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Fabrication of multi-well platform for efficient myogenic commitment of C2C12 cells via electrical stimulation

C2C12 근육 조직 전구세포의 분화 촉진을 위한 전기자극 멀티웰 플랫폼의 제작

2015년 2월

서울대학교 대학원

공과대학 화학생물공학부

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Abstract

Fabrication of multi-well platform for efficient myogenic commitment of C2C12 cells via electrical stimulation

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To engineer tissue-like structures, cells are required to organize themselves into three-dimensional networks that mimic the native tissue microarchitecture. Here, we present agarose-based multi-well platform incorporated with electrical stimulation (ES) to build skeletal muscle-like structures in a facile and highly reproducible fashion. Our observations showed that ES improved myogenic differentiation of C2C12 cells in two-dimensional monolayer culture and increased the diameter of the resulting myotubes. Furthermore, C2C12 skeletal muscle cells were encapsulated in

collagen/matrigel scaffolds in 3D manner, and electrical stimulation was applied for efficient myogenic commitment. ES also facilitated the formation of free-standing 3D muscle tissues with or without mechanical stretch at both ends of the gel structure. Consequently, we confirmed the transcriptional upregulations of myogenic related genes in the ES group compared to non-stimulated control group in our multi-well 3D culture platform. Interestingly, the cell-encapsulated gel consisted solely of matrigel did not give admirable myotube formation; however, collagen showed upregulated myosin heavy chain, muscle regulatory factor expression and prominent effect in building myotubular structures. Given the robust fabrication, engineered muscle tissues in multi-well platform may find their use in high-throughput biological studies drug screenings.

Keywords: C2C12, electrical stimulation, agarose-based multi-well platform, collagen, matrigel, encapsulation

***Student Number*: 2013-20976**

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PART 1. INTRODUCTION

Overview

Stem cells may play a significant role in regenerative medicine field. Ability to control their differentiation and commitment would be critical for clinical applications of stem cells. Embryonic stem cells can differentiate into various tissue types such as endoderm, neural and muscle tissue. Moreover, adult stem cells are found in almost all tissues. They are unipotent or multipotent and are usually raised to their tissue origin. Recent findings of stem cell application are now manipulated in the fields of cell therapy, drug discovery, tissue engineering to the delicate level [1-3].

Based on the pluripotency of the stem cells, stem cell differentiation can be controlled by multiple factors such as chemical, biological or physical stimulations. The studies of electrical stimulation of cells forming the organism basically based on the level of electroactive cells by regulation of cell-to-cell and cell-to-extracellular matrix interactions [4]. Many growth factors, pharmacological reagents-mediated signaling pathways participate in cell differentiation. Even though the proper building and functioning of derived cells are achieved through scaffold materials or cytokines, biomimetic stimuli including mechanical and electrical stimuli are now imperative for the fabrication of functional tissues. Electrical stimulation is

now regarded as one of the efficient methods for regulating the behavior of electrically active cells. Endogeneous electrical activity in early embryonic development is exuberant; differentiating embryonic cells can be biased to particular cell lineage. Electrical stimulation has been utilized by applying a confined electric current through the skin to undertake tactile sensation [5] and in stem cell differentiation of muscle cells [6]. Other applications include cardiac pacing, muscle strengthening, wound healing, and iontophoresis drug delivery [7, 8]. External electric field has been proven to induce cellular responses such as cell surface receptor redistribution, microfilament organization, intracellular calcium dynamics, and enhanced cell differentiation.

One of the earliest reports of responses to the electrical stimuli of cell dates back into 1964, Crain and his colleague observed bioelectrical activity of mouse cerebral neocortex fragments throughout their growth and differentiation while they were cultured *in vitro* [9]. During several weeks of culture, electrical stimulation via microelectrodes evoked much greater spike potential of cerebral cortex as well as the amplitude, regularity, and complexity.

Electrical stimulation control of myogenic differentiation

Contractile motion of skeletal muscle is mediated by acetylcholine release

from peripheral nervous system (motor neuron) on neuro-muscular junction. Upon stimulated release from motor neuron and binding to acetylcholine receptors on skeletal muscle, neurotransmitter medicated depolarization of the cell membrane and its propagation to T-tubules allows the release of divalent calcium from the sarcoplasmic reticulum, initiating muscle contraction. External electrical stimulate that can mimic the neurotransmitter-mediated skeletal muscle contraction has been demonstrated to work in a similar way. Recently, controlling the stem cell fate using electrical stimulations has been investigated numerously. A lot of studies have shown that electrical stimuli have a significant effect in cell behavior [10-13]. Endogenous electric fields ranging from a few mV/mm to hundreds of mV/mm are present in the regenerating and developing tissues, in the cell cytoplasm, or in the extracellular spaces [14-16]. There is little information on the roles of electrical stimulation in paracrine signaling, morphological change, or conformational change of ion channels and receptors. Despite the remaining of tricky questions, electrical stimulation in physiological levels could be achieved without any noticeable cytotoxicity. Electrical stimulation has shown effective results in stem cell lineage commitment. Differentiation rate of electrically stimulated cardiomyocytes from induced pluripotent stem cell group was observed more rapidly compared to electrically non-stimulated group [17]. Electrical stimulation

induces regular functional excitation and contraction. Amplitude of synchronous contractions and calcium current peak was observed [1, 2, 4, 18]. Electrically stimulated adipose-derived stem cells displayed an induced transcriptional profile more closely related to that of cardiomyocytes. Moreover, it is indicated that the bi-phasic stimulation is more effective than the monophasic stimulation [18, 19].

The target cells on myogenic differentiation are cardiomyocytes and skeletal myocytes. Cardiogenesis of human embryonic stem cells has been a constant challenge. Electrical stimulation impacts the cardiac protein expressions, transcription factors, population number and cell morphology. The effectiveness of electrostimulation to induce pre-commitment of fibroblasts into cardiomyocytes was evaluated by Genovese, J. A. *et al.* [20]. Serena *et al.* revealed that through the mechanisms associated with the intracellular generation of reactive oxygen species, electrical stimulation plays a key role in cardiac differentiation of hESCs [21]. There also was plausible long-term stimulation of hESC-derived cardiomyocyte clusters on thin-film microelectrode structures *in vitro* [22]. Regarding the alignment of cells in relation to the applied electrical stimulation, it seems the type of the cells appears to be an important factor to the response of the cells. Microelectrode array-compatible electrical stimulation platform was used to achieve cell orientation and the maturation of cardiomyocytes. Nonetheless,

any specific alteration of orientation or morphological change was not observed [23]. Mooney *et al.* proposed biomimetic electroactive cue manipulating the electrical properties of a carbon nanotube scaffold [24], suggesting nanoscale biomimetic materials would be applied to various electrophysiological tissue repairs. Bioactivity and survival of bone marrow-derived stem cells could promote the implanted spike wave electrical stimulation as a way of another clinical approach [25]. Collagen fibers around rat mesenchymal stem cells became disconnected and loosely re-organized only in response to a large electrical stimulus range about 10 V/cm. Interestingly, only the fibroblasts surrounded by collagen fibers had a tendency to align in the direction of electrical stimulation [26].

Electrical Fields (EFs) is also known to trigger the required activation of progenitor cell receptors. Stem cell migration, alignment, and elongation were observed in the guidance of physiological electrical field strength in response to direct-current electrical stimulations [21, 24, 27, 28]. Among several frequencies of electromagnetic fields, the most efficient osteogenic differentiation of human mesenchymal stem cells was observed at 50hz [29]. It was proven that the electrical stimulation applied to mouse muscle *in situ* caused increases in the cellular AMP:ATP ratio [30]. Another study revealed the electrical excitability of early embryonic stem cell-derived cardiomyocytes was involved with ATP-dependent K(+) channels [31].

Importance of calcium anion influx for modulation of differentiation fate also became noted [11]. Titushkin *et al*. closely examined the change of cell cytoskeletal elasticity subject to electrical stimulation [32]. Electro-biochemically induced actin reorganization and ERM linker protein in response to electrical stimulation has been shown. Significant inhibition of the linker protein could be controlled by energy depletion. Inhibition of the linker proteins by electrical stimulation brings the result of the membrane dissociation from the cytoskeleton and causes not only the elasticity change but also the morphological change. It was also proven that integrin-dependent electrocoupling mechanisms were involved in 3D cell adhesion and orientation [26].

Layer-by-layer coating method for stem cell differentiation has succeeded with stabilized fibronectin-coated gold nanoparticles in that the use of fibronectin-coated gold nanoparticles was found to be an excellent matrix for hESCs [12]. Therefore, the self-assembling peptide nanoscaffold could be applied in three-dimensional extracellular environment [13]. Peptide nanoscaffold can be a good way to improve the differentiation of stem cells into cardiac-like cells. Upon electrical stimulation, nanoparticle-based scaffolds will serve as good cellular materials. Numerous devices have been developed so far to regulate the differentiation processes and to differentiate the progenitor cells efficiently. Since the delicate patch-clamp technique has

been developed in 1981, clamp methods are broadly used in these days [33]. Even the whole-cell patch clamp recording method is used [34]. Additionally, two kinds of biochips having contact-promotive and -preventive microenvironments were created using microfabrication and laser-guided cell micropatterning techniques in 2012 [35]. Only the stem cells in contact with adjacent cardiomyocytes were induced to acquire electrophysiological properties for action potential formation similar to that of cardiomyocytes. Takayama *et al*. developed microactivity substrates with embedded electrodes, which allow ensemble electrical stimulation of a large number of embryoid bodies (EBs) simultaneously and effectively [36].

Application of electrical stimulation is now used as a practical method to study differentiation cues of the multipotent cells. Considering the economic point of view, the low cost advantages of electrical stimulation method is reliable. Physicochemical approach works will accelerate the application of stem cells and establish dependable differentiation control protocols utilizing electrical field.

PART 2. TWO-DIMENSIONAL CULTURE AND EVALUATION OF ELECTRICAL STIMULATION

2.1 Introduction

Skeletal myogenesis is a highly orchestrated terminal differentiation process in which the proliferating mono-nucleated myoblasts differentiate and fuse to form multi-nucleated myotubes. It is important to derive highly organized myotubes to make functionally operative load-bearing muscle tissues [37]. Skeletal muscle is composed of not only cellular components, but also of extracellular matrix, nerves and blood vessels. To activate muscle, the electrical signal sent from a brain flows and reaches the ion pump on plasma membrane of the muscle cells via neuromuscular junction. The propagation of action potential into the axonal end promotes the release of acetylcholine [38, 39]. The calcium release channel allows calcium anion to flow to the sarcoplasm. The increasing concentration of calcium ion stimulates a sliding interaction between the myosin and actin filaments, thereby contracting the muscle cell [38, 40]. There also have been numerous papers related to the effectiveness of electrical stimulus on embryonic or adult stem cell fate control [41-43]. Electrical stimulation (ES) pulse is shown to enhance C2C12 myogenic differentiation. Assembly of functional sarcomeres and contractile activity of the cells were accelerated with ES [44-

47]. On these prior studies, we became interested in examining how electrical simulation can be orchestrated to maximize the differentiation of C2C12 mouse myoblast cells into myotubes.

2.2 Materials and methods

2.2.1 Cell culture

The murine-derived muscle cell line (C2C12) was purchased from American Type Culture Collections (ATCC, US). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Life technologies, US) containing 10% Fetal Bovine Serum (Thermo Scientific, US), 1% Penicillin-streptomycin (Life technologies, US), and 1% L-glutamine (Life technologies, US) (growth media; GM). To induce the differentiation of myoblasts into myotubes, C2C12 cells at 80-90% confluency were shifted to DMEM containing 2% Horse Serum (Life technologies, US), 1% Pen-strep and 1% L-glutamine (differentiation media; DM). GM were replaced by fresh media every 2 days. DM were changed every day.

2.2.2 Electrical stimulation

For the application of electrical stimulation (ES), electrical stimulator (S88; GRASS, US) with monophasic square shape pulse was used (2 V/cm, 1 Hz

frequency, and 2 ms). Carbon electrodes (C-Dish; IonOptix, US) were used. ES was applied for 2 hrs/day. For myogenic fusion index, randomly captured five different fluorescent microscopic images for each condition were measured for quantification. ES study scheme is shown in figure 1.

2.2.3 Viability analysis

Apoptosis test for analysis of the interrelationship between cell viability and ES was performed. For 2D monolayer cultured cell, TrypanBlue (Life technologies, US) exclusion assay was performed. After 3 minutes incubation at room temperature with solution, cells were counted under the microscope and determine the proportion of the live cells.

2.2.4 Proliferation quantification

Proliferation quantification of two-dimensional cultured C2C12 cells were performed by hemacytometer cell counting method and 5-ethynyl-2'-deoxyuridine (EdU) staining. For manual cell counting, cells were harvested using trypsine EDTA 0.25% and make pellet by 5min centrifugation at 100xg. Resuspend pellet with 500 μl to 1000 μl of culture media because of the low cell density. To gain mean value and standard deviation, count several times using hemacytometer. EdU staining was performed by

following manufacturer's protocol. Labeling C2C12 2D monolayer cells with EdU. Incubate cells with 10mM EdU containing GM for 2 hours. Wash with 1X PBS and add 4% paraformaldehyde, afterwards, permeabilize and staining with reaction cocktail.

2.2.5 Quantitative Gene Analysis

Total RNA of each sample was extracted with Trizol reagent (Life technologies, US). RNA was reverse transcribed to cDNA using cDNA synthesis kit (TOPscript™; enzynomics, Korea). Real time PCR was performed with SYBR green (enzynomics, Korea) according to manufacturer's protocol using StepOnePlus real-time PCR system (Life technologies, US). Myogenic markers of interest included MHCd, MLP, MRF4, sarcomeric actin, and myogenin as shown in Table 1. Relative expression level of each gene was normalized by GAPDH housekeeping gene.

2.2.6 Immunofluorescence staining

To observe the morphology and the formation of myotubular structure, the 2D culture cells were fixed using 4% paraformaldehyde for 15 min and permeabilized with 0.1% (v/v) Triton X-100 (Sigma Aldrich, US) for 10 min. Afterwards samples were blocked with 1% (w/v) bovine serum albumin.

Samples were stained with Mouse monoclonal [MY-32] fast skeletal Myosin (Abcam, US) (1:150 dilution in PBS) for 1.5 hours at RT after three times of gentle PBS washing. After washing three times with PBS, primary antibody binding was visualized through the secondary antibody (goat anti-mouse IgG antibody) conjugated with Alexa Fluor 488 (1:500 dilution in PBS) for 1.5 hours at RT. For visualization of cell nuclei, 6-diamidino-2-phenylindole dihydrochloride (DAPI; Life technologies, US) staining was performed for 10 min consequently as recommended by the manufacturer's instructions. Each culture samples was observed through the fluorescence microscope (EVOS; Lifetechnologies, US).

2.2.7 Statistical analysis

A Student's t-test was used for statistical analysis. Numerical data are presented as the mean ± SD. The P value lower than 0.05 was considered statistically valid.

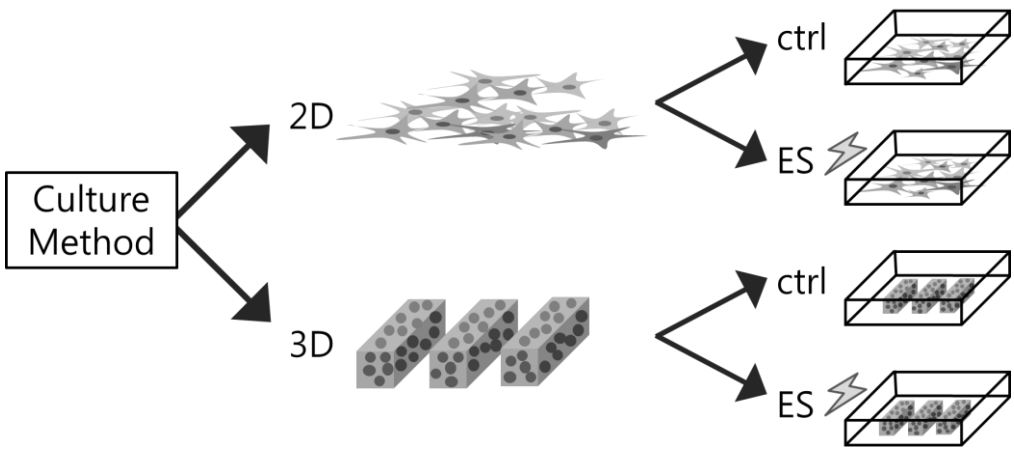


Figure 1. Schematic illustration of Electrical stimulation study. ES study was divided into 2D and 3D study. Elaborated 3D seeding geometry is depicted in supplementary data.

2.3 Results

2.3.1 C2C12 cell viability and proliferation under Electrical Stimulation

Electrical stimulation (ES) study scheme is shown in figure 1. We confirmed whether our ES regime (2V/cm, 1Hz, 2ms duration) have cytotoxicity or not and the influence of ES on cell proliferation. Figure 2A shows the time line of ES regime. Because of fast growth rate of C2C12 cell that have a doubling time of ~19 hours, we used low initial seeding density of 5 thousand cell/㎠. ES were applied for 48 hours continuously.

As shown in Figure 2B, cellular morphologies are same in both ES- and ES+ condition, but cell proliferation rate of electrically stimulated group was lower than that of the electrically nonstimulated. Quantification of cell population by direct counting illustrates arrested cell division (Figure. 2C) in accordance with bright field microscopic images (Figure. 2B). Besides, negative control group [ES -] displays exponential cell growth. Live cell percentage of control group is as follows: 99.22 ±0.01 %, 99.10 ±0.01 %, 98.32 ±0.01% on day1, day2 and day3, respectively. In the case of ES+ group, the percentage of live cells stayed relatively constant throughout the three days: 98.95 ±0.01 %, 98.29 ±0.01 %, 98.20 ±0.01%. Although ES affected cell proliferation rate, apoptotic test demonstrated that ES did not noticeably trigger C2C12 cell death. Figure 3 illustrates observation of

C2C12 cell proliferation rate for one week. The solid line indicates typical cell proliferation trend that have been delayed due to high cell density (Figure 3B). Cell cycle recovery was observed in dotted line that shows convalesced cell division rate after withdrawal of ES. Figure 3C designates are EdU staining that highlights S phase cells to show only actively replicating cells. The fact that EdU-attached cell population is bigger in nonstimulated group supports to the previous cell-count results.

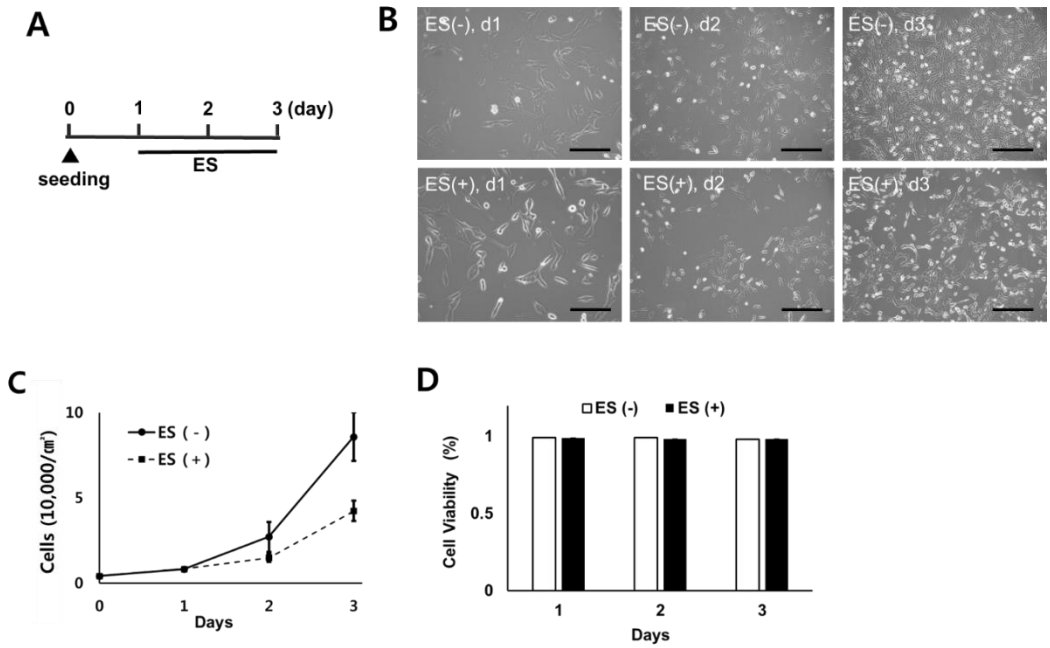


Figure 2. The observation of C2C12 cell proliferation and viability under ES. (*A*) C2C12 cells [ES(+)] underwent ES (2V, 2ms, 1Hz) from day 1 to day 3 continuously overnight under GM condition. Control groups [ES(-)] were cultured simultaneously without ES and compared. (*B*) Compared with microscopic image, electrically stimulated groups seem to have lower density of cells than control groups for entire 3 days. (*C*) The cell proliferation was arrested in ES(+) group. (*D*) Viability of both groups are nearly 100%. It shows that ES does not have cytotoxicity and the retard proliferation rate of electrically stimulated cells is not attributed to the cell death. Scale bar 300 μm.

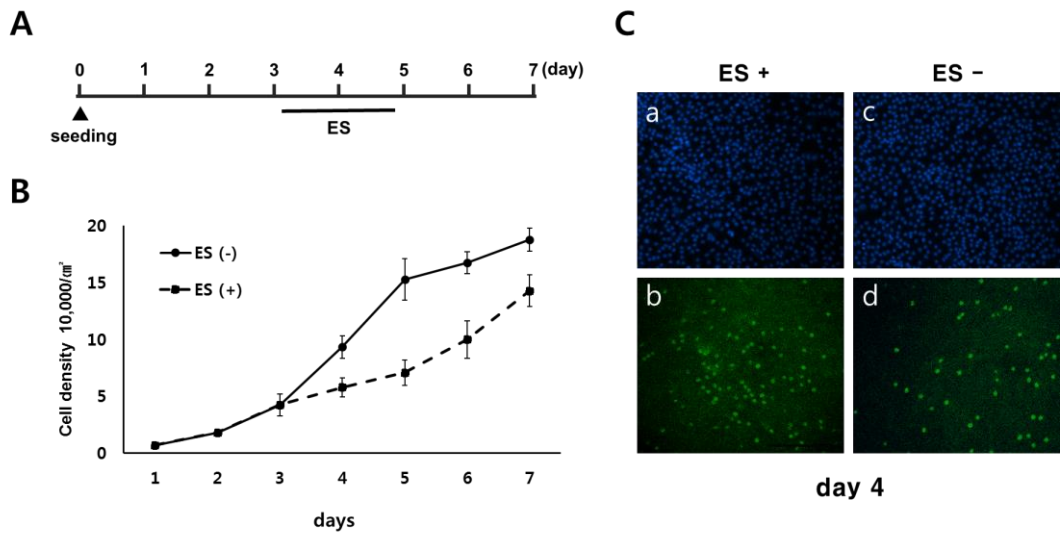


Figure 3. Application of ES on 2D monolayer C2C12 and EdU staining. To figure out whether ES completely arrests growth cell cycle, C2C12 cells underwent non-continuous ES with GM. Control groups [ES(-)] were cultured simultaneously without ES and compared. (*A*) ES was given only 48 hrs at 3rd and 4th day of seeding. (*B)* The growth of C2C12 cells was arrested during ES, but recovered the growth rate during non-ES period. The cell growth seems to be arrested only during the cells are exposed to ES. (*C*) EdU staining exhibits S phase DNA doubling cells at day 4. The proportion of EdU incorporated green fluorescence signal cells is bigger in nonstimulated group. Blue fluorescence DAPI indicates nucleus.

2.3.2 C2C12 differentiation comparison between control group and electrical stimulation group

Evaluation of efficacy of ES on myogenic differentiation in 2D culture was obtained through immunohistochemistry (Figure 4). In differentiation induction study followed by proliferation and apoptosis test, ES was applied intermittently, 2 hours per a day, as shown in figure 4A.

Myosin immunostaining results represents that electrical stimulation enhanced myotube formation and more rapid myogenic commitment (Figure 4(B-D)). Development of myotubes could occur without ES, but electrically stimulated C2C12 cells show longer and thick myotubular structure. We observed normal formation and scattered distribution of skeletal myotubes on the culture well plate after 5 days of culture with DM. There was no evidence of regular orientation of multinucleated myotubes in both electrically stimulated and nonstimulated groups. To quantitatively examine the effect of ES on differentiation of myogenic precursor cells, we measured the thickness of the individual myotubes and the number of nuclei in a single myotube. Measured thickness of myotubular structure is shown in Figure 4C. The mean thickness of DM cultured myotubes with ES is thicker than DM cultured nonstimulated groups' myotubes even though there were no differences between GM culture samples with or without ES. Under DM culture condition, the myotubular structures appeared with or

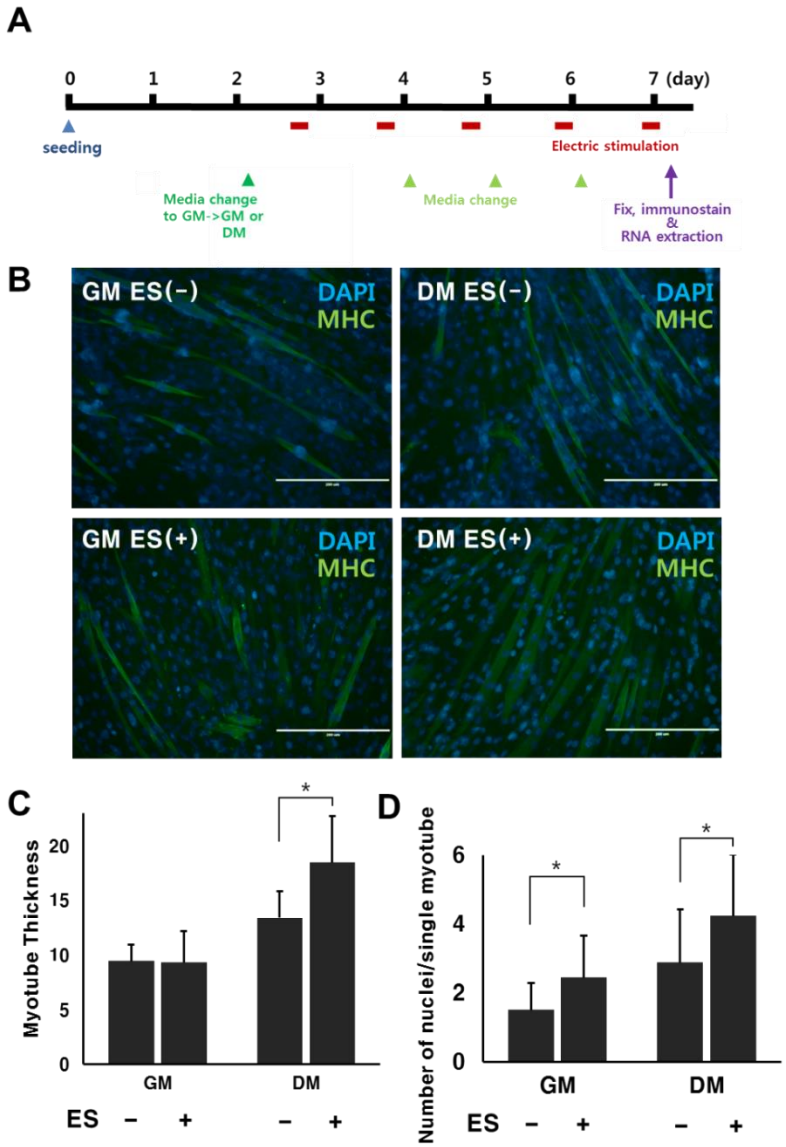


Figure 4. The evaluation of GM or DM cultured C2C12 cell differentiation through myosin heavy chain immunostaining. (*A*) ES regime for formation of C2C12 cell fusion. (*B*) Fluorescence microscopic image of each samples; growth media, GM; differentiation media, DM; nonstimulated, ES-; electrically stimulated, ES+. Blue fluorescence stands for nuclei and green for Myosin. Scale bar 200 μm. (*C*) Myotube thickness of 2D culture C2C12 in each culture condition. (*D*) Average number of nuclei per single myotube.

without ES. However, compared with nonstimulated group, electrically stimulated group has 50% more nuclei in one myotube (Figure 4D). As a negative control group, nonstimulated GM cultured sample [ES(-) GM] has very low myosin fluorescence intensity, and fluorescence-tagged sites had only 1 to 2 nuclei (Figure 4B). This implicates that with ES, 7 days of GM culturing can induce functional myotube. On the other hand, [ES(+) GM] condition had longer myosin fluorescent sites than those of nonstimulated group. In short, the group that had ES application in GM had more nuclei than the group in same GM condition without ES ($p < 0.05$). Active myotubular structure formation was witnessed even in GM with ES through microscopic observation (Supplementary fig.2).

Next, transcriptional expression of myogenic 2D cultured C2C12 with or without ES was evaluated by calculating $2^{-\Delta CT}$ (Figure 5). Relative fold induction of sarcomeric actinin, MHCd and MLP was analyzed. These proteins are related to elasticity and contractility of physiological muscle. In case of MHCd gene expression, approximately 30-fold induction was detected for ES group compared to control group in both GM and DM cluture condition. Likewise, sarcomeric actinin and MLP gene expression were also upregulated in ES group. Electrically stimulated C2C12 cells had noticeably enhanced myogenic gene expression level in the ES group.

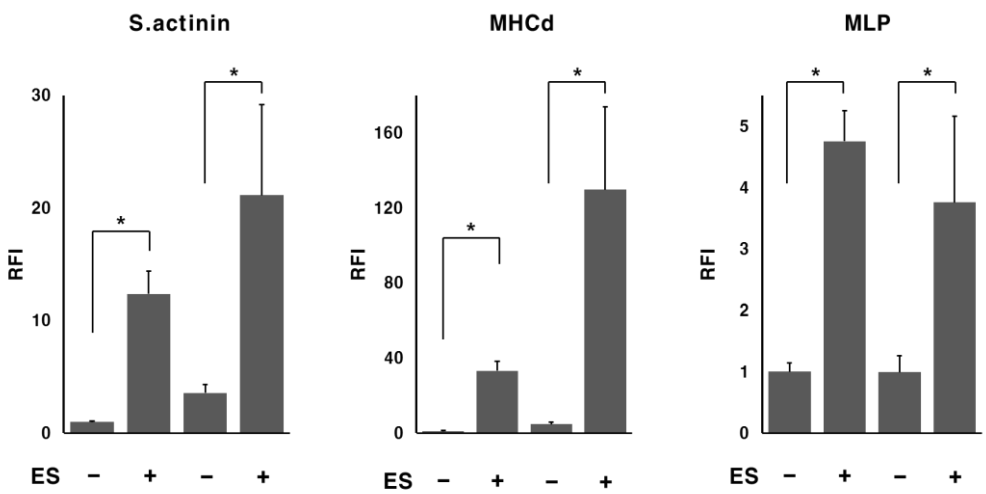


Figure 5. Real-time PCR analysis after ES for 5 days under GM and DM condition. (*A*) Microscopic images show that cells cultured under DM condition express myogenic marker genes better when they are exposed to ES. (*B*) Significantly different expression levels of myogenic regulatory factors (MHCd, MLP, S.actinin).

2.4 Discussion

To discover cell response regarding continuous electrical stimulus, we applied ES of 48 hours onto monolayer C2C12 cells. Compared to electrically nonstimulated cells, the proportion of the cell population in S phase of electrically stimulated group was smaller. Dilatory C2C12 cell proliferation of electrically stimulated group can be based on the inhibited cell cycle progression [48]. Although ES affected cell proliferation rate, apoptotic test demonstrated that ES did not trigger C2C12 cell death, and cellular physiological activity was recovered as soon as the ES was withdrawn. There was no evidence of regular orientation of multinucleated myotubes in both electrically stimulated and nonstimulated groups. Similarly, there also was no orientation of cardiomyocytes in any specific direction in the presence of ES [49].

Quantitative expression level analysis of MHCd, MRF4, MLP, sarcomeric actinin genes that pertain to myogenesis illustrates electrically stimulated cells are much more upregulated than nonstimulated cells. Similar study using carbon nanotube-embedded GelMA as ES substrate, also reported 50 to 100 times higher myogenic marker gene expression [50]. For more elaborated ES study, adequate onset timepoint of ES can be evaluated [44]. Furthermore, examination of exhaustion or attenuation of electrically stimulated engineered muscle tissue will be required.

PART 3. Three-dimensional Culture: Design of 3D Agarose-based Multi-well Platform and Its Application

3.1 Introduction

The fact that myotubes can develop into more matured sarcomeres and better contractility in respond to ES implied that cellular responses including transcriptional regulation, protein expression and glucose metabolism suggests that these findings need to be investigated in three-dimensional (3D) scaffold model.

The overall architecture of tissues in the body does not follow the two-dimensional cell culture, which overlooks important parameters such as mechanical cues, cell-to-cell and cell-to-matrix communication and thus, fails to reproduce cellular function observed in natural tissue. In their natural environment, cells are supported by extracellular matrix (ECM) in 3D manner. Optimizing the differentiation in the tissue biomaterials is fundamental for engineering functional tissues. Without appropriate supporting material, C2C12 cells tend to aggregate and adhere to each other. Various methods of mimicking 3D environment have been developed. The use of Velcro segments [44, 51] or glycoprotein-coated suture myoid methods [52-54] was expanded. Or collagen sponge scaffold is one of the easiest and most popular methods [55]. Methacrylated gelatin hydrogel and fibrin gel were utilized to make micropattern [56-59]. However, there is remaining

problem of whether these methods sufficiently simulate the 3D environment. Recently, muscle tissue engineering field is motivated to use C2C12 mouse myogenic precursor cells as a useful cell line by myoid [53, 54], cantilever [60], biocompatible gel encapsulation and electrospinning [61, 62] methods. It has been previously shown that tension mediated interactions (i.e., elaborate on stiff biomaterials) via RhoA and ROCK pathway have enhanced the skeletal muscle differentiation [63-65]. Therefore, we hypothesized that ECM-based scaffolds in conjunction with ES could confer optimal microenvironment for skeletal differentiation and myoid tissue assembly. In response to ES (2 V/cm, 1 Hz frequency, and 2 ms duration time), mature myotubes were formed. We further tested the influence of C2C12 cells on collagen, matrigel, or collage/matrigel hydrogels, and the results suggested that ECM does not exert significant influence on cellular differentiation under ES conditions. These results can have important implication in optimizing the engineering of skeletal muscle tissues.

3.2 Materials and Methods

3.2.1 Design of multi-well mold and making agarose-based multi-well

Acrylonitrile-butadiene-styrene resin 3D printing mold design was done by

AutoCAD (Autodesk, US) software. Dimensions of each single well were 5 mm × 2 mm × 3.5 mm (supplementary Fig.1), horizontal, vertical, and height respectively. 2% Agarose (Ultrapure Agarose, Invitrogen) solution made with sterilized ddH2O and was poured into the 3D mold. After solidification of agarose gel, multi-well platforms were transported to clean 6-well plate taken out of the ABS mold. Lastly, agarose-based multi-wells were sterilized UV light for 30 minutes in PBS solution to prevent dehydration. Before cell seeding, agarose multi-wells were incubated in growth media for 2 hours in 37℃ incubator. Agarose monomers, leaked out of agarose gel are washed after the incubation at 37℃ to provide suitable condition for normal cell culture.

3.2.2 3D Culture of C2C12 cells

Collagen based 3D construct was composed of Collagen type I (3.1 mg/ml; Advanced BioMatrix, US) adding with 10X PBS and was neutralized with 1M NaOH solution. 3D cell-encapsulation using matrigel was fabricated following the manufacturer's protocol. Briefly, Matrigel™ (BD Biosciences, US) stock solution was thawed slowly on ice block. Blank DMEM was added so that the concentration of matrigel solution was 2.5 mg/ml; corresponding to the concentration of collagen type I solution. All steps were done on ice to prevent pre-gelation of the collagen gel or matrigel.

Next, harvested C2C12 cell pellets were mixed gently with collagen solution, collagen/matrigel 1:1 mixture, and matrigel avoiding bubble formation. 0.2 Million cells per 20μl construct of cell encapsulating gel solution was loaded to agarose-based multi-well. Cell-encapsulated collagen/matrigel constructs were gelled in 37 ℃ incubator under a 5 % CO_2 atmosphere.

Minutien pins (Fine Science Tools, US) were used for manufacturing the muscle-like C2C12 gel structures. After 3-5 hours of gelation and contractions, cell-encapsulated 3D gels were transported from agarose-based multi-well to polydimethylsiloxane (PDMS) (Sylgard 184; Dowcorning, US) cured well plate. And for the pin-fixed samples, cell encapsulated constructs were pinned 5 mm apart at both ends of the gel.

3.2.3 Viability analysis

For 3D cultured cells, Live/Dead cytotoxicity kit (Life technologies, US) was used. To evaluate three-dimensionally clustered cell structures accurately, gels were sliced into a thickness smaller than 1 mm. After incubation of gels with working solution for 20 minutes while avoiding the light, cells were observed under fluorescence microscope (EVOS; Life technologies, US).

3.2.4 Contraction measurement on agarose-based multi-well

Bright field microscopic images of gel constructs were obtained by optical

microscope (Olympus, Japan) every two days. Low magnification microscopic images of the single gel structure were used for major axis length measurement. Major axis length of each single 3D cell encapsulated gels was analyzed by image J software (NIH, US).

3.2.5 Quantitative analysis

Except for additional step for digest 3D gel structures thoroughly with Trizol reagent using pestle, the whole RNA extraction and cDNA synthesis followed by real-time polymerase chain reaction process was conducted in the same manner.

3.2.6 Immunohistochemistry analysis of fabricated muscle tissue

First, we fixed the cell-laden gel samples with 4% paraformaldehyde for 30min at RT. To preserve the structures, samples were submerged in 20% w/v sucrose in PBS for 2 hours at RT. Small amount of OCT compound (CellPath, UK) was poured into the cryomold. Next, sample was placed so that the side is parallel to the bottom of the cryomold. Then, we filled the cryomold with OCT compound

For embedding procedure, we filled dewar with liquid nitrogen and poured

small container with approximately 2 cm of isopentane. The bottom of the isopentane container should immerse into the liquid nitrogen about 2-3 cm deep. Then place the cryomold into the container filled with isopentane using long handled forceps. When the OCT compound color changed into white and perfectly frozen, we wrapped the OCT mold with aluminum foil in an airtight bag and stored at -80℃. Cryostat sectioning was executed with cryotome (HM 505E; MICROM, Germany). Lastly, Cryostat sectioned samples followed the same immunostaining protocol as done for monolayer samples.

3.2.7 Statistical analysis

A Student's t-test was used for statistical analysis. Numerical data are presented as the mean ± SD. The P value lower than 0.05 was considered statistically valid.

3.3 Results

Owing to resistive property of agarose gel, C2C12 cells properly contract into gelation structure in a few hours. Collagen, matrigel and 1:1 mixture of collagen and matrigel encapsulated 3D environmental cell viability was assessed (Figure. 6A). In live/dead analysis of these matrices, red fluorescent dead cells are detected rarely in the group consisted of collagen only or matrigel only. Collagen and matrigel mixture had relatively poor viability compared to collagen- or matrigel-only constructs regardless of the application of ES. Cell aggregation constructs were maintained over 7 days without the loss of cell (Figure. 6B). As shown in figure 6C, major axis length of gel-encapsulated cell structures indicates C2C12 cells contract into smaller sizes, but there was no significant differences between control group and electrically stimulated group.

Cell-cell and cell-ECM interactions play a crucial role in modulating a number of processes, including gene expression, proliferation, and differentiation. Cell spreading and integrin binding appear to be important mediators of cellular function and signaling [66]. Gene expression quantification results demonstrated 1:1 mix of collagen and matrigel environment does not give admirable effect on myogenic- and contractile-related gene expression (Figure 7). This comes to the distinction with the report of Zhou *et al*. which represented the embryonic stem cell

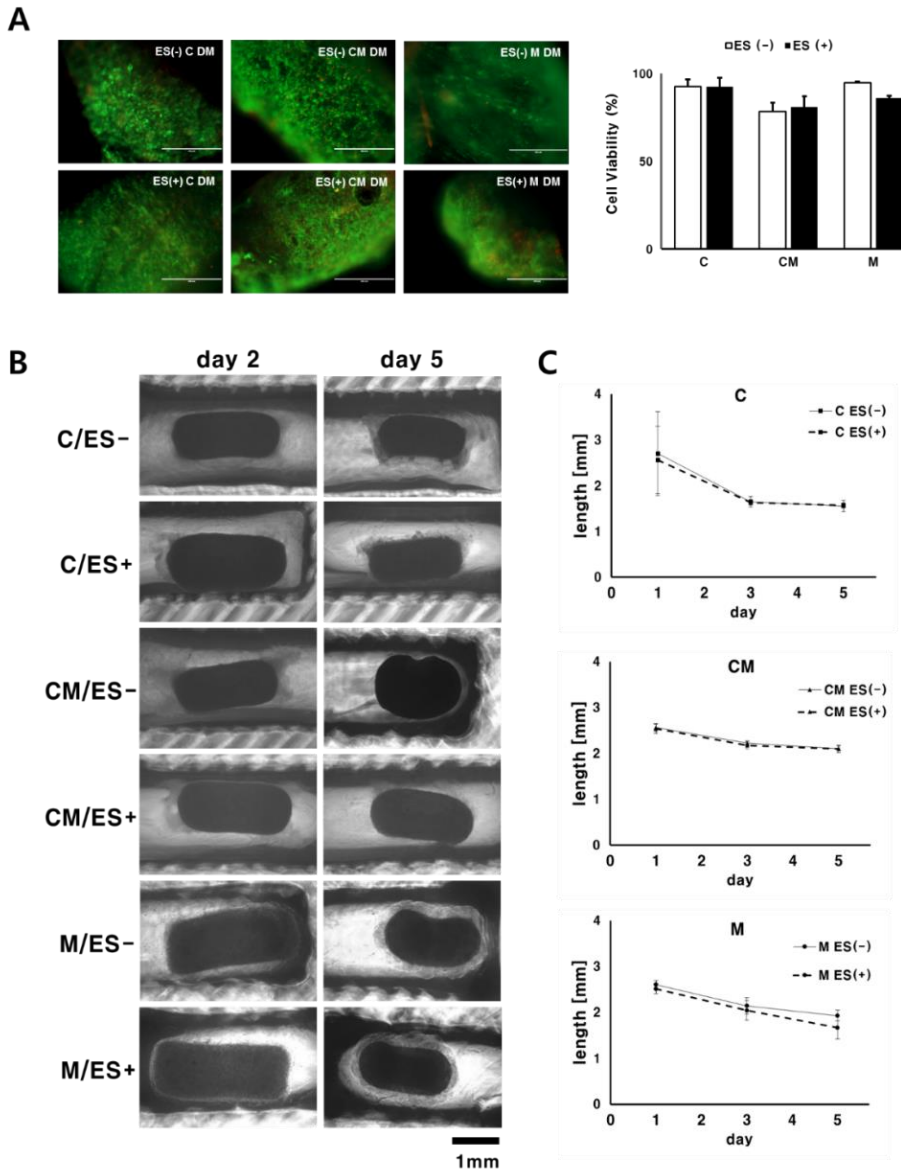


Figure 6. 3D cultured C2C12 cells underwent ES (2V, 2ms, 1Hz) from day 2 to 7 continuously overnight under 3 different kinds of scaffold. **A**: Live/Dead staining fluorescence microscopic observation and quantification of live cell population. Among three scaffolds, the CM showed low viability. **B**: Bright field image of representative C, CM M constructs at day 2 and 7. **C**: All three scaffolds have tendency to contract as time goes by. Abbreviations; collagen [C], matrigel [M], and collagen : matrigel = 1:1 mixed gel [CM] each.

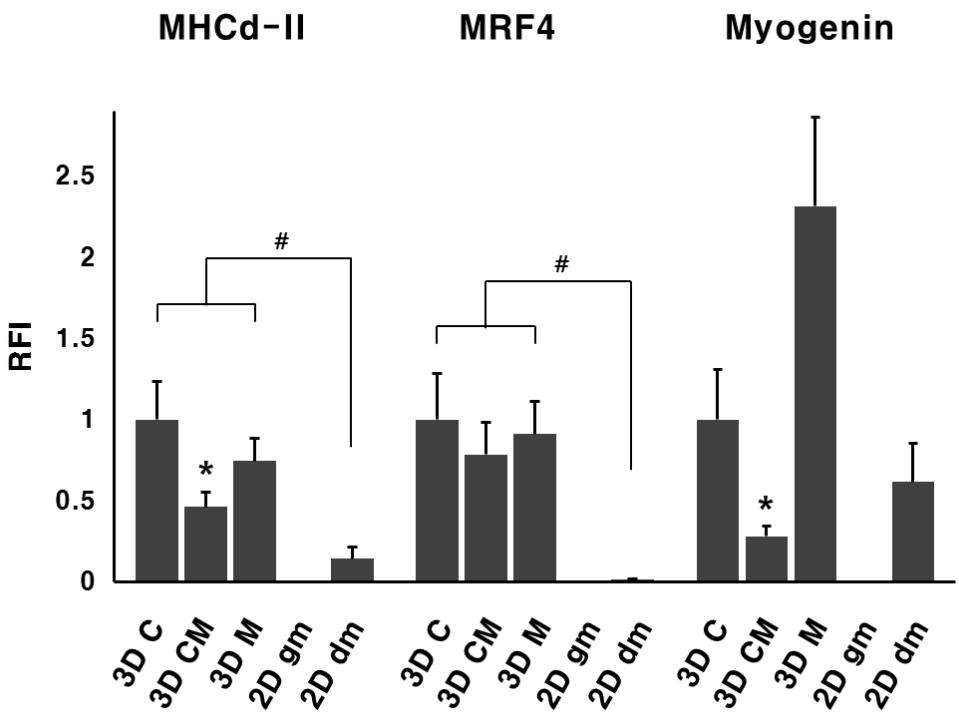


Figure 7. Transcriptional regulation analysis comparing two-dimensional GM and DM cultures and three-dimensional gel-encapsulation DM cultures.

differentiation was induced on the 4:1 v/v mix of collagen and matrigel, but not on the pure collagen [67]. As illustrated in Figure 7, more active transcriptional regulation of muscle regulatory factors such as MHCd and MRF4 was observed in 3D environment cells rather than in 2D environmental C2C12. 2D GM cultured group cells did not express myotube maturation associated genes. Because myogenin is related to early differentiation, relatively large folds that were perceived at matrigel encapsulated 3D cultured sample and 2D DM cultured sample designates matrigel-only gel induces rather slower myogenic differentiation than collagen-only constructs.

Highly oriented aligning morphologies of myoblast cells were observed in pinned samples (Figure 8A). Mechanical stretching of skeletal muscle organogenesis brought myotube orientation and longitudinal growth in the direction of strain. Submissive stretch of skeletal myoblasts cause electrical coupling with adjacent myocytes, suggesting the mechanical force can generate functional electromechanical junctions. To some extent, electrically stimulated 3D cells had higher myogenic transcriptional regulation compared with electrically and mechanically nonstimulated control group (Figure 8B). Gene expression analysis of MHCd, MRF4 and MLP was performed. Pin-anchored samples have 2 to 4 times higher transcriptional expression compared to samples that were not fixed to the pins in not stimulated samples. Additionally, the relative fold of not pinned ES(+)

group were 1.5 to 3 times higher than not pinned ES(-) samples ($p<0.05$). Especially, comparison of two conditions without electrical stimulus, ES – control and ES – pinned, shows significant differences between gene expression levels. Likewise, immunofluorescent myosin signal (green) of cross-sectional 1 week cultivated gel structure was much stronger in minutien pin-fixed samples (Figure 9).

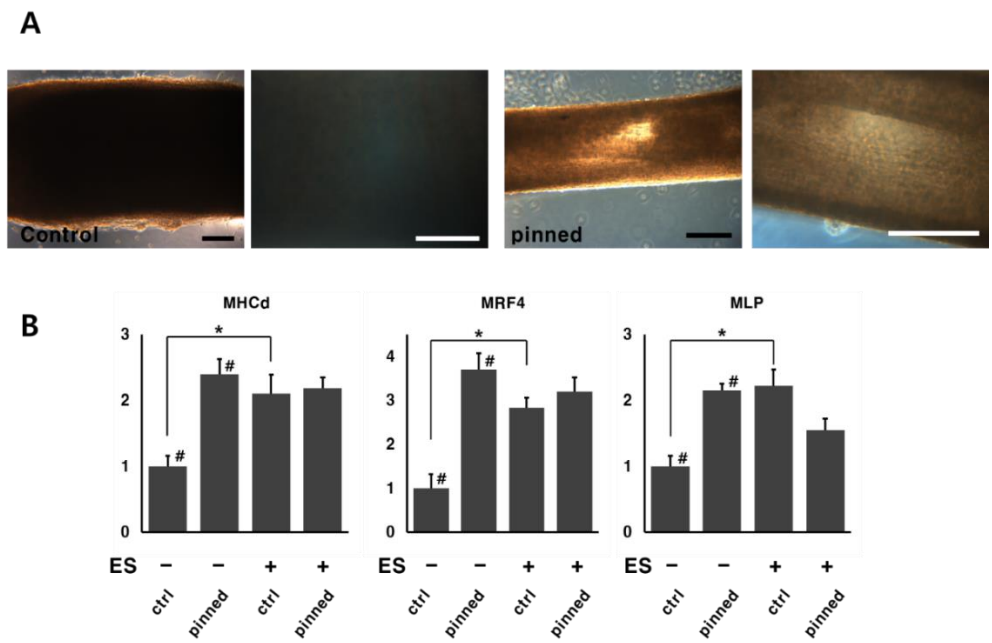


Figure 8. (*A*) Microscopic image of C2C12 3D structure with or withour pinned structure. (*B*) Relative fold induction of myogenic marker genes MHCd, MRF4, MLP.

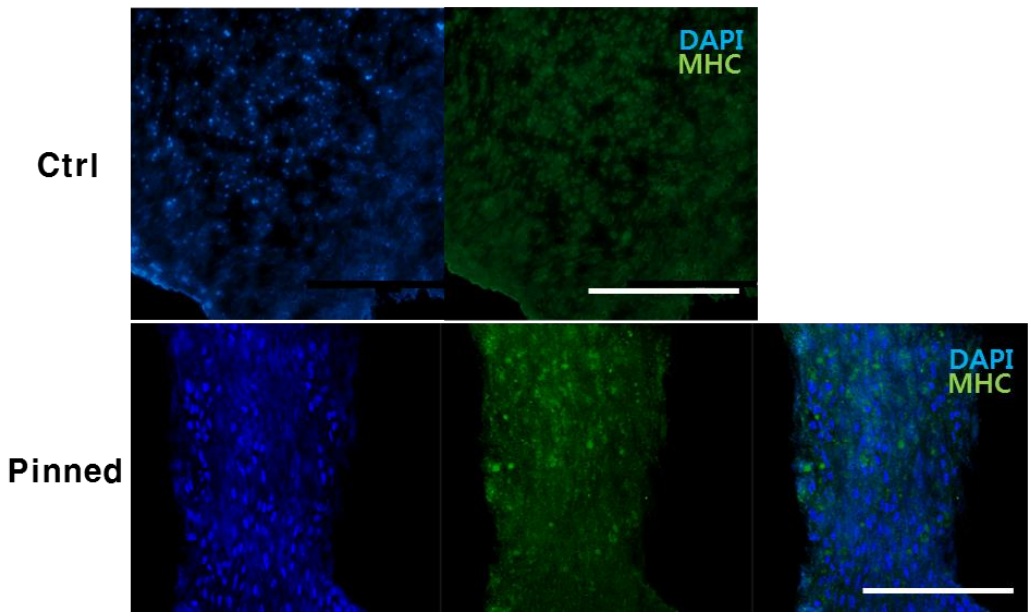


Figure 9. Immunohistochemistry of collagen-encapsulated 3D constructs. Blue: DAPI; green: Myosin. Scale bar 200μm.

3.4 Discussion

2D cell culture misses crucial factors such as mechanical cues, cell-to-cell and cell-to-matrix communication. In reality, cells are supported by 3D extracellular matrix (ECM). Design of cell encapsulation platform satisfying the biocompatibility is required.

Collagen type I is one of the commonly-used material for 3D cell culture because it is the most plentiful ECM protein in mammalian tissues [68]. Collagen I monomers assemble into stable 3D gels at physiological temperature and pH. Particularly, extra-strains are dissipated throughout the whole collagen gel structure [69]. Besides *in vivo*, collagen submit to fibrillogenesis and crosslinks into collagen networks which are prearranged distinctively in different tissues [70]. Cells facilitate collagen fibrils to have link with cell-oriented molecules and enzymes to form extra- and inter-cellular network [71]. Thus, we chose collagen gel as our pin-stretching model material. Muscle makes active tension upon stimulation, highly oriented aligning morphologies of myoblast cells were observed in pin-anchored 3D gel-encapsulated cell structures. Submissive stretch of skeletal myoblasts causes electrical coupling with adjacent myocytes, suggesting the mechanical force can generate functional electromechanical junctions [72, 73]. Martinello *et al*. observed considerable ATP release from differentiated myotubes via mechanical and electrical stimulation [74].

Agarose gel has multiple usages in biological research field by means of microbe nutrients, electrophoresis, and so on. As one of the hydrogels, agarose gel has also been employed in cell bioengineering method for cell motility examination or cell culture method as a form of solution in media or with ECM matrixes [75-77]. In the meanwhile, agarose resists adjustment of ECM proteins which is essential for cell adhesion [78, 79], it consequently does not allow cell attachment to agarose-based well. The absence of cell adhesion ligands in agarose gel enable easy detachment of collagen-encapsulated cell structure [77, 78, 80]. Thus, we translated this regime into a 3D agarose-based multi-well platform system. Owing to resistive property of agarose gel, C2C12 cells properly contract into gelation structure in a few hours. Cell aggregation constructs derived from agarose gel were maintained over one week (fig.6). For future work, localized coating of substrate surface with agarose will be able to effectively inhibit non-specific protein adsorption and fibroblast cell adhesion.

We aimed to mimic the *in vivo* environment to develop tissue engineered skeletal muscle and quantitatively investigated the functionality of 3D cultured cells from agarose-based multi-well. Furthermore, we evaluated whether electrical stimulation have triggered improvement of myogenic differentiation marker gene expression. The platform introduced in this paper can induce normal cellular metabolism and differentiation activity.

The first trial of suspending myotubes in collagen gel was performed by Strohman *et al*. [81]. Myoblasts were cultured on collagen-coated Saran Wrap, covering the cured Sylgard polymer and pinned down by stainless needles. Collagen fibrils are classically systematized across a length of 50-200 nm and allow the adhesive interplay contact of fibroblast cells [82]. Actual skeletal muscle cells mature from the fusion of dozens or hundreds of dense myoblasts [54]. We first examined the effects of ES regime C2C12 in 2D monolayers culture. Observations made by Marotta *et al*. implicated that ES at a frequency of 1 to 3 Hz triggered most monolayer C2C12 cells to contract. Otherwise, no contraction activity was observed at frequencies higher than 10 Hz [83]. In reference to this experiment that enriched contractile properties of myotube [84], we chose the following conditions as our ES regime: 1 Hz and 2 volt per cm for a duration time of 2 ms.

PART4. CONCLUSION

This paper observed effect of electrical stimulation on C2C12 cell proliferation and viability. C2C12 cells underwent ES (2V, 2ms, 1Hz) for 2 days and the proliferation was arrested in ES(+) group, but viability of both groups are nearly 100%. It showed that ES is not toxic to cells and low proliferation rate of ES(+) group is not due to the cell death.

More efficient differentiation induction was demonstrated by electrically stimulated group in GM and DM condition both. Consequently, the early development of the C2C12 myotube can occur due to electrical stimulation.

In 3D investigation, we designed agarose-based multi-well platform and utilized this platform as the mold that brings the gelation of cell-containing collagen solution without cell loss. We have not verified all geometries, aspect ratios of agarose mold well. But for more expeditious biochemical assays, longer length of agarose mold well can be designed in case of large number of cells are necessary. Besides, freshly isolated neonatal rat ventricle cardiomyocytes were seeded onto designed multi-well platform with same method of C2C12 seeding; i.e. collagen-encapsulation method, and it was also a reliable method (supplementary fig.3). 3D culture of cardiomyocytes also obtained by this platform. Agarose-based multi-well made it possible cardiomyocytes' free-standing and self-beating as well. Furthermore, other cell types may spread preferentially in other shapes.

Agarose-based multiwell-origin cell structure can also be used for study of stem cell incorporation into muscle tissue, muscle tissue engineering of cells from animals that have genetic mutations, or plasticity and function screening of muscle tissue from the satellite cells of grown mammals. If this platform model is adapted for evaluation of human tissue, it would be utilized to test the efficacy of specific gene therapy methods to treat disorders such as muscular dystrophy or malignant hyperthermia.

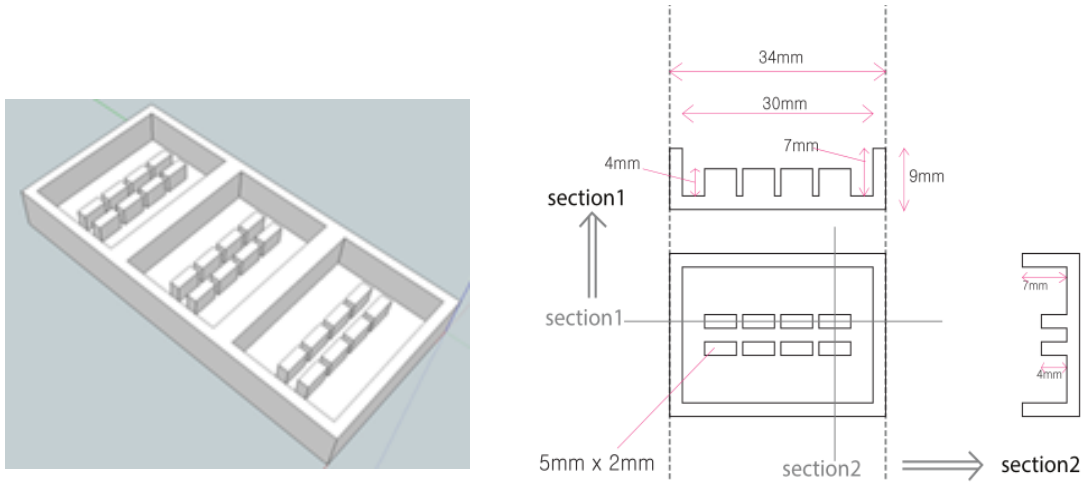
In summary, single unit structures of 3D myocyte constructs via agarose-based multiwell platform may be elaborated from primary culture of cells to fast or slow muscles of mammals. The excitability and contractility of muscle cell structures will be similar to that of which is expected from native muscle tissue under conditions of chronic denervation.

In addition, our agarose-based multi-well platform-origin 3D environment give rise to faster maturation of C2C12 than in the 2D monolayer culture system, as shown by the upregulation of myogenic marker genes. Cells facilitate ECM to have link with cell-oriented molecules and enzymes to form extra- and inter-cellular network [71]. Moreover, diverse application of various types of biocompatible gels and chemical crosslinking onto this platform to improve bioarchitecture of scaffold will be possible. Additionally, pin-anchoring method provides easy force transducer attachment for dynamic enhancement. Electrophysiologically and

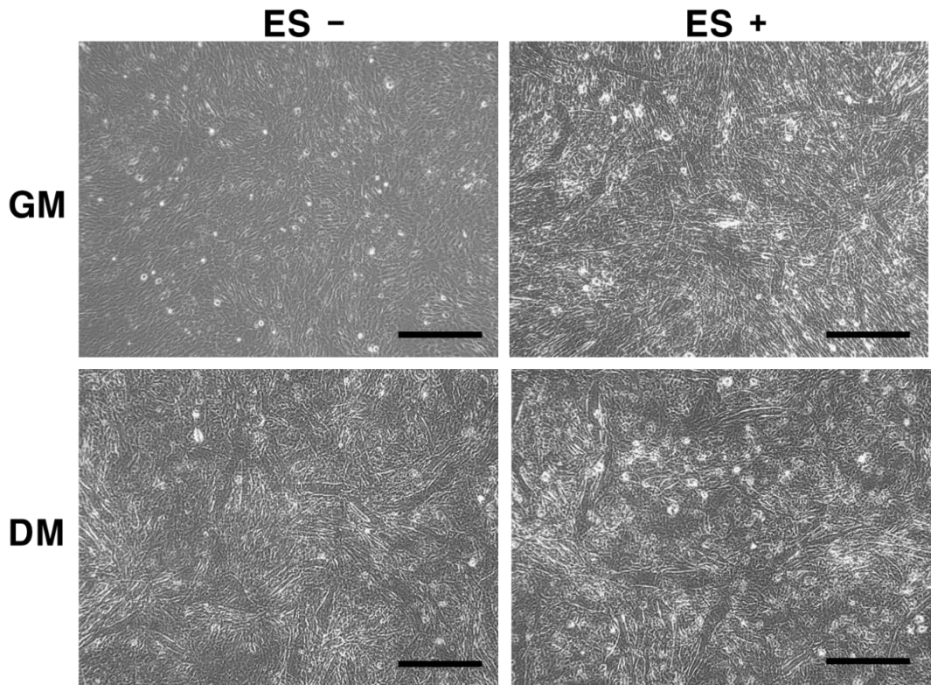
mechanically engineered muscle organoids might be applicable for improving the therapeutic efficacy of engineered muscle structures for salvation of infarcted muscle tissue.

Table 1. Primer list

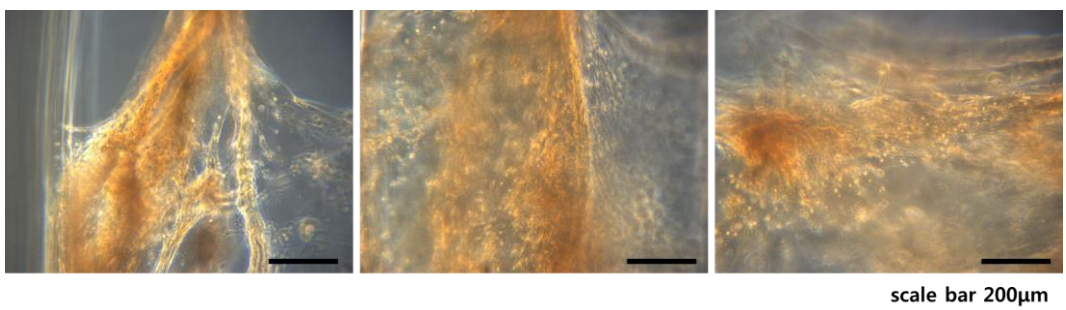
Gene	Forward primer sequence	Reverse primer sequence
GAPDH	5’-GTGGCAAAGTGGAGATTGTTGCC-3’	5’-GATGATGACCCTTTTGGCTCC-3’
MHCd	5’-GCGACAGACACCTCCTTCAAG-3’	5’-TCCAGCCAGCCAGCGATG-3’
MLP	5’-TGGGTTTGGAGGGCTTAC-3’	5’-CACTGCTGTTGACTGATAGG-3’
MRF4	5’-CGAAAGGAGGAGACTAAAG-3’	5’-CTGTAGACGCTCAATGTAG-3’
Sarcomeric actinin	5’-ATGGTAGGTATGGGTCAG-3’	5’-GATCTTCTCCATGTCGTC-3’
Myogenin	5’- TGTCTGTCAGGCTGGGTGTG-3’	5’-TCGCTGGGCTGGGTGTTAG-3’



Supplementary figure 1. Design of mould for agarose-based multi-well.



Supplementary figure 2. Bright field image of C2C12 2-dimensional culture for 7 day with or without electrical stimulation.



Supplementary figure 3. Collagen-encapsulated 3-dimensional culture of neonatal rat ventricle cardiomyocytes in agarose-based multi-wells.

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국문초록

근육 조직 전구 세포의 분화 촉진을 위한 전기 자극 멀티웰 플랫폼의 제작

실제 생체 조직과 유사한 구조를 만들기 위해서 세포는 조직의 마이크로 구조를 모사하는 3차원 네트워크로 구조화되어야 한다. 우리는 여기서 근육과 유사한 구조를 만들기 위해 간편하고 재현성 있는 방법으로 전기 자극을 가할 수 있는 아가로오스 기반의 멀티웰 플랫폼을 개발하였다. 먼저 2차원 배양 조건에서 전기 자극의 생적합성과 그로 인한 C2C12 세포의 근분화 촉진을 확인하였고, C2C12 근육 전구세포들을 콜라젠과 마트리젤 기반의 3차원 지지체에 넣고 전기 자극을 가하였다. 아가로오스 멜티웰에서 유래한 세포 인캡슐레이트 젤 구조는 전기 자극 하에서도 3차원적 지지가 가능하였으며, 2차원 배양 조건보다 멀티웰 플랫폼을 이용하여 만든 3차원 배양 세포에서 근분화 마커 유전자들이 더 활발히 발현됨을 관찰할 수 있었다. 특이할 점은 마트리젤로만 구성된 젤에서는 두드러진 근관세포의 형성을 볼 수 없었으나, 콜라젠으로 인캡슐레이션한 표본에서는 상향된 근분화 관련 마거 유전자들의 발현을 관찰할 수 있었다. 이러한 플랫폼의 제작을 통하여

조작된 근육 조직은 생물학적 연구나 빠른 약물 스크리닝, 바이오로보틱스에 응용될 수 있을 것이다.

주요단어 : C2C12, 아가로오스 기반 멀티웰 플랫폼, 전기 자극, 콜라젠, 마트리젤

학번 : 2013-20976