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Three-dimensional neuron–muscle constructs with neuromuscular junctions

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ABSTRACT

This paper describes a fabrication method of muscle tissue constructs driven by neurotransmitters released from activated motor neurons. The constructs consist of three-dimensional (3D) free-standing skeletal muscle fibers co-cultured with motor neurons. We differentiated mouse neural stem cells (mNSCs) cultured on the skeletal muscle fibers into neurons that extend their processes into the muscle fibers. We found that acetylcholine receptors (AChRs) were formed at the connection between the muscle fibers and the neurons. The neuron–muscle constructs consist of highly aligned, long and matured muscle fibers that facilitate wide contractions of muscle fibers in a single direction. The contractions of the neuron–muscle construct were observed after glutamic acid activation of the neurons. The contraction was stopped by treatment with curare, an neuromuscular junction (NMJ) antagonist. These results indicate that our method succeeded in the formation of NMJs in the neuron–muscle constructs. The neuron–muscle construct system can potentially be used in pharmacokinetic assays related to NMJ disease therapies and in soft-robotic actuators.

1. Introduction

Physical motion of our body is driven by contractions of skeletal muscles comprising bundles of highly aligned skeletal muscle fibers. These muscle fibers contract when neurotransmitters (acetylcholine) bind to a cluster of acetylcholine receptors (AChRs) at neuromuscular junctions (NMJs) [1]. Recently, *in vitro* reconstruction of NMJs has received widespread attention for drug development and pharmacokinetic screening to develop new treatments for neurodegenerative and degenerative muscle diseases, such as myasthenia gravis, Lambert–Eaton myasthenic syndrome and amyotrophic lateral sclerosis [2–5]. In order to analyze functions of NMJs, multiple *in vitro* co-culture systems composed of motor neurons and muscle cells have been proposed using two-dimensional (2D) culture on a dish [5–8]. However, the skeletal muscle fibers in 2D culture do not permit the development of *in vivo* muscle properties such as proliferation and contractility because the muscle fibers are attached to the 2D substrate during the cultivation [9–11]. Although several methods have been proven successful in fabricating three-dimensional (3D) free-standing muscle fibers [11–15], motor neurons directly added to the free-standing muscle fibers tend to slip down, preventing stable adherence and arrangement of the motor neurons on the muscle fibers for the formation of NMJs. Consequently, construction of NMJs in 3D muscle fibers has not been achieved.

In this paper, we propose a method to promote NMJ formation in 3D free-standing tissue constructs composed of skeletal muscle fibers and motor neurons. NMJs are formed on the muscle fibers within the constructs at contact points with motor neurons (Fig. 1). To fabricate these constructs, we use neurospheres composed of mouse neural stem cells (mNSCs) that are immobilized onto the muscle fiber bundle by reducing the volume of culture medium; this volume reduction allows continuous contact between the neurospheres and the skeletal muscle fiber bundle, preventing the neurospheres from slipping down and unstable adherence between the muscle fibers and the neurospheres. After adherence of the neurospheres to the muscle fiber bundle, we differentiate the mNSCs into neurons, forming NMJs. Owing to the high migration property of mNSCs during their differentiation process into neurons [16], the differentiation promises rearrangements of motor neurons to promote their stable contact with muscle fibers. We here investigate the NMJ formation on the neuron–muscle

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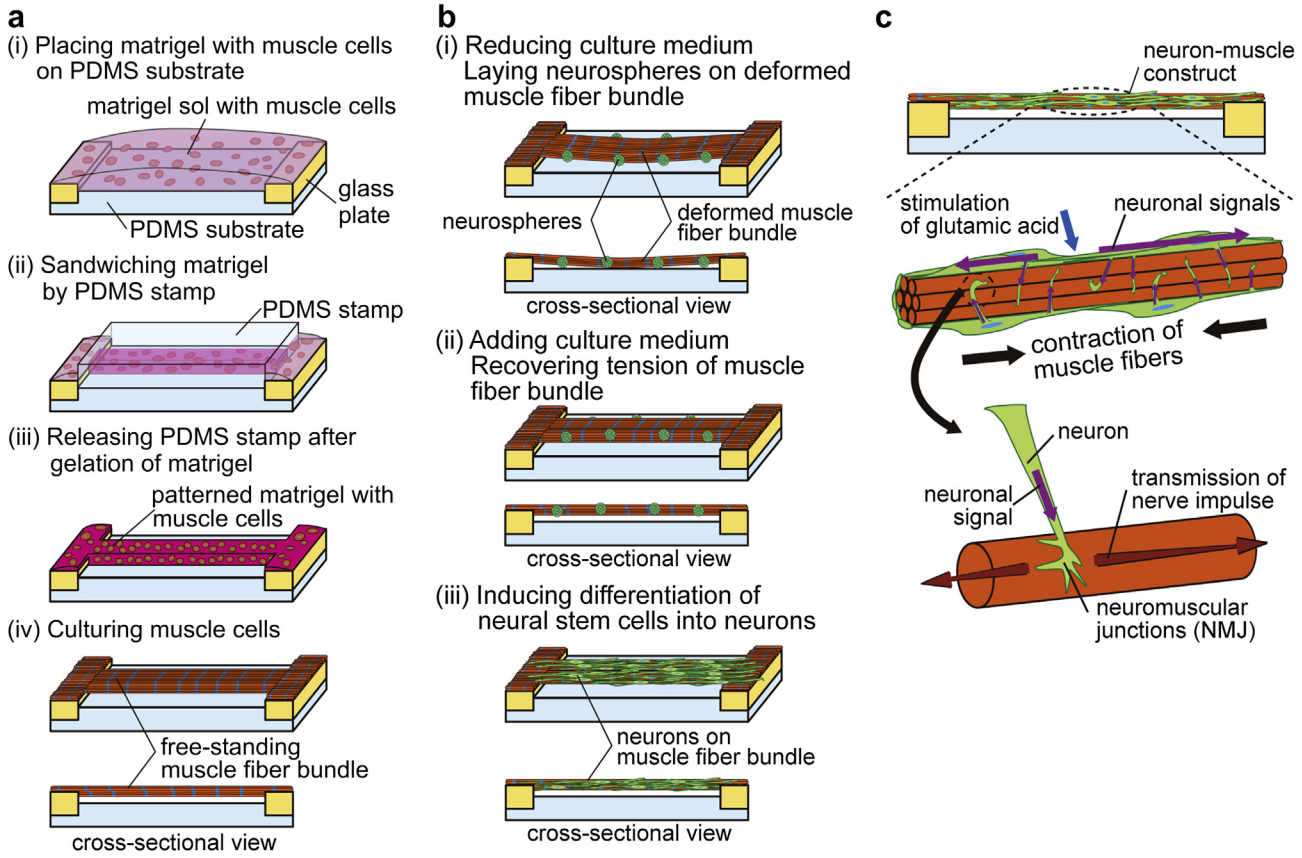

Fig. 1. Schematic of fabrication process of the neuron–muscle construct and an illustration of the contractile mechanism of the neuron–muscle construct. (a) Fabrication of a bundle of free-standing muscle fibers using a PDMS stamp with a striped pattern. (b) Formation of the neuron–muscle construct using a muscle fiber bundle and neurospheres. (c) Contractile mechanism of the neuron–muscle construct activated by neurotransmitters.

construct, and demonstrate that its contraction occurred in one direction with neurotransmitters when motor neurons in the neuron–muscle construct were activated.

2. Materials and methods

2.1. Cell culture

Skeletal muscle cells (C2C12 mouse muscle myoblasts, American Type Culture Collection) were maintained at 37 °C in a 5% CO_2 atmosphere in growth medium. The C2C12 growth medium consisted of Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. The C2C12 differentiation medium consisted of DMEM with 2% horse serum (HS), 100 U/ml penicillin and 100 μg/ml streptomycin.

mNSCs were prepared from the striata of ICR mice (embryonic day 13.5, Sankyo Labo Service Corporation, Inc.) by mechanical trituration, as previously described [17]. The mNSCs were cultured in growth medium composed of Neurobasal medium (Invitrogen) with B27 supplement without vitamin A (Invitorogen), human epidermal growth factor (20 ng/ml, Peprotech), basic fibroblast growth factor (20 ng/ml, Peprotech), 2 mM L-glutamate, 100 U/ml penicillin and 100 μg/ml streptomycin. After approximately 7 days in culture, the mNSCs proliferated to form neurospheres. The mNSCs were used for experiments within one or two passages. Neural induction medium for the mNSCs consisted of Neurobasal medium with B27 supplement without vitamin A, 2 mM L-glutamate, 100 U/ml penicillin and 100 μg/ml streptomycin. All mice were maintained in accordance with the policies of the University of Tokyo Institutional Animal Care and Use Committee.

2.2. Fabrication of 3D free-standing skeletal muscle fibers

A polydimethylsiloxane (PDMS) stamp and substrate were used to form the 3D free-standing skeletal muscle fiber bundle (Fig. 1(a)). The PDMS stamp and substrate features were defined by resin molds fabricated with a commercial stereolithography modeling machine (Perfactory, Envision Tec, Germany). Oxygen plasma treatment was used to make the PDMS stamp and substrate hydrophilic. The stamp and substrate were then washed in ethanol and exposed to ultraviolet light for 2 h to sterilize them. The surfaces were then treated using ethanol with phosphorylcholine-based (MPC) polymers (0.5 wt%, NOF Corporation) and heated at 65 °C for 1.5 h to block cell adhesion. For construction of free-standing muscle fiber bundle, the edges of the muscle fibers must be fixed with anchors [18–21]. We used glass plates coated with fibronectin as anchors for our muscle fibers. The glass plates were attached on the PDMS substrate. The surface of the glass plates was upper than that of the PDMS substrate to prevent contact between the muscle fiber bundle and the surface of the PDMS substrate.

Striped patterns on the PDMS stamp were used to define the dimensions of the muscle fiber bundle. The muscle cells were first suspended in a pre-gel solution of Matrigel (Becton, Dickinson and Company) between the PDMS stamp and the substrate. The Matrigel/cell suspension was then gelated in a 37 °C incubator for 15 min. Previous studies have demonstrated that Matrigel is an appropriate matrix material for muscle cells [19]. After releasing the PDMS stamp, we obtained striped patterns of Matrigel containing the muscle cells. We cultured the muscle cells within the Matrigel in the growth medium for 1 day and then changed the culture medium to differentiated medium. As a result, the muscle cells formed multinucleated muscle fibers fixed at both ends to the glass plates. This allowed the muscle fibers to retain their configuration and tension during the multinucleation; the maintenance of muscle fiber tension is important to keep its function [11].

2.3. Fabrication of the neuron–muscle constructs

Since fetal brain-derived mNSCs can differentiate into motor neurons [22], we induced differentiation of mNSCs on the free-standing muscle fibers bundle to fabricate neuron–muscle constructs (Fig. 1(b)). To attach neurospheres to the muscle fiber bundle, we distributed the neurospheres throughout the bundle. We then aspirated the culture medium around the bundle such that the surface tension of the remaining culture medium immobilized the neurospheres and caused the muscle fiber bundle to contact the PDMS substrate. This process allowed us to culture the neurospheres in direct contact with the muscle fibers. The muscle fiber bundle and neurospheres were maintained in this state for 1 h in a high-humidity environment to allow the neurospheres to fully adhere. After 1 h, we refilled the culture medium to release the muscle fiber bundle from the PDMS substrate. The