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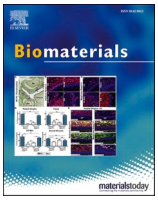

Harvest of quality-controlled bovine myogenic cells and biomimetic bovine muscle tissue engineering for sustainable meat production

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ABSTRACT

Alternative technology for meat production holds the potential to alleviate ethical, environmental, and public health concerns associated with conventional meat production. Cultured meat produced using cell culture technology promises to become a viable alternative to animal-raised meat for the future of the food industry. In this study, biomimetic bovine muscle tissue was artificially fabricated from myogenic cells extracted from bovine meat. Our primary culture method relies on three key factors; a sequential digesting process, enzymatic treatment with pronase, and coating with laminin fragment on culture dishes. This method allows the efficient collection of large numbers of primary cells from bovine cheek meat, purifies the myogenic cells from the cell mixture, and then continuously grows the myogenic cells in vitro. In addition, using our "quality control" methods, we were able to determine the "cell quality", including the proliferative and differentiation capability in each step of the primary culture. Furthermore, to mimic native bovine meat, the quality-controlled bovine myogenic cells were cultured on a micropatterned thermoresponsive substrate stimulating a native-like aligned structure of cells, which were then transferred onto a fibrin-based gel. This gel-based culture environment promoted structural and functional maturation of the myogenic cells, resulting in the production of bovine muscle tissues with sarcomere structures, native-like membrane structures, and contractile ability. We believe that these biomimetic features of "tissue-engineered meat" are important for the production of future cultured meat, which will need native-like nutrients, texture and taste. Therefore, our meat production approach will provide a new platform to produce more native biomimetic tissue-engineered meat in the near future.

1. Introduction

As the reported by the Food and Agriculture Organization (FAO) of the United Nations, the ethical and environmental concerns associated with meat production will increase as the rapidly growing world population is forecasted to increase meat consumption by 73% by 2050 [1]. The increased demand for livestock will exacerbate the effects of greenhouse gas emissions, as well as increased land and water usage [2–5]. In particular, the concern focuses on ruminants such as cows and sheep producing methane; a greenhouse gas with a 20-fold higher global warming potential than carbon dioxide [6,7]. In the near future, it is certain that the growing impact of livestock and animal-based food products will have a larger environmental footprint [8–10]. In addition, animals consume large amounts of food throughout their lives, and up to 97% of the calories are used for body maintenance [11,12]. The traditional meat production system uses a wide variety of agricultural crops for the production of non-edible parts of animals. Water consumption is also a serious concern in many parts of the world since, for example, 1 kg of beef requires more than 15,000 L of water over the lifetime of a cow used in food production. Therefore, traditional meat production systems are not sustainable in the future, and technological innovations are required to meet the growing global demand for meat consumption without sacrificing the environment.

Cultured meat can be produced in vitro using cells from animals, and is expected to become a practical alternative to traditional livestock husbandry [11,13–17]. This new innovative strategy for meat production gained widespread attention after the Mark Post group produced the first cultured meat prototype [18,19]. While it is expected that cultured meat production will be able to overcome the ethical and environmental issues associated with livestock-based meat production,

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this animal-free approach could also improve food safety, since cultured meat can be produced in a controlled sterile environment. Moreover, in the future this new production of meat could avoid potential risks in zoonotic disease transmission between livestock and humans. Whereas cultured meat production offers enormous benefits in terms of reduced environmental impact, improved human health, and ethical animal welfare, researchers in this new field first need to establish a process to harvest and expand various kinds of cells that will ultimately be chosen by preferences and tastes of customers (e. g., beef, pork, and chicken). To address this need, some previous studies have reported on the development of techniques for primary culture of many different kinds of myogenic cells [17,20–22]. These techniques will likely form the basis of our future success in sustainable meat production.

Tissue engineering has proven to be a key technology in this innovative system of meat production. For the last few decades, tissue engineering has made significant progress to produce native-like engineered tissues, mainly as transplantable tissues in regenerative medicine or as in-vitro tissue models for biological studies and drug discovery [23,24]. Human cell-based tissues have been the focus in these fields because of the potential therapeutic benefits [25,26]. In-vitro engineered human muscle tissue provides valuable tools to better understand human myogenesis, including development, growth, and regeneration [27–31], as well as to facilitate the discovery of new drugs for treatment of metabolic, neuromuscular, and dystrophic disorders [29,32]. Muscle tissue engineering is a promising new approach to make cultured meat more biomimetic. Indeed, cultured meat is expected to have all the same characteristics including taste, aroma, texture, and nutrients as traditional meat. In order to produce such a new authentic type of meat, tissue engineering can potentially provide muscle cells a culture environment suitable for structural and physiological maturation in in-vitro conditions. Our group also developed a tissue engineering method to produce human muscle tissue that mimics the structural and functional properties of native muscles [33]. In our approach, a micropatterned thermoresponsive substrate produces a biomimetic-aligned myofiber structure that is then transferred onto a fibrin-based gel containing Matrigel. The aim of this study was to demonstrate that our muscle tissue engineering technique is applicable for cultured meat production. Since bovine cells are not usually appropriate for biomedical research, they are not as commercially available as rodent and human cells. In this study, first an original primary culture method had to be developed to harvest myogenic cells from bovine meat. Next, these cells were cultured under strict quality control. In this way, bovine muscle tissue could be artificially produced based on our tissue engineering technique. Cultured beef, in particular, is expected to be in greater demand in the initial stages of the development of cultured meat production due to the larger ecological impact of traditional cattle farming [3,34]. We believe that our unique approach provides an exciting alternative to produce native-like bovine muscle tissue with biomimetic morphology and physiology.

2. Materials and methods

2.1. Primary cell culture derived from bovine meat

Skeletal muscle samples were collected from the cheek meat of Japanese Black cattle slaughtered and provided by Tokyo Shibaura Zoki (Tokyo, Japan). First, the surface of the cheek meat was disinfected by immersion of the whole muscle in 0.5% chlorhexidine gluconate (5% Hibitane, Sumitomo Dainippon Pharma, Osaka, Tokyo) for several minutes, and cut with a scalpel into samples (about 3 × 4 × 0.5 cm of skeletal muscle). The samples were disinfected again by washing with povidone-iodine solution (Meiji Seika Pharma, Tokyo, Japan) followed by rubbing ethanol (Yoshida Pharmaceutical, Saitama, Japan). After washing with Hanks' balanced salt solution (HBSS) (FUJIFILM Wako Pure Chemical, Osaka, Japan), the skeletal muscle sample was minced into pieces (1–2 mm) with a scalpel and scissors, then 2 g of the minced sample was placed in a 50 mL conical tube. For the enzymatic treatment, the sample was immersed in 10 mL HBSS containing 1 mg/mL pronase (Pronase from *Streptomyces griseus*, Sigma-Aldrich, St. Louis, MO, USA), incubated at 37 °C, and agitated at 100 rpm (Table 1) [35–37]. After 1 h, 10 mL HBSS with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) was added into the conical tube. Furthermore, to physically disperse the cells, the enzymatically treated sample was repeatedly pipetted (suction and discharge) 10–20 times first with a 25 mL measuring pipette and then 10–20 times with a 10 mL measuring pipette [22]. The dispersed sample was passed through a 40 μm cell strainer to remove large tissue segments and the cell suspension was centrifuged at 1000×*g* for 10 min. After removal of the supernatant, the precipitated pellet was resuspended with 10 mL Dulbecco's modified cultured medium-high glucose (DMEM) (FUJIFILM Wako Pure Chemical) with 10% FBS, 1% penicillin-streptomycin-amphotericin B (FUJIFILM Wako Pure Chemical), and 10 ng/mL basic fibroblast growth factor (bFGF) (KAKEN PHARMACEUTICAL, Tokyo, Japan). The suspended cells were placed in a φ10 cm culture dish and incubated for 1 h at 37 °C to remove any early adhering cells, such as fibroblasts. After pre-plating, the culture supernatant was transferred to a 5–6 of φ10 cm cultured dish coated with iMatrix-511. The coated dish was prepared by incubating it with Easy iMatrix-511 (Nippi, Tokyo, Japan) for 1 h at 37 °C. The supernatant was cultured in growth medium for 6–7 days. The medium was changed the day after seeding and every 1 or 2 days thereafter. To assess the effect of the enzyme, 0.25% Trypsin-EDTA (FUJIFILM Wako Pure Chemical) was used instead of the pronase solution. To evaluate the effect of various coating materials, the original iMatrix-511 (concentration: 0.55 μg/cm^2) was replaced with gelatin (Sigma-Aldrich), Matrigel (BD Biosciences, San Jose, CA, USA), type-I collagen (Sigma-Aldrich), type-IV collagen (Sigma-Aldrich), human recombinant laminin-111 (BioLamina, Sundbyberg, Sweden), human recombinant laminin-221 (BioLamina), or human recombinant laminin-511 (BioLamina). All coating was carried out under the same conditions (0.55 μg/cm^2, 37 °C, 1 h) (Table 2).

2.2. Proliferation and differentiation of bovine myogenic cells in repeated passaging

The primary bovine cells were cultured on normal cell culture dishes to increase the population through several passages. The primary cells were unable to adhere on culture dishes without a coating with biomolecules, such as iMatrix-511. On the other hand, since they adhered and proliferated after passage even on non-coated dishes, they were seeded to non-coated dishes and cultured in DMEM containing 10% FBS, 10 ng/mL bFGF and 1% penicillin-streptomycin-amphotericin B for several days. Before reaching confluence, they were harvested by treatment with trypsin and repeatedly seeded at a density of 1 × 10^6 cells/dish (φ10-cm cultured dishes) to expand the cell population over 10 passages. To confirm the differentiation capability at each passage, they were seeded at a density of 1 × 10^5 cells/cm^2 on normal dishes and cultured for 3–4 days in the growth medium (10% FBS-DMEM). This procedure of repeated passaging was independently performed three

Table 1
List of enzymatic treatments for harvest of primary bovine myogenic cells..

Enzymatic treatment	
Enzyme	Concentration
Collagenase type-2	0.5 mg/mL
Collagenase type-2 + Trypsin-EDTA	0.5 mg/mL + 0.25 w/v%
Collagenase type-4	0.5 mg/mL
Collagenase type-4 + Trypsin-EDTA	0.5 mg/mL + 0.25 w/v%
Pronase	1 mg/mL
Trypsin-EDTA	0.05 w/v%, 0.25 w/v%

Table 2
List of coating materials for culture of primary bovine myogenic cells..

Coating teatment	
Material	Condition
Gelatin	All coating was carried out under the same conditions. (0.55 μg/cm^2, 37 ℃, 1 h)
Matrigel	
Fibronectin	
Mouse laminin	
iMatrix-511	
Type-1 collagen	
Type-4 collagen	
Laminin 111	
Laminin 211	
Laminin 221	
Laminin 411	
Laminin 421	
Laminin 511	
Laminin 521	
Atelocollagen	

times for the primary culture. In addition, after 10 passages the cells were also incubated for 3 days in DMEM containing 2% horse serum (Thermo Fisher Scientific) (2% HS-DMEM) as a differentiation medium.

2.3. Preparation of micropatterned thermoresponsive substrate

In this study, a commercially available thermoresponsive cell culture dish "UpCell" (CellSeed, Tokyo, Japan) was used. The micropatterned thermoresponsive substrate was prepared with the previously reported method of polymer patterning [33]. To produce the micropatterns on a hydrophilic polymer, acrylamide (AAm) aqueous solution (50 w/w%) (FUJIFILM Wako Pure Chemical), containing a water-soluble photo--initiator camphorquinone (7,7 dimethyl-2,3-dioxobicyclo[2.2.1] heptane-1-carboxylic acid) (1 w/w%) was poured into a culture dish and visible light was irradiated for 7, 10 or 20 min onto the culture surface through an appropriate photomask. Stripe-shaped micropatterns (the width of non-irradiation and irradiation regions were 50 μm) were fabricated by grafting polyacrylamide (PAAm). To optimize the patterned dish for bovine myogenic cells, visible light was irradiated for three different time periods (7, 10, and 20 min).

To confirm the formation of micropatterns on the substrate, fluorescently labeled fibronectins were immobilized on the patterned surface, then the relative immobilized amounts on the two different polymer patterns was estimated from fluorescence intensity [38]. HyLyteFluor488-lebelled bovine fibronectin (10 μg/mL in phosphate buffered saline (PBS)) (Cytoskeleton, Denver, CO, USA) was incubated with the culture substrate at 37 °C for 6 h. After being washed thoroughly with PBS, the difference in the protein immobilization on the surface was confirmed by microscopic imaging using a fluorescence microscope (ECLIPSE Ti2–U) (Nikon, Tokyo, Japan).

2.4. Structural and functional maturation of bovine muscle tissue

To investigate the relationship between micropatterning and the resulting orientation of the bovine cells, the cells were first seeded at a density of 2×10^4 cells/cm^2 on a normal non-patterned dish or a patterned dish (irradiation time: 7, 10, and 20 min). After culturing in growth medium for 5 days, the medium was replaced with a differentiation medium (2% HS-DMEM) and cultured for 2 days. To produce muscle tissue with fibrin-based gel, bovine myogenic cells were seeded at a density of 1×10^6 cells/dish onto the patterned substrate (irradiation time: 20 min, seeding area: 15 × 15 mm) and cultured in growth medium. After 4 days, Matrigel-containing fibrin gel (Fib/Mtr gel) was formed with a mixture of fibrinogen (from bovine plasma, 20 mg/mL, 2 mL) (Sigma-Aldrich), thrombin (from bovine plasma, 20 U/mL, 500 μL) (Sigma-Aldrich), Matrigel (500 μL) (BD Biosciences) and $CaCl_2$ solution (8 mM, 1 mL) and poured onto the cells (900 μL per a dish). Finally, a square-shaped silicon ring was inserted into the mixture to prevent gel shrinkage. Gel formation was achieved by incubating the culture dish for 30 min at 37 °C, and then adding a growth medium (10% FBS-DMEM with anti-fibrinolytic agents, aprotinin (500 KIU/mL, from bovine lung) (FUJIFILM Wako Pure Chemical) and 6-aminocaproic acid (2 mg/mL) (FUJIFILM Wako Pure Chemical)) to the culture dish. After 4 days, the medium was replaced with a differentiation medium (2% HS-DMEM with anti-fibrinolytic agents) and cultured for 7 days. To release the bovine muscle tissue from the culture substrate, the samples were incubated at 20 °C for 30 min, and then placed upside down in the growth medium on a 6-well culture plate [33].

2.5. Immunofluorescence staining

Bovine myogenic cells were placed on cover glasses coated with concanavalin-A (LKT Laboratories, St. Paul, MN, USA) just after the harvest (Day 0) and after 7 days of culture (Day 7). The cover glasses were washed once with PBS, then 0.075% poly-L-lysine solution (500 μL) was dropped onto the glasses (Sigma-Aldrich) for 10 min at RT. After washing twice with PBS, the cover glasses were covered with 0.5 mg/mL of concanavalin-A solution (50 μL) and dried in an incubator at 50 °C [39,40]. The suspension of bovine cells was placed on the coated cover glass and incubated for 30–60 min at RT. At passages 3, 5, and 10, the same procedure was repeated to investigate the changes in myogenic cell population during the repeated passages. The attached cells were fixed with 4% paraformaldehyde for 15 min at RT, and then permeabilized with 0.5% Triton-X solution (Sigma-Aldrich). After washing three times with PBS, the cells were incubated in blocking solution (1% bovine serum albumin (BSA) (Sigma-Aldrich)) for 30 min at RT, and treated with primary antibodies [PAX7 (1:200) (sc-81648, Santa Cruz Biotechnology, Dallas, TX, USA), MYOD (1:1000) (sc-377,460, Santa Cruz Biotechnology), or MYF5 (1:200) (ab125301, Abcam, Cambridge, MA, USA)] diluted with 0.1% BSA at 4 °C overnight. After washing with PBS, the cells were treated with fluorescently labeled secondary antibodies (1:1000) (Thermo Fisher Scientific) at RT. After 1 h, the cover glasses were incubated with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific) for nucleus staining. Images were acquired using an FV1200 inverted confocal microscope (Olympus, Tokyo, Japan), and analyzed using FV10-ASW software. ImageJ software was used to count the numbers of each type of primary cell.

To assess myotube formation, desmin- or myosin heavy chain (MHC)-positive multinucleated cells were stained fluorescently. After fixation and permeabilization, the fixed cells were incubated in blocking buffer (2% BSA in PBS) for 30 min at RT, and treated with anti-desmin antibody (1:1000) (MA5-13259, Thermo Fisher Scientific) or anti-MHC antibody (1:500) (MAB4470, R&D Systems, Minneapolis, MN, USA) diluted with 2% BSA at 4 °C overnight. After washing with PBS, the cells were treated with fluorescently labeled secondary antibodies (1:1000) (Thermo Fisher Scientific) at RT. The areas of fluorescent regions were analyzed using ImageJ software.

Muscle tissues with the Fib/Mtr gel were fixed with 2% paraformaldehyde overnight at 4 °C [33]. After fixation, samples were incubated in blocking solution (5% BSA with 0.2% Triton-X 100) (Sigma-Aldrich) for 12 h at RT. The tissue samples were treated with primary antibodies (sarcomeric α-actinin (1:500) (ab9465, Abcam), laminin (1:500) (ab11575, Abcam), desmin (1:500) (MA5-13259, Thermo Fisher Scientific)) at 4 °C overnight, and then treated with fluorescently labeled secondary antibodies (1:1000) (Thermo Fisher Scientific) for 2 h at RT. For nuclei staining, tissue samples were

incubated with Hoechst33258 (Dojindo Laboratories, Kumamoto, Japan) for 10 min at RT. Between all steps, samples were washed three times with PBS. Images were acquired using an FV1200 inverted confocal microscope and analyzed using FV10-ASW software.

2.6. Analysis of real-time polymerase chain reaction (PCR)

Total RNA was isolated from bovine myogenic cells using ISOGEN II (Nippon gene, Toyama, Japan). cDNA was generated using SuperScript® III Reverse Transcriptase (Thermo Fisher Scientific). The abundance of transcripts was assessed by real-time, quantitative PCR (qPCR) analysis using the Fast SYBR® Green Master Mix (Thermo Fisher Scientific) and the gene-specific primer pairs listed below. The qPCR was performed with an Applied Biosystems ViiA™7 real-time PCR system. All the expression levels were normalized to *β-Actin* (*ACTB*).

The forward (F) and reverse (R) primers used were as follows:

MYOD-F, 5′-ACGTCTAGCAACCCAAACCAG-3′,

MYOD-R, 5′-TGCAGGCCTTCGATATAGCG-3’; *MYF5*-F, 5′-AAGTTGCTCTGATGGCATGC-3′, *MYF5*-R, 5′-AGACGCTGT-CAAAACTGCTG-3’; *ACTB*-F, 5′-TGCGGCATTCACGAAACTAC-3′, *ACTB*-R, 5′-TGTTGGCGTAGAGGTCCTTG-3’.

2.7. Western blotting analysis

Cells were lysed by using M-PER Mammalian Protein Extraction reagent (Thermo Fisher Scientific) [41]. Insoluble material was removed by centrifuging at 15,000×*g* at 4 °C for 5 min. Protein concentrations were determined using Pierce BCA Protein Assay (Thermo Fisher Scientific) according to manufacturer’s instructions. Heat-denatured protein samples (5 μg) were separated on NuPAGE 4–12% SDS-polyacrylamide gels (Thermo Fisher Scientific) and blotted on to PVDF membranes (Merck, Darmstadt, Germany). After blocking with 2% ECL Prime Blocking Reagent (Cytiva, Tokyo, Japan) in Tris-Buffered Saline containing 0.05% Tween20 (TBST) for 1 h at RT, the membrane was incubated at 4 °C overnight with an antibody against MYOD (Santa Cruz Biotechnology) (1:500), or MYF5 (Abcam) (1:10,000) in 2% blocking buffer with constant shaking. After washing with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or anti-mouse IgG (Abcam) (1:10,000) for 1 h at RT. Housekeeping controls were detected with an antibody against β-actin (Abcam) (1:10,000) and HRP-conjugated anti-rabbit IgG antibody. Chemiluminescence was performed using ECL Prime Western Blotting Detection Reagent (Cytiva) and images were captured using a ImageQuant LAS 4000 mini System (FUJIFILM).

2.8. Macroscopic and microscopic observation of bovine muscle tissue contractions

Spontaneous contraction of bovine muscle tissue was recorded using a video camera (30 frames/sec). The shrinkage distances of the muscle tissue were calculated using the motion analysis tool ViewPoint (Glenallan Technology Inc., Clinton, NY, USA) [33]. To induce muscle contraction, the bovine muscle tissues were electrically stimulated at 64 days after the seeding onto the culture dish. Two carbon electrodes (C-Dish; IonOptix, Milton, MA, USA) were immersed into the medium, then an electrical pulse stimulation (EPS) was applied (voltage: 10 V, frequency: 1 or 15 Hz, duration time: 10 msec) using an electrical pulse generator (IonOptix). The EPS-induced muscle contraction was recorded at 30 frames/sec by a CCD camera with phase-contrast microscopy. The resulting tissue contraction distances were calculated using the motion analysis tool ViewPoint.

2.9. Assessment of myotube orientation

The myotube orientation was evaluated with a directional histogram constructed using the “directionality” function in the ImageJ software [33]. The parallel alignment from the axis of the stripe patterns was denoted as 0° and the perpendicular alignment was denoted as 90°. A flat histogram indicated a completely isotropic orientation, and a histogram with a clear peak indicated a respective orientation.

2.10. Statistical analysis

Data are expressed as the mean±standard deviation. Statistical analysis was performed with Student’s t-test (Fig. 1). Multiple comparisons were analyzed by one-way ANOVA with the Dunnett’s test (Figs. 4 and 5). Statistical significance was considered at $^*P < 0.05$. Statistical processing was performed using R Software.

3. Results

3.1. Highly efficient primary culture of bovine myogenic cells harvested from bovine meat

In studies of skeletal muscle biology, several methods have been reported for the isolation of cells from skeletal muscles [17,20–22]. These methods use similar steps that involve the mechanical mincing of the muscle, followed by enzymatic digestion to harvest the muscle cells. Based on these methods, we have originally developed a new primary culture method by modification of previously reported methods. The important factors in our primary culture method is the sequential mincing using two different sizes of pipettes, the enzymatic treatment with pronase, and use of a culture dish coated with the laminin-like fragment, iMatrix-511. Specifically, minced meat segments were treated with pronase and triturated alternately using a 25 mL measuring pipette and then a 10 mL measuring pipette to form progressively smaller pieces of meat (Fig. 1). To harvest the myogenic cells, the meat segments were treated with several commonly used enzymes including collagenase and trypsin. Specifically, collagenase (type 2 and 4), trypsin and their various mixtures were used to compare these effects on the harvest of myogenic cells from bovine meat (Table 1). Then, from among these representative enzymes, we found that the use of trypsin was quite effective to harvest a large number of cells. However, the treatment with pronase allowed us to harvest an even larger number of cells, when compared with trypsin. Therefore, pronase was ultimately selected for use in this study. Pronase is known as a highly effective enzyme to release satellite cells from muscle tissue [22], and in this study some advantages were observed for bovine myogenic cells. As an example, cell numbers collected through pronase treatment were remarkably higher than that using trypsin (Fig. 1b). In addition, the enzymatic treatment influenced not only the number of cells collected, but also the adhesion behavior of the isolated cells. When cells were treated with pronase and seeded at a density of 2×10^6 cells/dish onto culture dishes coated with iMatrix-511, the number of adherent cells on the dishes were much higher than with trypsin (Fig. 1c). The cells treated with pronase adhered and proliferated for 7 days, whereas most of the cells treated with trypsin needed the longer culture time to adhere to the culture dishes. This indicated that the pronase treatment was the best choice to maintain the adhesive and proliferative capability of the cells. Moreover, it was demonstrated that the iMatrix-511 coating did not have any direct influence on the cell adhesion between trypsin and pronase treatments. When cells from both treatments were incubated in culture dishes coated with other cell adhesive proteins including collagen, laminin and Matrigel, the bovine cells treated with pronase always adhered more rapidly compared with cells treated with trypsin, regardless of the coating materials (Fig. 1d). Therefore, in our primary culture method, the treatment with pronase enabled the most effective collection of bovine myogenic cells with higher rates of adhesion and proliferation potential. On the other hand, even when the primary myogenic cells were harvested by pronase treatment, they were unable to adhere on normal cell culture dishes without a biomolecule coating. Therefore, biomolecule coating on culture dishes was necessary to

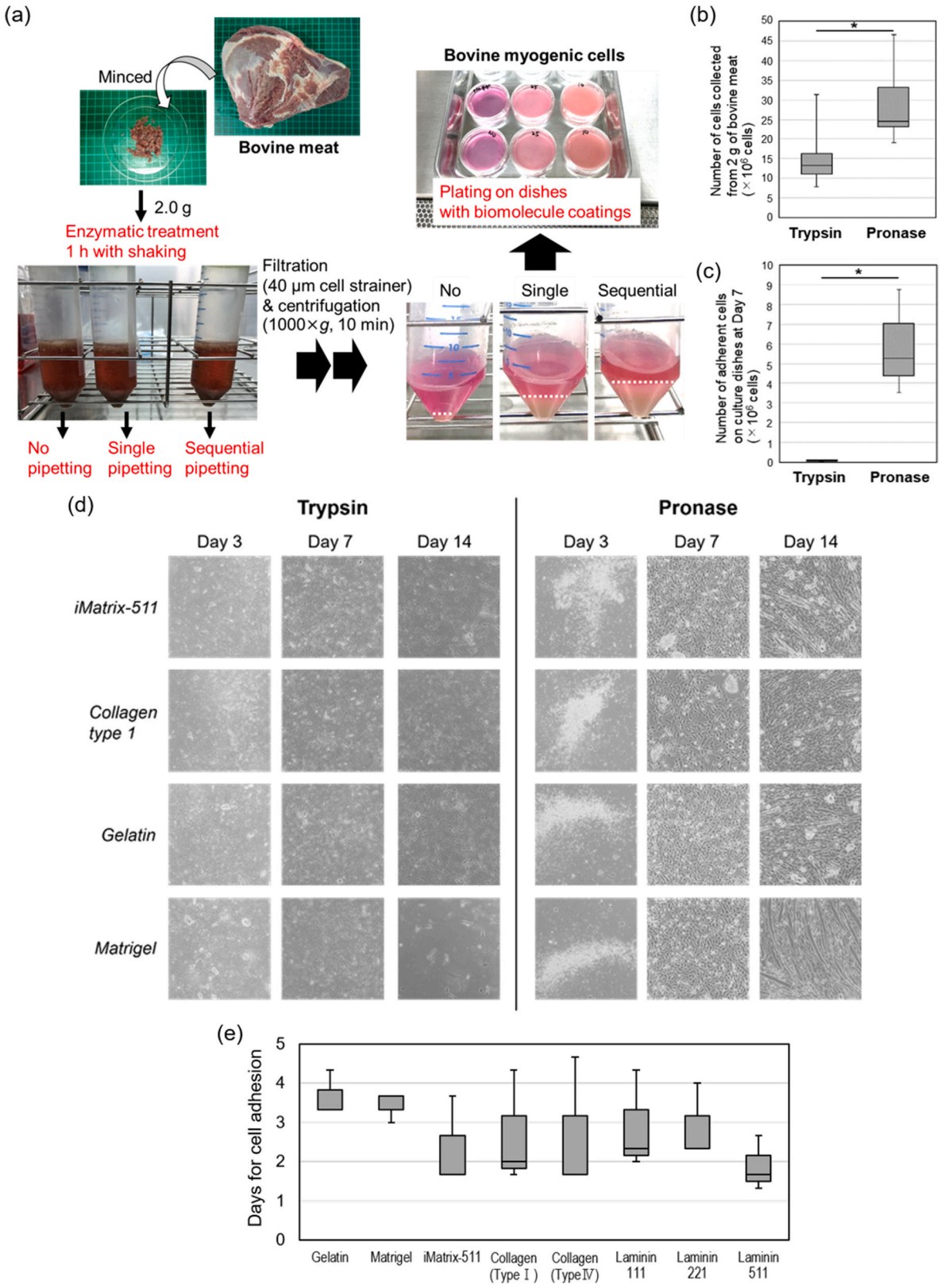

Fig. 1. Harvest and culture of primary bovine myogenic cells treated with trypsin or pronase in our primary culture method. (a) Primary culture process efficiently harvested myogenic cells from bovine meat through both physical and enzymatic treatments. The minced samples (2 g) was enzymatically treated in a conical tube (50 mL) and chopped by pipetting (single: only using a 25 mL measuring pipette, sequential: sequentially using both 25 mL and 10 mL measuring pipettes). After filtration and centrifugation, the precipitates which contained the target cells were harvested. The dotted lines indicate the amounts of harvested cells in the tubes. After resuspension of the precipitates in growth medium, cells were plated in culture dishes with various biomolecule coatings for primary culture of bovine myogenic cells. (b) Number of primary cells collected from 2 g of bovine meat. (n = 6) (c) Number of adherent cells on culture dishes coated with iMatrix-511 at Day 7. (n = 3) (d) Microscopic images of adherent cells on culture dishes with various biomolecule coatings. The images were taken at Days 3, 7, and 14 after seeding of the primary cells. (e) Comparison of days required for adhesion of cells treated with pronase on culture dishes with various coatings. (n = 3).

culture the primary cells. As shown in Fig. 1e, while the cells were able to adhere to all of the dishes after a few days, laminin-like materials including iMatrix-511 were the most efficient in promoting adhesion on the culture dishes. All coating materials used in this study are listed in Table 2.

More importantly, the coating with iMatrix-511 also enhanced the differentiation of myogenic cells into myotubes. For example, Fig. 2 shows that the bovine cells differentiated efficiently into myotubes on the laminin fragment-coated dishes, compared with cells on gelatin-coated dishes. This indicated that the bovine myogenic cells harvested by our primary culture method maintained their differentiation capability and the biomolecule coating influenced the differentiation rate. Although the iMatrix-511 coating was effective to promote myotube differentiation, it was difficult to clarify the effect of the coating using the cells harvested with pronase treatment. As describe above, they had much stronger adhesive properties and a greater capability to differentiate into myotubes than with the trypsin treatment. As a result, they rapidly formed a number of large myotubes on all of the culture dishes coatings, even with gelatin or collagen. Therefore, just to confirm the advantage of using iMatrix-511 coating on differentiation capability, myogenic cells treated with trypsin were cultured on all of the coated dishes. Interestingly, even after only one-time passaging the bovine cells adhered on normal culture dishes without any biomolecule coating, whereas biomolecule coating was required for adhesion of the primary cells. As shown in Fig. 2, they adhered and differentiated into myotubes on non-coated dishes. Whereas there was no significant difference in differentiation rate between non-coated and gelatin-coated dishes, iMatrix-511 coating promoted myotube differentiation of the bovine myogenic cells. In this experiment, bovine cells treated with trypsin were used to understand the effect of biomolecule coating on differentiation, since the cells treated with pronase rapidly formed myotubes on all of the culture dishes. On the other hand, due to the capability of the pronase treatment to create a high level of differentiation, the primary cells treated with pronase were typically used in the experiments described below.

3.2. *Quality confirmation of primary bovine myogenic cells collected from meat*

In the primary cell culture process, the cells are treated with several kinds of physical and chemical methods to harvest the largest number of cells and simultaneously to consistently harvest cells with the same level of quality. To confirm the quality of the harvested cells from our primary culture method, PAX7, MYF5, and MYOD were fluorescently stained as representative myogenic protein markers to determine the population of myogenic cells in the harvested cell mixture. Importantly, this quality confirmation method indicated that the iMatrix-511coating purified the myogenic cells. Whereas approximately 30% of the originally harvested cells were non-myogenic cells [negative: PAX7(−), MYF5(−), and MYOD(−)], after 7 days of the incubation MYF5-, or MYOD-positive cells had increased to more than 90% (Fig. 3a–d). This indicated that only myogenic cells, including muscle satellite cells and myoblasts selectively adhered and proliferated on the culture dishes in our primary culture process. Primary cells harvested from skeletal muscles often contain mixed populations of myogenic cells, fibroblast, adipocytes, and immune cells. Therefore, it is very advantageous to selectively collect only bovine myogenic cells. Furthermore, this method can be used to not only understand the population of myogenic cells in the cell mixture, but also confirm the quality of individual harvested cells at each step in the primary culture process. When we independently processed a primary culture, it resulted in a similar population for each type of cell (Fig. 3d); which showed that our primary culture method could harvest myogenic cells with a comparable purity each time.

One of the main characteristics of myogenic cells is the capability to differentiate into myotubes, so it is important to confirm the quality of the myogenic cells. To induce myotube differentiation in in-vitro culture, the growth medium is usually replaced with a low serum medium, such as 2% HS-DMEM. In this study also, the primary myogenic cells showed a strong differentiation capability when cultured in the differentiation medium. However, the bovine cells harvested in this study rapidly differentiated into a number of large myotubes and then detached from the culture surface after only a few days, probably due to the high differentiation capability of the bovine cells. Consequently, the detachment of myotubes made it difficult to evaluate the differentiation

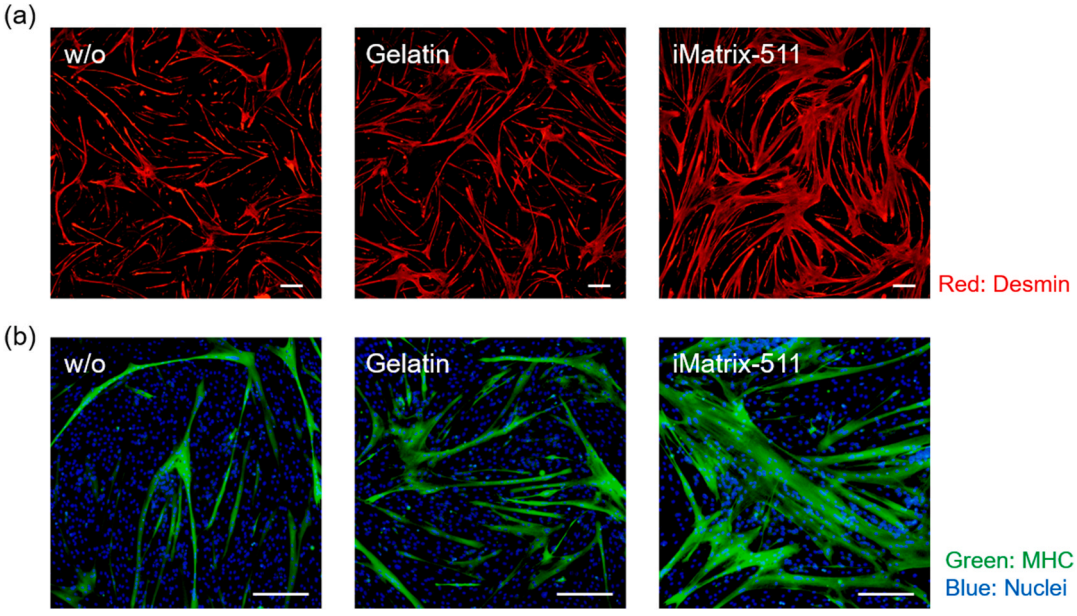

Fig. 2. Effect of biomolecule coating on differentiation of bovine myogenic cells harvested using our primary culture method. The cells were harvested with trypsin treatment and cultured for two passages before induction of differentiation. The cells were cultured on normal culture dishes without coating, culture dishes coated with gelatin or iMatrix-511 for 3 days in differentiation medium (2% HS-DMEM). Desmin and myosin heavy chain (MHC) were fluorescently stained to identify differentiated myotubes ((a): desmin, (b): MHC). Scale bar: 200 μm.

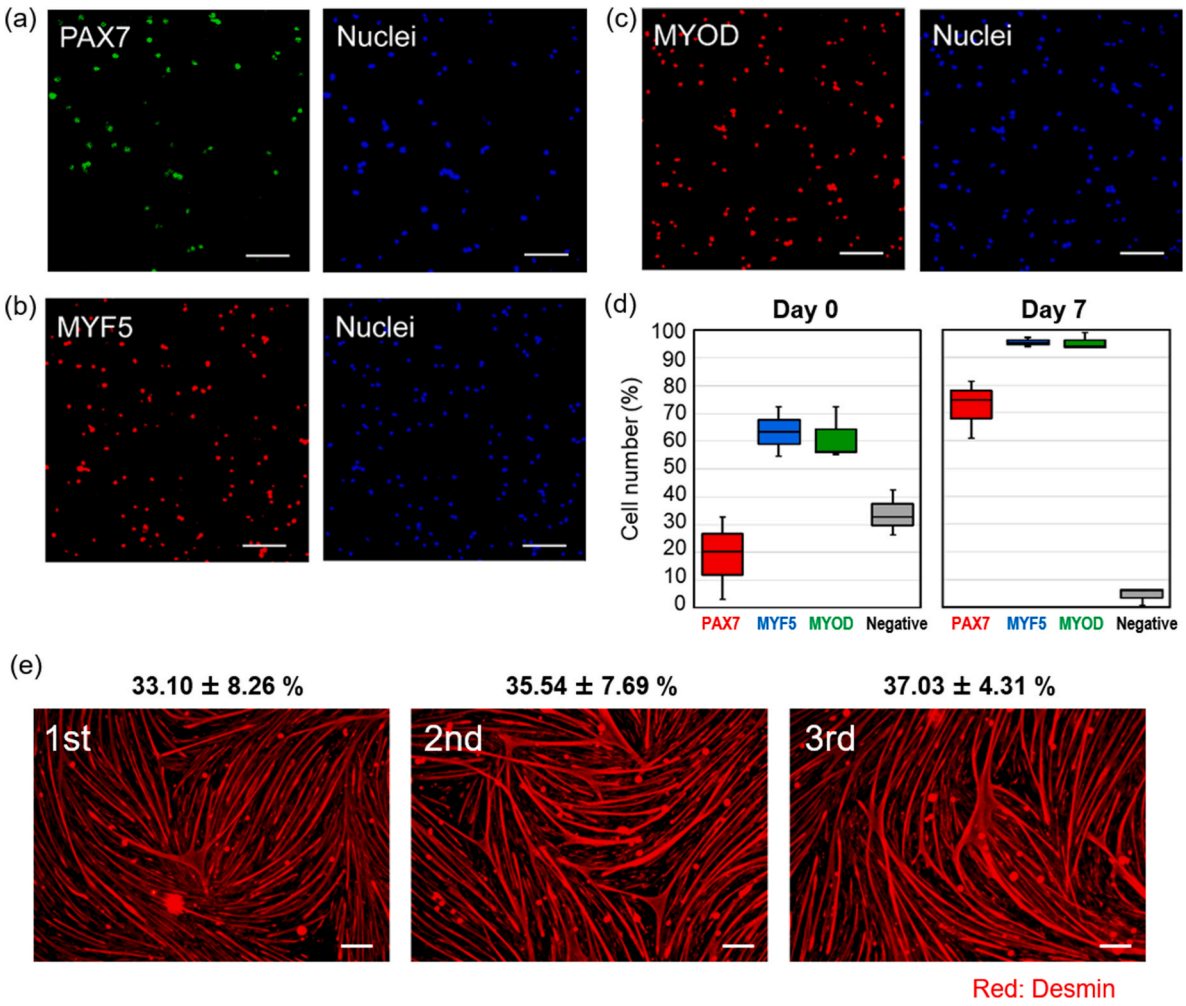

Fig. 3. Quality confirmation of primary bovine myogenic cells harvested from bovine meat. (a–c) Fluorescence images of myogenic cells stained with (a) PAX7, (b) MYF5, and (c) MYOD after 7 days on iMatrix-coated dishes. Nuclei were also stained blue in all images. Scale bar: 100 μm. (d) Population of bovine myogenic cells just after harvesting from meat (Day 0) and after 7 days in-vitro culture on culture dishes (Day 7). (e) Representative images of differentiated myotubes from individually harvested myogenic cells using our primary culture method. Scale bar: 200 μm. Three independent processes of our primary culture were performed (first, second, and third trials), and areas of desmin-positive myotube formation were determined to compare the differentiation rates between the three trials. The percentage of the areas were analyzed using ImageJ software and shown above each image. Averaged value: 35.23±1.62%. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

capability. On the other hand, myogenic cells at 100% confluency will spontaneously differentiate into myotubes, even when cultured in growth medium. Therefore, in this study the confluent cells were continuously cultured for 10 days in a growth medium (10% FBS-DMEM). As shown in Fig. 3e, the primary cells harvested from bovine meat differentiated into myotubes in the growth medium. Importantly, since these slowly forming myotubes were stable on the culture dishes, it demonstrated that this culture process was appropriate for quality confirmation of the primary cells from the point of view of differentiation efficiency. Furthermore, to quantitatively evaluate the differentiation rate, the area of desmin-positive myotubes was analyzed using ImageJ software. In this study, three samples were prepared from each of the primary culture, and three different locations in each sample were imaged to analyze the average area of myotube formation. In independent primary culture trials, each cell differentiated into desmin-expressing myotubes at the same level (differentiation rate: 33.10 ± 8.26% in the first trial, 35.54 ± 7.69% in the second trial, and 37.03 ± 4.31% in the third trial). It showed that this quality confirmation method could be used to determine the differentiation capability of primary cells harvested from bovine meat. In addition, this result also demonstrated that our primary culture method allowed us to collect primary bovine cells that had similar proliferative and differentiation capabilities each time myogenic cells were harvested from bovine meat.

3.3. *"Lifetime" of primary bovine myogenic cells for cultured meat production*

To constantly produce cultured meat from primary bovine cells, a large number of myogenic cells must be collected. However, after several passages it often causes a reduction in the performance of primary cells, including proliferation and differentiation [42]. Therefore, it is very important to maintain the proliferative and differentiation capabilities of primary cells in an in-vitro culture. To determine the useful "lifetime" of primary bovine myogenic cells, the cells harvested using our method were passaged several times using a normal 2D culture method. In this experiment, the cells were seeded onto normal culture dishes during repeated passaging since they adhered and proliferated even on non-coated dishes. As shown in Fig. 4a and b, these cells continued to proliferate with repeated passaging. According to our calculations, at least 2.5×10^{13} cells were collected from 2 g of bovine meat simply by the culturing and passaging procedure. Whereas the population doubling level continuously increased, the rate of increase

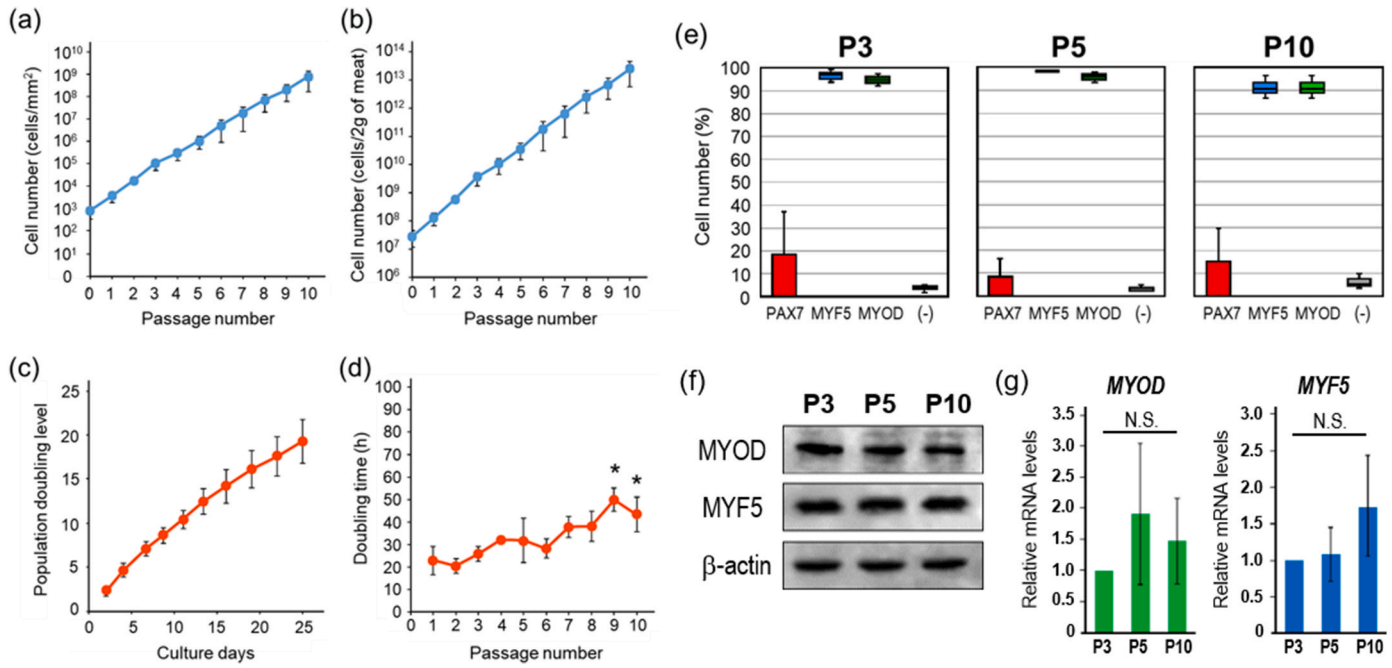

Fig. 4. Sustainable capability for proliferation of bovine myogenic cells in in-vitro growth over several passages. Primary bovine cells were independently harvested three times starting from bovine meat to determine the average values. (a, b) Numbers of bovine myogenic cells at passages 0 to 10. (c, d) Population doubling level and doubling time of bovine myogenic cells after 25 days of repeated passaging. The significant difference shown in Fig. 4d was determined by comparing with the doubling time at passage 1. (e) Population ratio of myogenic cells expressing PAX7, MYF5 or MYOD at passages 3 (P3), 5 (P5), and 10 (P10). (f) Western blot analysis for expression of myogenic proteins at P3, P5, and P10. Three independent primary culture and proliferation procedures were carried out and western blotting was repeatedly performed in each proliferation process. (g) Relative mRNA levels of *MYOD* and *MYF5* in myogenic cells at P3, P5, and P10. (N.S.: not significant).

was slightly reduced in the latter half of the repeated passaging (Fig. 4c). In fact, when the doubling time was compared between cells at each passage, only the doubling time of the cells between passages 9 and 10 was significantly different from that of the cells at passage 1 (Fig. 4d). This indicated that the proliferative capability of the bovine myogenic cells was maintained at the same level between passages 1 to 8. In addition, even after passage 9, they continued to proliferate after each subsequent passage. Although the change in doubling time indicated that the cells had lost half their proliferative capability at passage 9, the proliferation rate during the repeated passages (passages 1 to 10) was approximately 6.5×10^5.

To estimate the impact of repeated passages on myogenic characteristics of the cells, the expression of representative myogenic markers was analyzed using immunofluorescence staining, real-time qPCR, and western blotting. Importantly, whereas the myogenic cells remarkably lost PAX7 expression at passage 3, there was no difference in the population of myogenic cells compared with non-myogenic cells between passages 3, 5, and 10 (Fig. 4e). Fig. 4f indicates that the expressions of MYOD and MYF5 were maintained during the proliferation period. In addition, there were no significant differences in mRNA levels of MYOD and MYF5 (Fig. 4g). These indicates that the repeated passaging allowed expansion of myogenic cells that expressed two representative myogenic proteins in this culture period. Generally, there is concern that the population ratio of a specific type of cell could decreased during a large cell expansion. In fact, when the myogenic cells reached confluence once and were incubated for several days with 100% confluency, it induced a remarkable decrease in mRNA levels of MYOD and MYF5. Therefore, in considering cell density using our primary culture system, the myogenic cells were able to continuously proliferate in a simple 2D culture method. In the future, this will be very advantageous for the collection of a large number of myogenic cells from small samples of bovine meat dedicated for cultured meat production.

In general, most kinds of primary muscle cells often lose the ability to differentiate after several passages. In the production of cultured meat, even if myogenic cells continue to proliferate during the several passages without the ability to differentiate, they will not be incorporated into morphologically and functionally mature muscle tissues. Therefore, to evaluate the "lifetime" of the myogenic cells, differentiation capability was determined here with a quality confirmation method. In this study, as described above, while culturing in the differentiation medium (2% HS-DMEM), the myogenic cells rapidly formed a number of large myotubes and then detached one by one from the dishes. Therefore, the proliferating cells at each passage were cultured in the growth medium (10% FBS-DMEM) and then differentiated myotubes were fluorescently stained to confirm the change in differentiation capability. The differentiation efficiency was not significantly different between passages 3 and 5 (Fig. 5a and b). On the other hand, the cells at passage 10 formed only a few myotubes (Fig. 5a and b); indicating that after several passages, the differentiation capability of the cells had been reduced. To compare the differentiation efficiency at each passage, areas of desmin-positive myotubes were analyzed from the fluorescence images using ImageJ. Fig. 5c shows that the cells at passages 8, 9, and 10 had a reduced differentiation capability. On the other hand, the bovine myogenic cells at passage 7 still showed the same differentiation capability as the cells at passage 1. In addition, although the differentiation capability was reduced after the several passages, the cells at passage 10 still significantly differentiated into myotubes by incubating in a typical differentiation medium such as 2% HS-DMEM (Fig. 5d). That is, the bovine myogenic cells harvested by our primary culture method were able to continuously proliferate while maintaining their differentiation capability in a long-term culture. This is very advantageous to be able to collect a significant number of cells for production of large-scale tissue-engineered meat in the future.

3.4. Fabrication of biomimetic aligned orientation of bovine myofibers

The aim of this study is to demonstrate the potential of our tissue engineering technique for cultured meat production. To mimic the

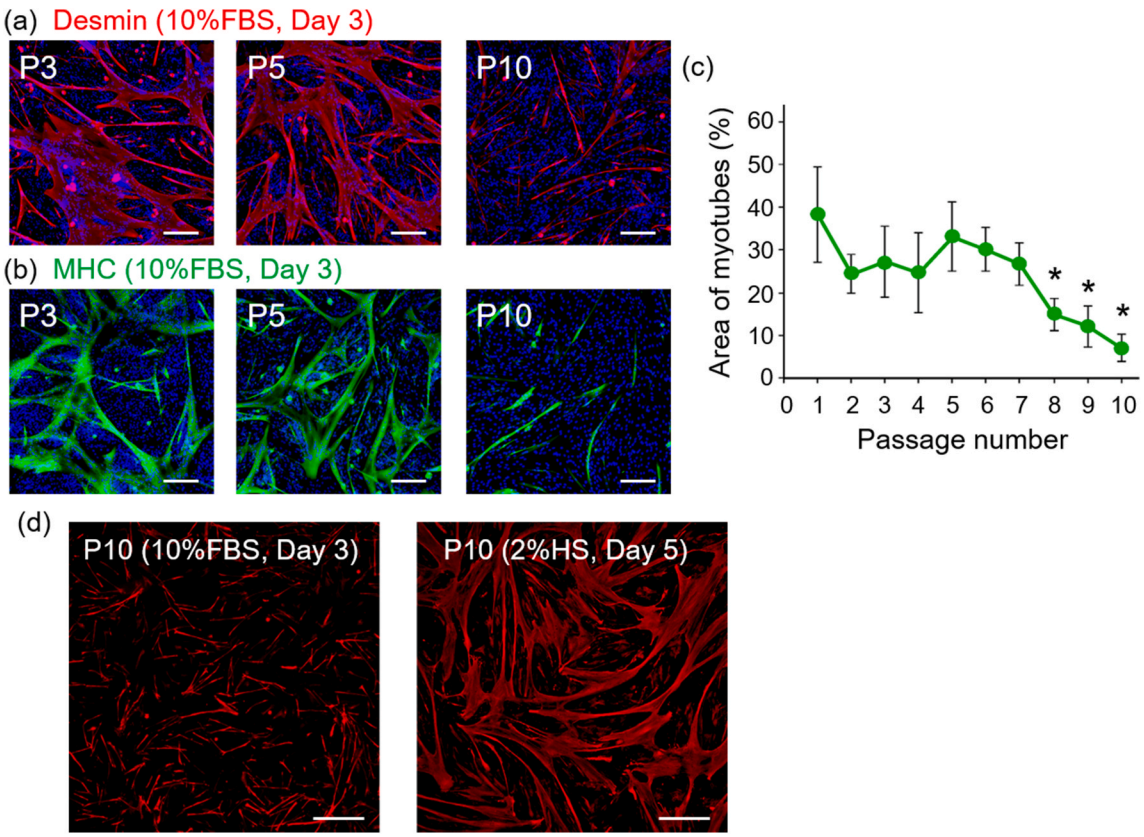

Fig. 5. Sustainable differentiation capability of bovine myogenic cells in in-vitro growth over several passages. Primary bovine cells were independently harvested three times starting from bovine meat to determine the average values. (a, b) Representative fluorescence images of myotubes at passages 3 (P3), 5 (P5), and 10 (P10) (red: desmin, green: MHC, blue: nucleus). The cells at each passage were seeded at the same density and cultured under the same conditions to determine the differentiation capability of myogenic cells at each passage. Scale bar: 200 μm. (c) Areas of myotube formation to compare differentiation capability of cells at each passage. The cells were cultured in 10% FBS-DMEM for 3 or 4 days and then desmin-positive myotubes were stained fluorescently. The significant difference was determined by comparing with the area of differentiated myotubes found in passage 1. (d) Fluorescence images of desmin-positive myotubes differentiated from bovine myogenic cells at passage 10 (P10). The cells were incubated in the growth medium 10% FBS-DMEM for 3 days (10%FBS, Day 3) or incubated in the growth medium overnight and then in the differentiation medium 2% HS-DMEM for 5 days (2%HS, Day 5). Scale bar: 500 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

morphology of myofibers in bovine meat, the bovine myogenic cells harvested in this study were first cultured on micropatterned thermoresponsive substrates [33,43]. Our group has previously developed a thermoresponsive cell culture substrate allowing us to fabricate a sheet-shaped cellular assembly, a "cell sheet" by simply lowering the culture temperature to 20 °C. Based on this cell sheet technology, a micropatterning approach was used on the thermoresponsive poly (*N*-isopropylacrylamide) (PIPAAm)-grafted substrate to regulate the alignment of human myoblasts and transfer them to a fibrin-based gel [33,43]. In this study, this microfabricated biomaterial was used to regulate the alignment of bovine myogenic cells. However, the behavior of bovine cells including adhesion and proliferation were different from those of human myogenic cells. Furthermore, as described above, bovine myogenic cells prefer to be cultured on dishes coated with iMatrix-511. Therefore, to regulate the cell alignment, the micropatterning was optimized by changing the photo-irradiation time in the micropatterning process (Fig. 6a). In our previous study, we confirmed that the micropatterns were fabricated at a nanoscale thickness [44]. Therefore, the difference in thickness between the two pattern regions is likely too small for cells to recognize. On the other hand, as shown in Fig. 6b, these two patterns have different affinities for protein immobilization. Since the cells can recognize the difference in immobilized proteins underneath themselves, human myoblasts formed aligned structures on the micropatterned substrate that was fabricated by using 7 min of photo-irradiation [33]. However, bovine myotubes showed the same kind of unregulated orientation on the patterned substrate optimized for human myogenic cells (photo-irradiation: 7 min), as that on a non-patterned substrate (Fig. 6c and d). The longer photo-irradiation induces the formation of thicker polymer patterns on the thermoresponsive surface and allows the bovine cells to be aligned more strictly with the direction of the patterns. Consequentially, the cells were well aligned on the patterned substrate prepared with 20 min of irradiation, whereas a 10 min-irradiation proved unable to form to the aligned structure (Fig. 6e and f). Taken together, the patterning designed for bovine cells was successfully fabricated by adjusting the photo-irradiation condition, and finally the aligned bovine myotubes were produced with greater accuracy using the micropatterned culture substrate in this study (Fig. 6f).

3.5. Maturation of functional bovine muscle tissue within a gel-based environment

Several previous studies demonstrated that biomaterial-based culture environments were necessary to promote functional maturation of in-vitro engineered muscle tissues [29,33]. In this study, using the thermally-induced cell detachment technique, the aligned cells on the patterned dish were transferred to a fibrin-based gel. As described above, in the differentiation process, rapidly growing myotubes were often detached from the culture dish within a few days. Before the unexpected detachment, a fibrin-based gel was fabricated on the aligned cells and then the cells were released from the surface by lowering culture temperature to 20 °C (Fig. 7a) [23,44]. Consequently, the

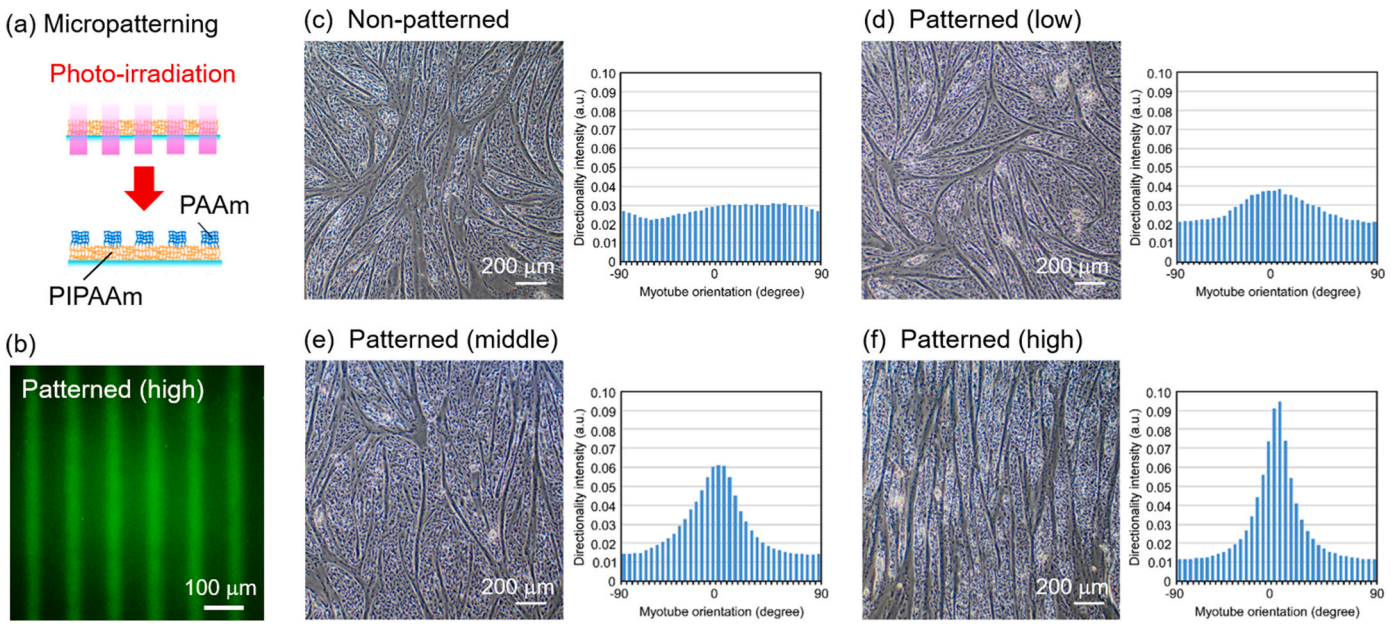

Fig. 6. Regulation of bovine myotube alignment using micropatterned thermoresponsive culture substrates prepared with different patterning conditions. (a) Schematic illustration of photo-induced micropatterning of PAAm on a PIPAAm-grafted substrate. (b) Fluorescence image of fluorescently-labeled fibronectin immobilized on the micropatterned substrate (photo-irradiation time: 20 min). (c–f) Microscopic images of myotubes differentiated on (c) non-patterned or (d–f) patterned substrates (photo-irradiation time: (d) 7 min, (e) 10 min, and (f) 20 min) were taken on Day 2 after induction of differentiation. Histograms of orientation of the myotubes were analyzed from six samples in each group.

aligned cells were further cultured on the gel environment (Fig. 7b). In our previous study, the fibrin gel containing Matrigel was effective to promote maturation of human myofibers [33]. Also in this study, the bovine cells were cultured in the same environment. On the other hand, the medium condition was adjusted for bovine muscle tissue production. Interestingly, more matured bovine myofibers were produced in 10% FBS-DMEM, compared with that in 2% HS-DMEM (Fig. 7c and d). In general, growth medium such as 10% FBS-DMEM is replaced with low serum medium to induce differentiation into myotubes and here the bovine muscle tissues were cultured once in 2% HS-DMEM. On the other hand, after differentiation was initiated, the medium was replaced again with 10% FBS-DMEM in the subsequent culture process. Consequently, compared with culture in 2% HS-DMEM (Fig. 7c), larger myofibers were formed at a higher density by culture in 10% FBS-DMEM (Fig. 7d). Although in our previous studies, engineered human muscle tissues were cultured in 2% HS-DMEM during the whole process of functional maturation, this study demonstrated that bovine muscle tissues preferred to be cultured in 10% FBS-DMEM. This result indicated that bovine myofibers probably require more nutrient supplement for maturation in vitro. In addition to myofiber formation, it is important that the myofibers remained aligned on the gel throughout the differentiation process (Fig. 7e). Consequently, these aligned myofibers showed sarcomere formation, suggesting that they were functionally mature (Fig. 7f and g). In addition, laminin was localized surrounding the myofibers. It is well known that a similar basement membrane-like structure is also found in native skeletal muscles and is very important for their physical strength in the body [30,45]. Therefore, this biological aspect is probably advantageous to biologically and physically mimic native muscle tissues. In addition, cell nuclei were localized at the gap between the sarcomere and the membrane-like laminin layer (Fig. 7g). In native muscles, cell nuclei migrate from the center of a myofiber to the outside of the sarcomere structure during myogenic maturation and this arrangement of nuclei indicates microstructural maturation of native myofibers in the body. The self-arrangement of myofibers shown here implies that our tissue production method was effective to form native tissue-like microstructures within the engineered bovine muscles.

To mimic the physiology of bovine muscles before becoming meat, engineered bovine muscle tissue should have the ability to contract. As described above, our muscle tissue formed sarcomere structures that are essential for muscle contraction. Consequently, after several days of culture in this gel environment, the muscle tissue began to contract spontaneously (Supplementary Video 1). Although this irregular twitching contraction is not usually seen in native muscle tissue, some previous studies have reported that this kind of contractile behavior was often observed in the early stage of in-vitro production of engineered muscle tissue [30,31,45]. Surprisingly in this study, our bovine muscle tissue was able to produce significantly strong contractions that they could be observed without the use of microscope (Supplementary Video 2). As described in Fig. 7a and b, a square-shaped silicon ring was incorporated in the fibrin-based gel to prevent shrinkage of the gel, because functionally mature myofibers often cause the gel to shrink. That is to say, the silicon ring structure forced it to maintain the original shape of the gel. Nevertheless, this bovine muscle tissue contracted at the submillimeter-scale that can be visually confirmed (Fig. 8a). In the case of human muscle tissue produced using our method, the average displacement was approximately 80 μm where it was impossible that it could be visually confirmed [33]. Our bovine muscle tissue has a much higher contractile ability compared with the human muscle tissue. Although the contractile behavior indicated the muscle contraction was uncontrollable by electrical or chemical stimulation, long-term culture (e.g., 64 days) eliminated the spontaneous contraction. This probably indicated that this muscle tissue matured sufficiently to organize the muscle contractions. When being simulated at a frequency of 1.0 Hz, the bovine muscle tissue produced a twitch contraction according to the electrical stimulation (Fig. 8b, Supplementary Video 3). On the other hand, as the frequency increased to 15 Hz, the muscle tissue produced tetanus contraction significantly larger than the twitch contractions (Fig. 8c, Supplementary Video 3). These contractile behaviors indicated that our bovine muscle tissue was physiologically functional as native muscle tissue. On the other hand, the EPS-induced contraction was smaller than the contraction observed in the early stage of maturation. Therefore, although the engineered bovine tissue produced in this study had structural and functional biomimetic properties, in the future we need to find a method to prevent the reduction in functionality and

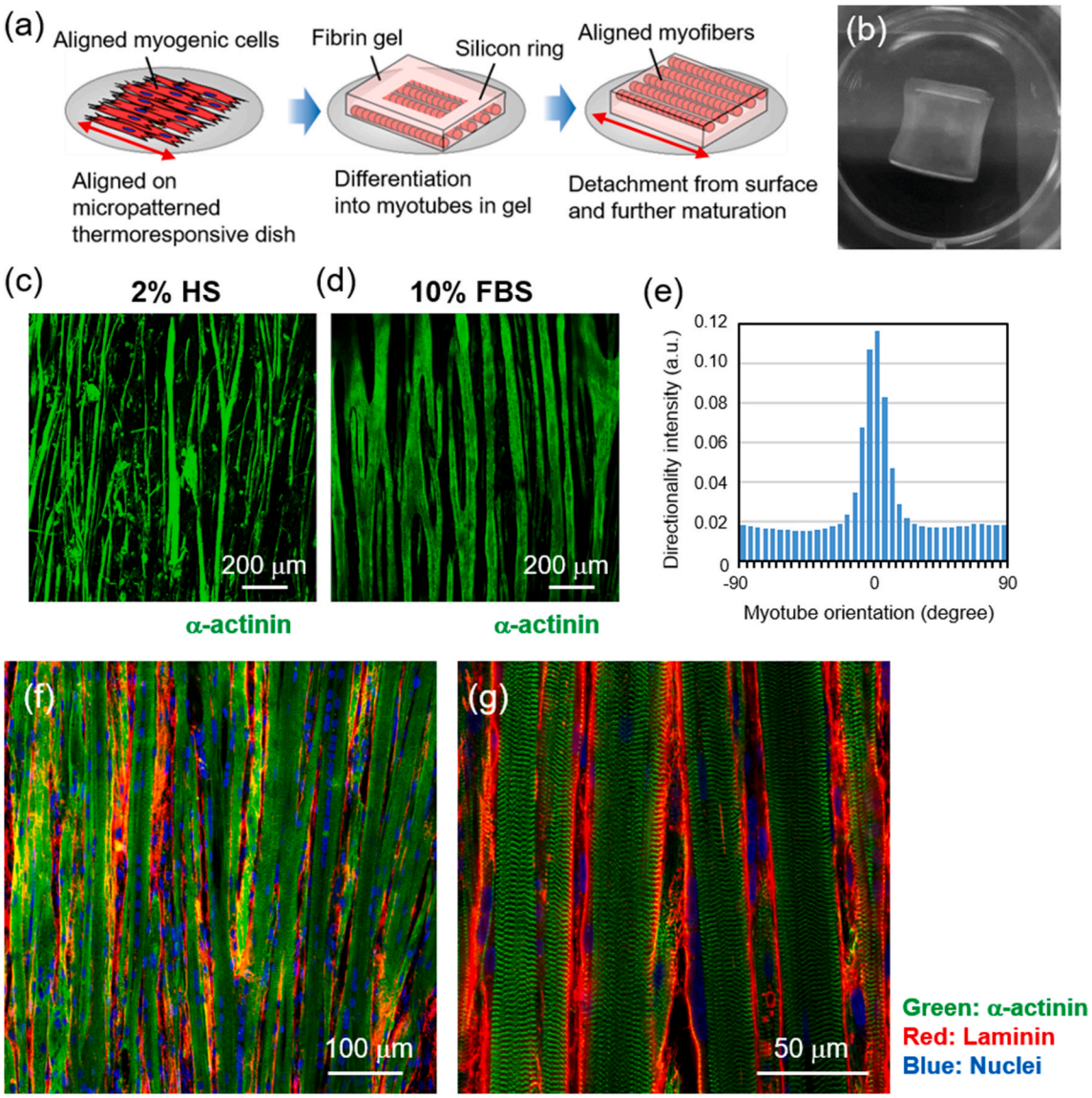

Fig. 7. Biomimetic morphology in bovine muscle tissue produced on a fibrin-based gel environment. (a) Schematic illustration of the transfer process of aligned myogenic cells from a micropatterned dish to a fibrin-based gel. The thermoresponsive substrate allows the aligned cells to release from the surface by lowering the culture temperature. After the cells attached to the fibrin-based gel, the tissue was incubated at 20 °C for 30 min and then the gel was placed upside down to a new culture dish. (b) Photograph of a bovine muscle tissue on a fibrin-based gel. The size of the gel was 15 × 15 mm. (c, d) Fluorescence images of myofibers produced by culture in (c) 2% HS-DMEM or (d) 10% FBS-DMEM. (green: α-actinin) (e) Histogram of orientation of desmin-positive myofibers on the fibrin-based gel. Orientation analysis was performed for three samples and the histogram shows the averaged intensity. (f, g) Fluorescence images of a functionally mature bovine muscle tissue with sarcomere formation and membrane-like localization of laminin (green: α-actinin, red: laminin, blue: nuclei). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

control the submillimeter-scale contractions during the long-term culture.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.biomaterials.2022.121649

4. Discussion

Cultured meat production is a promising approach to alleviate the ethical, environmental, and public health concerns associated with conventional meat production [3,19]. Plant-based meat made only from plant ingredients is another alternative to animal-based meat [10,46]. The innovative food manufacturing techniques developed by two leading companies, Impossible Foods and Beyond Burges, can produce alternatives to beef that has the taste and texture of traditional beef [47]. On the other hand, tissue engineering researchers are focusing on future next concepts in food technology [16,34]. Muscle tissue engineering is expected to produce cultured meat made of bovine myogenic cells. In conventional approaches, engineered muscle tissue is mainly produced for tissue modeling in the field of muscle physiology and drug discovery for muscle diseases [27,28]. A number of researchers, including ourselves have reported on artificially producing human muscle tissue that can mimic native muscle [28–30,33]. We believe that our native-like tissue production process (growth from muscle progenitor cells to matured myofibers) can mimic as-yet recognized native-like nutrients inside the cultured meat and replicate the native-like texture of meat. In this study, we demonstrated that our tissue engineering method has the potential to produce "tissue-engineered meat"; that is to say, bovine muscle tissue that is nearly indistinguishable from native muscle tissue. Cell sheet-based tissue engineering allowed to mimic the aligned structure of bovine myofibers by using a micropatterned

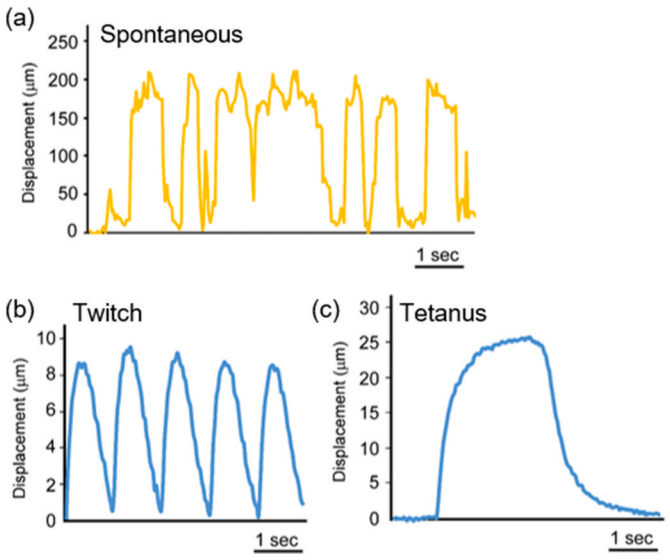

Fig. 8. Representative contraction displacement profiles of the bovine muscle tissue at different stages of the maturation process. (a) Displacement profile of spontaneous contraction without EPS application at 7 days after induction of differentiation. The displacement was analyzed from Supplementary Video 2. (b, c) Displacement profiles of EPS-induced contractions at 64 days after induction of differentiation. EPS were applied at frequencies of (b) 1 Hz or (c) 15 Hz to induce contraction of the bovine muscle tissue. These displacements were analyzed from Supplementary Video 3.

thermoresponsive substrate, and then by lowering the culture temperature the aligned cells could be transferred to the fibrin gel containing Matrigel for functional maturation. Consequently, engineered bovine muscle tissue with biomimetic structure and function was successfully produced by our tissue engineering approach in this study. The tissue-engineered meat formed an aligned structure, a membrane-like laminin structure, and showed native-like nucleus localization. Moreover, the functionally mature myofibers showed unidirectional contraction induced by electrical stimulation. Interestingly, the bovine muscle tissue immediately showed a remarkably high contractile ability. This was probably because our primary culture method initially harvested bovine myogenic cells with high proliferative and differentiation capability. On the other hand, while the spontaneous contraction had subsided, the electrically stimulated contractions were also reduced after the long-term culture. Therefore, our technique needs to be improved to maintain a higher contractile ability, which would result in the production of a more native-like tissue-engineered meat.

In tissue modeling for drug discovery, replicating the native-like tissue physiology within engineered tissue is more important than scaling-up the size of the engineered tissue. On the other hand, for cultured meat production, scaling-up is one of the most important factors; a piece of beef with the size of 15 × 15 mm is not satisfactory for a meal. Importantly, our primary culture method allowed us to rapidly collect a large number of bovine myogenic cells with differentiation capability from a small sample of muscle tissue. This is very advantageous to continuously produce tissue-engineered meat on a large-scale. However, to realize the benefits of alternative meat to replace animal-based foods, an important challenge is to reduce the associated cost for large-scale cell culture. The significant volume of culture medium and various kinds of growth factors and hormones are expected to be required for large-scale cell culture. These initial costs have made cultured meat very expensive, so a new culture method needs to be developed without use of expensive biomolecules. Although this study produced cell sheet-based muscle tissue with biomimetic microstructures and function, future bovine muscle tissue needs to be scaled up to produce cultured meat that is acceptable as a popular food item. In our previous study, we demonstrated that 3D human muscle tissue was producible by layering multiple muscle sheet tissues [43]. Therefore, this tissue engineering method will be a promising approach for scaling-up future tissue-engineered meat. Although this method is advantageous because it contains no biomaterials inside the 3D muscle tissue, it is time-consuming to prepare a large number of muscle sheets and stack them one-by-one. In addition, to prevent necrosis inside the cell-dense 3D tissue, vasculature structures are required to sufficiently supply oxygen and nutrients for long-term culture [48–50]. Therefore, in future work these problems remain a limitation for producing bovine meat on a large-scale. Although challenges remain, our primary culture method does allow us to harvest a large number of myogenic cells sufficient to produce cultured meat on a large-scale since they maintain a highly proliferative and differentiation capability over long-term culture.

Components of animal origin and various nutrients traditionally used in media for cell culture are still need and in this study, for example, serum collected from fetal bovines and horses were supplemented in the culture media for cell growth, differentiation, and maturation. On the other hand, cultured meat production holds the potential to improve ethical and environmental concerns by avoiding the use of livestock animals. Therefore, it is ideal that cultured meat can be produced using animal-free culture methods and without the use of serum components [51]. Various nutrients for livestock are usually derived from grains, and this agriculture production has its own environmental footprint. Although cultured meat production is expected to significantly reduce the grain production used for livestock, basal medium such as DMEM contains several nutrients such as glucose, amino acid, and vitamins, and these nutrients essential for cell culture are also mainly extracted from grains. Therefore, future nutrient substitutes such as components extracted from algae should be incorporated in future meat production [52]. The other challenge is to select an appropriate composition material for a 3D scaffold [34,53]. Several animal-derived biomaterials have often been used as scaffolds for tissue engineering, since they closely mimic their natural physiological niche and promote maturation of artificial tissues in in-vitro cultures. However, considering the main focus of alternative meat production, it should avoid to use any animal-derived biomaterials. These challenges taken together will require new innovations to improve the scalability and minimize dependence on animal sources to upgrade tissue-engineered meat to become a more attractive option for public meat consumption.

5. Conclusion

This study demonstrated the feasibility of bovine muscle tissue engineering, based on our original primary culture method and cell sheet-based tissue engineering approach. Our primary culture method allowed us to efficiently harvest and proliferate bovine myogenic cells while maintaining their differentiation capability. New methods to confirm the quality were also developed, which demonstrated that our primary culture method enabled an efficient harvest of bovine myogenic cells, critical for the stable production of bovine tissue-engineered meat. Furthermore, in this study, structurally and functionally biomimetic muscle tissue was successfully produced based on our tissue engineering technique. We believe that the biomimetic features of our muscle tissue are important to produce native-like tissue-engineered meat in the future. Currently cultured meat technology is still limited, therefore, only a small amount of cultured meat has been grown in a laboratory. Although the challenges of scaling-up and cost reduction are significant for a sustainable supply of cultured meat in the future, our technique reported here has the potential to become a key part of its success as a future food technology. Finally, these techniques for bovine muscle tissue engineering will also be applicable to the production of physiologically and functionally mature human muscle tissue that is required for the studies of muscle physiology and diseases in the future.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contribution

Hironobu Takahashi: Conceptualization, Methodology, Validation, Investigation, Visualization, Writing-Original draft preparation. Azumi Yoshida: Conceptualization, Methodology, Validation, Investigation, Visualization, Writing-Original draft preparation. Botao Gao: Methodology, Investigation. Kumiko Yamanaka: Methodology, Investigation. Tatsuya Shimizu: Writing-Review & Editing, Supervision.

Declaration of competing interest

Tatsuya Shimizu is a stakeholder in CellSeed Inc. Tokyo Women's Medical University receives research funds from CellSeed Inc.

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