Construction of functional artificial skeletal muscle tissue by regulation of cell-substrate interaction using myogenic C2C12 cells

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Content

Abstract

Construction of functional artificial skeletal muscle tissue models is valuable for medical investigation of muscle diseases. Especially in the field of pharmaceutical development, it is urgent to develop an experimental model and evaluation method for the replacement of animal experiments. Although there are several approaches to construct an artificial skeletal muscle tissue, these have not succeeded in the construction of the artificial skeletal muscle tissue which is comparable to *in vivo* tissues. This study challenged this goal by focusing on cell microenvironment. Within this microenvironment, cell-substrate interaction such as cell-extracellular matrix (ECM) interaction is an essential factor for the expression of cell phenotype, behaviors, and functions.

Chapter 2 presents the investigation data of the effects of ECM and supplementation of a small molecule, which has been reported to enhance α7β1 integrin expression (SU9516), on cell migration speed, cell fusion rate, myoblast (mouse C2C12 cells) differentiation, and contractile force generation of tissue-engineered artificial skeletal muscles. When cells were cultured on various ECM coated-surfaces, an enhancement in the migration speed was observed. At the same time, the myotube formation (differentiation ratio) decreased under all conditions except for cells cultured on Matrigel coated-surfaces. In contrast, SU9516 supplementation elevated both the myotube width and differentiation ratio. Following combined culture with a Matrigel-coated surface and SU9516 supplementation, myotube width was further increased. Additionally, the physical force generation of the artificial skeletal muscle tissue could be improved using SU9516 and Matrigel. As a result, this chapter demonstrated that a combination of Matrigel (ECM) and SU9516 supplementation induced the generation of robust myotubes and improved the contractile force generation of artificial skeletal muscle tissues.

Chapter 3 demonstrates an *in vitro* tissue-engineering system to construct the functional

skeletal muscle tissue through soft substrate and signal inhibition. Depending on the substrate stiffness and ECM, self-organization of C2C12 cells were induced, but the levels of differentiation and myotube construction in the clump-shaped tissue were not sufficient. To improve myogenic differentiation, bone morphogenetic protein (BMP) inhibitors were applied during myogenic differentiation. These supplementations resulted in the enhancement of differentiation ratio and myotube hypertrophy in two-dimensional cell cultures. Furthermore, during the engineering of ring-shaped tissue using soft substrate, immunofluorescence staining revealed that the cells in the ring-shaped tissue generated higher myotube construction than those in the clump-shaped tissue. Following combined establishment with ring-shaped tissue and BMP inhibitor, both skeletal muscle differentiation ratio and myotube hypertrophy were enhanced. Myogenic maker expression in the self-organized tissue was significantly improved by BMP inhibitor treatment. These observations indicate that this procedure using the soft substrates and the BMP inhibitor may provide a novel functional artificial skeletal muscle.

Overall, these methods provide a straight-forward process to enhance myogenesis and improve the function of tissue-engineered artificial skeletal muscles by regulation of cell-substrate interaction.

Chapter 1: General introduction

As a cutting-edge technology, artificial tissue construction *in vitro* is a kind of synthetic technique that combines biochemistry, biology, materials, chemistry, physics, and applied engineering. Various methods for constructing functional skeletal muscle tissue have been challenged and reported by many researchers. However, it has not been achieved to construct completely functional artificial tissues *in vitro*.

1.1 The necessity of the functional artificial skeletal muscle tissue *in vitro* model

In the scope of medical research and pharmaceutical development for patients with injured, diseased, and age-related muscle dysfunction, cultured cells have been used as a first model to evaluate biological reactions (1-2). Generally, these evaluation systems have been constructed as a two-dimensional (2D) culture model because it is not only easy to observe but also has a low cost. However, it is difficult for the 2D culture model to mimic *in vivo* skeletal muscle phenotype, mainly due to lack of native muscle microenvironment. On native skeletal muscle tissue, the cells are three-dimensionally exposed to other cells, extracellular matrix (ECM), and mechanical force (3). Furthermore, one of the most critical functions of skeletal muscle is contractile force generation, which depends on cell differentiation and maturation. These myogenic developments are also determined by the cell microenvironment. In fact, Ito *et al*. have reported that the drug response differs between 2D and three-dimensional (3D) culture models (4). Therefore, it is important to understand the disease development and drug efficacy at 3D tissue level. For instance, skeletal muscular dystrophy is a common chronic disease (5). The underlying mechanism of it has not been fully identified yet because of its high complexity. It has been already reported that the central mechanism includes inflammation, genetic deficiency, immune response, infection, and trauma. Muscle disorder is mainly manifested as muscle fatigue, pain, muscle strength

decline, muscle atrophy, and muscle hypertrophy. It is especially difficult to evaluate disease caused by such complex factors because the environment in 2D culture model significantly differs from *in vivo*. The 3D culture model enables evaluation at a more natural physiological environment and simulates the functional structure of muscle tissue, resulting in more efficient research on the disease. Currently, most of the drugs are evaluated with a 2D culture model *in vitro*, but they often fail during animal experiments or do not maintain the drug efficacy for the long term. These findings suggest that the 2D culture model is insufficient as an evaluation method. Furthermore, if the functional muscle can be constructed, the damage to tissues and organs caused by diseases such as diabetes, spinal cord injury, heart disease, and kidney failure can be cured as a regenerative medicine. Therefore, the construction of functional artificial skeletal muscle tissue (3D culture model) *in vitro* can provide reliable methods for medical research and pharmaceutical development (6) (**Fig. 1.1**).

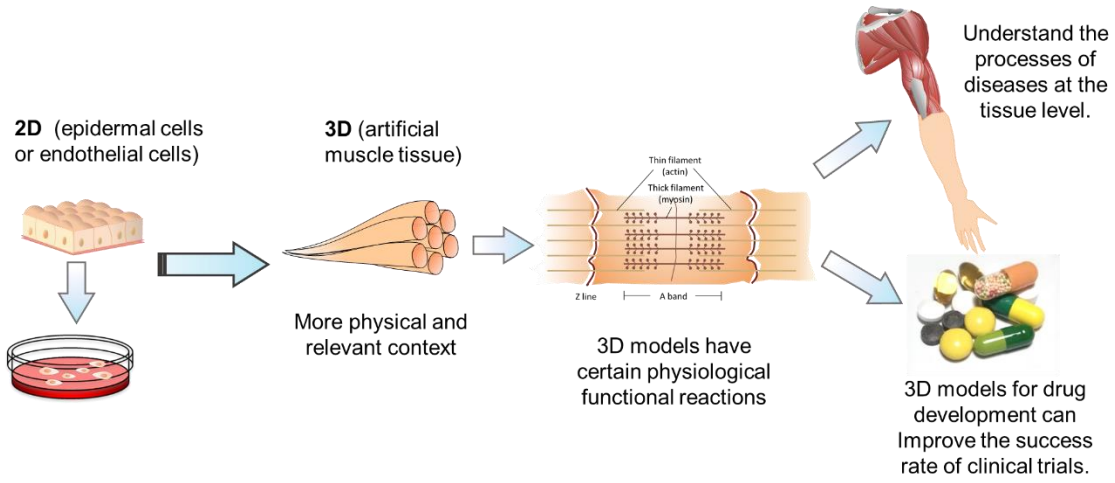

Fig. 1.1 Schematic diagram showing the application of 3D culture

1.2 Skeletal muscle tissue-engineering

Skeletal muscle tissue engineering is an important technique for the construction of artificial skeletal muscle tissue. In the past, many research groups have developed various methods for

the construction of artificial skeletal muscle tissues. For instance, artificial scaffolds such as biodegradable sponges (7) and hydrogels composed of fibrin (8) and/or Matrigel (9, 10) have been employed. In recent years, a method of construction of an artificial skeletal muscle tissue using 3D cell-printing technology with hydrogel ink has also been reported (11). Although these methods succeeded in the development of artificial skeletal muscle tissues, there are still some problems with the differentiation ratio, contractile force generation, procedure, and manufacturing cost. For instance, the construction of artificial skeletal muscle tissue with hydrogel containing various ECMs, certain ECM may be in excess, resulting in the inhibition of myogenic differentiation. To approach this problem, chapter 2 describes the effect of cell-ECM interaction on myogenesis. This study evaluated the effects of ECM and integrin expression regulatory reagent (SU9516) supplementation on migration, proliferation, differentiation, myotube formation, and contractile force generation of the artificial skeletal muscle tissues (**Fig. 1.2**).

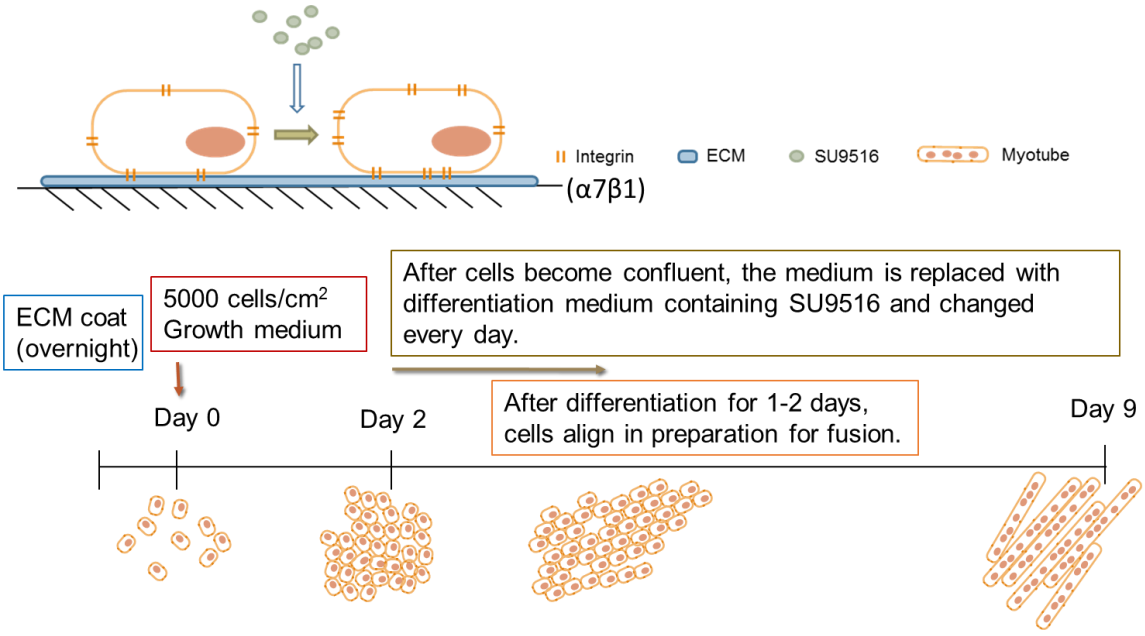

Fig. 1.2 Schematic illustration showing the differentiation process of myotubes in the presence of ECM and SU9516.

Furthermore, since the native skeletal muscle tissue is constructed with a high cell density, the use of artificial scaffolds and hydrogels may interfere with cell-cell interactions, thereby

resulting in the inhibition of myotube formation. Thus, the generation of functional artificial skeletal muscle tissue is associated with the density and differentiation state of skeletal muscle cells within artificial muscle tissues. To challenge this problem, chapter 3 focuses on the establishment of functional artificial skeletal muscle tissue. This method induces the self-organization according to optimal ECM and stiffness on the culture surfaces, resulting in the development of artificial skeletal muscle tissue without artificial material between the cells (**Fig. 1.3**).

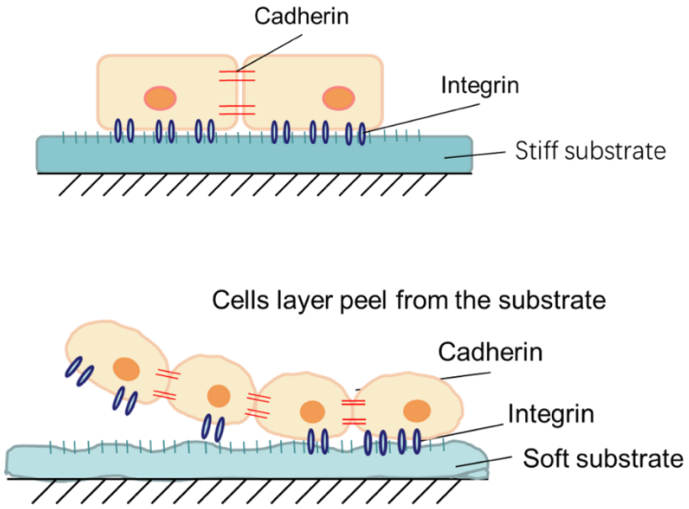

Fig. 1.3 The processes of self-organization according to the stiffness of the culture surface.

1.3 Skeletal muscle development and myogenic differentiation

In the human body, muscle tissue largely accounts for the total weight. The skeletal muscle is a form of striated muscle tissue, which exhibits stronger contractility than other types of muscle (cardiac muscle), while it is difficult to maintain over time (12). Skeletal muscle is a voluntary muscle that is controlled by signals from the brain, and it is consisted out of the thousands of aligned skeletal myofibers in the same direction. Similar to most tissues, skeletal muscle tissue is composed of several types of cells, mainly including polynuclear

myotubes and myosatellite cells. *In vivo*, myosatellite cells play an essential role in the growth and repair of muscle tissue (13). However, *in vitro* models do not reproduce the muscle repair by myosatellite cells, and there are still many unknown factors. Thus, constructing useful 3D model is very important for these studies.

Myosatellite cells are also known as myogenic progenitor cells (MPC), which were firstly described by Mauro in 1961 (14). In healthy skeletal muscle, myosatellite cells maintain dormant state and reside between the plasma and basement membranes (15). To induce the myofiber formation, the quiescent myosatellite cells are activated and differentiate into myoblasts. As a precursor of myotubes with the ability to proliferate and differentiate, the myoblast fuses with an adjacent cell to form small nascent myotubes. Subsequently, these myotubes are fused with other myotubes and form large mature myofibers with dozens to hundreds of nuclei **(Fig. 1.4)**. During the skeletal muscle development, some muscle developmental marker genes (e.g., MyoD, Myf-5, and myogenin) are expressed in different stages. Thus, evaluation of the gene expression of these markers is vital to understand the degree of muscle differentiation.

C2C12 cells represent a mouse myoblast cell line (16). These cells are capable of rapid proliferation under high-serum conditions and of differentiation into myoblasts under low-serum conditions. As an important cell model for myogenic research, C2C12 cells have been widely applied to biological research (e.g., regeneration, transplantation, and drug discovery). Koh *et al.* directly transplanted the C2C12 cells into the heart at syngeneic mice and observed skeletal muscle cell behavior and polynuclear myotube formation (17). Lima *et al.* reported the construction of a microplate assay system for measuring cell death in C2C12 cells (18). Thus, the C2C12 cells are characterized in different aspects. However, most of these researchers have reported that 2D culture systems are inferior to mimic *in vivo* behaviors. Therefore, the construction of functional artificial skeletal muscle tissue using C2C12 cells *in vitro* is a meaningful research.

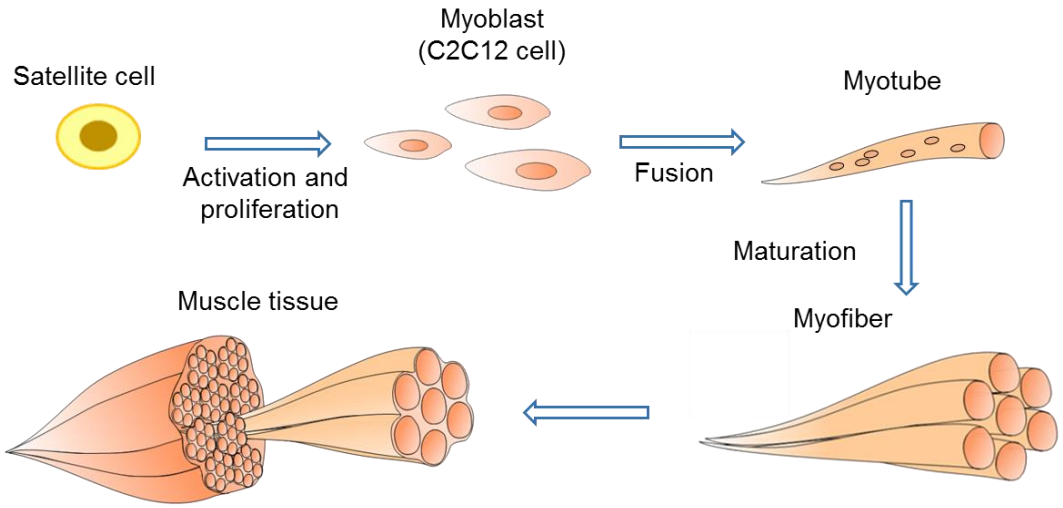

Fig. 1.4 The process of myogenic differentiation

C2C12 cell differentiation is regulated by various factors such as cytokines, nutrients, and protein synthesis. The previous research at 2D culture conditions has shown that inhibition of the BMP signaling pathway induces an escape from the cell cycle and entering the differentiation stage (19). In the proliferation and differentiation processes of myoblast cells, BMP signal is activated via BMPR (bone morphogenetic protein receptor) on the cell surface, and the signal transduction is further processed by phosphorylation of Smad 1/5/8 proteins (20, 21). Ultimately, this signaling pathway induces the Id1 gene expression and osteoblast differentiation (22, 23). Conversely, the inhibition of the BMP signaling pathway induces muscular differentiation, cell maturation, and intercellular fusion (24). Therefore, the BMP inhibitor accelerates myogenic differentiation and facilitates the functional myotube construction because it blocks intracellular Smad 1/5/8 protein activation (25, 26). Furthermore, BMP signaling pathway also mediates other non-Smad signaling pathways such as P38-MAPK, ERK, and PI3K/Akt, and establishes the crosstalk with multiple signal pathways to regulate the cell migration, differentiation, and maturation (27-30) (**Fig. 1.5**). Thus, the regulation of the BMP signaling pathway is important for the construction of functional artificial skeletal muscle tissue.

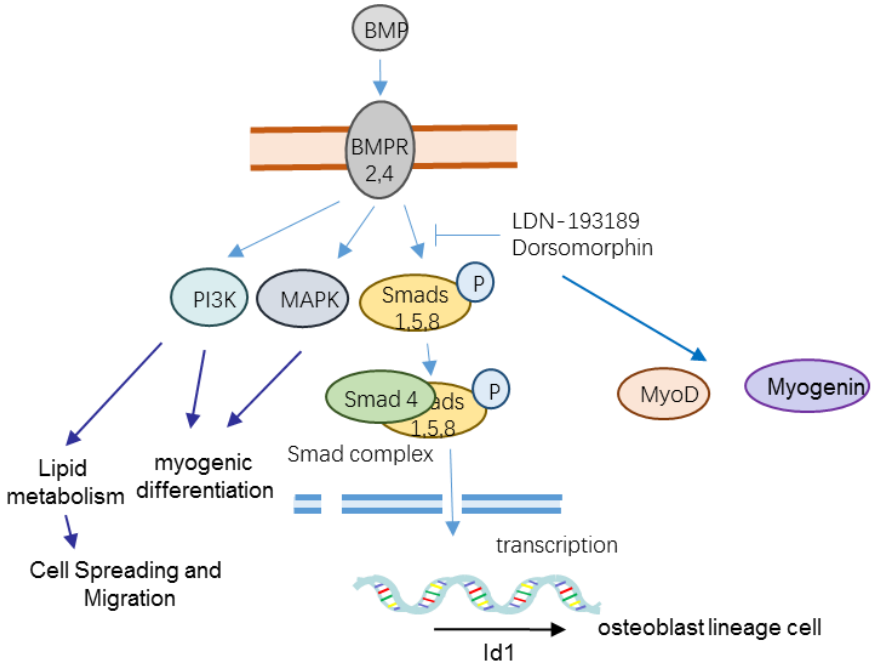

Fig. 1.5 BMP signaling pathway in myotube formation

1.4 Extracellular matrix

The extracellular matrix (ECM) is a 3D complex network of macromolecules, which fills the space between cells, tissues, and organs. The ECM contributes to the formation of a basement membrane, which is a protein network foundation for cells and tissues (31, 32). *In vivo,* ECM is secreted by cells, and its components depend on the cell types. As the ECM for the skeletal muscle, a basal lamina surrounds the myofibers. The basal lamina is composed with Laminin and Collagen 4, and are further sheathed by the endomysium containing Fibronectin and Collagen 1, 2 and 5 (33). ECM has not only a high capacity for water retention and lubrication but also participates in several cellular functions such as adhesion, signal transmission, proliferation, differentiation, and apoptosis (34-36). *In vivo*, except for blood cells, most cells maintain homeostasis with ECM-mediated adhesion. In addition, ECM can also store various growth factors, which play an important role for environmental transmission processes in the cell-cell and cell-external interactions.

Besides biochemical interactions between cells and ECM, the cellular response to mechanical properties with ECM maintains the normal cellular functions. The ECM receives mechanical stresses such as tension and compression, and also protects and regulates cells and tissues *in vivo*. Therefore, optimal ECM is required for the maintenance of skeletal muscle tissue homeostasis. During skeletal muscle regeneration, the ECM of the basal lamina and endomysium plays an essential role in the migration, proliferation, and differentiation of myoblasts (37, 38). Thus, the research of ECM regulation in C2C12 cell behavior is important to construct functional skeletal muscle tissues using C2C12 cells *in vitro*. Additionally, the stiffness of ECM depends on the tissues and organs, and it is also avital parameter for the cell behavior. However, the combined effect of ECM and stiffness on C2C12 cells is still unknown.

1.5 The cell-substrate and cell-cell interactions

Generally, the cell-substrate interaction such as cell-ECM interaction is constructed with non-cellular solid-state component networks and neighboring cells. These interactions are accompanied by various physical and biochemical reactions **(Fig. 1.6)**. On the cell-ECM attachment, whether actin-linked junction or hemidesmosome, ECM proteins and integrins are indispensable (39). As a receptor on the cell surface that binds to ECM and links the cytoskeleton, integrin activates the signaling pathways to form a complex crosstalk for regulation of cellular behavior (40). For most cells, such as muscle and epidermal cells, their survival depends on the attachment to the ECM. The separation of these cells from ECM induces programmed cell death, and, therefore, many diseases are associated with integrin dysfunctions (41-43). The integrin is a transmembrane heterodimer with various subtypes that depends on the type of cells and binds to specific ECM. For instance, the integrin α7β1 (main integrin type on C2C12 cells) mainly links Laminin, which is a component of the basement

membrane to the intracellular actin cytoskeleton and regulates migration and myogenesis (44, 45). Moreover, the cell-substrate junction directly reacts to mechanical stress, such as stiffness of cell tension. This physical microenvironment is consisted of the combination of substrates such as ECM and cell pseudopodia, and it regulates cell behavior (46, 47). Ng *et.al.* showed that substrate stiffness regulates the integrin accumulation on the cell surface and influences cell adhesion and cytoskeleton spreading (48). Finally, it was shown that C2C12 myoblast diffusion and cell morphology change via integrin activation, which is crucial in the early stage of myogenesis. Therefore, in order to improve the myogenic differentiation of artificial skeletal muscle tissue, low molecular compound (SU9516) was used to optimize the integrin expression, which is described in chapter 2.

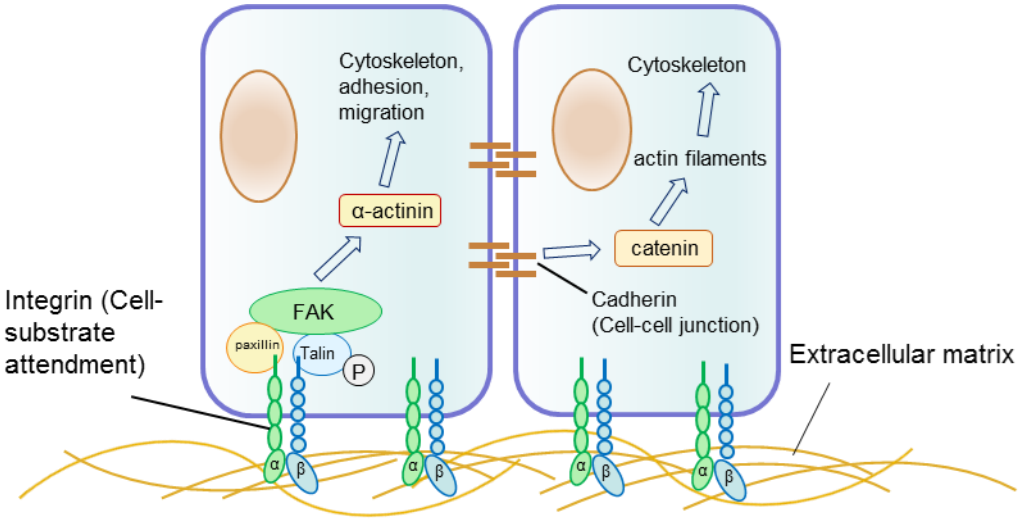

Fig. 1.6 The schematic diagram of the cell microenvironment

Cell-cell adhesion mediated by various molecules is an equally important factor in regulating differentiation and proliferation of cells. *In vivo*, a part of these contacts is mediated by cadherin family, which is maintained in a calcium-dependent manner. The extracellular domain of cadherin interacts with cadherin molecules on neighboring cells in a homotypic way. Furthermore, the cadherin anchors intracellular actin filaments and

cytoskeleton through catenin and plays an important role in cell rearrangement during development (49).

In healthy muscle, transmembrane proteins like integrins link the ECM to the actin cytoskeleton and regulate cell behaviors (adhesion, migration, and signal transduction). Therefore, it seems that the changes of balance between cell-cell and cell-substrate by adjusting substrate stiffness induce various cell behaviors. Taking it into consideration, the self-organization was induced to develop more native artificial skeletal muscle tissue, which is described in chapter 3.

The ultimate goal of skeletal muscle tissue engineering is development of the functional artificial skeletal muscle *in vitro* that is comparable with *in vivo* situation. In this study, the regulatory effects of cell-substrate interaction for the construction of functional artificial skeletal muscle tissue were investigated *in vitro*.

Chapter 2: The effect of cell-extracellular matrix interaction on myogenic characteristics and artificial skeletal muscle tissue

2.1 Introduction

Artificial skeletal muscle plays an important role in various aspects, as they can be used in the understanding of muscle physiology (50) or evaluation of drug targeted to muscle (51). They also have potential to be used in regenerative medicine (52), bio-actuators (53) or meat alternatives (54), and the efforts are made to construct artificial muscle that have the function resembling the muscle *in vivo*. Generally, *in vitro* culture systems have been used to verify the drug efficacy by investigating their effect on the myotubes characteristics (55). However, *in vitro* culture systems exhibit different characteristics to *in vivo* skeletal muscle systems and their functionality is misrepresented mainly due to lack of cell maturation and muscle architecture. Active tension generation capability is one of the most important functions of skeletal muscles. Thus, artificial skeletal muscle tissues used for drug discovery should possess the architecture of native muscle *in vivo*, including highly differentiated cells and well-matured and well-fused muscle fibers, surrounded by ECMs.

Cells can recognize and interact with their environment through membrane receptors specific to different ECMs. The heterodimeric integrins play main role on cell-ECM interactions (56). Skeletal muscle cells, composing up to 40% the human body mass, are constructed with high density and well directed contractile myofibers. The relationship of the ECM with the cytoskeleton is essential for homogeneity of the sarcolemma network, supporting appropriate myofiber construction, and accurately transmitting the forces produced by muscle architectures (57). The integrin is part of a linkage system with the basal lamina, which includes Laminin and Collagen 4, and are further sheathed by the endomysium which contain Fibronectin and other type of Collagens (58). It was reported that the

expression level, subtype, and activation state of the integrins and the components of basal lamina are specifically regulated for myogenic cell activation and proliferation, migration and differentiation (59-62).

During myoblast differentiation and maturation, the cell-ECM interaction between the basal lamina and endomysium have significant role in skeletal muscle regeneration (63, 64). Composition of ECM affects the physiological properties of myogenic cells. For instance, Laminin enhances myogenic differentiation (65), whereas Fibronectin inhibits it (66). Furthermore, the combination of cell microfilament system and the extracellular matrix not only maintains the structure of the myofibers, but also establishes the foundation for the physiological functions of the muscle tissue (57, 67, 68). Thus, the regulation of cell-ECM interaction in myoblasts is important to construct artificial tissue engineered skeletal muscle using myoblasts *in vitro*. A number of integrins, including Laminin receptor $\alpha_7\beta_1$ integrin, are expressed by myoblasts (59, 60). Within muscle fibers, the $\alpha_7\beta_1$ integrin is distributed along the sarcolemma at costameres, and is expressed at neuromuscular and mytotendinous junctions in skeletal muscle (69, 70). The $\alpha_7\beta_1$ integrin has central role in maintaining the function and structures which contribute to muscle development and physiology (71). Declined α_7 integrin expression has been reported in Laminin-related muscular dystrophies, and dystrophin-deficient *mdx* mice did not survive past 4 weeks of age (72, 73). On the contrary, overexpression of $\alpha_7\beta_1$ integrin in *mdx* mice also elevated other cytoskeleton-associated proteins in integrin focal adhesions (74). This increase in the adhesion complexe components suggests that the regulation of $\alpha_7\beta_1$ integrin expression is important in maintaining skeletal muscle integrity.

It is well known that SU9516, a cyclin-dependent kinase 2 (CDK2) inhibitor, plays an important roles in the anti-cancer therapeutics, serving as compound for cyclin-dependent kinase2 (CDK2)-inhibitor, which is known to reduce cell proliferation, increase apoptosis and induce mitochondrial injury in various cancer cell lines (75, 76). In recent year, SU9516 is

reported to increase of $\alpha_7\beta_1$ integrin complex protein expression in mice and human myotubes (77, 78). Furthermore, treatment with SU9516 improved skeletal muscle function and reduced aberrant pathology in the mouse model for myopathy (78). From these reports, this chapter hypothesized that a combination of optimal ECM composition with SU9516 supplementation would improve the function of tissue-engineered artificial muscle.

The ultimate goal for tissue-engineered artificial skeletal muscle is to construct with comparable contractile properties and bioactivity *in vivo*. The association of sarcomeres and the contractile functional units in striated muscle, is required for contractile activity. Shimizu *et.al.* have previously reported a three-dimensional culture model of mouse skeletal muscle, where modified microdevice were used to construct the tissue-engineered skeletal muscles containing striated myotubes (79). In this method, the cell-ECM mixture is used for constructing skeletal muscle tissues. Therefore, this method was applied to assemble artificial skeletal muscle. This chapter examined the effects of ECM compositions and SU9516 supplementation on migration, proliferation, differentiation and fusion of C2C12 myoblast cells and active force generation of the artificial skeletal muscle tissues constructed.

2.2 Materials and Methods

2.2.1 Cell culture

C2C12 cells (within 10 passages; ATCC, Manassas, USA) were seeded at the density of 2000 cells/cm^2, and cultured in low glucose Dulbecco′s modified Eagle′s medium (DMEM; Nacalai Tesque, Kyoto, Japan) with 10% fetal bovine serum (FBS; EQUITECH-BIO, Kerrville, USA), Penicillin-Streptomycin Mixed Solution (Nacalai Tesque), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid (HEPES; DOJINDO, Kumamoto, Japan) at 37°C in a 5% CO_2 incubator. To induce myogenic differentiation, growth medium was replaced with differentiation medium containing high glucose DMEM and 4% calf serum. Cells were grown at 37°C, 5% CO_2. The medium change was performed every two days.

2.2.2 Cell migration assay

C2C12 cells (5000 cells/cm^2) were seeded in 35-mm polystyrene dish (Control; Greiner Bio-one, Frickenhausen, Germany) and ECM-coated dishes (1 μg/cm^2 Fibronectin, 5 μl/cm^2 Matrigel Growth Factor Reduced (BD), 1 μg/cm^2 Collagen 1, 1 μg/cm^2 Collagen 4, 1 μg/cm^2 Laminin), and cultured with growth medium. On subsequently experiment, same ECM protein amounts were used. Thereafter, the C2C12 cells were observed under BZ-9000 microscope (Keyence, Tokyo, Japan) equipped with a 5% CO_2 incubation chamber (Keyence). The cells were cultured with growth medium for 1.5 h, and time-lapse images were captured every 1 min. For each condition, 10 cells were randomly, a single cell in the captured images

was tracked, and the migration speed was calculated using Image J software.

2.2.3 Measurement of the cell viability, myogenic differentiation ratio, myotube width and myotube length

C2C12 cells (5.0×10^4 cells/cm^2) were seeded in 35-mm tissue culture dishes (control) or ECM-coated dishes at 2 days before induction of differentiation. After 2 days of culture in growth medium, the medium was replaced with differentiation medium (day 0) and the cells were further cultured for 7 days in a 5% CO_2 at 37°C. The cells on day 7 were collected, and the number of living cells were counted by the trypan blue exclusion method. Furthermore, the cells were washed with phosphate-buffered saline (PBS), and then fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. The cells were washed twice with PBS, permeabilized in PBS containing 0.1% Triton X-100 (MP Biomedicals) for 5min, washed with PBS for 15 min and blocked with Block Ace (Wako) for 90 min. The treated cells were incubated with a primary antibody against α-actinin (Sigma-Aldrich, St. Louis, Mo, USA) for 60 min, washed three times with TBS and then immersed in PBS containing an Alexa fluor 594-conjugated secondary antibody. After washing three times with TBS, the cells were incubated with 4′6-diamidino-2-2phenylindole (DAPI, Nacalai Tesque) for 20 min. Finally, the cells were observed using BZ-X710 fluorescence microscope. Microscopic images of five fields in each separate wells/sample were captured. The α-actinin positive myotube width, myotube length, and the number of DAPI-positive nuclei were measured using ImageJ software (NIH, Bethesda, MD, USA). The differentiation ratio was defined by the following equation: Differentiation ratio = (number of DAPI-stained nuclei in α-actinin-stained myotubes in the field)/(number of DAPI-stained nuclei in the field).

2.2.4 SU9516 treatment

C2C12 cells were exposed to SU9516 (2 and 5. 3-[1-(3*H*-Imidazol-4-yl)-methy-(Z)-yliden-e]-5-methoxy-1, 3-dihydro-indol-2-one, 0.01, 0.1, 1 or 10 μM; MedChemExpress, Pennsylv-ania, USA) during the induction of myogenic differentiation. The following day, the myotube width and differentiation ratio were measured.

2.2.5 Quantitative real-time RT-PCR analysis

Cells were sampled 9 days after culturing with various conditions for RNA isolation. RNA was extracted using RNAiso Pus (TakaRa bio, Shiga, Japan), and cDNA was synthesized using First Strand cDNA Synthesis Kit Rever Tra Ace-α (TOYOBO, Osaka, Japan). The cDNA was used for quantitative real-time polymerase chain reaction (PCR) analysis. The analyses were performed using primers shown in Table.2.1. The real-time PCR mixture (10 μl) included SYBR Green Master Mix (Applied Biosystems, California, USA), gene-specific primers, and a cDNA template and was reacted in StepOnePlus^{TM} Real-Time PCR (Applied Biosystems). The following PCR conditions were used: 95°C for 20 s, 40 cycles at 95°C for 3 s, 60°C for 30 s, 95°C for 15 s, and 95°C for 15 s.

Table 2.1. Primer sequences for real-time RT-PCR analysis

Target gene	Primer sequence
M-cadherin	FW: 5′- ATGTGCCACAGCCACATCG-3′
	RV: 5′ -TCCATACATGTCCGCCAG-3′
Integrin α7	FW: 5′- GAGGTGGCTGCTGGTGGGCG-3′
	RV: 5′ -CTCCTTCTGCACGTTAGCTC-3′
Integrin β1	FW: 5′- GACCTGCCTTGGCGTCTGTGC-3′
	RV: 5′ -CCAAAGTAGAAAGCAGGGAG-3′
GAPDH	FW: 5′- CTACCCCCAATGTGTCCGTC -3′
	RV: 5′- GCTGTTGAAGTCGCAGGAGAC -3′

2.2.6 Contractile force measurement of the muscle tissues

Contractile force of tissue-engineered skeletal muscle was measured by using microdevices, as reported previously (30). Briefly, the cell in hydrogel solution was prepared. The hydrogel solution contained fibrinogen from bovine plasma (10 mg/ml, F8630, Sigma-Aldrich), Matrigel (354234, Corning), 2 × DMEM, cell suspension (2×10^6 cells/ml), and thrombin from bovine plasma (50 U/ml, T4648, Sigma-Aldrich) at the volume ratio of 50 : 25 : 50 : 121 : 4. For the conditions of without Matrigel, a volume ratio of 75 of fibrinogen was mixed. Forty-five μl of the solution was poured into the dumbbell shaped pocket on the device placed in 1 well of a 6 well plate and the devices were incubated at 37°C to solidify the hydrogel. Then, 7 ml of the growth medium was poured into the well to immerse the whole device and then cultured. After 2 days, the medium was exchanged for differentiation medium, DMEM supplemented with 4% calf serum, 1% Penicillin-Streptomycin, 2.0 mg/ml

6-Aminocaproic acid (A2504, Sigma-Aldrich) and 10 μM of SU9516. The final DMSO concentration in the differentiation medium was 0.1 % because the SU9516 was dissolved in DMSO. After 6 days, the muscle tissues constructed on the devices were electrically stimulated electrically to achieve maximum tetanic force. An electrical stimulus of 20 V at 30 Hz with 2 ms wide pulses was used. The displacement of the tip of the microposts was observed by using a microscope (BX53F, Olympus, Osaka, Japan). Images were captured before and after the stimulation, and the displacement was measured.

2.3 Results

2.3.1 Effect of ECM proteins on migration, proliferation, differentiation, and fusion of C2C12 cells

To investigate the effects of different ECM proteins on C2C12 cells the C2C12 myoblasts were cultured on the Fibronectin, Laminin, Collagen 1, Collagen 4, and Matrigel coated culture surface. Time-lapse analysis showed that the C2C12 cells on each ECM coated surface migrated with higher speed than that of cells on the control tissue culture surface (**Fig. 2.1A**). Next, to examine the effect of ECM protein on the cell viability of C2C12 cells, the cells were cultured in differentiation

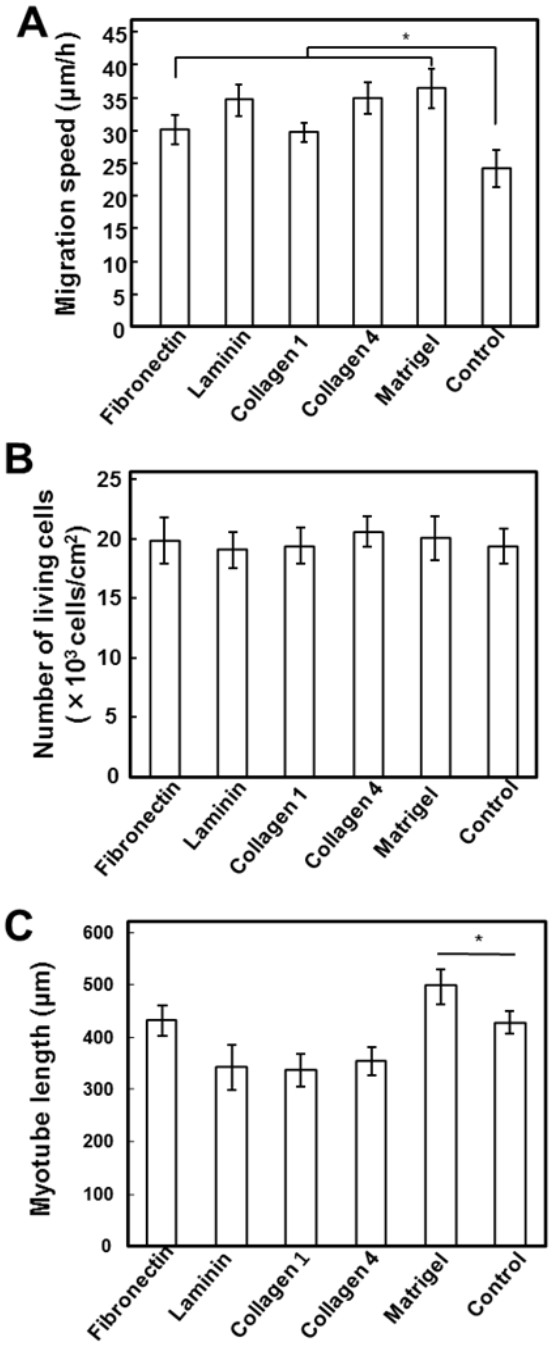

Fig. 2.1 Effects of different ECM proteins on C2C12 cells. (**A**) Migration speed of C2C12 cells on various ECM-coated culture surfaces. (**B**) Cell viability of C2C12 cells on various ECM-coated surfaces. Data are the means and SD of triplicate experiments. (**C**) Myotube length of C2C12 cells on various ECM-coated surfaces. Data are the means and SD of triplicate experiments. *P < 0.05. Control: Polystyrene surface.

medium for 7 days. As shown in Figure 2.1B, the number of living cell did not alter among each ECM protein and control tissue culture surface. Figure 2.1C show myotube length on each ECM protein and control tissue culture surface. Compared with the control, the Matrigel coated-surface increased the myotube length, while it decreased or remained unchanged in other ECM coated-surfaces. Finally, differentiation ratio and myotube width were examined on day 7, respectively. As a result, obvious increases on the ECM proteins-coated surface were not observed compared to the control tissue culture surface. (**Fig. 2.2**).

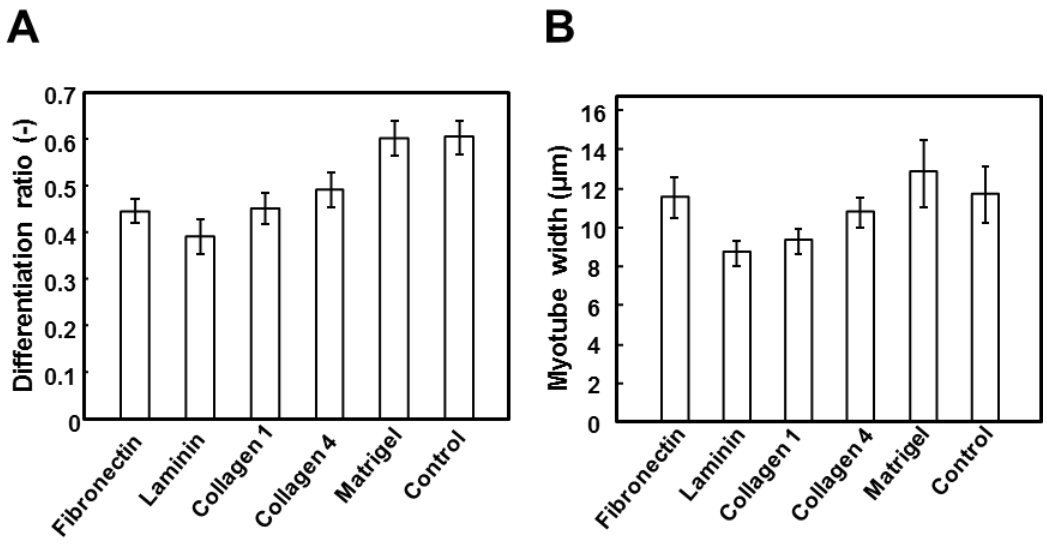

Fig. 2.2 Differentiation of C2C12 cells on various ECM-coated culture surfaces. After induction of C2C12 differentiation, immunofluorescence staining and quantitative analyses were performed to clarify the differentiation ratio (**A**) and myotube width (**B**) for α-actinin and DAPI staining. Control: Polystyrene surface.

2.3.2 Effect of SU9516 supplementation on myogenic differentiation

Next, the effect of different concentration of SU9516 on C2C12 cell differentiation was determined. When SU9516 was added to the growth medium, the proliferation rate significantly decreased in a dose-dependent manner (data not shown). In differentiation medium, the myotube width increased at 10 μM (**Fig. 2.3A**). Furthermore, the differentiation

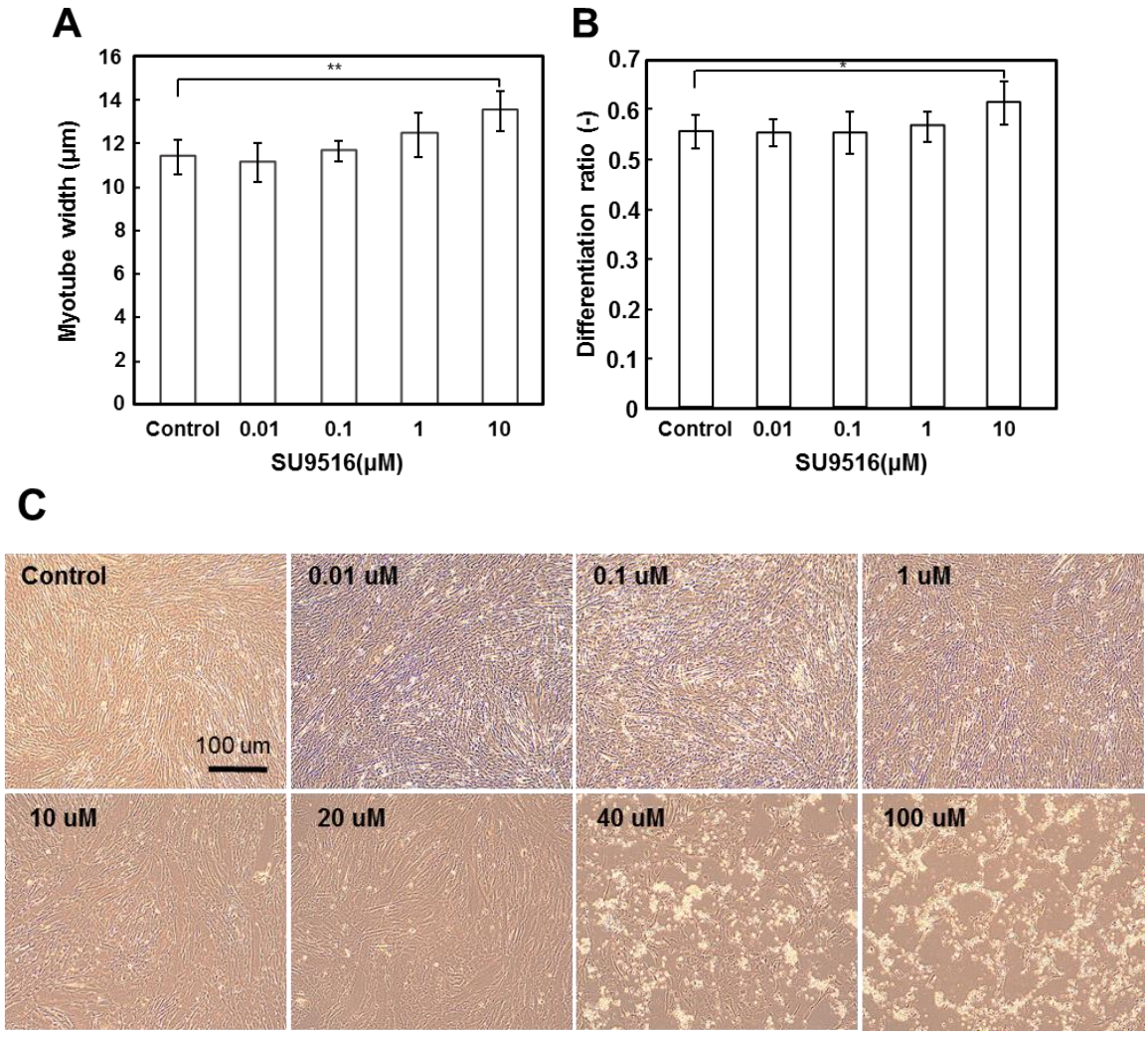

Fig. 2.3 Effects of SU9516 concentration on myogenic differentiation. Quantitative image analysis of the myotube width (A) and differentiation ratio (B) on day 7; data are expressed as mean ± SD (n = 3); **p < 0.01 vs 0 μM. Control: SU9516 (-).

ratio slightly increased in these SU9516-supplemented C2C12 at 10 μM (**Fig. 2.3B**). However, at over 40 μM, the myogenic differentiation was inferior (**Fig. 2.3C**). Therefore, concentration of 10 μM SU9516 was used for further experiments.

2.3.3 Combined effects of Matrigel and SU9516 supplementation on myogenic differentiation

The combined effects of Matrigel with SU9516 supplementation on differentiated C2C12 cells were investigated (**Fig. 2.4**). The sole SU9516 supplementation and that with a Matrigel-coated surface induced hypertrophy whereas, SU9516 supplementation with a Laminin-coated surface did not significantly alter myotube width (**Fig. 2.4A, B**). SU9516 supplementation increased the differentiation ratio, independent of the ECM-coated surfaces (**Fig. 2.4C**). Nuclear number analysis revealed that the frequency of the nuclei in large myotubes (with > 20 nuclei) was significantly higher in SU9516-supplemented cells compared with control cells, suggesting that SU9516 is involved in myonuclear accretion which promotes myotube formation (**Fig. 2.4D**). Therefore, when C2C12 cells were cultured with Matrigel-coated surfaces in differentiation medium containing SU9516, further enhancement of myotube hypertrophy was successfully achieved (**Fig. 2.4**).

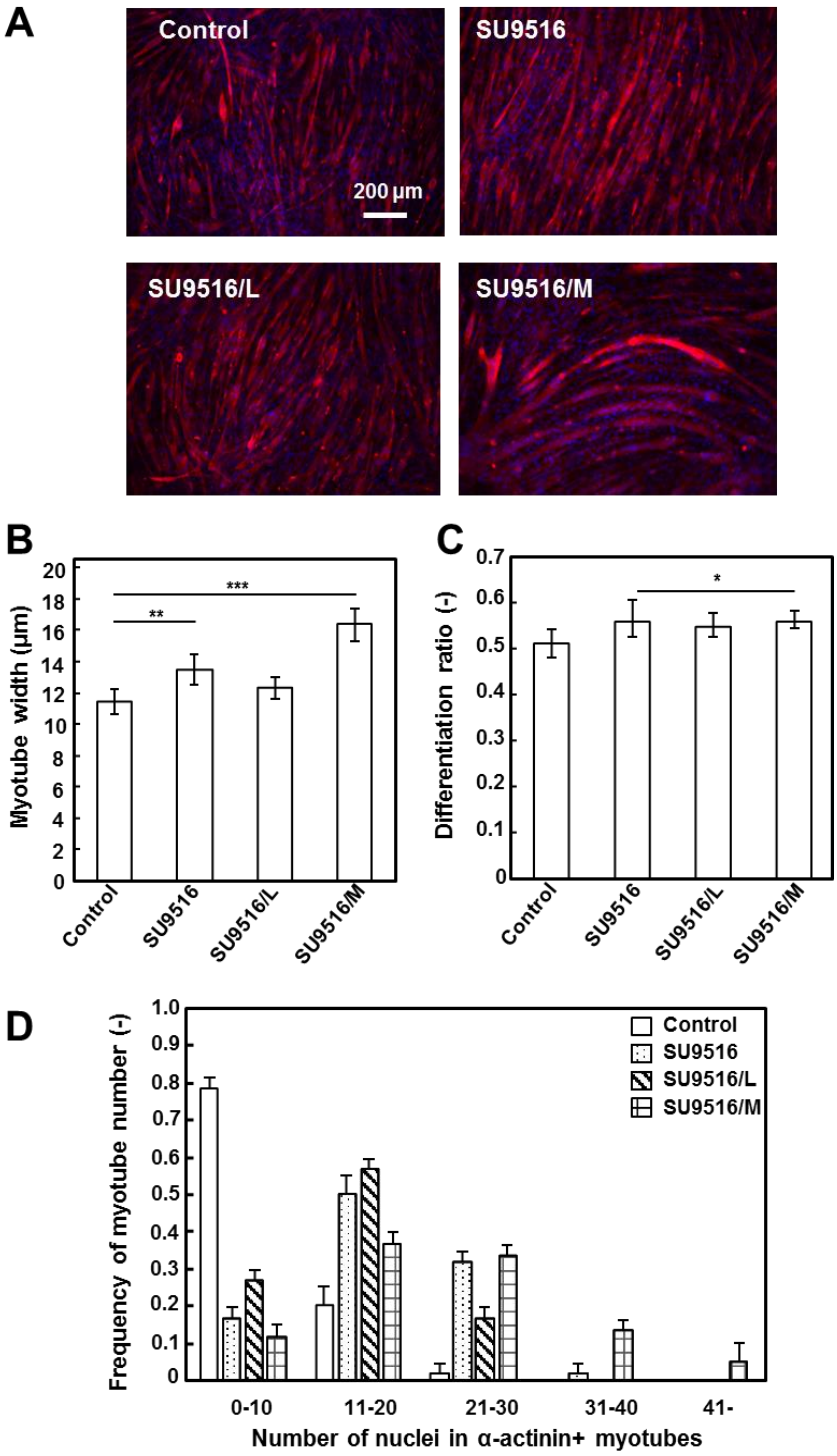

Fig. 2.4 Combined effect of Matrigel and SU9516 supplementation on myogenic differentiation. (**A**) Fluorescence microscopic images of myotubes on day7. Red, α-actinin-positive myotubes; blue, DAPI-stained nuclei. (**B, C**) Quantitative image analysis of myotube width (**B**) and differentiation ratio (**C**) on day7 data are expressed as mean ± SD (n = 3); *p < 0.05, **p < 0.01, ***p < 0.001. (**D**) Fraction of total myotubes with number of nuclei in α-actinin-positive myotubes. Data are the means and SD of triplicate experiments. Control: Polystyrene surface/SU9516 (-); SU9516/L: Laminin coated surface/SU9516 (+); SU9516/M: Matrigel coated surface/SU9516 (+).

2.3.4 The expression of relative mRNA

To further explore the role of interactions between cell-cell and cell-ECM in myogenic cells, C2C12 myoblasts keep differentiating on PS surface and Matrigel coated surface were treated with or without SU9516 before being processed for RT-PCR analysis. Figure 2.5A showed that SU9516 addition to myogenic differentiation culture induced gene expression of M-cadherin. In addition, as shown in Figure 2.5B and 2.5C, marked increment of integrin α7 and integrin β1 accumulations were observed in SU9516 treated cells, especially in the combination of Matrigel and SU9516 condition.

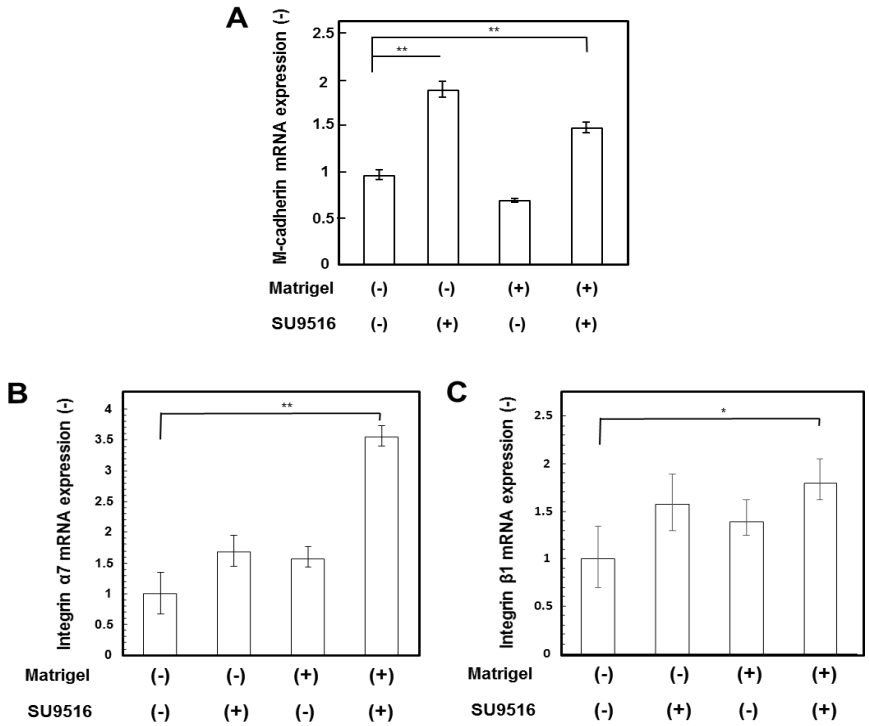

Fig. 2.5 Gene expression levels in C2C12 cells treated with or without Matrigel and/or SU9516. (**A**) M-cadherin, (**B**) Integrin α7, (**C**) Integrin β1.

2.3.5 Effect of Matrigel and SU9516 supplementation on artificial skeletal muscle tissue constructs

Artificial skeletal muscle tissue constructs were constructed on the microdevices for measuring muscle contractile forces. The procedure for their construction is shown in Figure 2.6A. Next, the effects of Matrigel and SU9516 on artificial skeletal muscle tissue constructs and its functions were investigated. When Matrigel and/or SU9516 were added to the medium, the artificial skeletal muscle tissue constructs showed an increase in width (**Fig. 2.6B**). In addition, the width of artificial muscle tissue constructs in cultures with Matrigel and SU9516, was evidently larger than other tissue culture conditions (**Fig. 2.6B**). The histology of the artificial skeletal muscle tissue constructs after 8 days of culture in differentiation medium with Matrigel and/or SU9516 is shown in Figure 2.7A. On the control culture (without Matrigel and SU9516) cells, indiscernible striation patterns and myotubes were not detected with a striation pattern consisting of sarcomeric α-actinin. Culturing with Matrigel or SU9516-supplemented cultures induced a discernible striation pattern. Moreover, adding the Matrigel and SU9516, clearly induced the formation of striation patterns in the myotubes.

For investigation of contractile force generation, the artificial skeletal muscle tissue constructs were stimulated by electrical pulses. The contractile force generation in response to electrical pulse stimulation at 20 V with 2 ms pulse width at 30 Hz is shown in Figure 2.7B. The artificial skeletal muscle tissue constructs under the Matrigel and SU9516 supplementation produced significantly higher physical force compared with artificial skeletal muscle tissue constructs under the control culture. On the contrary, artificial skeletal muscle tissue constructs under the Matrigel or SU9516 supplementation produced low

physical force. These results indicated that Matrigel or SU9516 supplementation moderately enhanced the contract force. Furthermore, the combined supplementation of Matrigel and SU9516 successfully enhanced the physical force generation of artificial skeletal muscles, significantly higher than control cultures.

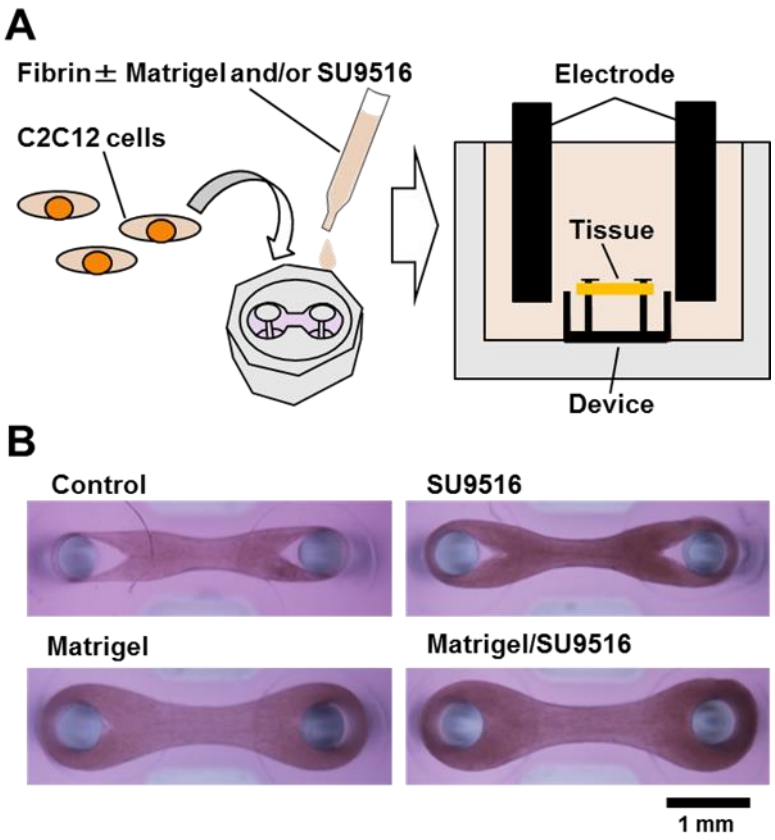

Fig. 2.6 Combined effects of Matrigel and SU9516 supplementation on artificial skeletal muscle tissues. (**A**) Schematic diagram of artificial skeletal muscle tissue construction and electrical stimulation. (**B**) Top view image of an artificial skeletal muscle tissue cultured on the device, supplemented with or without Matrigel and SU9516. The scale bar reads 1 mm. Control: Matrigel (-)/SU9516 (-).

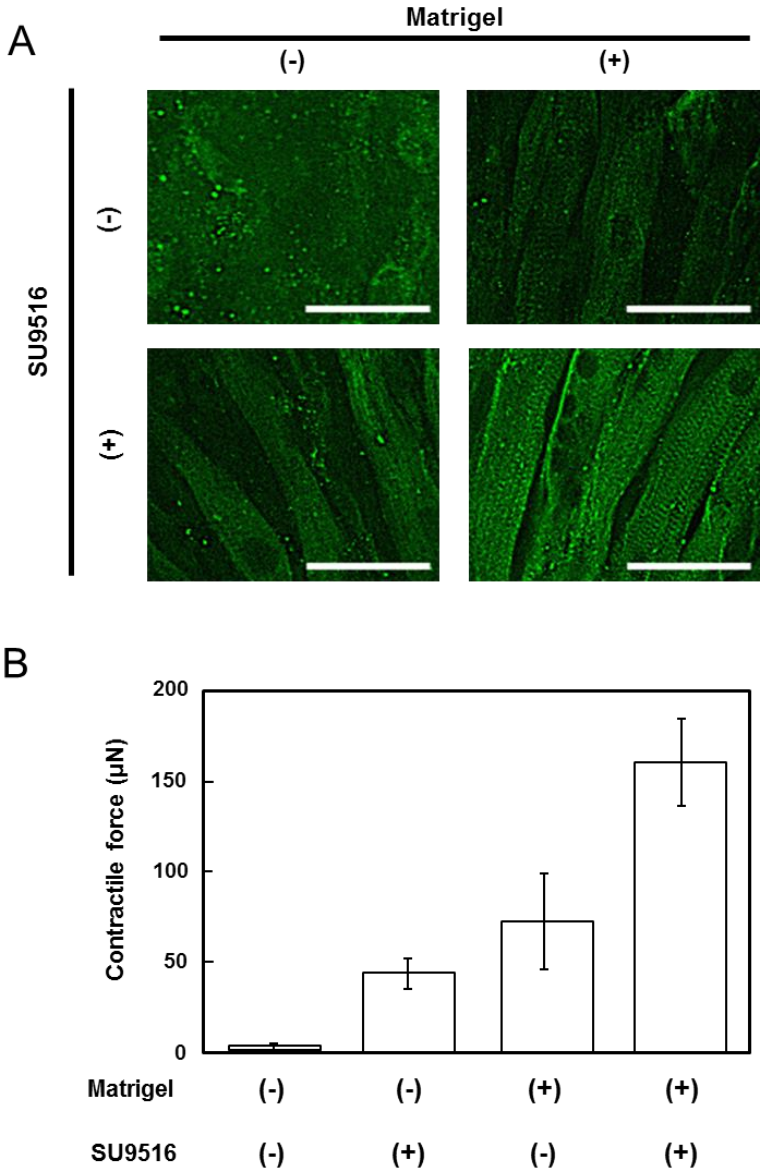

Fig. 2.7 Effects of adding C2C12 cells together with Matrigel and SU9516 on the contractile force of myotubes. (**A**) Representative fluorescence microscopic images of myotubes. After differentiation, the artificial skeletal muscle tissues were fixed and then stained with α-actinin antibody and Alexa488-conjugated secondary antibody. Scale bars: 50 μm. (**B**) Contractile forces of artificial skeletal muscle tissues; data are expressed as mean ± SD (n = 3).

2.4 Discussion

This chapter demonstrated that the physical force generation of artificial skeletal muscle tissues could be improved by use of combination of SU9516 and Matrigel, which contribute to the creation of a suitable myogenic environment *in vitro*. For drug screening and testing, the use of established cell lines, such as mouse C2C12 cells provide reproducible and repeatable systems, which resulted in the reduction of animal studies. Furthermore, drug screening using artificial skeletal muscle tissue would contribute as a useful pre-screening method before animal studies that could reduce the cost, time and the number of animals killed. The interaction of cell-ECM regulates cell behaviors be acting as a signal mediator (58). Level of integrins, such as $\alpha_7\beta_1$, $\alpha_9\beta_1$ and $\alpha_5\beta_1$, which are thought to be related to myogenic differentiation, were upregulated by supplementation of cytokines (78, 80, 81). Among them, $\alpha_7\beta_1$ is known to play an important role in myoblast differentiation (82), and it has been reported that enhancement of $\alpha_7\beta_1$ integrin expression ameliorated muscular pathology and improved muscle function in mouse model (78). It could be hypothesized that the combination of increasing expression of integrin $\alpha_7\beta_1$ and optimization of ECM protein might further induce skeletal muscle hypertrophy. Therefore, chapter 2 focused on the effects of ECM proteins and integrin expression level on the behavior of C2C12 cells in differentiation processes including cell migration, proliferation, differentiation and fusion into myotubes *in vitro*. To prove our hypothesis, C2C12 cells were cultured on various ECM substrata. However, C2C12 cells neither enhanced proliferation, differentiation ratio nor myotube width during differentiation culture (**Fig. 2.1, 2.2**), suggesting that effect of ECM on cellular behaviors are multifaced, and that ECM alone is not enough to induced skeletal

muscle hypertrophy. Ito et al. (83) reported that the migration speed of C2C12 cells was not increased by cultivation on the ECM-coated surfaces *in vitro* (83). In conflict to their report, the migration speed of C2C12 cells cultured on various ECM-coated surfaces was increased in this study. The mechanism of this difference is still unknown, but it may relate to the expression of integrin induced by ECM proteins on the culture surfaces. Ito et al. used ECM pre-coated tissue culture vessels to investigate for effect of the ECM proteins. The concentration of ECM proteins on the culture surface may the behavior of C2C12 cells, and the regulation of cell attachment on the culture surface, mainly through modulation of integrin expression and it may enhance of the migration speed. It has reported that C2C12 cells express various types of integrins (84). However, only Matrigel-coated surface and no other individual ECM-coated surfaces including Fibronectin, Laminin, Collagen 1, Collagen 4, enhanced or maintained the migration, proliferation, myotube length, myotube width, and differentiation of C2C12 cells (**Fig. 2.1, 2.2**). Matrigel consists of basement membrane extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. The major component of basement membrane is Laminin, and it includes Fibronectin, Collagen 4, Entactin, Heparin sulfate proteoglycans and Nidogen (85). Lee et al. (86) reported that co-immobilization of Fibronectin and Laminin induced enhancement of myogenic differentiation of C2C12 cells compared to single immobilization of either Fibronectin or Laminin alone. On the contrary, our data showed that C2C12 cells cultured on Matrigel did not significantly enhance the differentiation. However, the mechanism underlying the effect of Matrigel on the C2C12 cell behaviors remains to be elucidated. The relationship between ECM proteins and cell behaviors is complicated, and further studies need to clarify the mechanism.

It has been reported that SU9516 increased the expression levels of $\alpha_7\beta_1$ integrin in C2C12

cells. Additionally, they showed that SU9516 promotes myofiber regeneration through facilitated expression and activation of $\alpha_7\beta_1$ integrin (78). In this study, SU9516 supplementation increased not only expression of $\alpha_7\beta_1$ integrin but also myotube width and differentiation ratio during differentiation of C2C12 cells (**Fig. 2.3, Fig. 2.5**). Although the mechanism remains to be clarified, it was reported that commitment to muscle differentiation and prevention of apoptosis require activation of signal transduction pathways, including cell-cell interaction molecules. Notably, M-cadherin markedly regulated myoblast interaction and plays an important role in terminal differentiation of skeletal muscle cells (87). This chapter showed that SU9516 addition to myogenic differentiation culture induced gene expression of M-cadherin (**Fig. 2.5**). However, differentiated skeletal muscle cells cultured with SU9516 increased myotube width, whereas enhancement of myogenic differentiation ratio was slightly lowered (**Fig. 2.3**). This may be attributable to the increased cell adhesion via M-cadherin as a consequence of SU9516 supplementation, and myotube formation was promoted but the M-cadherin expression levels did not suffice for myogenic differentiation. Interestingly, when C2C12 cells were cultured on Matrigel-coated surfaces with SU9516, enhancement of myotube width were further observed. (**Fig. 2.4**). Although the Laminin is a major component in the Matrigel, the myotube width were not dramatically enhanced when C2C12 cells were cultured on Laminin-coated surfaces with SU9516. This result indicated that not only Laminin but also other component (e.g. Collagen, Fibronectin) as a Matrigel is important for myogenic differentiation. Furthermore, cells maintain their homeostasis by maintaining a balance between cell-cell interaction and cell-substrate interaction; thus, the combined treatment of Matrigel with SU9516 may suitably regulate this balance during myotube construction. These results prompted us to combine Matrigel with SU9516

supplementation for construction process of the artificial skeletal muscle tissue (**Fig. 2.6, 2.7**).

Active tension generation capability is extremely important for drug screening based on contractility data, because it demonstrates both the microenvironments including ECM and the differentiation levels including the myogenic modulatory factors, and contractile proteins. However, there have been very few quantitative analyses to clarify the contractile properties of artificial muscle tissues. In the present study, the contractile force generated by the artificial skeletal muscle tissues was measured to clarify the effect of ECM and SU9516 supplementation on active tension generation capability. When artificial skeletal muscle tissues were cultured on Matrigel with SU9516 supplementation, substantial contractile force generation was observed (**Fig. 2.7B**). Although the effect of Matrigel with SU9516 supplementation on the myotube width was significant, the differentiation ratio was insignificant (**Fig. 2.4C**). The contractile force is associate with myogenic fusion as well as myogenic differentiation. However, it can be difficult to explain that these enhancements of contractile force were only caused by myotube width changes. This difference may be attributable to the culture niche during differentiation phase of C2C12 cells. Recent studies report that the niche cues can fundamentally alter the cell fate (88). On 2D culture, the Matrigel and SU9516 supplementation induced the expression of $\alpha_7\beta_1$ integrin and M-cadherin (**Fig. 2.5**). These adhesion proteins have been reported to perform the important roles on myogenic behaviors, as described above. Similarly, it is considered that these adhesion proteins were induced by Matrigel and SU9516 supplementation on 3D culture condition. Furthermore, the C2C12 cells cultured in 3D culture, more cells might be adhered to each other because the cells were spatially arranged. In fact, Grabowska *et al*. reported that

C2C12 cells cultured on 3D condition showed higher expression of adhesion proteins than the C2C12 cells cultured on monolayer condition (89). The enhancement of adhesion proteins may affect in the promoting of differentiation of C2C12 cells. Construction of artificial skeletal muscle tissue involves cells being embedded in fibrin gel, adopted to maintain the bundle shapes containing aligned myotubes. This construction procedure with fibrin gel is one of standard methods for tissue engineering. Hinds *et al* (90) showed that the artificial skeletal muscle tissues with fibrin gel significantly improved contractile function. However, the artificial skeletal muscle tissues constructed using fibrin gel alone generated lower physical force and sarcomere structures were not observed. This indicates that Matrigel and SU9516 play an important role in differentiation of C2C12 cells in the artificial skeletal muscle tissues. In this study, SU9516 supplementation resulted in improved contractile force generation of artificial skeletal muscle tissue, and the combined treatment of Matrigel with SU9516 supplementation, significantly improved the active tension generation compared to the control tissue cultures (**Fig. 2.7B**). These results indicated that regulation of cell-ECM interaction of tissue-engineered skeletal muscle enhanced contractile force generation.

In conclusion, this study demonstrated that a combination of Matrigel and SU9516 supplementation enhanced the skeletal muscle hypertrophy and improved active tension generated by artificial skeletal muscle tissues. These findings indicate the impact of tissue culture using Matrigel and SU9516 supplementation on engineered skeletal muscle formation and function.

Chapter 3: Self-organization of mouse myoblast cells mediated by soft substrates and improvement of myotube formations in artificial skeletal muscle

3.1 Introduction

For some diseases, 2D cell culture model is not enough for clinical trials to simulate the disease environment *in vivo*. In order to meet the requirements of muscle function level for the drug development, various 3D muscle tissue models with histological feature have been proposed in recent years, which mimicked the environment *in vivo* and participated in normal functional response in stereoscopic structure (91, 92). However, cell differentiation and maturation on these artificial tissues are not enough for the muscle tissue model, and the construction procedures are also cumbersome.

In drug developmental field, skeletal muscle cells in two-dimensional (2D) culture are used to evaluate the drug efficacy as a cell assay (93). In general, to assess the skeletal muscle, not only the differentiation ratio and myotube structure such as the sarcomere should be analyzed, the gene and protein expression are also as evaluation indexes. However, these evaluation indexes are not sufficient to reflect drug efficacy *in vivo* skeletal muscle, mainly due to the difference in the cell microenvironment. One of the fundamental functions of the muscle is the contractile force that also reflects these various indexes such as gene expression and protein expression. Thus, contractile force is the most appropriate index to evaluate the drug efficacy. Although the contractile force can be measured in two-dimensional culture condition, it is desirable to measure the contractile force of tissue-engineered skeletal muscle tissue that is closer to the native condition. In fact, Ikeda et al. showed that the epigenetic drug promotes

myogenic differentiation of mouse C2C12 myoblast cells in two-dimensional culture conditions, but this differentiation ratio was not correlated with contractile force of tissue-engineered skeletal muscle tissues which prepared in three-dimensional culture conditions (94).

In recent years, tissue-engineered skeletal muscle construction for contractile force evaluation have been reported which using primary muscle myoblasts (95), myoblast cell lines (96), and inducted myogenic cells from induced pluripotent stem cells (97). To construct these tissue-engineered skeletal muscle tissues, several researchers have applied decellularized scaffolds (98) and hydrogels such as fibrin gel (99). However, these methods are very troublesome, thus a method for constructing tissue more simply is required. Furthermore, in native skeletal muscle tissue *in vivo*, cell-cell interaction is one of the important factors to maintain the tissue function and homeostasis (100). Artificial materials such as scaffold and hydrogel may interfere with the cell-cell interaction, resulting in the low differentiation ratio, inhibition of myotube formation, and low contractile force. Consequently, in order to construct the functional tissue-engineered skeletal muscle model, it is important to construct a tissue in which no artificial materials are contained. In recent researches, organ buds have been established with soft substrates, and this phenomenon was dependent on substrate stiffness (101). This procedure is the most promising approach to construct the artificial tissues because the cells can migrate on their suitable. However, the contractile force from tissue-engineered skeletal muscle has been low compared with that *in vivo*. This may be explained by the function of artificial muscle tissue, which is decided by not only the cell differentiation and myotube fusion, but also myotube orientation. To induce myotube orientation, the artificial anchor points have been applied to mimic the tendon at the end of

the muscle bundle and regulated the orientation of internal myotubes in 3D structure (102). In short, controlling of myotube formation and orientation is a prerequisite for the ideal muscle contraction force.

As another problem, low myogenic differentiation in the tissue-engineered skeletal muscles is serious. Different from smooth muscle, skeletal muscle is composed of multi-nucleated striated muscle fibers, in which the clear expression of sarcomere structure is the sign of mature skeletal muscle. Ito et al, reported that most differentiated cells are observed at the periphery of the tissue-engineered skeletal muscle constructs (103). They discussed that this reason may be attributed to the distribution of oxygen, nutrient and extracellular matrix (ECM). BMP signaling has an inhibitory effect on the formation of multi-nucleated myotubes, especially in the late stage of differentiation. It inhibits the transcriptional activities of myogenic marker proteins such as MyoD and Myogenin to control premature myogenic differentiation (104, 105). Consequently, the activation of the signaling pathway on C2C12 cells induces the Id1 expression and the osteoblast differentiation and inhibits the myogenic differentiation (106). BMP inhibitors such as Dorsomorphin and LDN-193189 not only block the intracellular Smad 1/5/8 proteins activation but also accelerate terminal differentiation and facilitate the functional myotube generation (107, 108). Furthermore, several studies indicated that these inhibitors can affect non-Smad signaling, such as P38-MAPK, ERK and PI3K/Akt, and establish the crosstalk between different signaling pathways (109-111). Thus, this established crosstalk promoted the myotube differentiation and maturation (112).

In this chapter, the author purposed to develop a novel procedure for the construction of functional artificial skeletal muscle tissues based on regulation of cell-substrate interaction by using soft substrate and the BMP inhibitor.

3.2 Materials and methods

3.2.1 Cell culture

Mouse C2C12 myoblast cells (ATCC, Manassas, USA) were maintained undifferentiated state in growth medium (GM) which composed of low-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS) and Penicillin-Streptomycin Mixed Solution (Nacalai Tesque, Kyoto, Japan). The cells were used within 10 passages. The C2C12 cells were induced to differentiate in differentiation medium (DM) which composed of high-glucose DMEM with 4% calf serum (CS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid (HEPES; DOJINDO, Kumamoto, Japan) and Penicillin-Streptomycin Mixed Solution (Nacalai Tesque, Kyoto, Japan).

3.2.2 Preparation of soft substrates

To induce the self-organization, soft substrates were prepared with polyacrylamide (PA) hydrogel according to the previously reports (113). Briefly, clean and dry circular cover glasses with diameters of 15-mm (Matsunami, Osaka, Japan) were treated with 3-methacryloxypropyltriethoxysilane (ShinEtsu, Tokyo, Japan) for 1 h. To remove the cover glasses easily, the slide glasses were treated with dichlorodimethylsilane (Nacalai Tesque) for 10 min. Cover glasses and slide glasses were washed with ultrapure water, wiped with ethanol, and dried. Then, the prepolymer solution was dropped on treated slide glass and cover glass was placed on top to sandwich the prepolymer solution. The prepared glasses were incubated at least for 30 min at room temperature. The substrate elasticity was regulated

by mixture ratio of acrylamide and bisacrylamide, and the quantitative values of elasticity were measured by atomic force microscope (Nanowizard 3, JPK Instruments, Berline, Germany) (**Table 3.1**). After gelation, the 15-mm cover glasses were removed from silane-coated slide glasses and put into 24-well plate (**Fig. 3.1**). These cover glasses were washed with 50 mM HEPES buffer and were added Sulfo-SANPAH solution. Then UV light (312 mm) irradiated to the cover glasses for 10 min.

Table 3.1. Modulus of Elasticity after Polymerization of Relative Acrylamide and Bis-Acrylamide Concentrations

Stiffness (kPa)	Acrylamide (ml)	Bis-acrylamide (ml)	Water (ml)
1	1.25	0.15	8.6
10	2.5	0.5	7
20	2	1.32	6.68
40	2	2.4	5.6

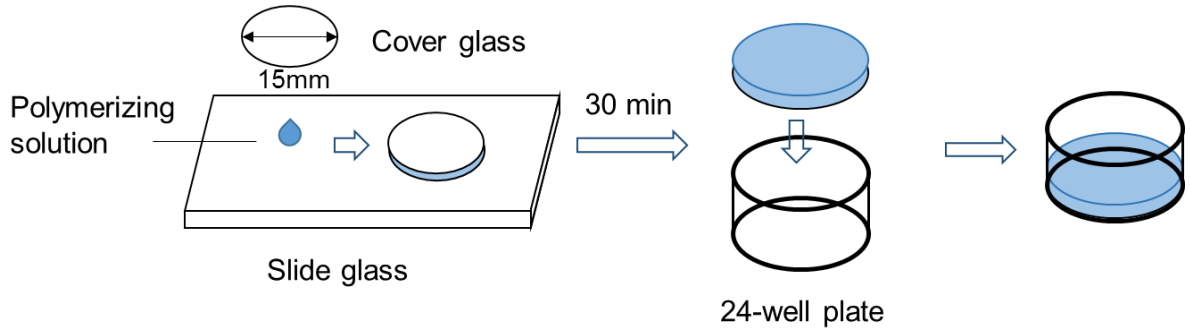

Fig. 3.1 The process of soft substrate construction

3.2.3 Induction of self-organization

For inducing self-organization, acrylamide substrates of various rigidity were prepared, and various extracellular matrix (ECM) components (1 μg/cm^2 Fibronectin, 1 μg/cm^2 Laminin, 1 μg/cm^2 Collagen 1, 1 μg/cm^2 Collagen 4 and 5 μl/cm^2 Matrigel) were coated. Next, predetermined number of cells (1×10^6 cells/well) were seeded on the acrylamide surfaces, and the cells were cultured to induce the self-organization for 72h. To determine the suitable seeding number for self-organization, C2C12 cells at different densities were seeded on acrylamide surfaces which were put into the wells of 24-well plates (Greiner Bio-one, Frickenhausen, Germany). Additionally, the contraction of C2C12 cells on acrylamide surfaces with growth medium (GM) or differentiation medium (DM) was evaluated by measuring their surface areas. Triplicate samples from culture of 1×10^6 cells/well were photographed at 10, 20, 25, 30, 45, 72, 168 and 240 h, and the area of the C2C12 cells was measured by using ImageJ software (NIH, Bethesda, MD, USA).

3.2.4 Construction of ring-shaped tissue

To construct the C2C12 cell rings, a polycarbonate plug (diameter, 5 mm) was positioned at the center of the well of 24-well acrylamide substrate plate. C2C12 cells (4×10^6 cells/well) were seeded into the gap between the well wall and polycarbonate plug. After cells seeding for 3 days, the ring-shaped tissue was completely constructed. The cellular ring was removed from the polycarbonate plug and inserted around two stainless steel pins as anchors (distance, 6 mm), which were pinned on a PDMS layer (SYLGRDR^{TM} 184 Silicone Elastomer; Dow Corning, Michigan, USA).

3.2.5 Immunofluorescence staining

The cells or thin sections (20 μm) were washed with phosphate-buffered saline (PBS), and then fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature followed by treating with Triton X-100 (MP Biomedicals) for permeabilization. The specimens of cells were blocked with Block Ace (Wako) for 90 min and then the blocked specimens were incubated with a primary antibody against myosin heavy chain (MHC; Sigma-Aldrich, St. Louis, Mo, USA) for 60 min. After washing with Tris-buffered saline (TBS), the specimens were immersed in PBS containing an Alexa Fluor 488-conjugated secondary antibody for 60 min. Also, cell nuclei were stained with 4′6-diamidino-2-2phenylindole (DAPI, Nacalai Tesque) for 20 min. After washing with PBS, the specimens were observed using BZ-X710 All-in-One fluorescence microscope (BZ-X710; Keyence, Osaka, Japan).

3.2.6 Measurement of the myotube width and differentiation ratio

The images of three fields in three separate samples were randomly captured using fluorescence microscope. The MHC-positive myotube width was measured using ImageJ software. The differentiation rate was calculated as follow: Differentiation rate = (number of DAPI-stained nuclei in MHC-positive myotubes in the field)/(number of DAPI-stained nuclei in the field).

3.2.7 BMP inhibitors treatment

C2C12 cells were exposed to various concentrations (0.01, 0.1, or 1 μM) of BMP inhibitors (LDN-193189(4-(6-(4-(piperazin-1-yl) phenyl)pyrazolo[1,5-a]pyrimidin-3-yl) quinolone (Stemgent) and Dorsomorphin (Calbiochem)) during the induction of myogenic differentiation. The following day, the differentiation ratio and myotube width were measured.

3.2.8 Quantitative real-time RT-PCR analysis

The mRNA in cells was extracted using RNAiso Plus (TakaRa bio, Shiga, Japan) 3 days and 7 days after self-organization, and mRNA was reacted of reverse transcription using First Strand cDNA Synthesis Kit (Rever Tra Ace-α: TOYOBO, Osaka, Japan). Then, DNA amplification by quantitative real-time polymerase chain reaction (PCR) was performed with StepOneTM Real-Time PCR system (Applied Biosystems, California, USA) by using SYBR Green Master Mix (Applied Biosystems) and gene-specific primers **(Table. 3.2)**.

Table 3.2. Primer sequences for real-time RT-PCR analysis

Target gene	Primer sequence
MyoD	FW: 5′- TACAGTGGCGACTCAGATGC-3′ RV: 5′ - TCACTGTAGTAGGCGGTGTC -3′
Myogenin	FW: 5′- ATGCACTGGAGTTCGGTC -3′ RV: 5′ - AGGCAACAGACATATCCTCC -3′
M-cadherin	FW: 5′- ATGTGCCACAGCCACATCG-3′ RV: 5′ -TCCATACATGTCCGCCAG-3′
GAPDH	FW: 5′- CTACCCCCAATGTGTCCGTC -3′ RV: 5′- GCTGTTGAAGTCGCAGGAGAC -3′

3.3 Results

3.3.1 Induction of self-organization

To induce the efficient self-organization, the effects of ECM and substrate stiffness on the cell layer peeling and shrinking were investigated. Compared with the control, cells on the soft substrates showed peeling and/or shrinking behaviors depending on the stiffness of the substrate, and formed clump-like constructs on several ECM conditions (**Fig. 3.2A**). However, almost conditions formed small and multiple clumps, only the combined substrate with Matrigel-coated and 1kPa promoted effective self-organization. Next, to estimate the cell seeding number for efficient self-organization, C2C12 cells were seeded into the wells of 24-well plate. Although the self-organization was successfully induced at soft substrates, while the clump formation was not efficient in all except for cells cultured on 1×10^6 cells/well and 1 kPa (**Fig. 3.2B, C**). To construct a tissue with larger size, the cultivation area was expanded to examine whether self-organization could be induced. As a result, the self-organization was not induced at above 6-well plate (**Fig. 3.3**).

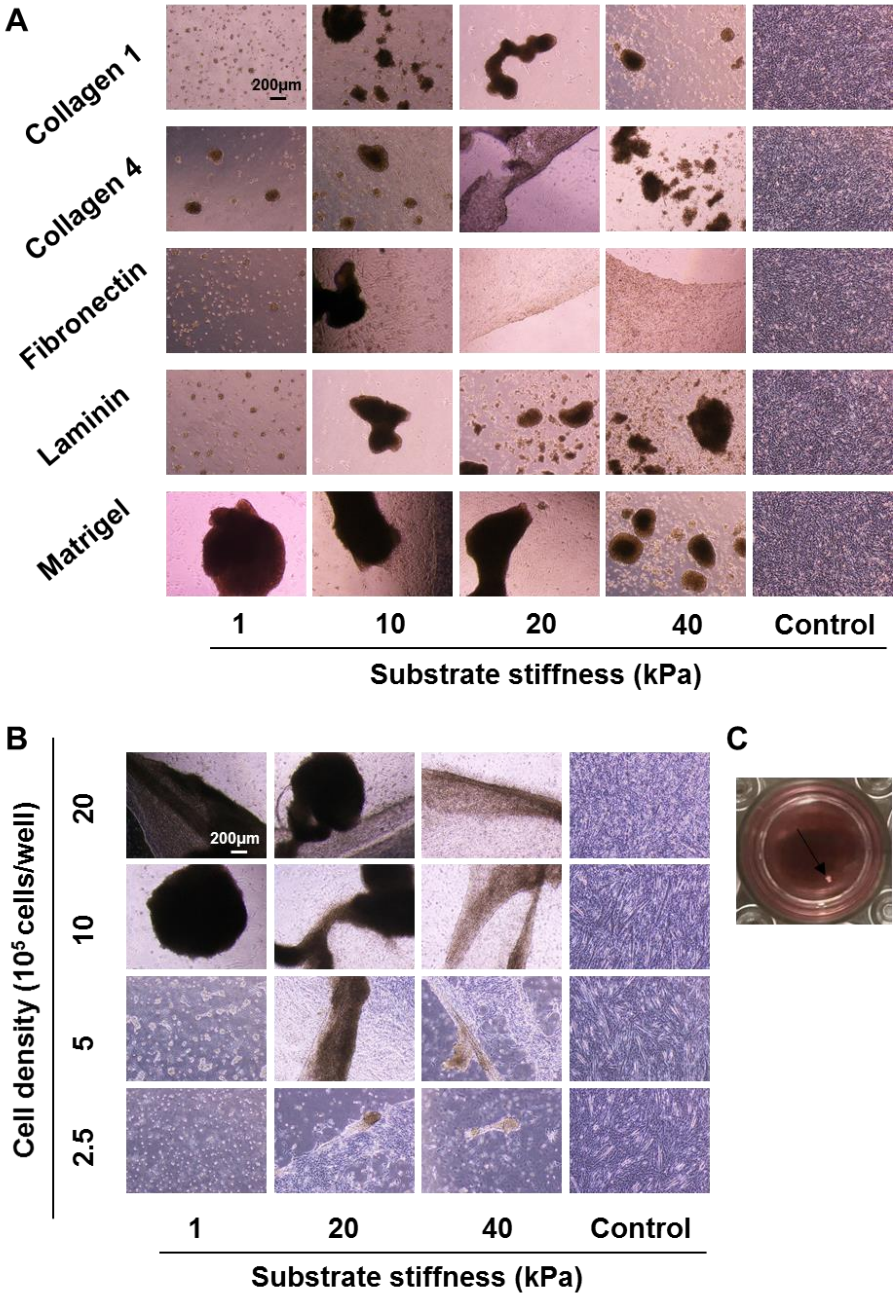

Fig. 3.2 The effects of cell microenvironment on self-organization. (**A**) Cells grown on soft ECMs coats with stiffness ranging from 1 to 40kPa, the changes of cell layers after 72h of differentiation culture. (**B**) Cells were seeded with different density on soft Matrigel coat with stiffness of 1, 20, 40kPa, the changes of cell layers after 72h of differentiation culture. (**C**) The tissue indicated by black arrow formed at 1×106 cells/well on 1kPa Matrigel coat after 7 days differentiation culture. Control: Polystyrene surface.

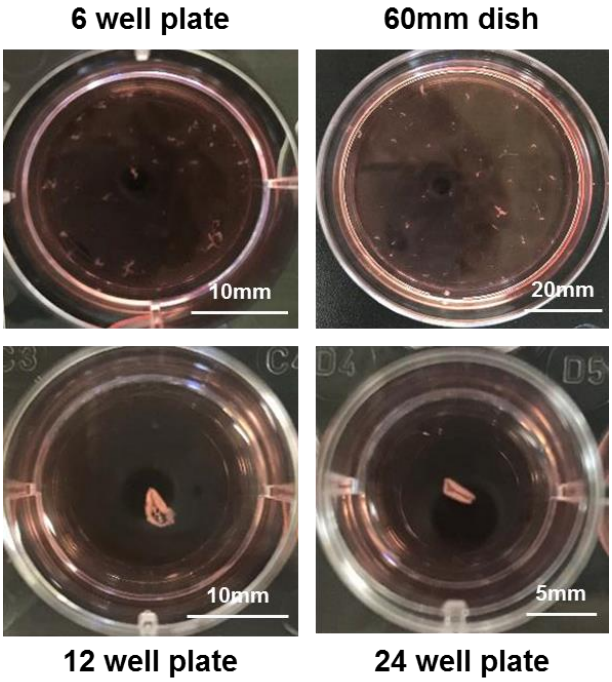

Fig. 3.3 The effects of cultivation area on self-organization. The C2C12 cells were inoculated on 60mm dish, 6-well plate, 12-well plate and 24-well plate at a density of 5×105 cells/cm2.

During self-organization, medium is an important factor to induce efficient contraction. As shown in Figure 3.4, C2C12 cells started contraction within several hours, and the areas of cell layers cultured with growth medium (GM) or differentiation medium (DM) were rapidly reduced until 45 h. Although the initial contraction speed of cell layers with GM was slightly high, they reached to the same areas at 45 h. Therefore, in subsequent experiments, the DM was used in self-organization to induce the cell differentiation as soon as possible.

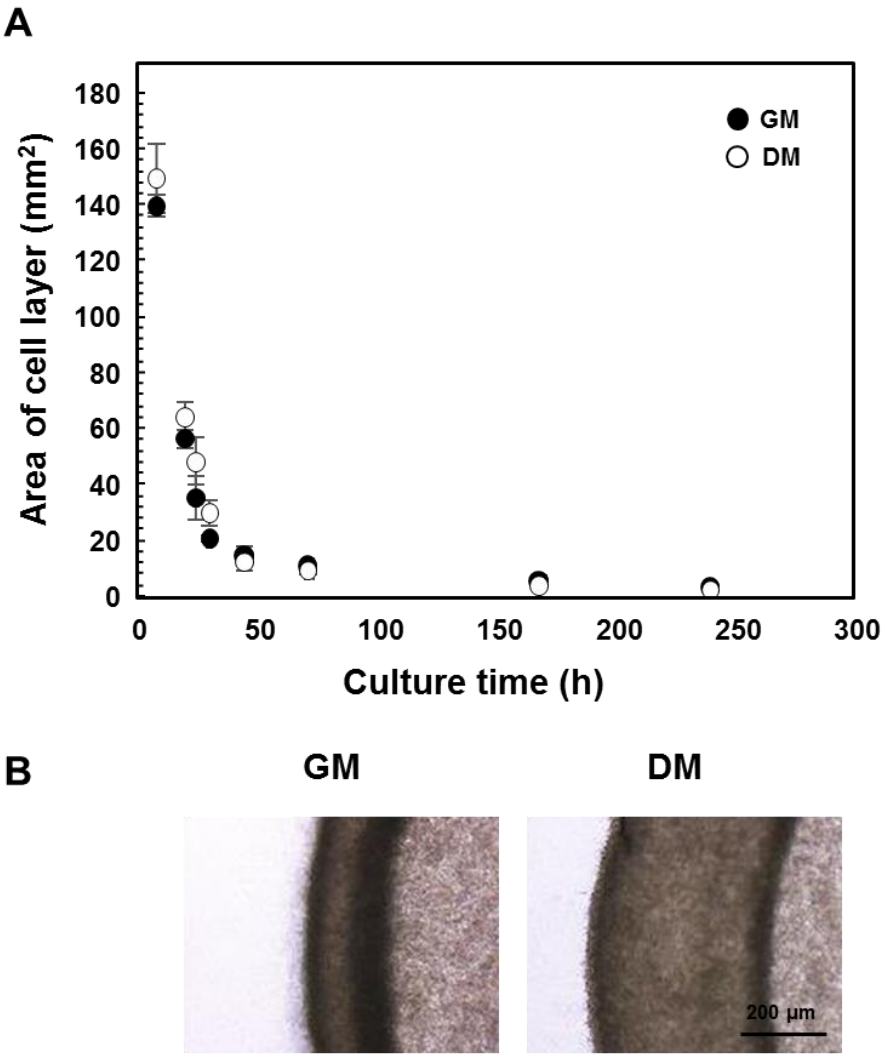

Fig. 3.4 The effect of medium on contraction of cell layers. (**A**) Time course of the area of cell layers in different mediums (top view). (**B**) The cell layers show contraction edges with different thickness in different mediums. GM: growth medium; DM: differentiation medium.

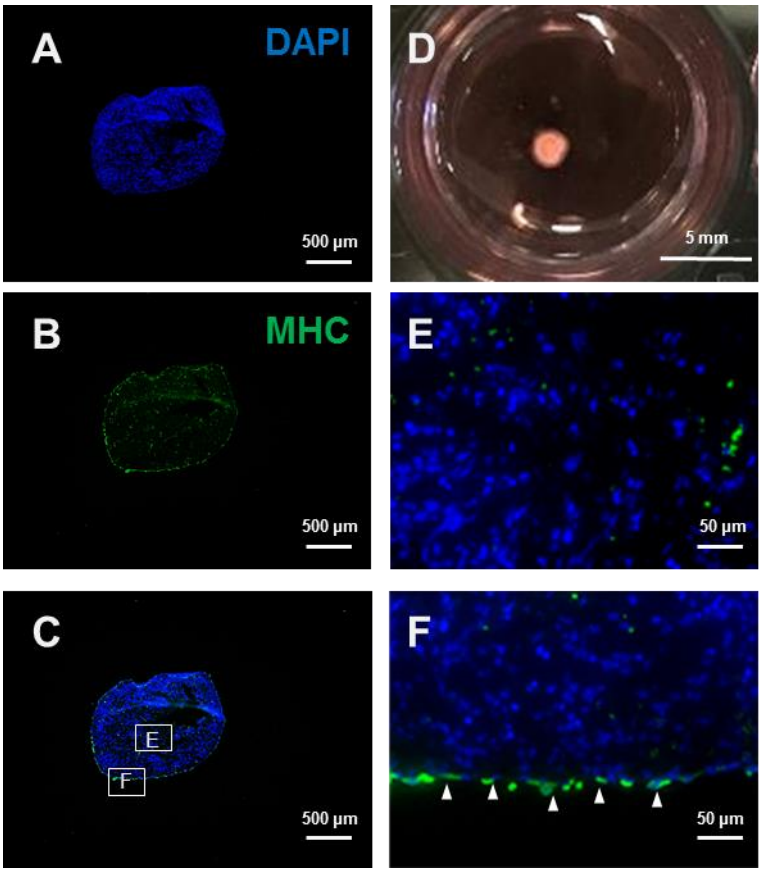

Fig. 3.5 Immunostaining and morphological characterization in 3D culture. (**D**) Spherical structure formed on Matrigel and soft substrate with 1kPa after 7days of differentiation culture. (**A-C**) Staining of the spherical tissues sections. Scale bar = 500μm. (**E, F**) The distribution of differentiated cells in the center (**E**) and the edge of section (**F**). Scale bar = 200μm. The Myosin heavy chain (MHC, green) and DAPI (blue) were used in the experiment.

The morphological characteristics and immunofluorescence stained section of the self-organized tissue after cultivation for 7 days are shown in Figure 3.5. The uniform cell distribution were observed in the tissue. While, myogenic differentiation marker MHC-positive cells almost located at the peripheral region of the tissue. These results indicated that this procedure is insufficient for myogenic differentiation despite induction of

efficient self-organization.

3.3.2 Effects of BMP inhibitors on myogenesis

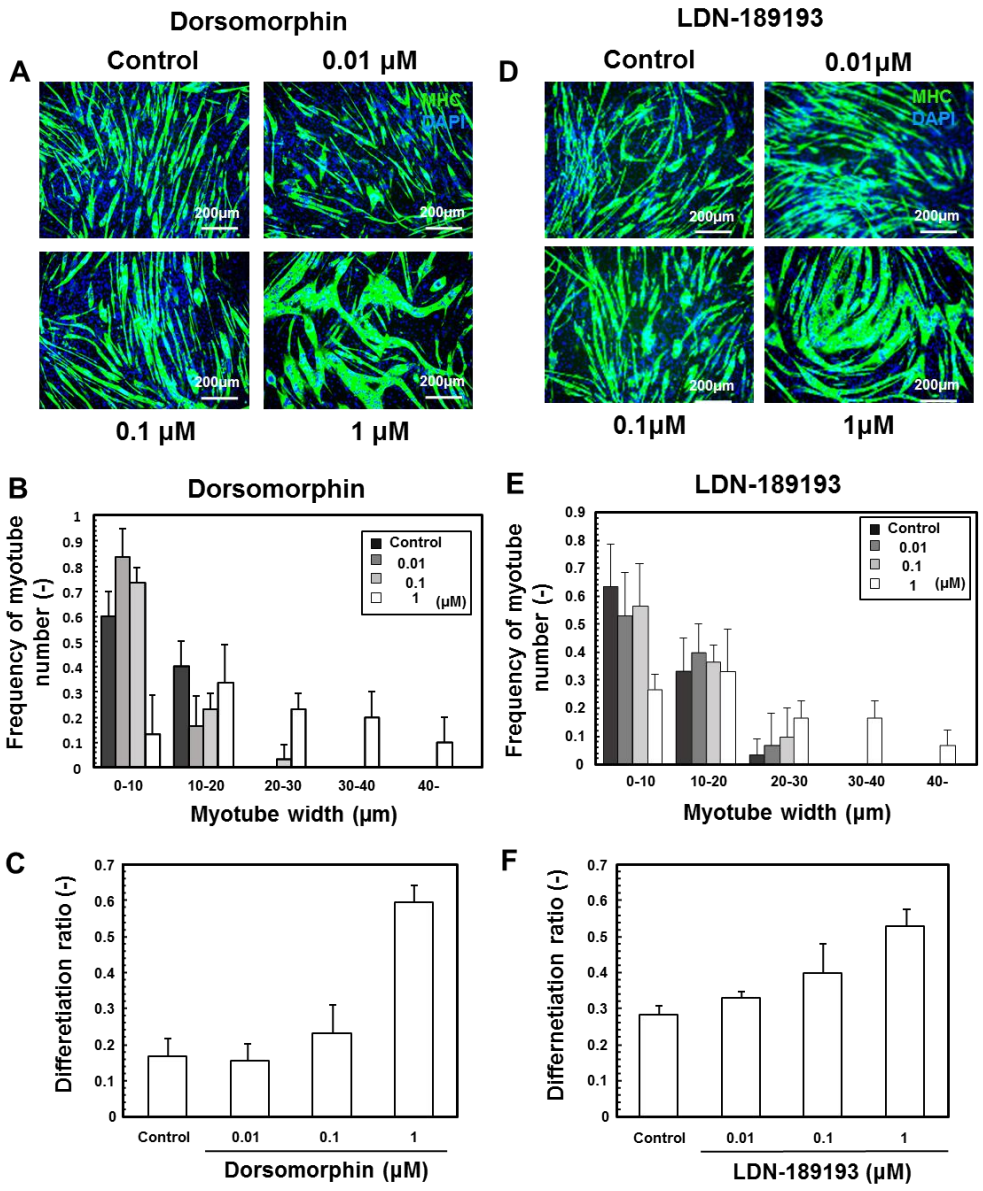

Fig. 3.6 Effects of BMP inhibitor treatments on the myogenesis in 2D culture. Distribution of myotubes after 7 days of differentiation culture in different concentration of BMP inhibitor treatments. (**A-C**) Dorsomorphin, (**D-F**) LDN-189193, Scale bar = 200μm. Control: Dorsomorphin (-) or LDN-189193 (-).

To enhance myogenic differentiation, the effects of different concentration of BMP inhibitors (Dorsomorphin, LDN-193189) on C2C12 cell differentiation and myotube formation were determined. When these BMP inhibitors were added to the differentiation medium at 1 μM, myotube hypertrophy was induced (**Fig. 3.6A, D**). Quantitative analysis revealed that the frequency of larger myotube number (with > 30 μm) in the condition supplemented with 1 μM BMP inhibitors was significantly higher than that in control condition (**Fig. 3.6B, E**). Furthermore, the C2C12 cells cultured with the BMP inhibitors at 1 μM significantly improved the differentiation ratio (**Fig. 3.6C, F**). Therefore, concentration of 1 μM BMP inhibitors were used for further experiments.

3.3.3 Construction and differentiation of artificial skeletal muscle rings

As shown in Fig. 3.5, the self-organized clump like tissue was insufficient for myogenic differentiation. In the living body, the muscle tissues are anchored to bone by tendons, the myogenic differentiation may be enhanced by not only soluble factors but also physical tension. Therefore, the ring-shaped tissue was developed by using self-organization and artificial tendons were constructed by using pins to mimic the native muscle. The procedure for construction of their shape is shown in Figure 3.7A. On day 7, there were not obvious differences in the bundle-shaped tissues between control and BMP inhibitor supplementation (**Fig. 3.7B**). However, compared with the control, immunofluorescence results showed that the cells which cultured with BMP inhibitors dramatically fused and formed larger myotubes in tissues **(Fig. 3.7C, D**). Therefore, when the artificial skeletal muscle tissue were cultured in DM which containing BMP inhibitor, myotube hypertrophy was successfully achieved in the

tissue.

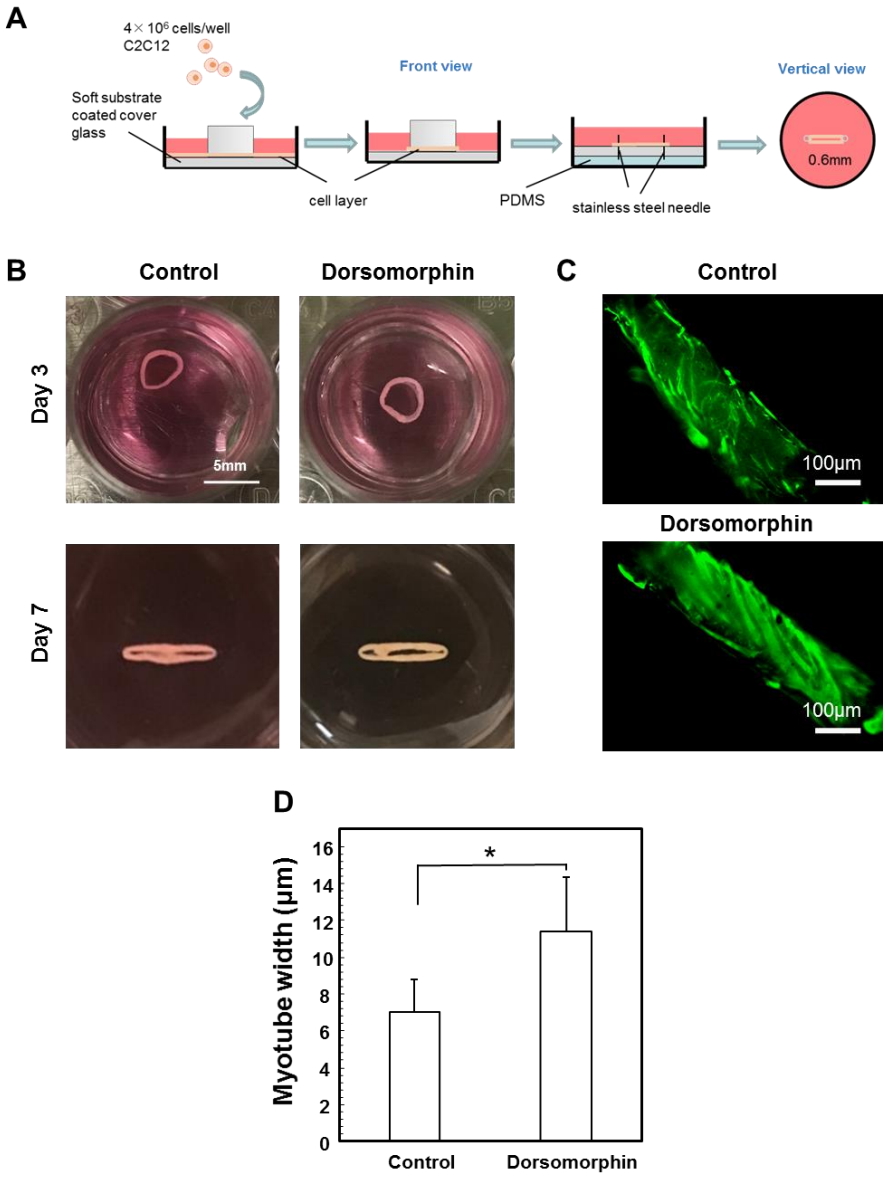

Fig. 3.7. Construction of artificial skeletal muscle tissues with tendons and histology analyses. (**A**) Schematic diagram of bundle tissue production. (**B**) After cells seeding for 3 days cellular ring was assessed and completely anchored into muscle bundles on day7. (**C**) Myosin heavy chain (MHC, green) staining of self-assembled tissue with or without BMP inhibitor. Scale bar = 100μm. (**D**)Quantitative analyses of myotube width in bundle tissue. *P < 0.05 relative to control group. Control: Dorsomorphin (-).

To analysis the effect of BMP inhibitor supplementation on the C2C12 cell differentiation, the gene expression levels of myogenic markers were evaluated (**Fig. 3.8**). On day 7, C2C12 cells under the BMP inhibitor supplementation significantly increased gene expression levels of myogenic markers compared with C2C12 cells under the control culture. Furthermore, both MyoD and Myogenin levels were increased by BMP inhibitor supplementation on day 3, suggesting that BMP inhibitor promoted myogenic differentiation from the early phase of differentiation. Taken together, the BMP inhibitor supplementation was successfully achieved to induce the C2C12 differentiation and hypertrophy in the self-organized muscle tissue.

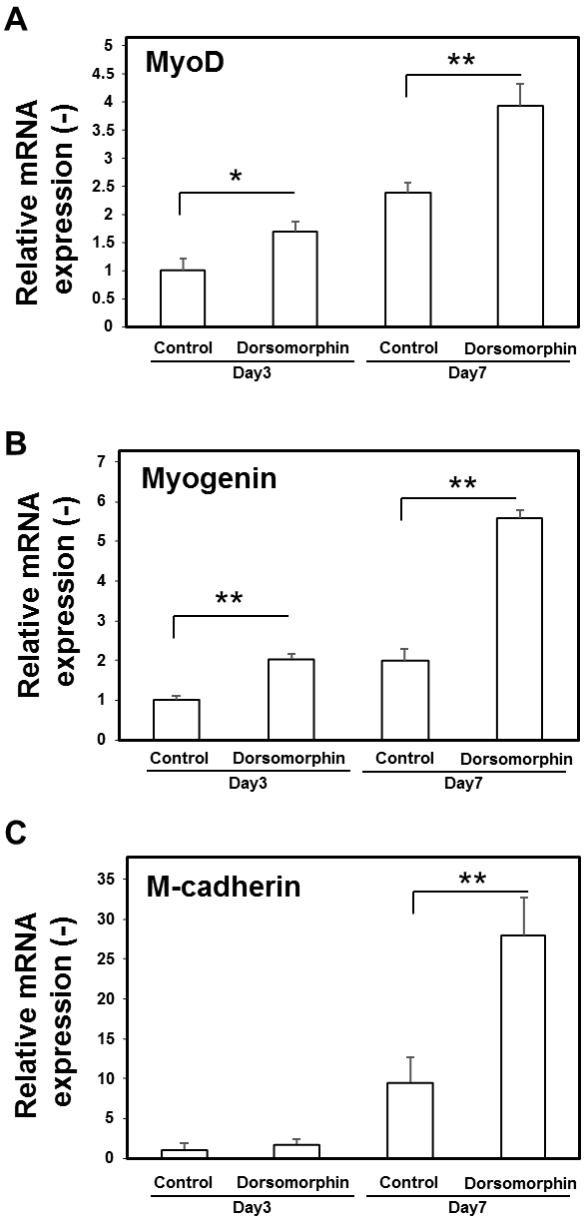

Fig. 3.8 The expressions of Myogenic marker genes in artificial skeletal muscle tissues after 3 days and 7 days of inducing differentiation (with or without BMP inhibitor) were analyzed by RT-PCR. (**A**) MyoD (**B**) Myogenin (**C**) M-cadherin. *P < 0.05, **P < 0.01 relative to control groups at same day. Control: Dorsomorphin (-).

3.4 Discussion

ECM plays an important role in cell maturation and myofiber formation. To construct the artificial skeletal muscle tissue that close to living body, several studies have been reported to mimic the microenvironment in the past few years (114, 115, 116). This study purposed a novel method that induced self-organization by using soft substrates and BMP inhibitor supplementation. In the cell microenvironment, cadherin as an adhesion factor mainly maintains the intercellular force, supports the cell-cell junction, signal transduction and arrangement of mature myotube. Additionally, the cell-substrate interaction induces the activation of integrin-dependent signaling pathway. Integrin locates at the cell membrane and anchors to the ECM, regulates cell adhesion, extension and migration (117-122). Matrigel as the analogue of ECM contains some basement membrane proteins that bind to C2C12 cell, and preserve many kinds of growth factors which indirectly regulate the cell-cell and cell-external environment (123, 124). *In vivo*, skeletal muscle tissues are surrounded by ECMs, which exhibit different elasticity in gastrocnemius and tendons (125). The substrate stiffness can affect the morphology of cell pseudopodia in a 2D environment. On the soft culture surface, cells easily adhere to the substrate, and the intracellular contraction force is decreased. This is responsible for the low substrate resilience and decreased tension at the anchorage site (126, 127). Therefore, the investigation of substrate stiffness and ECM in self-organization *in vitro* is essential to construct the functional skeletal muscle tissues.

In this research, 1×106 cells/well (24-well plate) had proven to be the optimum density for efficient self-organization (**Fig. 3.2B**). However, the efficient myogenic differentiation could not be induced in the clump like tissue (**Fig. 3.5**).

It has reported that BMP inhibitors (Dorsomorphin, LDN-189193) induced cells to break

away from the cell cycle and precocity (128). Figure 3.6 indicated that BMP inhibitor affected cell fusion and differentiation ratio. During proliferation and differentiation of myoblast, BMP signaling is receipted by BMPR on cell surface and activates the Smad signaling pathway. Then the signaling is transmitted into the nucleus, controls the Id1 gene expression and induces osteoblast differentiation (129-132). Several researches have been reported that Dorsomorphin selectively interfered with the interaction between BMPR and its downstream signaling pathway (133). It disrupts Smad1/5/8 phosphorylation mediated by intracellular BMP and prevents osteogenic differentiation (134). In terms of myogenesis, the BMP signaling pathway is inhibited, and C2C12 myoblasts converted their differentiation direction into that of muscular cells. Furthermore, cells maturation and intercellular fusion are induced (135) (**Fig. 1.5**).

Subsequently, the bundle shape tissues with anchors were constructed by using the forming ring shape tissue (**Fig. 3.7**). Compared with C2C12 cells clumps, obvious myotube structures could be observed in these tissues. It may relate to the shape of tissues, because the ring shape tissue has a larger surface area than the cell clump, which results in high utilization of oxygen, nutrients and growth factors. In addition, BMP inhibitor supplement increased myotube width during differentiation of C2C12 cells. Gene expressions of myogenic regulatory factors (MyoD, Myogenin) showed the significant effect of Dorsomorphin on differentiation (**Fig. 3.8**). Dorsomorphin induced the M-cadherin expression and enhanced the wider multi-nuclei myotubes formation. In brief, the myogenesis was enhanced by BMP inhibitor supplementation in the bundle shape tissues.

In this research, an optimal condition for self-organization was found through investigating the effects of substrate stiffness and ECM. The artificial skeletal muscle tissues were

successfully constructed by using soft substrate and BMP inhibitor supplementation. This procedure may represent a powerful tool for the study of biology and pathology.

General conclusion

In this study, a straight-forward method for the construction of functional artificial skeletal muscle tissue with the regulation of the cell-substrate interaction was established.

In chapter 2, the five candidate ECMs (Fibronectin, Laminin, Collagen 1, Collagen 4, and Matrigel) were tested in the 2D culture. These ECMs did not significantly enhance proliferation, differentiation ratio, and myotube width during differentiation culture, and some ECMs even have inhibitory effects. However, the artificial skeletal muscle tissue constructed with Matrigel and SU9516 supplementation exhibited more defined sarcomere structure and stronger contractile force. These results demonstrated that a combination of Matrigel and SU9516 supplementation enhanced skeletal muscle maturation and improved the function of the artificial skeletal muscle tissues.

In chapter 3, the author evaluated the effects of substrate stiffness, ECMs, and biochemical factors on self-organization and myotube formation of artificial skeletal muscle tissues, respectively. Cells on a rigid substrate formed a cell layer due to strong cell-substrate interaction, while a soft substrate reduced the cell-substrate interaction and induced self-organization. Therefore, an easy-to-use procedure for the construction of artificial skeletal muscle tissue was established by the regulation of cell-substrate interaction with the soft substrate. Moreover, the BMP inhibitor promoted the myogenesis and myotube formation in the 3D culture system.

Overall, to construct a functional artificial muscle tissue that mimics that in the living body, the cell-substrate interactions were regulated. Different from other ECMs (Fibronectin, Laminin, and Collagen), Matrigel enhanced or maintained C2C12 cell behaviors such as

migration, proliferation, and differentiation. Moreover, by the enhancement of specific integrin subtype with cyclin-dependent kinase 2 inhibitor (SU9516) supplementation, the artificial skeletal muscle tissues cultured with the Matrigel showed higher contractile activity. In addition, the suitable combination of soft substrate and ECM were determined to construct artificial skeletal muscle tissue. Finally, the construction of bundle shape tissue and BMP inhibitor supplementation during differentiation provided the enhancement of cell fusion and myogenesis in the tissue structure. Thus, these findings indicate that regulation of cell-substrate interaction is an effective way to establish functional artificial skeletal muscle tissue.

For extending the current research toward future application, the following problems should be considered:

1. In chapter 3, in order to obtain the artificial skeletal muscle tissue with a higher cell number, the author attempted to induce self-organization by the culture area expansion. However, the cell layers were easy to be fragmented and could not influence self-organization completely. Moreover, the expansion of tissue size by increasing cell density delayed the contraction because of excessive cells. At the same time, it is challenging to maintain cell survival in tissues for a long time.

2. The utilization rate of oxygen and the nutritious of inner cells of artificial skeletal muscle tissue is low, and the differentiation ratio of internal and external cells is not consistent. Therefore, to overcome these issues, further attempts should be taken into account for the development of efficient methods.

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
CDK	Cyclin-dependent kinase
CS	Calf serum
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DM	Differentiation medium
Dors	Dorsomorphin
ECM	Extracellular matrix
FBS	Fetal bovine serum
GM	Growth medium
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
PA	Polyacrylamide
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
TBS	Tris-buffered saline

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Acknowledgements

I would like to express my gratitude to all who make it possible for me to complete this Ph. D. thesis.

First of all, I would like to express my deepest gratefulness to my supervisor, Professor Takuo Kawamoto (Radioisotope Research Center, Kyoto University) for giving me the opportunity to study in Kyoto University. Thanks for his support and valuable advices for my life and study in Japan. With his help, I can complete my Doctoral course.

I would like to express my sincere gratitude to Asst. Prof. Masanobu Horie (Radioisotope Research Center,) for his technical assistance, paper revision, as well as submission, and providing me the basic training of cell culture and Instrument operation. With his strict instruction, I have gained a lot.

I would like to express my heart-felt gratitude to Sumire Nagasaka, Saki Ohsumi, Kazunori Shimizu, Hiroyuki Honda (Department of Biomolecular Engineering, Graduate School of Engineering, Nagoya University) for their technical assistance.

I would like to express my sincere thankfulness to Radioisotope Research Center of Kyoto University for providing me all of the environment and equipment for studying and experiment. I would also like to express my gratefulness to Professor Ikuji Takagi, Asst. Prof. Fujimoto, Hiroyuki, Mr. Takashi Nishimoto, Mr. Noritaga Kakishita, Ms. Masami Watanabe, Mr. Fumihiko Ishizuka, and Ms. Masako Kobayashi for their help for my study and life in Japan.

I would also like to express my sincere gratefulness to my current group members, Mr. Takuya Sugiura, Miss. Haruka Yamazaki for their kind cooperation during my experiments,

and making my lab-life exciting and unforgettable.

I also express my gratefulness to all of my international and Chinese friends for their helps and supports during these three years.

In addition, I would like to thank Journal of Bioscience and Bioengineering for its recognition of my research (Chapter 2: The effect of cell-extracellular matrix interaction on myogenic characteristics and artificial skeletal muscle tissue).

Finally, very special thanks would go to my family, especially my parents, for their loving consideration and great confidence in me all through these years.

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