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Development and evaluation of a removable tissue-engineered muscle with artificial tendons

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Tissue-engineered skeletal muscles were potentially useful as physiological and biochemical *in vitro* models. Currently, most of the similar models were constructed without tendons. In this study, we aimed to develop a simple, highly versatile tissue-engineered muscle with artificial tendons, and to evaluate the contractile, histological and molecular dynamics during differentiation. C2C12 cells were embedded in a cold type-I collagen gel and placed between two artificial tendons on a silicone sheet. The construct shrank and tightly attached to the artificial tendons with differentiation, finally detaching from the silicone sheet within 1 week of culture onset. We successfully developed a tissue-engineered skeletal muscle with two artificial tendons from C2C12 myoblasts embedded in type-I collagen gel. The isometric twitch contractile force (TCF) significantly increased during differentiation. Time to Peak Tension (TPT) and Half-Relaxation Time (1/2RT) were significantly shortened during differentiation. Myogenic regulatory factors were maximally expressed at 2 weeks, and subsequently decreased at 3 weeks of culture. Histological analysis indicated that myotube formation increased markedly from 2 weeks and well-ordered sarcomere structures were observed on the surface of the 3D engineered muscle at 3 weeks of culture. These results suggested that robust muscle structure occurred by 3 weeks in the tissue-engineered skeletal muscle. Moreover, during the developmental process, the artificial tendons might contribute to well-ordered sarcomere formation. Our results indicated that this simple culture system could be used to evaluate the effects of various pharmacological and mechanical cues on muscle contractility in a variety of research areas.

[**Key words:** Tissue-engineered muscle; C2C12; Muscle contractility; Myogenic regulatory factors; Myogenesis]

Skeletal muscle is a tissue that is characterized by the presence of multinucleated cells, and functions as an actuator for physical movement as well as a shock absorber of external mechanical forces. In addition to these features and biomechanical functions, skeletal muscle is involved in important physiological functions such as protein storage, glucose homeostasis, and maintenance of body temperature. Furthermore, skeletal muscle is a highly sensitive tissue that responds to external stresses such as physical activity and injury. Its unique adaptability allows for changes in muscle mass size, fiber type, and metabolic parameters, depending upon the amount or intensity of physical activity.

Although skeletal muscle has been the focus of much research, the process of skeletal muscle cell maturation in response to external cues is not well understood. Elucidation of the functional relevance of the skeletal muscle differentiation process is of particular interest and would be helpful in developing more effective training regimes and therapies (1). Therefore, a useful experimental model is necessary to further research efforts in this area. To date, many experimental models have been proposed for skeletal muscle research. In particular, skeletal muscle studies using tissues derived from animals, such as rats or mice, have been conducted. However, in addition to the various ethical issues, it is difficult to control experimental conditions and directly estimate the involvement of physiological effects on muscle cells because exercise induces whole-body responses such as blood flow and immune system adaptations.

In light of this, many studies have employed skeletal muscle cell lines such as C2C12 or L6. These cell lines are excellent systems because of their ability to proliferate in the mitotic phase and to differentiate into multinucleated myotubes (2,3). Moreover, the use of established cell lines for *in vitro* culture experiments may relieve researchers from the above-mentioned ethical issues (2,4). Furthermore, the active tension of established cell lines has been successfully measured using UV-crosslinked collagen film (2). Nevertheless, muscle function in a two-dimensional (2D) monolayer cell culture differs substantially from native muscle function *in vivo*. In fact, tissue-specific architecture, mechanical and biochemical cues, and cell–cell communication is lost under simplified conditions (5,6).

Recently, three-dimensional (3D) engineered muscles have been constructed; these functional models are anticipated to be used for the development of skeletal muscle *in vitro* (7–13). Unlike 2D culture, 3D engineered muscles can possess high functional activity. By using fibrin gels, Huang et al. (10) successfully engineering high

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functioning 3D muscle tissues, which displayed greater tetanic force (805.8 ± 55 μN) compared to previous *in vitro* models. Since 3D cultures likely mimic the functions of living tissues better than monolayer cultures (5), tissue-engineered skeletal muscle might exhibit characteristics more representative of fetal skeletal muscle. Thus, 3D tissue modeling *in vitro* is necessary to understand the basic functions and regulatory mechanisms that occur *in vivo* (7). Such models would be applicable to research focusing on muscle physiology, biology, and tissue engineering (8).

Although there have been numerous advancements in tissue engineering, one remaining challenge involves the fixing of the ends of tissues. Consequently, considerable variation in quality is observed in 3D muscle tissues. Furthermore, the fixation of both ends of the muscle may enhance its 3D structure. Engineered tissues that are constructed by self-organization without fixing both ends of the muscle are difficult to evaluate as 3D structures during the early differentiation stage. Therefore, fixation of the muscle ends using artificial tendons is necessary to establish a stable 3D engineered muscle. Moreover, considerable technical proficiency and funds are needed for the construction of these 3D tissue-engineered muscles. To enable universal accessibility, the generation of a simple, cost effective and standard 3D engineered muscle is important (6,14). In this study, two artificial tendons at the both ends of engineered muscle introduced to be fixed and handled firmly. The tendons were made up of acellular porcine blood vessel and they have high affinity to the cells in collagen gel of engineered muscle since the acellular tissue mainly consists of collagen fibers. Thanks to the pinhole of artificial tendons, the engineered muscle could be easily set to the measurement apparatuses for the quantitative evaluation of contraction and relaxation kinetics.

The evaluation of contractile dynamics from a histological and molecular viewpoint is also important for the construction of 3D engineered muscle. The contractility of 3D engineered muscle is affected by its structure (2). Moreover, muscle differentiation can be evaluated by investigating the expression of marker genes that play distinct roles during myogenesis (15). Among the marker genes, myogenic regulatory factors (MRFs) play a predominant role (16). MyoD, myogenin and Myf6 are transcriptional factors of the basic helix-loop-helix family. MyoD appears in the early stages of myogenesis, whereas myogenin appears in later differentiation and acts more specifically in myoblast fusion (17). Myf6, also known as MRF4 or herculin, acts in late myogenic differentiation (18). These transcription factors are up-regulated during myogenesis (19) and can serve as marker molecules for the different stages of maturation (15,20). Therefore, it is important to evaluate the MRFs in the development of 3D engineered muscle. However, the relationship between contractile development and histological and molecular dynamics in 3D engineered muscle is not well known.

The goal of the current study was to generate a simple, highly versatile 3D engineered muscle with artificial tendons and evaluate its contractile dynamics during differentiation from a histological and molecular viewpoint.

MATERIALS AND METHODS

C2C12 cell culture Mouse C2C12 myoblast cells were cultured in growth medium (GM) that consisted of High-glucose Dulbecco's modified Eagle's medium (DMEM: Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS: Thermo Fisher Scientific), 1% antibiotic antimycotic solution (Sigma–Aldrich, St. Louis, MO, USA). Differentiation medium (DM) consisted of DMEM supplemented with 7% horse serum (HS: Thermo Fisher Scientific), 1% antibiotic antimycotic solution.

3D culture in collagen gel We used C2C12 cells (ECACC, Salisbury, UK) from normal adult C3H mouse muscle (21) in this study. The 3D culture was based on the method reported by Yamasaki et al. (22). Elastase-treated porcine aortae were used as the artificial tendons. Porcine aortae were purchased from local research biotool company (Tokyo Shibaura Zoki Co, Ltd., Tokyo, Japan) and their elastic fibers and cells were removed by gentle stirring in 0.15 mg/ml elastase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in pH8 0.01 M Tris buffer with 10 mM $CaCl_2$ and 0.02% NaN_3 for 72 h. The treated aortae were rinsed by 80% ethanol for 72 h and stored in PBS until use at 4°C. The tissue-derived collagen was cut into circular form (3 mm in diameter) and was drilled in the center (1 mm in diameter) using a biopsy punch (Biopsy Punch, Kai Medical, Seki, Japan) to prepare the artificial tendons. The artificial tendons were placed on the surface of a silicone sheet, which was then placed on a polycarbonate plate and the tendons were fixed using stainless-steel pins placed 12 mm apart (Fig. 1A). The C2C12 cells were embedded in a cold type-I collagen gel (Cellmatrix; Nitta Gelatin, Osaka, Japan) solution at a density of 1.0×10^7 cells/ml; cell density was determined using a hemocytometer. A 100 μl cold suspension of C2C12 cells was added between and on the surface of the two artificial tendons (Fig. 1B). After gelation of the cell suspension at 37°C in an incubator, the construct was placed in GM and cultured for two days at 37°C in a 5% CO_2 atmosphere. To enhance muscle differentiation, the cell suspension was replaced with DM. Samples were obtained at 1, 2 and 3 weeks (1 WK, 2 WK and 3 WK) after the onset of GM culture.

Twitch contractile dynamics of the 3D engineered muscle Contractility was assessed using an electrical stimulation system (22). Platinum plated electrodes (3 mm × 18 mm) were attached to a polycarbonate jig and an electrical pulse generator (WF1974; maximum output voltage 10 Vp-p, range of duty ratio 0.01%–99.99%; NF, Yokohama, Japan) was connected to the electrodes and a resistor was inserted in series with the electrical circuit to facilitate monitoring of the electrical current. An amplified (As-904; gain × 15; maximum output voltage 150 V; NF) electrical signal was applied to the medium through the electrodes. The waveform of the applied voltage and current was monitored with an oscilloscope (54810A; Agilent, Santa Clara, CA, USA).

The protocol for measuring contractility of the 3D engineered muscle was adapted from Yamasaki et al. (22). Contractility was assessed using a custom-made measurement apparatus by an electrical stimulation. The engineered muscle was removed from the stainless-steel pin and set on a measurement apparatus to evaluate isometric contractility. Isometric muscle contraction was induced by electrical pulse stimulation, and the force was measured using load cell (Fig. S1). Briefly, the electric pulse was generated by function generator (WF1974, NF Corporation, Yokohama, Japan) with high speed bipolar amplifier (HSA4101, NF Corporation) and applied to the culture medium through two platinum electrodes. The force generated by the cultured muscle was measured by the ultra small-capacity load cell (LVS-5G, Rated capacity 50 mN, Kyowa Electronic Instruments, Tokyo, Japan) and captured to PC through the strain amplifier (DPM-711B, Kyowa Electronic Instruments) and data logger (PCD-320A, Kyowa Electronic Instruments). The variables measured were twitch contractile force (TCF, peak twitch force) and time-dependent twitch parameters, such as time-to-peak twitch (TPT, from onset of electrical impulse to time of peak twitch) and one-half relaxation time (1/2RT, from time of peak twitch to 50% recovery time). Isometric twitch contractile forces were generated with electric pulses of ~1.0 V/mm at 0.5 Hz for 2 ms and determined after subtraction of the passive baseline force from the total force values. Passive baseline force was measured as the average baseline passive force preceding the onset of stimulation. During contractility measurements, the temperature of the 3D engineered muscles was maintained at 37 ± 1°C using a heated aluminum platform. Data files were recorded for each peak twitch force trace at 5000 Hz.

Analysis of myogenic gene expression Total mRNA from the 3D engineered muscle was obtained using RNAqueous-4PCR (Ambion, Austin, TX, USA). The cDNAs were obtained by reverse transcription of 1 μg of total RNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). The primer sequences were as follows: myoD (myoD1-771F: ACTACAGTGGCGACTCAGATGC, myoD-977R: TGGAGATGCGCTCCACTATG), myogenin (myog-523F: GGTGCCCAGTGAATGCAACT, myog-673R: GCTGTCCACGATGGACGTAAG), myf6 (myf6-468F: TGCACCGGCTGGATCAG, myf6-609R: CTGGAATGATCCGAAACACTTG), GAPDH (GAPDH-498F: TGCACCACCAACTGCTTAG, GAPDH-674R: GGATGCAGGGATGATGTTC).

The mRNA expression level was detected by quantitative RT-PCR in a fluorescent temperature cycler (Applied Biosystems StepOne Real Time PCR System). To determine the relative change in each mRNA in RT-PCR, the housekeeping gene GAPDH was used as a control template. Samples were incubated in the Applied Biosystems StepOne Real Time PCR System. PCR amplification was performed using SYBR green fluorescence (Power SYBR Green PCR Master Mix; Applied Biosystems) under the following conditions: one cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Emissions were determined after each cycle, and the synthesis of each gene mRNA was quantified using StepOne Software V 2.1. PCR reactions were conducted in triplicate.

Histology 3D engineered muscles were rinsed in phosphate-buffered saline (PBS) and fixed in 10% formaldehyde for 1 day. The fixed strips were dehydrated using an ethanol gradient and embedded in paraffin. Serial cross sections (4 μm thick) were cut on a microtome in both transverse and longitudinal directions. The sections were stained using hematoxylin and eosin (H&E).

Transmission electron microscopy (TEM) analysis was conducted to characterize the ultrastructure of the 3D engineered muscle. The constructs were fixed for 4 h at 4°C in 2.5% glutaraldehyde solution. The muscle samples were then rinsed three times with cacodylate buffer (pH 7.4) containing 7.5% sucrose, postfixed in 1% osmium tetroxide for 2 h at room temperature, dehydrated in graded concentrations