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

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Abstract. To engineer tissue-like structures, cells are required to organize themselves into threedimensional networks that mimic the native tissue micro-architecture. Here, we present agarose-based multi-well platform incorporated with electrical stimulation to build skeletal muscle-like tissues in a facile and highly reproducible fashion. Electrical stimulation of C2C12 cells encapsulated in collagen/matrigel hydrogels facilitated the formation 3D muscle tissues. Consequently, we confirmed the transcriptional upregulations of myogenic related genes in the electrical stimulation group compared to non-stimulated control group in our multi-well 3D culture platform. 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

1. Introduction

Skeletal myogenesis is a highly orchestrated process in which the proliferating mono-nucleated myoblasts differentiate and fuse to form multi-nucleated cells. Skeletal muscles govern body movement with their contraction abilities, and they are composed of oriented multicellular myotubes (Handschin et al. 2014). Thus, loss of skeletal muscle function caused by various diseases, such as muscular dystrophy, is a major problem limiting the body movements (Emery 2002). Recently, stem cell-based approaches to engineer highly organized myotubes for functionally operative load-bearing muscle tissues (Bischofs and Schwarz 2003). Furthermore, engineered skeletal muscles tissues may have potential applications in regenerative medicine, drug screening, and future myoid tissue-based cell assays (Cezar and Mooney 2014).

The mouse skeletal muscle cell line C2C12, which can differentiate into myotubes, is a widely used model for the building of functional skeletal muscle tissue. C2C12 myoblasts form myotube networks of random size and alignment in vitro when cultured in a conventional culture dish. They have various width, orientation, and low differentiation rate. There have been many efforts to
improve myotube formation of C2C12 cells. These include myoblast alignment by topographical constraints, mechanical stretching, chemical patterning, modulation with substrate stiffness. In addition, in order to improve the myotube formation and maturation, 3D culture models have been used. Similarly, electrical stimulation (ES) pulse is shown to enhance C2C12 myogenic differentiation. Assembly of functional sarcomeres and contractile activity of the cells has been shown to be accelerated with ES (Fujita et al. 2007, Kaji et al. 2010, Langelaan et al. 2011, Manabe et al. 2012).

The tissues in the body overlook important parameters such as mechanical cues, cell-to-cell, and cell-to-matrix communication and thus, 2D culture platform fails to reproduce cellular function observed in natural tissue. In their natural environment, cells are supported by extracellular matrix (ECM) in 3D manner. Without appropriate supporting material, C2C12 cells tend to aggregate and adhere to each other. Tissue engineering strategies have allowed the development of various methods of mimicking 3D environments. The use of synthetic components, such as Velcro segments (Langelaan et al. 2011, van der Schaft et al. 2013) or glycoprotein-coated suture myoid methods (Dennis and Dow 2007, Dennis et al. 2001, Huang et al. 2005), have been applied for skeletal tissue engineering. Alternatively, naturally-derived collagen sponge scaffold have been highly utilized for a muscle differentiation platform (Radisic et al. 2004). Recently, biosynthetic biomaterial, such as methacrylated gelatin was combined with fibrin gel to make micropattern substrates for efficient muscle differentiation (Ahadian et al. 2012, Carey 1920, Hosseini et al. 2012, Nagamine et al. 2010). However, there is remaining problem of whether these biomaterials can sufficiently simulate the 3D environment. Furthermore, composition of ECM can significantly influence the cell fate. For this reason, we hypothesized that the identification of optimal ECM-based scaffolds in conjunction with ES could confer optimal microenvironment for skeletal differentiation and myoid tissue assembly.

In this study, we have developed multi-well platform to examine various ECM-compositions for muscle differentiation. In particular, we studied the effects of the ECM composition on C2C12 cells differentiations and the formation of a functional muscle tissue. Thus, we monitored the evolution of C2C12 cells encapsulated in various ECM-based hydrogels during 5 days in differentiation medium and analyzed the myoid tissue formation. Furthermore, we then evaluated the effects of the presence of the ES on myotube formation by the C2C12 cells in optimized ECM microenvironment. C2C12 cells were encapsulated in ECM-hydrogels, and muscle differentiation was examined in response to ES (2 V/cm, 1 Hz frequency, and 2 ms duration time). Our multi-well platform may find their use in high-throughput biological studies involving the identification of optimal differentiation microenvironment for various tissues.

2. Materials and methods

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). 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-
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2.2 Multi-well platform fabrication

Acrylonitrile-butadiene-styrene (ABS) resin-based 3D printed mold design was performed by AutoCAD (Autodesk, US) software. Dimensions of each single well were 5 mm×2 mm×3.5 mm horizontal, vertical, and height respectively (Fig. 1). 2% Agarose (Ultrapure Agarose, Invitrogen) solution was poured into the 3D printed mold. After agarose solidification, multi-well platforms were transported into 6-well plate with PBS and further sterilized with UV light for 30 minutes. Before cell seeding, agarose multi-wells were incubated in growth medium for 2 hours in 37°C. Agarose monomers were washed after the incubation to provide a suitable condition for cell culture.

2.3 Electrical stimulation and differentiation

For electrical stimulation, electrical stimulator (S88; GRASS, US) with monophasic square shape pulse (2 V/cm, 1 Hz frequency, and 2 ms duration) and carbon electrodes (C-Dish; IonOptix, US) were used. Electrical stimulation was applied for 2 hrs/day.

2.4 Viability analysis

For 2D monolayer cultured cell, Trypan Blue (Life technologies, US) exclusion assay was performed. After 3 minutes of incubation at room temperature with solution, live cells were counted under the microscope. 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 less 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).

2.5 Cell proliferation analysis

Proliferation analysis was performed by simply counting cells under microscope or 5-ethynyl-2'-deoxyuridine (EdU) staining. For manual cell counting, cells were harvested using 0.25% trypsin-EDTA and counted under bright field microscope. To assess cell proliferation, Click-iT EdU Assay Kits (Invitrogen) were used according to manufacturer’s instruction. In brief, C2C12 cells were seeded on glass bottom dish (SPL lifescience) and incubated with 0.1% 5-methynyl-2'-deoxyuridine (EdU) for 3 hours. Click-iT reaction cocktail were added on the dish for 30 min and washed with distilled water. To label total cell nucleus, 4’,6-diamidino-2-phenylinolde (DAPI) was added after EdU staining.

2.6 Luciferase assay

The myogenic enhance factor (Mef-2c) gene of interest was cloned. C2C12 cells were transfected with Luc-Mef-2c via electroporation (Neon Transfection System; Life Technologies, US). The activity of luciferase was measured by a luminometer.

Table 1 Primer sequences used in real-time PCR analysis
2.7 3D Culture of C2C12 cells

Collagen type I (3.1 mg/ml; Advanced BioMatrix, US) was neutralized with 10X PBS and 1M NaOH to make 2.5 mg/ml collagen hydrogel. For matrigel-hydrogel, Matrigel™ (BD Biosciences, US) was thawed and DMEM was added to make final concentration 2.5 mg/ml. For collagen/matrigel hydrogel, collagen (2.5 mg/ml) was mixed with matrigel (2.5 mg/ml) in 1:1 ratio. 200 μl ECM-solutions (collagen, matrigel, or collagen/matrigel) containing 2 × 10⁵ cells were loaded onto agarose-based multi-well. Cell containing ECM-hydrogels were incubated in 37°C incubator for 30 minutes. Constructs were maintained with myogenic differentiation medium for upto 7 days. Minutien pins (Fine Science Tools, US) were used to stabilize developed myoid tissues. For myogenic fusion index, randomly captured five different fluorescent microscopic images for each condition were measured for quantification.

2.8 Real-time PCR 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™; enzyomics, 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.

2.9 Immunostaining

For myoid constructs, samples were cut into 10 μm sections with cryostat (HM 505E; MICROM, Germany) and processed for immunostaining. For cells on monolayer culture, cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% (v/v) Triton X-100 (Sigma Aldrich, US) for 10 min. Cells 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 followed by 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. Images were taken with EVOS (Liftotechnologies, US).

2.10 Statistical analysis

All data are represented as mean±standard deviation (SD). Statistical significance was determined by student’s t-test with *p<0.05, **p<0.01.
3. Results

3.1 Design of multi-well platform with electrical stimulation

The master molds of multi-well for myoid tissue culture were fabricated with acrylonitrile-butadiene-styrene (ABS) resin-based 3D printed system. AutoCAD software was used to make dimensions of each single well with 5 mm×2 mm×3.5 mm horizontal, vertical, and height respectively (Figs. 1(a)-(b)). Designed mold was filled with agarose solution and placed in 8-well plate with carbon electrodes. Each agarose platform was designed to maintain 8 myoid tissues with single pair of carbon electrodes for simultaneous ES as shown in Fig. 1(d).

3.2 Electrical stimulation response of C2C12 cells

Cells respond to their physical surroundings and external signals such as ES. We initially confirmed whether our ES setting routine (2V/cm, 1Hz, 2ms duration) have cytotoxic effect or modulate cellular behavior. C2C12 cells were plated at 5x10^3 cells/cm² and ES was applied after 24 hours of plating for next 48 hours (Fig. 2(a)). As shown in Fig. 2(b), cellular morphologies were same in both electrical stimulation (ES+) and without electrical stimulation (ES-) condition, but cell proliferation rate of ES+ group was lower than that of the ES- group. Cell counting showed that ES- group resulted in exponential growth, while ES+ group showed delayed cell proliferation (Fig. 2(c)). To examine further, whether lower cell counting in ES+ group was the result of cell death, cell viability analysis was performed as shown in Fig. 2(d). In the case of ES+ group, the percentage of live cells stayed relatively constant throughout the culture. Although ES
affected cell proliferation rate, apoptotic test demonstrated that ES did not noticeably trigger C2C12 cell death. To examine whether the ES dependent cellular arrest is a permanent phenomenon, we examined the cellular responses after ES for longer period (Fig. 2(e)). Cells were cultured for 7 days and ES was applied from D3 to D5 for 2 days. ES+ groups displayed delayed proliferation (dotted line); however, cell proliferation rate was recovered after the withdrawal of ES. EdU staining, which indicates the S phase cells, confirmed that ES+ groups showed fewer positive nuclei (Fig. 2(f)). This confirmed that ES effectively slows down the cell proliferation without modulating the cell death.

3.3 Electrical stimulation dependent myogenic differentiation

To evaluate the efficacy of ES on myogenic differentiation, ES was applied intermittently (2 hours per a day, up to day 7) on C2C12 cells cultured with GM or DM. Immunostaining of myosin heavy chain (MHC) showed that ES+ enhanced myotube formation and induced rapid myogenic commitment of C2C12 cells cultured in both GM and DM (Fig. 3(a)). ES+ group displayed longer and thicker myotubular structure. To quantitatively examine the myotube quality, we measured the
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Fig. 3 Electrical stimulation dependent myogenic differentiation. (a) Bright field images and MHC and multi-nuclei (DAPI) staining of C2C12 cells maintained in GM or DM and exposed to ES+ or ES- (b) Quantitative analysis of myotube thickness and number of nuclei per fused myotubes (c) Mef-2C activity was quantified by luciferase activity (d) Real-time PCR analysis of muscle-related genes (Scale bar 200 μm)

thickness of the individual myotubes and the number of nuclei in a single myotube. The mean thickness of DM cultured myotubes ES+ was thicker than that of DM induced myotubes with ES-. However, cells maintained in DM, ES+ group had 50% more nuclei than ES- group in each myotube (Fig. 3(b)). For a negative control, ES- GM cultured sample had weak myosin fluorescence intensity and fluorescence-tagged sites with only 1 to 2 nuclei. On the other hand, ES+ GM sample showed cell fusion and had longer myotubular structures than those of ES- group. Active myotubular structure formation was witnessed even in GM. This implicates that with induction of ES, GM culture was also able to develop myotubes. As the quantification shows, more nuclei were observed when cells were subjected to ES in the case of GM and DM. We further evaluated ES dependent myogenic enhance factor (Mef-2C) activity by transfecting the cells with Mef-2-C plasmid with luciferase reporter. ES was applied for 2 hours and luciferase assay was performed to quantify the promoter activity. Several hours (i.e., 2 hours) of ES were sufficient to derive the Mef-2C promoter activity as shown in Fig. 3(c). Finally, gene expressions of the sarcomeric actinin, MHCd and MLP were analyzed by real-time PCR with ES+ or ES- in both GM and DM cultured conditions (Fig. 3(d)). These genes are known to be related to elasticity and contractility of physiological muscle. In the case of MHCd gene expression, approximately 30-fold induction was detected for ES+ group compared to ES- group in both GM and DM cultured conditions. Likewise, sarcomeric actinin and MLP gene expressions were increased in ES+ group.
Fig. 4 Real-time PCR analysis after 5 days under DM condition in collagen (C), matrigel (M), or 1:1 mixture of collagen/matrigel (CM).

Fig. 5 ECM-based 3D structures and electrical stimulation. (a) C2C12 cells were encapsulated in C, M, or CM and cultured under ES+ or ES- (b) Viability analysis by Live/Dead staining (Scale bar=200 μm) (c) Bright field images C, M, and CM cultured under ES+ or ES-. C/ES- : Collagen without electrical stimulation. C/ES+: Collagen with electrical stimulation. M/ES-: Matrigel without electrical stimulation. M/ES+: Matrigel with electrical stimulation. CM/ES-: Collagen/matrigel without electrical stimulation. CM/ES+: Collagen/matrigel with electrical stimulation (Scale bar=1 mm) (n=4)
3.4 ECM-based 3D structures and electrical stimulation

First, we analyzed the effects of ECM composition on myogenic gene expressions. Fig. 4 shows gene expression of MHCd, MRF4, and myogenin of C2C12 cells encapsulated in 3D ECM-based hydrogel structure. Overall collagen environment showed the highest expression of myogenic genes (Fig. 4). Interestingly, hydrogel consisted of both collagen and matrigel showed the lowest differentiation efficiency of C2C12 cells. We can speculate that low differentiation efficiency of C2C12 cells in collagen-matrigel hydrogel was a result of the diluted concentration of each ECM component. Collagen and matrigel components were not able to synergistically influence C2C12 cells and enhance differentiation of C2C12 cells.

Next, we analyzed the responsiveness of 3D cultured C2C12 cells to electrical stimulation. C2C12 cells were encapsulated in collagen, matrigel, or 1:1 mixture of collagen and matrigel. Hydrogels constructs were formed within agarose multi-well and ES was applied for duration of 5 days (Fig. 5(a)). Myoblasts remained within hydrogel constructs. The effects of ES on C2C12 cells within hydrogel constructs were examined by monitoring the viability and hydrogel constriction (Fig. 5(b)). Similar to 2D analysis, ES did not cause any significant cytotoxicity of C2C12 cells in hydrogel constructs; however CM hydrogel induced minute cytotoxicity to cells. Furthermore, as shown in Fig. 5(c), hydrogel contractions were observed due to cell-ECM mediated cytoskeletal tension in a time-dependent manner. From bright field microscopic images, it was shown that...
C2C12 cells encapsulated in CM hydrogel were able to contract the hydrogel the most compared to other ECM-based hydrogels. Previously, ES has been shown to induce myotube contractions, as ES can stimulate functional sarcomeres assemblies and increase contractile activity of the cells. However, we did not observe ES-dependent contraction of C2C12 encapsulated hydrogels (Fig. 5(c)).

Evaluation of myogenic differentiation and initial analysis with ES concluded that collagen hydrogel was optimal environment for skeletal tissue engineering. Therefore, further analysis with ES was performed with collagen hydrogel with mechanical stretching (pin-anchored to the agarose well). Prior to ES introduction, cell-seeded collagen hydrogels were either cultured in free floating or pin-anchored to the agarose well. Highly oriented aligning morphologies of myoblast cells were observed in pinned samples (Fig. 6(a)). In addition, pinned samples showed increased detection of MHC as demonstrated by immunostaining. Furthermore, mechanical stretching of skeletal muscle organogenesis brought myotube orientation and longitudinal growth in the direction of strain. Stretching of skeletal myoblasts showed higher myogenic transcriptional regulation compared with free floating control (Fig. 6(b)). However, free floating constructs exposed to ES showed comparable levels of myogenic gene expressions when compared to pinned samples (with mechanical stretching). Interestingly, ES on mechanically stretched sample did not result in augmented expression of myogenic genes (Fig. 6(b)).

4. Discussion

Continuous ES can alter cellular metabolism positively or negatively, with a possibility of inducing the apoptotic response and reduction of cell viability in a frequency- and voltage-dependent manner (Thelen et al. 1997). 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 (Marotta et al. 2004). Otherwise, no contraction activity was observed at frequencies higher than 10 Hz (Marotta et al. 2004). In reference to this experiment that enriched contractile properties of myotube (Thelen et al. 1997), we chose the following conditions as our ES regime: 1 Hz and 2 volt per cm for a duration time of 2 ms. We then examined the apoptotic activity in the ES samples. The apoptosis stain revealed that the tested ES regimen did not increase apoptosis (Fig. 1), a result consistent with those of Pedrotty et al. (2005). However, proportion of the cells in the a proliferation phase (S phase) was reduced via ES, and our ES regime inhibited cell cycle progression (Wang et al. 2003). ES dependent cell cycle progression inhibition was not a permanent phenomenon. Cell cycle progression was recovered as soon as the ES was withdrawn.

When the C2C12 cells are confluent, the cells fuse, become multinucleated and develop functional myotubes, which contract spontaneously (Blau et al. 1983, Kislinger et al. 2005). Development of functional myotubes in 2D culture of C2C12 takes 2 weeks in differentiation medium. In our study, ES clearly enhanced myogenic gene expression of C2C12 cells. ES is known to enhance the maturation of the muscle cells. Quantitative gene expression level analysis of MHCd, MRF4, MLP, sarcomeric actinin genes expressions were upregulated by ES (Fig. 3(d)). In addition to myoblast cells, ES has shown to increase cardiomyocyte function (Chan et al. 2013). In the present study, the optimized regime for ES may be different from in vivo motor neural stimulation. In addition, recent studies have reported that co-culture of PC12 cells can enhance myoblast fusion (Ostrovidov et al. 2014). In the future, in vitro skeletal tissues strategies will have
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to be adapted and refined for optimal ES similar to motor neurons and neuronal cellular microenvironments.

For 3D investigation, we designed agarose-based multi-well platform and utilized this platform retain cell-containing ECM hydrogels with ES. Agarose gel has multiple usages in biological research field by means of microbe nutrients, electrophoresis, and so on. Agarose gel does not allow cell adhesion and allows minimal interactions with ECM-hydrogels (Dumitriu 2012, Nelson et al. 2007, Yang et al. 2009). Thus, we fabricated agarose-based multi-well platform that would allow 8 small wells [with dimension 5 mm×2 mm×3.5 mm] within a single platform. Each multi-well platform was immersed into the carbon electrode-containing tissue culture plates. C2C12 cells were encapsulated within collagen, matrigel, or collagen/matrice. We observed that even with ES, C2C12 cells within ECM-hydrogels were maintained over one week (Fig. 4). Previously, remodeling of the cellular environment by physical stimulation has been extensively explored, and induction of cellular development by biomimetic physical stimulation is closely related to transcriptional signal transduction cascades (McDevitt et al. 2002). For this reason, we examined the ES dependent contractility of C2C12 cells within ECM-hydrogels by measuring the lengths of the tissue constructs. We observed that ES dependent C2C12 cell was only evident with matrigel. C2C12 cells within collagen containing scaffolds showed no ES dependent contraction. This is probably due to a strong cell-mediated collagen contraction that resulted in the shrinkage of constructs independent of ES. Indeed, at day 2, C2C12 within Matrigel maintained its original shape, while collagen and collagen/matrice constructs shrunk significantly.

We further demonstrate that mechanical stretching can facilitate the myogenic commitment using collagen as model biomaterials. Without mechanical stretching, the collagen constructs had formed poorly developed myofibrils, with sarcomeres scattered in the cytoplasm, resulting in non-functional muscle fibers without contraction (Fig. 6). In contrast, in mechanically stretched groups the muscle fibers were functional and structural analysis identified well developed MHC arranged into myofibrils in the cytoplasm. These data clearly suggested that ECM-environment not only acted as a signaling cue for protein expression, but ECM-mediated mechanical stretching promoted the assembly of appropriate ultrastructural features. Interestingly, when we applied ES on the mechanically stretched groups, we did not observe synergistic effects. The exact mechanisms of stretch-mediated mechanical signals coupled with ES mediated electrical signals into cellular responses need to be investigated.

5. Conclusions

We aimed to develop an optimal microenvironment platform for skeletal muscle tissue engineering.

- ES on C2C12 cells on 3D culture, and our agarose-based multi-well platform gave a rise to faster maturation of C2C12 cells.
- Coupling of ES with mechanical stretching could provide multi-modalities for optimal muscle tissue formation conditions.
- We envision that this platform can be applied to diverse screening applications with various types of biocompatible hydrogels.
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