Fabrication of Contractile Skeletal Muscle Tissues Using Directly Converted Myoblasts from Human Fibroblasts

Short title: Tissue engineering using directly converted myoblasts

Kazunori Shimizu[1,*], Saki Ohsumi[1], Tsunao Kishida[2], Osam Mazda[2], Hiroyuki Honda[1,3]

[1] *Department of Biomolecular Engineering, Graduate School of Engineering, Nagoya University, Nagoya 464-8603, Japan*

[2] *Department of Immunology, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan*

[3] *Innovative Research Center for Preventative Medical Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan*

[*] Correspondence: shimizu@chembio.nagoya-u.ac.jp

Tel: +81-52-789-3213, Fax: +81-789-3214

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Abstract

Transplantation of stem cell-derived myoblasts is a promising approach for the treatment of skeletal muscle function loss. Myoblasts directly converted from somatic cells that bypass any stem cell intermediary stages can avoid the problem of tumor formation after transplantation. Previously, we reported that co-transduction with the myogenic differentiation 1 (*MYOD1*) gene and the v-myc avian myelocytomatosis viral oncogene lung carcinoma derived homolog (*MYCL*) gene efficiently converted human fibroblasts into myoblasts. Although the directly converted myoblasts efficiently fused into multinucleated myotubes *in vitro* and *in vivo*, it is not clear whether they have the contractile ability, which is the most significant phenotype of the muscle. In the present study, we aimed to examine the *in vitro* contractile ability of the myotubes differentiated from the directly converted myoblasts by the overexpression of *MYOD1* and *MYCL*. We fabricated three-dimensional (3D) tissues on a microdevice for force measurement. The 3D culture enhanced the differentiation of the myoblasts into myotubes, which were confirmed by gene expression analysis of skeletal muscle-related genes. The tissues started to generate contractile force in response to electrical stimulation after 4 days of culture, which reached approximately 12 μN after 10 days. The addition of IGF-I decreased the contractile force of the 3D tissues, while the use of cryopreserved cells increased it. We confirmed that the tissues fabricated from the cells derived from three different donors generated forces of similar magnitude. Thus, directly converted myoblasts by the overexpression of *MYOD1* and *MYCL* could be a promising cell source for cell therapy.

INTRODUCTION

The skeletal muscle works as an actuator for physical movement and is involved in various significant physiological functions, including maintenance of body temperature and glucose homeostasis. The skeletal muscle is composed of multiple bundles of contractile myofibers, matured multinucleated myotubes that are formed by the fusion of mononucleated myoblasts. Loss of skeletal muscle function is associated with traumatic injury, and some congenital, inherited, and degenerative diseases; this function loss greatly reduces the quality of life of affected patients. Transplantation of stem cell derived myoblasts is a promising approach for the treatment of this condition, because the myoblasts will form myofibers after transplantation (1, 2).

The transgenic overexpression of the myogenic differentiation 1 (*MYOD1*) or paired box 7 (*PAX7*) gene in human induced pluripotent stem cells (iPSCs) enhances the differentiation of human iPSCs into myoblasts (3-6). Thus, iPSC-derived myoblast populations are considered as significant cell sources for the regenerative therapy of skeletal muscles. However, they may contain residual undifferentiated cells that have the potential of forming tumors after transplantation.

Meanwhile, the use of directly converted myoblasts from somatic cells, which bypass any stem cell intermediary stages, would avoid the problem of tumor formation. Since a pioneering study in 1987 (7), it has been well established that the expression of *MYOD1* in fibroblasts converts them into myoblasts (8-10). Thus far, several research groups including ours have reported the conversion of human somatic cells into myoblasts using a strategy based on the expression of MYOD1 (9, 11-14).

In a previous study, we showed that co-transduction with the *MYOD1* gene and the v-myc avian myelocytomatosis viral oncogene lung carcinoma derived homolog (*MYCL*) gene efficiently converted human fibroblasts into myoblasts (14). The myoblasts directly converted by the overexpression of *MYOD1* and *MYCL* genes (dMBs) fused more efficiently into multinucleated myotubes *in vitro* than the myoblasts converted by transducing the *MYOD1* gene only. We also showed that the dMBs formed a myofiber-like structure *in vivo* in mice. However, it is not clear whether the myotubes differentiated from the dMBs have contractile ability, which is the most significant phenotype of muscle cells. In the present study, therefore, we aimed to examine the contractile ability of the myotubes differentiated from the dMBs *in vitro*.

MATERIALS AND METHODS

Cells. Normal human dermal fibroblast (HDF) cells used in this study were all purchased from Toyobo (Osaka, Japan) and DS Pharma Biomedical (Osaka, Japan). The

PLAT-GP packaging cell line was purchased from Cell Biolabs (San Diego, CA). These cells were cultured in a DMEM medium containing 10% fetal bovine serum (FBS), 100 mM non-essential amino acids (NEAA), 100 U/mL penicillin, and 100 μg/mL streptomycin (complete medium).

Retroviral vectors. The coding sequence for the human *MYOD1* gene was amplified by RT-PCR using KOD-Plus-Neo DNA polymerase (Toyobo) and *MYOD1*-specific primers (FW 5-cagtgtggtggtacggggctatctacagcttgggttgg-3, Rv 5-accggcgctcagctgggggggcatttaagttcaatct-3). The amplified fragments were cloned into the EcoRI-digested pMXs vector (Cell Biolabs) using the Invitrogen GeneArt Gene Synthesis service (ThermoFisher Scientific, Waltham, MA). pMXs vectors containing v-myc avian myelocytomatosis viral oncogene lung carcinoma derived homolog (*MYCL*), and GFP genes were provided by S. Yamanaka (Kyoto University). Retrovirus vectors were obtained as described elsewhere (15). Briefly, PLAT-GP packaging cells were seeded in 100 mm gelatin-coated dishes at a density of 5×10^6 cells/dish, cultured overnight, and co-transfected with 2.5 μg each of pCMV-VSV-G and one of the pMX plasmids containing transcription factor genes described above, by means of X-treme Gene 9 regent (Roche Applied Science, Penzberg, Germany) diluted in 500 μL of Opti-MEM. Twenty-four hours later, the culture supernatant was changed and fresh antibiotic-free culture medium provided. After culturing for another 24 h, the supernatant was collected and filtered through a 0.45 μm-pore filter.

Infection and induction into myoblasts. Fibroblasts were resuspended in a complete medium and seeded at 1.31×10^4 cells/cm^2. The next day, the supernatant containing each retroviral vector was mixed, supplemented with 4 μg/mL polybrene, and transferred to the fibroblast culture from which the culture supernatant had been removed immediately before infection. Twenty-four hours later, the virus-containing medium was changed with fresh αMEM containing 5% horse serum (HS), non-essential amino acids (NEAA), 10 ng/mL IGF-1, 100 U/mL penicillin, and 100 μg/mL streptomycin (Differentiation Medium, DM). On day 4, the cells were treated with trypsin and collected by centrifugation. The harvested cells were resuspended in a cell preservative solution (Nacalai Tesque, Kyoto, Japan) and aliquoted in cryogenic vials. The vials were frozen at −80 ℃ and stored in liquid nitrogen until further use.

Differentiation of the dMBs into myotubes in 2D or 3D. The frozen vials were thawed by gentle agitation in a 37 ℃ water bath. After washing the dMBs with DM,

the dMBs were seeded at 2.0×10^4 cells/cm^2 and cultured overnight at 37 ℃ under a humidified atmosphere of 5% CO_2 and 95% air to remove the unattached damaged cells.

For differentiation in 2D, the attached dMBs were harvested, suspended in DM, and reseeded in a six-well plate at 2.0×10^4 cells/cm^2. The medium was changed every other day.

For differentiation in 3D, the microdevices for measuring muscle contractile force were used in the manner reported in our previous study (16). Briefly, the microdevices were placed at the center of a well of a six-well plate and sterilized under UV light for 1 h. The dumbbell shaped pocket and two microposts on the device were covered with 2% Pluronic F-127 solution (P6867, Invitrogen) and kept overnight at 4 °C to prevent tissue adhesion to the device. After removing the solution, the device was used for constructing skeletal muscle tissues. The attached dMBs were harvested and mixed with hydrogel. We defined the total volume of the cell-hydrogel mixture as 1 V. Ice-cold fibrinogen from bovine plasma (10 mg/mL, F8630, Sigma-Aldrich, Darmstadt, Germany), 2×DMEM, and Matrigel (354234, Corning, New York, NY, USA) were mixed in a microtube on ice with a volume ratio of 0.2:0.2:0.1. Then, 0.484 V of DM containing dMBs of concentration 2×10^6 cells/mL was added to 0.5 V of the hydrogel mixture. Finally, 0.016 V of thrombin from bovine plasma (50 U/mL, T4648, Sigma-Aldrich) was added to the mixture. A total of 45 μL of the resulting solution was poured into the dumbbell shaped pocket on the device and incubated at 37 °C for solidification. The tissues were cultured in DM containing 2.0 mg/mL 6-Aminocaproic acid (A2504, Sigma-Aldrich). The medium was changed every other day.

Real-Time RT-PCR. The total RNA was extracted from the muscle tissues using Nucleospin RNA (MACHEREY-NAGEL, Duren, Germany) according to the manufacturer's instructions. First-strand cDNA was prepared from the extracted RNA by using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan), according to the manufacturer's instructions. Quantitative real-time RT-PCR of cDNA was performed using StepOnePlus real-time PCR System (Applied Biosystems, CA, USA) with TaqMan®Advanced Master Mix (Applied Biosystems). The primers and dye probe for human β-actin (Table 1), human Myogenin (Table 1), human *CKM* (Hs00176490_m1), human *DMD* (Hs00758098), human *MYH3* (Hs00159463_m1), and human *MYH6* (Hs01101425_m1) were purchased from Applied Biosystems. Each 20 μL reaction mixture contained 2 μL of cDNA (100 ng), 1 μL of each primer probe, and 10 μL TaqMan Advanced Master Mix. Samples were incubated at 95 ℃ for 10 min for initial denaturation, followed by 40 cycles of denaturation at 95 ℃ for 15 s, and then annealing

and extension at 60 ℃ for 1 min. Relative changes in the gene expression were calculated according to the comparative threshold cycle (CT) method and normalized against β-actin, which was used as the endogenous control gene. All experiments were performed in triplicate.

Contractile properties of the muscle tissues. For measurement of the tetanic force, the muscle tissues constructed on the device in a well of a six-well plate were electrically stimulated as reported previously (16). An electrical stimulus of 1.1 V/mm at 30 Hz with 2 ms wide pulses (C-Pace EP, IonOptix, Westwood, MA, USA) and electrodes for the six-well plate (C-CLD6WACN, IonOptix) were used. The displacement of the tip of the microposts was observed using an upright microscope (BX53F, Olympus, Osaka, Japan). The electrical stimulation was applied to each tissue for 5 s, the images of the micropost tips were acquired both without and with the stimulation, and the displacement was measured using ImageJ.

For monitoring the contractile responses of the tissues to the electrical stimulation with different frequencies, the cultured muscle tissues were electrically stimulated at 1 and 30 Hz for 5 s. The movement of the tip of the microposts was monitored using the upright microscope with a 50 times magnification (BX53F) equipped with a camera (DP). The acquired videos were analyzed by a software for motion analysis (PV STUDIO 2D ver. 2, L.A.B., Miyazaki, Japan).

Fluorescent immunostaining. The tissues were fixed in 4% para-formaldehyde (Wako, Osaka, Japan) for 30 min, washed three times for 10 min each with phosphate-buffered saline (PBS), and permeabilized for 10 min with 0.3% Triton X-100/PBS (Sigma-Aldrich). We then subsequently washed them three times for 10 min each with PBS and blocked with a blocking solution (10% goat serum, 0.01% TritonX-100/PBS) for 30 min at room temperature. The tissues were applied with the primary antibody (monoclonal Anti-α-actinin, A7811, Sigma-Aldrich) for 3 h at room temperature. Then, the tissues were washed three times for 10 min each with PBS and applied with the secondary antibody (CF488A Goat Anti-Mouse IgG (H + L), 20018, Biotium, Fremont, CA) and 4′,6-diamidino-2-phenylindole (DAPI) for 2 h at room temperature. After washing three times for 10 min each with PBS, the stained tissues were observed using BZ-X700 (KEYENCE, Osaka, Japan).

Statistical analysis. Statistical analysis was conducted using Microsoft Excel (Redmond, WA) and BellCurve for Excel (Social Survey Research Information Co.,

Ltd., Tokyo, Japan). The statistical significance was determined by a t-test and one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Electrical stimulation to 2D cultured myotubes differentiated from dMB. Electrical stimulation has been used to induce contraction of myotubes derived from various types of myoblasts, including C2C12 murine myoblasts (17-20), primary human skeletal muscle cells (21), and human iPSC-derived myoblasts (3). Thus, first, we differentiated dMBs to myotubes in a two-dimensional (2D) plane culture and applied electrical stimulation to induce the contraction. Although the multinucleated myotubes were observed as we reported previously (14), we could not find any contracting myotubes in response to the electrical stimulation between day 4 to 10 through observation under phase contrast microscopy.

Fabrication of three-dimensional (3D) tissue engineered skeletal muscle tissues. The 3D culture of skeletal muscle cells enhanced the maturation of myotubes by an increase in the expression of skeletal muscle related genes (22) or by membrane localization of dystrophin (23). Next, we cultured and differentiated dMBs in 3D to examine their contractile ability. We fabricated 3D skeletal muscle tissues on the microdevices with two microposts as the anchor of the tissue, according to the setup developed in our previous study (16). The dMBs mixed with the hydrogel solution were seeded on the device and cultured for 10 days. As shown in Fig. 1A, ribbon-shaped tissues formed between the microposts by self-organization. The distance between the two posts decreased as the culture developed over time. Fig. 1B shows the distance between the two posts. The distance decreased between days 2 and 4 (3849.9±135.1 μm on day 2, 3118.0±262.2 μm on day 4); it continued to decrease slowly after day 4 till day 10 (2390.9±384.7 μm on day 10).

As the 3D tissues could be formed using dMBs, the expression levels of skeletal muscle-related genes in the tissues, including *MYOG*, *CKM*, *DMD*, *MYH3*, and *MYH6* genes, were determined during tissue formation. The expression levels of these genes in cells that were cultured in 2D were also determined and compared with those of cells cultured in 3D. Fig. 2 shows that the expression levels of all five genes increased time dependently for both 3D and 2D cultures. Although there were no significant differences between these cultures for all genes on day 2, the expression levels for the 3D culture became significantly higher than those for the 2D culture for all genes on day 10.

Contractile force measurement of the fabricated 3D tissues. Subsequently, we investigated whether the fabricated 3D tissues have contraction abilities in response to the electrical stimulation. On days 0, 4, 6, 8, and 10, we applied electrical stimulation to the tissues at 30 Hz, measured the displacement of the microposts, and calculated the contraction force. As shown in Fig. 3A, the fabricated 3D tissues had a contractile ability and the generated force increased with time dependently: the tissues generated 1.6±1.8 μN on day 4, 4.7±3.4 μN on day 6, 9.7±4.6 μN on day 8, and 12.2±5.3 μN on day 10. Contractile properties of the tissues on day 10 were evaluated by applying the electrical stimulation for 5 s at 1 and 30 Hz. As shown in Fig. 3B, a brief contractile response (twitch) was observed at 1 Hz and a lasting contraction (tetanus) was observed at 30 Hz. Immunofluorescent staining of α-actinin using the tissues on day 10 shows that the tissues contained several myotubes with a sarcomere structure (Fig. 3C).

Although the contractile myotubes were not observed in 2D, the fabricated 3D tissues were observed to generate a contractile force (Fig. 3). There is a possibility that the contractile myotubes in the 2D culture could not be found owing to the small displacement and/or small number of cells. By using the 3D culture, we could measure the sum of the forces of contracting myotubes without needing to find them under the microscope. Furthermore, the results of gene expression analysis of skeletal muscle-related genes in Fig. 2 strongly suggest that the differentiation of dMBs to myotubes was enhanced by using the 3D culture of dMBs as reported for different myoblasts (22, 23). In an earlier study, it was reported that the use of fibrin gel, which has a stiffness comparable to the native muscle, and the application of static tension to the C2C12 tissues enhanced fusion, differentiation, and maturation (24). The conditions for the 3D culture used in this study may increase the number of contractile dMB-derived myotubes and/or their contraction force; we used a fibrin-Matrigel mixed gel as a substrate for the 3D culture of dMBs, which is comparably stiffer to the native muscle than the polystyrene used for the 2D culture, where the tissues were cultured between two flexible posts under static tension. Thus, the use of the 3D culture made it possible for us to observe the contractile ability of dMB-derived myotubes.

Effects of IGF-1 addition or cryopreservation processes on the contractile force of the 3D tissues. dMBs are a promising cell source not only for transplantation for cell therapy but also for establishing an *in vitro* physiological tissue model. The contractile force of *in vitro* 3D skeletal muscle tissues is useful as an index for drug screening. The force generated by the 3D tissues fabricated with dMBs was smaller than

that of tissues fabricated from other myoblasts, such as C2C12 and human primary myoblasts. For example, on day 6, a force of 4.7±3.4 μN was generated for dMBs (Fig. 3), 58.4±9.7 μN for C2C12 (16), and 36.8±8.5 μN for human primary myoblasts (our unpublished data). It could be explained by the low number of contractile myotubes in the tissues formed by dMBs; we observed by the immunofluorescent staining of α-actinin that the number of myotubes with sarcomere structure in the 3D tissues of dMBs was much less than that of C2C12 and human primary myoblasts.

As a robust drug screening system, it would be better to use the tissues that generate a relatively larger force because the small force may be buried in unintended noise during the measurement. For the setup of contractile force measurement used in this study, it is empirically difficult to distinguish the difference of active tension less than 1 μN accurately. To examine the effects of certain drugs on the contractile force, the change of the magnitude in the contractile force is measured. Thus, the use of tissues which generate larger force would increase the sensitivity and accuracy of the system and be helpful to detect the small change clearly.

IGF-I, a peptide growth hormone, contributes to skeletal muscle growth and differentiation (25). The addition of IGF-I increased the contractile force of tissue engineered skeletal muscle (16, 26-28). Thus, we examined the effects of the addition of IGF-1 on contractile forces of tissues fabricated with dMBs. As shown in Fig. 4A, unexpectedly, the addition of IGF-1 at 10 and 100 ng/ml decreased the active tension of the tissues: the group to which IGF-I was added at 10 ng/ml yielded a relative value of 0.14 and the group to which IGF-1 was added at 100 ng/ml yielded 0.21. IGF-I is involved in the production of extracellular matrix (ECM) including collagen in fibroblasts (29, 30). As not all fibroblasts were converted to the myoblasts, fibroblasts still remained in the tissues (14). Although further experiments such as expression analysis of ECMs in the tissues or measurement of tissue stiffness are required, it is possible that the addition of IGF-I may induce the production of ECM from the fibroblasts and the 3D tissues become stiffer, thereby decreasing the strength of the contraction force. Gene expression analysis of skeletal muscle-related genes or counting the cell number in the tissues would also be helpful to understand the effects of IGF-1.

We also examined the effects of the cryopreservation processes of cells on the contractile force of the tissues. Both dMBs that had undergone cryopreservation and those that had not were used to fabricate tissues. As shown in Fig. 4B, the tissues fabricated with the cryopreserved dMBs generated a 3.6 times higher force than those fabricated with non-cryopreserved dMBs. It was still unclear why the cell population with cryopreservation generated a higher force. We supposed that, by freezing the cells, the

damaged cells by the retrovirus infection may be effectively removed from the cell population for tissue fabrication, resulting in enhanced differentiation in the 3D tissues. Further analyses of the constructed 3D tissues are necessary to understand this phenomenon. Meanwhile, considering dMBs as the cell source of choice for cell therapy, it is meaningful that the cryopreservation process shows apparent positive effects in the differentiation of dMBs. Namely, we could prepare and store frozen cellular stocks of dMBs in advance to their actual clinical use.

Effects of donor difference on the contractile force. Finally, we investigated whether the tissues fabricated from the cells of different donors generate contractile forces. We used different fibroblasts from three different donors (Table 2) and the same virus solution obtained from one experiment. As a result, all the tissues generated active tension regardless of the difference in donor (Fig. 5). These results support the findings that the myotubes differentiated from dMBs generate a force in response to the electrical stimulation. However, it was observed that the time course trends and maximum values of the contractile force differed among the donors. The tissues from donor A and B had a peak in the contractile force on day 10 while the tissues from donor C had a peak on day 6. These differences may be attributed to the differences in the original fibroblasts; however, the specific mechanisms are still unclear. We will investigate the reasons for this in detail in the future; this may provide useful information for clinical use.

In conclusion, in this study, we examined the contractile ability of myotubes differentiated from dMBs. By using a 3D culture, we could observe the contraction of myotubes in response to electrical stimulations. As the myotubes differentiated from different dMBs developed from different donors exhibited a contractile ability, we could conclude that dMBs would be a promising cell source for cell therapy.

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Table 1. Primers used in this study.

Gene	Fluorescent dye	Probe Sequence	Primer Sequence
hACTB	VIC	CTTTGCCGATCCGC	F: CCCCGCGAGCACAGA
			R: CCACGATGGAGGGGAAGAC
hMyogenin	FAM	CAGGGGATCATCTGCT	F: CAGCGAATGCAGCTCTCACA
			R: GCTGTCCACGATGGAGGTG

Table 2. Donors of the fibroblasts.

Donor	Sex	Age	Race	Part
A	Male	30	Caucasian	Abdomen
B	Female	38	Caucasian	Abdomen
C	Female	22	Black	No data

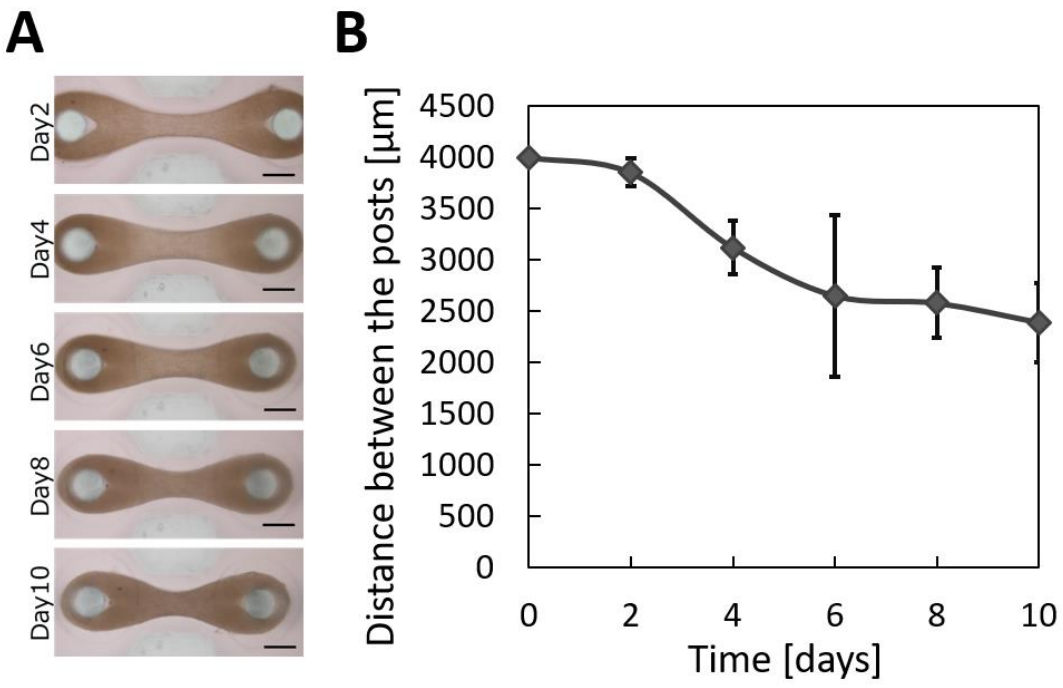

FIG. 1. 3D tissues fabricated with dMBs. (A) Top view of the tissues on day 2, 4, 6, 8, and 10. Scale bars represent 500 μm. (B) Distance between the tips of two microposts. Results represent mean±SD (n=14).

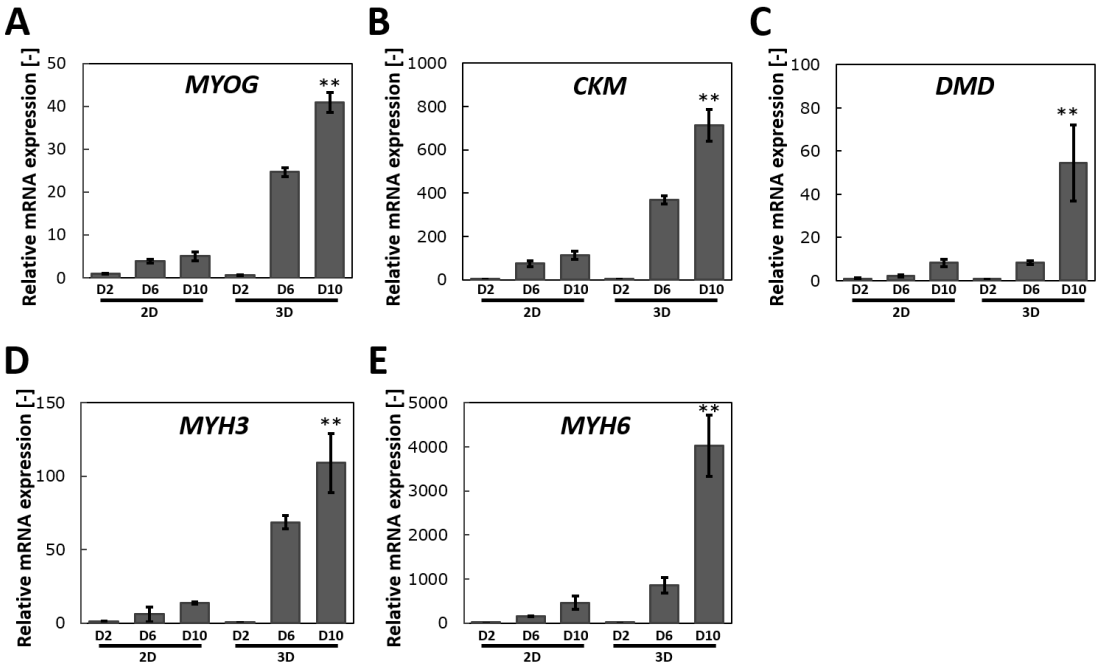

FIG. 2. Expression analysis of skeletal muscle related genes. Expressions of (A) *MYOG*, (B) *CKM*, (C) *DMD*, (D) *MYH3*, and (E) *MYH6* genes were obtained using real time PCR. Expression of these genes were compared between 2D and 3D cultures on day 2, 6, and 10. Results represent mean±SD (n=3). ** p<0.01 versus D6 of 3D and D10 of 2D.

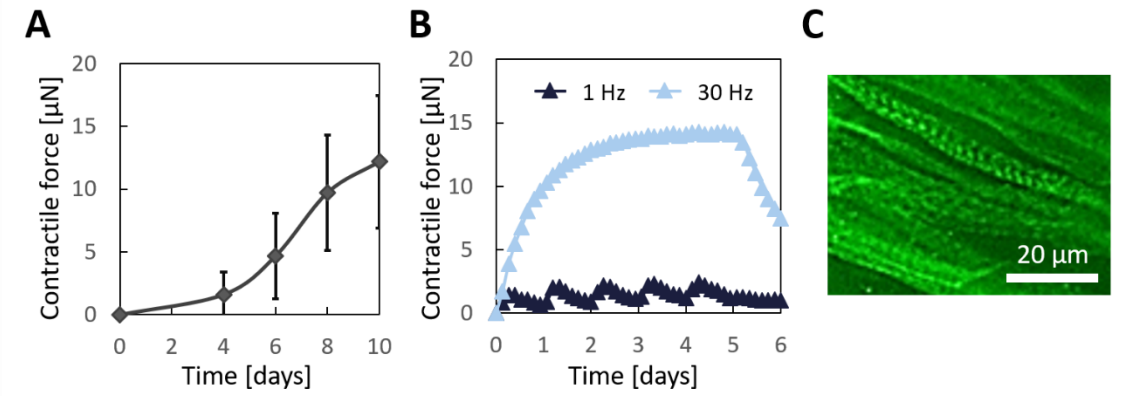

FIG. 3. Contractile force of the 3D tissues. (A) Time course of contractile force. Results represent mean±SD (n=15). (B) Contractile force of the tissues in response to 1 Hz or 30 Hz of electrical stimulation for 5 s. (C) Immunofluorescence staining of myotubes in the 3D tissues. Green indicates α-actinin.

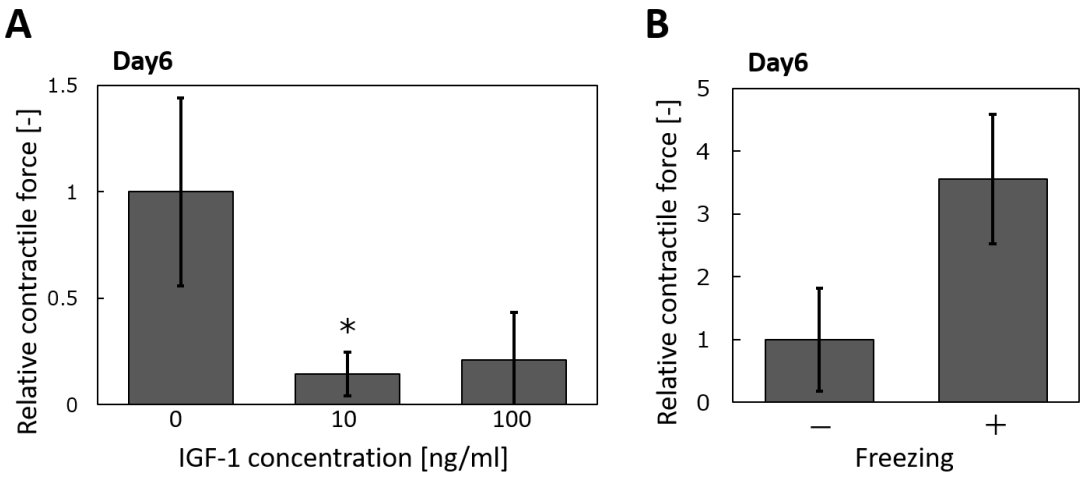

FIG. 4. Change in contractile forces by tissue fabrication processes. (A) Effects of IGF-I addition at 10 and 100 ng/ml on the contractile force of the 3D tissues. Results represent mean±SD (n=3-4). *p<0.05 versus 0 ng/ml. (B) Effects of cryopreservation process on the contractile force of the 3D tissues. Results represent mean±SD (n=3). p=0.0512 with and without freezing.

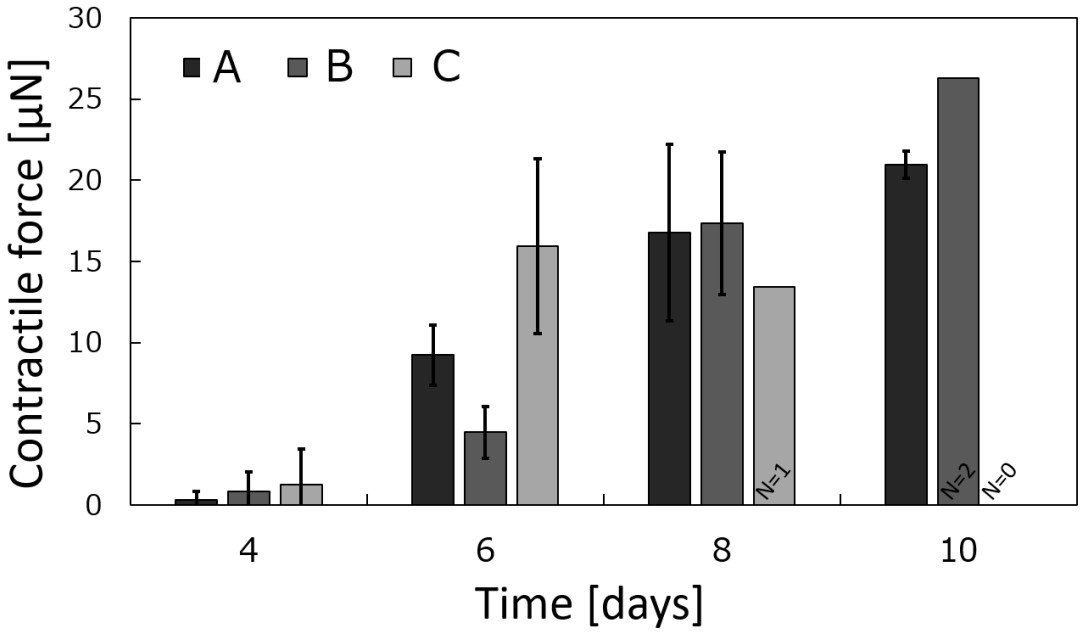

FIG. 5. Contractile force of the 3D tissues fabricated with dMBs derived from fibroblasts from three different donors. Three or four tissues for each condition were used for the contractile force measurement on day 4. Some tissues were detached from the microposts during the experiments. Results represent mean±SD (n=4 for A and C, n=3 for B) unless mentioned otherwise.