bovine serum (Sigma–Aldrich), 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and 5 ng/ml basic fibroblast growth factor (ReproCell) in 5% CO₂ at 37 °C.

2.4. RT-PCR

Total RNA was isolated from sorted cells using the TRI reagent (Sigma–Aldrich). cDNA was generated from 0.5 μg of total RNA using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). The RNA was previously treated with the DNase (Invitrogen). RT-PCR was performed using the Applied Biosystems StepOne Real-Time PCR System. PCR was performed in duplicate with reaction volumes of 10 μl containing Fast SYBR Green Master Mix (Applied Biosystems), forward and reverse primers, and the cDNA template. Data were analyzed using a comparative critical threshold (Ct) method, where the amount of the target normalized to the amount of the endogenous control relative to the control value is given by 2^−ΔΔCt. The primers were as follows: HPRT: 5’-ctcagcaacgggacataaa-3’ (forward), 5’-gggctgactctggyaaacagc-3’ (reverse), Pax7: 5’-ctcagtgatttgcattgaccg-3’ (forward), 5’-agagggtctcttgg-3’ (reverse).

2.5. Muscle injury and cell transplantation

To induce skeletal muscle regeneration, mice were anesthetized with isoflurane and their hind-limbs were shaved. 100 μl of cardiotoxin (CTX; 10 μM in 0.9% NaCl; Sigma–Aldrich) was injected into the tibialis anterior (TA) muscle using a 29-gauge needle. For transplantation experiments, 8–10-week-old WT mice were used as recipients and were injured with CTX 24 h before transplantation of cells into TA muscles. SM/C-2.6+ satellite cells were sorted by flow cytometry from EGFP-expressing mice and were cultured for 5 or 6 days, and were transplanted into injured muscle. At 14 days after injury, mice were euthanized and frozen sections were prepared.

2.6. Cryosections

Mouse TA muscles were dissected and frozen in isopentane (Wako) that was cooled with liquid nitrogen. Using a cryostat...
Fig. 1. Recombinant collagenase causes less damage to muscle mononuclear cells. (A) After mouse muscles were processed with ColG and ColH and conventional collagenase II, mononuclear cells were isolated from these muscles and subjected to FACS analysis. A PI-FACS histogram was generated to detect membrane damage. (B) Quantitative analysis of the percentage of PI⁺ dead cells. n = 3. Error bars, s.e.m.; *p < 0.05.

Fig. 2. Satellite cells isolated using recombinant collagenase retain surface antigens. (A) After mouse muscles were processed with ColG and ColH, mononuclear cells were isolated from these muscles and subjected to FACS analysis of SM/C-2.6 expression. The purple box represents the satellite cell population. The SM/C-2.6⁺ fraction was divided into the high positive fraction (pink box) and the low positive fraction (blue box). (B) Expression of integrins on the cell surface was evaluated by flow cytometry. Green lines indicate α7 and β1 integrin expression pattern. Gray histograms represent the matched control. (C) After mouse muscles were processed with conventional collagenase II, mononuclear cells were isolated from these muscles and subjected to FACS analysis of SM/C-2.6 expression. The purple box represents the satellite cell population. (D) Expression of integrins on the cell surface was evaluated by flow cytometry. Green lines indicate α7 and β1 integrin expression patterns. Gray histograms represent the matched control. (E) Quantitative analysis of mRNA expression of Pax7. The mRNA expression of each gene was normalized using HPRT mRNA expression. n = 3. Error bars, s.e.m.; ***p < 0.001.
(Leica), the frozen TA muscles were sectioned transversely at a thickness of 8 μm. Sections from the widest part of the TA muscles were attached to MAS-coated slide glasses (MATSUNAMI). The cryosections were kept at −80 °C until they were immunostained.

2.7. Immunofluorescence

Immunohistochemistry was performed using the cryosections described above. For immunocytochemistry, primary satellite cells were cultured on 8-well chamber slides (MATSUNAMI) coated with Matrigel. Tissue sections or cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 10 min at room temperature, and then permeabilized with PBS containing 0.2% Triton X-100 (Sigma–Aldrich) for 15 min at room temperature. After blocking with Power Block Universal Blocking Reagent (BioGenex) or a M.O.M. kit (Vector Laboratories), the fixed cells were incubated with primary antibodies overnight at 4 °C. After washing, bound primary antibodies were labeled with fluorescence-conjugated secondary antibodies for 1 h at room temperature. The immunostained samples were mounted with Mounting medium containing DAPI (Vector Laboratories). The primary and secondary antibodies were as follows: anti-laminin α2 (Sigma–Aldrich), anti-Pax7 (SantaCruz), anti-collagen I (Abcam), anti-collagen IV (Abcam), anti-collagen VI (Abcam) and mouse/rabbit/rat IgG-Alexa488 or -Alexa594 (Life Technologies).

2.8. Statistical analysis

Differences between samples were assessed by using the Student’s two-tailed t test for independent samples.

3. Results

3.1. Recombinant collagenase causes less damage to muscle mononuclear cells

We confirmed the expression of collagen I, IV, and VI in the endomysium and around Pax7-positive satellite cells of muscle sections (Fig. S1 in Supporting information). To clarify the effect of recombinant collagenase on the isolation of satellite cells, we

![Figure 3](image-url)

**Fig. 3.** Satellite cells proliferate in vitro using recombinant collagenase. (A) Immunostaining of Pax7 in sorted cells cultured on Matrigel for 5 days. Cells were stained with an anti-Pax7 antibody [38] and DAPI [42] to label undifferentiated cells and nuclei, respectively. (B) The relative numbers of cells were calculated. The cell number in the Matrigel culture was set to 1.0. n = 3. Error bars, s.e.m.; ***p < 0.001. (C) The relative numbers of Pax7+ cells were calculated. The Pax7+ cell number in the Matrigel culture was set to 1.0. n = 3. Error bars, s.e.m.; ***p < 0.001.
prepared ColG and ColH, and conventional collagenase II [21,22]. These ColG and ColH were mixed at an optimal ratio, adjusted to the equivalent enzymatic activity of that of conventional collagenase II. Satellite cells were isolated from limb muscles of 8–12-week-old C57BL/6 mice digested with ColG and ColH or conventional collagenase II. We added Dispase-II to the recombinant collagenases as a neutral metalloprotease because this greatly improved the efficiency of satellite cell isolation (Fig. S2 in Supporting information). To investigate cell damage caused by collagenase treatment, mononuclear cells were stained with PI to identify apoptotic cells by FACS (Fig. 1A). The percentage of PI$^+$ apoptotic cells decreased using ColG and ColH (Fig. 1B). These data suggest that the digestion of skeletal muscle with recombinant collagenase causes less damage to mononuclear cells, including satellite cells.

### 3.2 Satellite cells isolated using recombinant collagenase retain cell surface antigens

To evaluate the collagenase digestion on satellite cells, mononuclear cells negative for CD31 and CD45, which are markers of endothelial cells and lymphocytes/leukocytes, respectively, were gated among total cells, and then the population of satellite cells positive and negative for SM/C-2.6 and Sca-1, respectively, was analyzed [26] (Fig. 2A purple box). Satellite cells isolated with ColG and ColH highly expressed SM/C-2.6 (Fig. 2A pink box). A SM/C-2.6$^{high}$ population were not observed using muscles digested with various other types of conventional collagenase II (Additional file 1: Fig. S2).

To investigate other surface antigens preserved using recombinant collagenase, we next examined the expression of integrins in SM/C-2.6$^+$ satellite cells. We focused on $\alpha 7$ and $\beta 1$ integrins, which are responsible for adhesion and survival/proliferation of satellite cells [6,8]. We divided the SM/C-2.6$^+$ cell fraction into high positive fraction (Fig. 2B pink box) and low positive fraction (Fig. 2B blue box). Expression of $\alpha 7$ and $\beta 1$ integrins was clearly observed in the SM/C-2.6$^{high}$ fraction compared with the SM/C-2.6$^{low}$ fraction (Fig. 2B). We confirmed the expression of SM/C-2.6 in satellite cells isolated after muscle digestion with collagenase type II as a control (Fig. 2C), and $\alpha 7$ and $\beta 1$ integrins were either highly expressed or lowly expressed in these SM/C-2.6$^+$ cells (Fig. 2D). The expression level of Pax7 mRNA in SM/C-2.6 fraction prepared with the conventional collagenase type II was in between the SM/C-2.6$^{high}$ and SM/C-2.6$^{low}$ fractions (Fig. 2E). Taken together, these results indicate that satellite cells isolated using ColG and ColH retain surface antigens and Pax7 expression, suggesting that recombinant collagenase is a useful tool to isolate satellite cells with minimal damage and a high survival potency.

### 3.3 Satellite cells isolated using recombinant collagenase proliferate in vitro

To analyze the effect of recombinant collagenase, isolated satellite cells were cultured under proliferative conditions. Satellite cells were isolated from the SM/C-2.6$^{high}$ fraction separated using ColG and ColH (Fig. 2A pink box). SM/C-2.6$^+$ cells isolated using conventional collagenase II (Fig. 2D purple box) were used as a control. There was no significant difference in the number of satellite cells obtained by these two methods. However, the number of cultured satellite cells dramatically increased using ColG and ColH.
(ColG and ColH: 1527 cells ± 448.9 cells, conventional collagenase II; 412.3 cells ± 227.3 cells) (Fig. 3A and B). Furthermore, relative number of Pax7+ undifferentiated cells also increased (ColG and ColH: 557.3 cells ± 225.1 cells, conventional collagenase II; 155.6 cells ± 107.7 cells) (Fig. 3A and C). These results indicate that satellite cells isolated using recombinant collagenase retained high proliferation potency and Pax7 expression in culture.

3.4. Isolated satellite cells using recombinant collagenase have an improved regenerative capacity

To investigate the regenerative capacity of satellite cells isolated using recombinant collagenase, cultured satellite cells from EGFP-expressing mice were injected into the TA muscles (5 × 10⁴ cells per muscle) of 8–10-week-old C57BL/6 mice. Twenty-four hours before cell transplantation, recipient C57BL/6 mice were injected with CTX to induce regeneration. Two weeks after the injection, we investigated the contribution of two methods to muscle regeneration via immunodetection of EGFP+ fibers (Fig. 4). Upon transplantation, satellite cells isolated using ColG and ColH displayed more EGFP+ fibers than those isolated using conventional collagenase II. These results suggest that satellite cells isolated using recombinant collagenase improve the regeneration efficiency in vivo.

4. Discussion

A number of studies have used collagenase to isolate somatic stem cells from various tissues [27,28]. A conventional collagenase II digestion has been commonly used and reported as a simple and cost-effective method. It has never been reported that the comparison of conventionally used collagenase II and recombinant collagenase based on purity and yield of isolated stem cells. In addition, the expression of cell surface markers is critical after the enzymatic digestion. In this study, we checked the protocol previously published to isolate satellite cells from mouse skeletal muscle and found that isolated satellite cells using ColG and ColH retained cell surface antigens (Fig. 2). These satellite cells significantly proliferated in vitro and displayed high regeneration efficiency when transplanted in vivo (Figs. 3 and 4). Taken together, recombinant collagenase is a useful tool to isolate satellite cells with an improved potency for cell survival, proliferation, and regeneration.

Collagen fibers are the major components of the extracellular matrix. Skeletal muscle tissue is composed of several types of collagen [29]. Recombinant collagenase digested fibril collagens, such as collagen I, II and III [25,30]. Further, satellite cells isolated with recombinant collagenase showed the high expression of SM/C-2.6 and are clearly distinguished from cells expressed SM/C-2.6low, based on the FACS pattern. Our results also showed that α7 and β1 integrins remained intact on the surface of satellite cells isolated by recombinant collagenase. It has been previously reported that integrins are important for the survival, proliferation, and differentiation of various cells [9,10]. β1 integrin is important in the satellite cell niche that maintains the quiescent state of satellite cells and promotes their proliferation and self-renewal via fibroblast growth factor signaling [31]. Inactivation of β1 integrin in embryonic muscles causes defects in muscle cell migration, fusion, and sarcomere assembly [32]. Myoblast cell lines derived from satellite cells highly express α7 integrin. α7β1 integrin regulates the conversion of satellite cells into myofibers. Moreover, enhanced expression of α7 integrin in dystrophic skeletal muscle promotes satellite cell proliferation and activation [6,8]. These findings suggest that the expression of cell surface molecules plays an important role in the process of development, proliferation, and differentiation. In this study, satellite cells isolated by recombinant collagenase adhere and proliferate in vitro due to the preservation of integrins and/or other molecules. Future studies aimed at dissecting the cell surface expression of molecules, such as Syndecan 3/4, M-cadherin, and Notch, will contribute to understand the growth and survival pathways [33–36].

Our findings suggest that ColG and ColH increased the yields of satellite cells from skeletal muscle (Fig. 1). Collagenase is also used in islet isolation from human pancreas for transplantation surgery of type 1 diabetes [24,37–43]. It was recently reported that proteases with trypsin-like activity strongly influence islet isolation from the human pancreas [23]. Conventional collagenase II has a trypsin-like activity, and the degree of contaminated activity depends on the company and product batch, whereas recombinant collagenase does not. Previous study suggests the unknown toxicity of conventional collagenase in adipose-derived stem cells [27]. To data, clinical grade recombinant collagenase G and collagenase H are available. A recent study reported that purified recombinant collagenase H and thermolysin can efficiently dissociate rat pancreatic tissues and separate functional islets from acinar cells [25,30]. The results of our study may be beneficial for establishing safe stem cell isolation from other tissues and can be used as fundamental data for clinical applications. Transplantation of autologous satellite cells is considered as a potential therapeutic approach in muscular dystrophy and age-associated muscle atrophy. The protocol we provided will contribute the clinical application of isolated satellite cells, including drug screening and transplantation.

5. Conclusion

We demonstrated that the satellite-cell isolation using recombinant collagenase retain surface antigens and Pax7 expression, and have a high regenerative capacity. Our results provide an efficient method of satellite cell preparation using recombinant collagenase with a high cell yield, viability of cells, and regeneration potency to fit the biological raw material criteria.

Conflict of interest

All authors declare no conflict of interest associated with this manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.reth.2017.06.001.

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