Effect of Mechanical Stretching on the Maturation of 3-D Fascicle-like Muscle Tissue

by

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ABSTRACT

Skeletal muscle is well known for a rapid adaptation to mechanical environmental changes. Understanding the effects of the mechanical stimulation such as stretching to muscle tissues is important for understanding the nature of muscle development and muscle diseases. While there are many reports studying the effects of stretching on 2-D cultures in vitro, few research groups have investigated their effects in 3-D muscle strips with high volumetric density. Here, we used the muscle strips that have fascicle-like shape, high volumetric density, and optimal alignment in 3-D. We present that the fascicle-like muscle tissue will have stronger performance and more matured structure in response to particular stimulation. We applied the static and cyclic stretching, electrical stimulation and two different co-stimulation. Both the static and cyclic stretching induced stronger cell-ECM adhesions of 3-D cultured muscle cells. The static tension at day 9 caused striated actin of the muscles, but the cyclic tension at day 1 caused weakening of actin structure with less alignment. Similarly, the stretching could affect other proteins, related to muscle development. Therefore, the effect of the stretching to the muscle tissue is highly time-dependent, and it is important to find optimal timing for efficient training. We also show that muscle performance by the co-stimulation is higher than by the electrical stimulation alone. Although conditions of the each mechanical and electrical stimuli were identical, the performances were changed only by phase shift between the two stimuli. We still do not know the exact mechanism, but our results support the potential use of high-performance engineered muscle tissues for moving bio-robots or drug testing platforms.

Thesis Supervisor: Dr. H. Harry Asada
Title: Ford Professor of Mechanical Engineering

To my Parents

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CHAPTER 1: INTRODUCTION

1.1 DEVELOPMENT OF SKELETAL MUSCLE

It is well-documented that chemical cues are important for the development of skeletal muscles. However, electrical and mechanical cues are also critical in developing muscles by activating muscle growth, but the exact mechanisms are still investigated. In order to know how the electrical or mechanical stimulation affect the muscles, we have to understand muscle development, which is complex process including myogenesis and myofibrillogenesis.

During embryonic development, there is a multistep differentiation process to form skeletal muscle. This differentiation process of skeletal muscle depends highly on regulation of related genes at each step. The myogenic progenitor cells in the somites differentiate into myoblasts and secrete MyoD and Myf5. Then, myoblasts fuse into a long contractile multi-nucleated myotube. This process is called myogenesis. Myogenesis is induced by Myogenin, Mrf4, and MyoD [1]. Myotubes eventually mature into myofibril, which have expressions of Desmin, MyH, and striated actin and α-actinin [2].

The process of myofibrillogenesis, which forms myofibrils, is complicated, because making sarcomeric structures inside the myofibril are highly complex. Researchers still have not been able to figure out exactly how the contractile structures are formed. The current hypothesis for assembling myofibrils has three steps. First, mini-sarcomeres composed by alpha-actinin Z-bodies, actin filaments, and nonmuscle myosin II are constructed in premyofibrils. After the structures of premyofibrils align, titin and muscle myosin II filaments are added. Finally, mature myofibrils are formed by eliminating nonmuscle myosin II and increasing the distance between z bodied to around 2.5 μm [3].

1.2 SKELETAL MUSCLE IN TISSUE ENGINEERING

Researchers have tried to make artificial muscle tissues to use their functionality as actuators, drug-test platforms, or transplant. We want to make artificial muscle tissues with high performance when they are stimulated by electrical or mechanical stimulation. In muscle tissue engineering, most of the work related to muscle tissue engineering have been done on cardiac muscles for two reasons. The cardiac cells do not have to form elongated myotubes like skeletal muscle, and people are critically ill with heart diseases. Based on previous efforts, researchers

have succeeded in making the artificial cardiac muscle tissues in a variety of forms. However, those cardiac studies used muscle cell sheets, so these were based on 2-D culture technique. For instance, Nawroth et al made the artificial jellyfish. They fabricated about 20 μm thin Polydimethylsiloxane (PDMS) film and cultured cardiac myocytes on it by means of stamping the fibronectin in special pattern. As a result, they constructed freely swimming micro-robot, mimicking structure of a jellyfish [4]. In the same year, Chan et al developed the miniaturized walking bio-bot (biological robot). They used a 3-D printer to build a small hydrogel cantilever, and cultured contractile cardiac cell sheet on it. The muscle contraction, stimulated by an electrical field, bent the cantilever, and the cantilever induced locomotion [5].

Recently, people started to recognize the importance of skeletal muscle research. Nowadays, the age-related changes of skeletal muscle and the effects of exercise on aging muscle are becoming more important [6]. Skeletal muscles are also more suitable for bio-bots as an actuator because of controllability, since contraction of cardiac cells often happens spontaneously and has lower special resolution than skeletal muscles. In addition, skeletal muscles can regenerate themselves easily by proliferation, and they can produce persistent and strong force called tetanus. Therefore, skeletal muscles are suitable to use as an actuator, and people have tried to make artificial skeletal muscles. Vandenburgh et al was the first who made contractile myofibril in vitro from myoblasts [7]. They put the cells on the collagen-coated wells in 2-D, but the gel was dehydrated and collapsed by fast gel degradation. Strohman et al developed 3-D muscle tissue [8]. They made the differentiated muscle sheet with collagen, and rolled it up to form 3-D muscle tissue, called myooid. This roll-up method has been popularly used to make 3-D muscle tissues [9-11]. However, this method loses significant volumetric density, and the method is not suitable for scalability. To imitate what happens inside muscles in vivo, it is important to make artificial muscles in 3-D which have high cell density and aspect ratio.

We constructed 3-D artificial muscles in similar environment with a fascicle in vitro [12]. A fascicle is the functional unit of muscles, and the fascicle-like shape with high aspect ratio makes the muscles more developed. Skeletal muscles in vivo consist of aligned long myotubes in parallel, and the alignment plays a important role in development as a physical cue. [13]. Since skeletal muscle organ has a hierarchical structure, the fascicle-shape muscles can be scale up by bundling the artificial fascicles to utilize as an actuator. Each matured myofiber can contract by

excitation from motor neurons, and groups of the myofibril form muscle fascicle. Each fascicle is wrapped with epimycium, and forms the final elongated muscle organ. In this study, we investigated the effects of moderate stimulation for higher performance through the fascicle-like muscles, which allow more in vivo-like tests than the previous studies.

1.3 Effects of Muscle Training by Stretching

Skeletal muscle is well known for a rapid adaptation to mechanical environmental changes. If we exercise hard, then muscles will bulk up by muscle growth. Based on this idea, we trained the 3-D artificial muscles by the static and cyclic stretching to make stronger muscles in chapter 2 and 3 of this thesis. People have been curious how the exercise affects the skeletal muscle, but exact mechanism is still investigated.

By cellular signaling such as the mechano-transduction, mechanical stimulation such as stretching induces different intercellular signaling and gene expression related to muscle development, growth, and survival. While there are many reports studying the effects of stretching on 2-D cultures in vitro [14-17], few research groups have investigated their effects in 3-D muscle strips with high volumetric density [18, 19]. For instance, muscles under tension were investigated using myocytes in vitro cultured on 2-D flexible membranes. Soltow et al gave cyclic strain to myotubes for 5 days, and they stopped the stretching for 3 days. During the stretching, myotube size increased with induction of Akt signaling. However, the cessation of the stimulation caused protein degradation, reduction in myotube size, and decrease of Akt signaling [14]. Clarke et al. measured the high level of bFGF expression by mechanical stretch. The detected bFGF in the medium was released from surrounding stretched muscle cells, and it induced proliferation [15]. Also, Soltow et al. applied cyclic stretching for 1 hour to mouse muscle cell (C2C12). They figured out that mechanical stretch induced nitric oxide (NO) production, and the produced NO activated proliferation [16]. In addition, Goto et al.'s study showed that 96 h mechanical stress increased cellular protein concentration of rat skeletal muscle cells (L6). This result can be related to gain of muscle mass by exercise [17].

Although the prior works showed that stretching is beneficial to muscle, there were some negative results. Cheng et al. overly stretched myocytes, and number of apoptotic death cells increased 21-fold [20]. Because most of the studies that caused the negative effects gave over-

stretching (more than 15% strain), it is significant to give moderate stretching to muscles for inducing positive effect by stretching.

Together with development of tissue engineering, recent advanced studies deal with stretching 3-D muscle tissue. Zimmermann et al. developed a new method of 3-D muscle tissue, which is the ring-shaped tissue with cardiac myocytes and collagen gel. They also put the two small rods in the ring, and stretched the tissue by broaden the gap for 7 days. Therefore, they succeeded to get highly differentiated cardiac muscle cell having contractibility [18]. Clause et al. used the same method with Zimmermann to give stretching to cardiomyocytes. They observed activation of cell proliferation by stretching via p38-MAP Kinase Phosphorylation [19]. Instead of cardiac cells, Powell et al. put skeletal muscle cells into the silicon rubber mold with the two pins at the both ends. After 24 hours, collagen gel was compacted and the tissues were detached from the mold other than the two pins. Powell's team applied mechanical stretch by pulling one of the pins. After the stretching, they observed more dense myofibers by deeper nutrient diffusion and smoothing the tissue with reducing collagen cross-linking [21].

Even the making of artificial muscle tissues are very advanced tissue engineering technology, they still have some limitation. First, a performance of artificial muscle tissues was too lower than actual muscles from animals. Second, the previous tissues were mostly composed of gel instead of muscle cells, so they had too low cell density to make enough force. Third, most of the functional artificial muscles had been made by cardiac cells instead of skeletal muscle cells, since development of cardiomyocytes are much easier than skeletal myocytes. Thus, we applied the stretching to the fascicle skeletal muscle tissues to improve those limitations. Because the fascicle-like muscles have no hard contact in 3-D, the stretching can be delivered to the cells much efficiently than previous studies without hindrance. Furthermore, these advanced techniques allow to apply the stretching to more developed muscles and in vivo like controllable environment. Understanding the effects of applying stretching to muscle tissue is important for understanding the nature of muscle development and muscle diseases.

1.4 Optogenetics of Cells

In animals, muscles usually contract when they get signals from the neurons. However, in vitro, it is much more difficult to control muscle movement by neurons, because making connections between neurons and artificial muscles tissue is challenging. Thus, biologists needed

alternative to control the muscle contraction and get desired muscle behaviors. In the past, most people used electrical stimulation to mimic the neurons. This technique is limited because the electrodes contact the system, so it could cause by-products and hard to use in closed system. Also, the electrodes are difficult to apply fast-moving systems, and do not have spatial control.

Optogenetics is the fusion of optics and genetics to allow high speed, precision control for even freely moving animals. The idea arose initially from neuroscience for precise control. In 2010, Deisseroth et al succeed in expressing Channelrhodopsin in neurons by transfecting them [22]. Channelrhodopsin-2 (ChR2), membrane-bound light-sensitive ion channel, is popularly used for optogenetic control of neurons, because it is a precise non-specific cation ion channels such as sodium and potassium ions, and this ion flux induces depolarization of neurons [23]. This cation ion flux by illumination also evokes muscle contraction in the channelrhodopsin-2-transfected muscle cells, but the exact mechanism for this phenomenon is still investigated (Fig. 1.2). By changing the light exposure time, we controlled the muscle to have desired twitch or tetanus contractions. Precise control of artificial muscle tissue will enable to get freely moving bio-bot with muscle actuator in future.

1.5 The Scope of the Thesis

State-of-the-art artificial muscle tissues are not strong enough to be used as actuators or drug-test platforms. Thus, the goal of this thesis is finding an optimal protocol to make stronger artificial muscle tissues by applying electrical or mechanical stimulation. Unlike previous studies, we effectively applied the stimulation to the fascicle-like skeletal muscle tissues in 3-D, which have high cell density and no hard contact.

Chapter 2 and 3 cover the effects of static and cyclic stretching to the fascicle-like muscle tissue, respectively to find optimal training conditions of the muscles. Overall description of the stretching system and condition is also mainly introduced in chapter 2. Chapter 2 also shows more mature structure proteins of the muscles by the stretching. Both chapter 2 and 3 contain that efficient static and cyclic stretching produce stronger cell-ECM adhesions of the muscle cells, even though they are embedded in 3-D soft gel. In chapter 4, we will focus on optimal stimulation to produce higher force of matured muscle. We show the improving muscle contraction by stimulating through the co-stimulation, i.e. imposing stretching in combination with electrical stimulation. The description of the co-stimulation system and the method of

quantifying contraction are covered in the same chapter. This thesis presents how to mature muscle by stretching and the optimal stimulation to produce the best performance of artificial muscles.

1.6 Figures and Captions

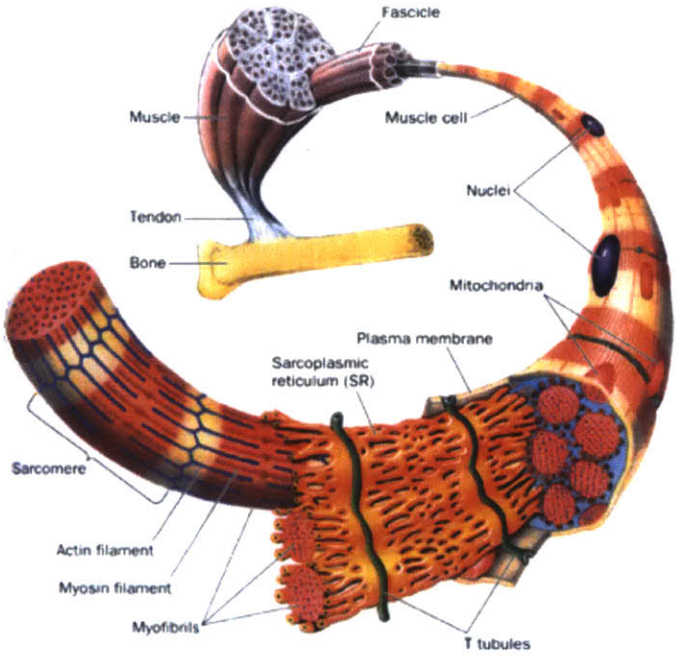


Fig. 1.1 | Hierarchical structure of skeletal muscle organ.[24]

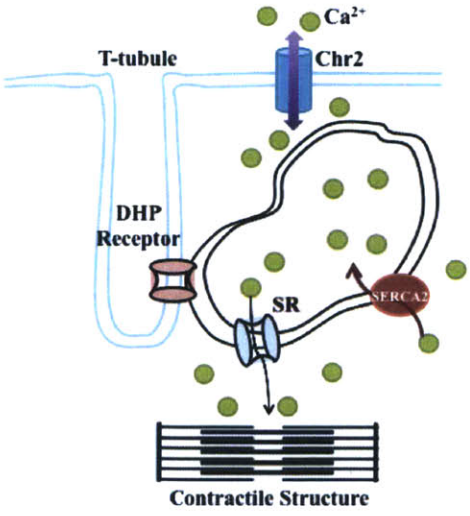


Fig. 1.2 | Possible mechanism for muscle contraction led by illumination. Channelrhodopsin 2 is opened by light, and this induces cation ion flux into cell cytoplasm. Then, DHP receptors sense the cell membrane depolarization, and stored calcium ions are released from sarcoplasmic reticulum (SR). Current hypothesis is the calcium ions are diffused to contractile structure, and it causes muscle contraction. SERCA2 helps restoration of SR.

CHAPTER 2: CHARACTERIZATION OF STATICALLY STRETCHED 3-D SKELETAL MUSCLE TISSUES

2.1 INTRODUCTION

Live muscle tissue has the potential to be a new type of actuator, having numerous degrees of freedom, regenerative capabilities, and a wide range of sizes (5 um ~ 1 m) and force generation (~5 μN ~ 1 kN) [25]. These advantages can potentially be used for multi-DOF micro surgical tools, sub-millimeter to millimeter scale robots, and other machines that are difficult to build with traditional actuators. An ideal engineered muscle tissue requires adequate size and form factor, highly aligned multi-nucleate cell structure, and fully differentiated, mature construction [26]. In the last decade a number of research efforts have been reported on engineered muscle tissues, but conventional studies have been limited to 2D environments [27, 28], muscle tissues in vivo [29], electrical stimulation [30, 31], and cardiac muscle cells [32]. Also, the artificial muscle tissues are by far inferior to the natural muscle in terms of output strain, stress, and controllability. Breakthroughs must be made to produce practically useful muscle actuators that are fully mature and functional as well as controllable.

Skeletal muscle is a highly organized and hierarchical tissue. It consists of fascicles that are a bundle of skeletal muscle fibers, and research of fascicle-like structure will be necessary to scale up muscle actuators in the future. We have developed a device closer to in vivo, which can culture myoblasts into fascicle-like structures. Specifically, the device allows muscle cells to grow away from contact with stiff surfaces, only anchoring at both ends. In addition, the structure of the developed device provides slight tension to muscle tissues because the hydrogel scaffold compacts after seeding the device. With these conditions, we have already observed that the muscle tissues mature more than 2D cell culture. We supposed that the stretching of the muscle tissue by the device might cause more mature muscle tissue to form. Therefore, we applied additional moderate external stretching to muscle tissue for understanding a role of physical force in muscle development.

Optogenetic coding is a relatively new technology that makes excitable cells light sensitive [33]. The authors group was the first to successfully activate skeletal muscle strips with "light" by transfecting skeletal muscle cells with Channelrhodopsine-2 [34]. Unlike the traditional electric activation, which uses a pair of electrodes inserted into the muscle tissue, the

optogenetic activation of muscles is non-invasive, non-contact, and wireless control and, more importantly, allows for activating densely arrayed muscle strips one by one separately or as a group in a coordinated manner. This opens up new possibilities of skeletal muscle actuators. High spatiotemporal resolution of optical, wireless control would make a bundle of muscle strips a high DOF, or even distributed DOF, actuator.

In the following, a new 3D fascicle-like skeletal muscle actuator is introduced, and its tension application mechanism is described. Experimental results confirm that stretching enhances muscle differentiation and maturation. Optogenetic control experiments are also conducted for the 3D fascicle-like muscle tissue. "Tetanus", a prolonged muscle contraction, is achieved for the first time for the light activated skeletal muscle tissues.

2.2 Fascicle-like Muscle Tissue

There are several functional requirements for the artificial muscle tissues as an actuator. First, the muscle tissue should produce durable and usable force, so it should have high density of matured muscle cells. Second, the force production should be controllable. Third, it should have a 3-D simple structure for easy implantation to many different types of devices. Finally, the scaffold of the tissue should help muscle cells be aligned well, because the alignment and elongation guidance promote muscle development. In this thesis, we used the fascicle-like muscle tissue [12]. The muscle tissue made by this new technique is a 5 mm long and 0.5 mm thick well-aligned tissue in 3D environment. This special tissue is only anchored at both ends, and there is no hard contact in the middle of the tissue. Therefore, this enables improved diffusion of medium into the muscle tissue than previous works, and the whole muscle strip has same strain rate.

There were multiple steps to make the fascicle-like device. Steel pins, which nominal diameter was 508 μm, were inserted into the mold that printed by 3-D printer (Dimension 1200es, Stratasys). After poured Polydimethylsiloxane (PDMS) was solidified in the mold, removing the steel pins made the tubular holes in the PDMS. 5 mm holes were formed by punch, and the holes were used as a reservoir for medium. The PDMS chips were cut into small pieces with 3 holes each, and bonded with 0.5 mm thin PDMS film (Fig. 2.1).

Before putting the cells into the fabricated chips, the chips were autoclaved in order to prevent contamination. One of the key techniques for the fascicle-like device was the sacrificial

molding to make tubular shape of the muscle tissue. The material for the sacrificial mold should keep solid at blow one temperature and form desired shape. However, it should be melted at high temperature to remove the sacrificial mold. So, we used 5% gelatin solution melted by 37°C growth medium and solidified below than 37°C. The solution also contained thrombin to use fibrin as the extracellular matrix (ECM), and 0.05 M NaOH solution for pH adjustment. This gelatin solution was poured into the PDMS chips with pin, and the chips were in the refrigerator for 30 minutes to accelerate gelatin solidification. The trypsinized C2C12 mouse myoblasts were centrifuged, and the cell pellet was mixed with growth factor reduced matrigel (356231, BD Matrigel), fibrinogen (Sigma-aldrich), and cold growth medium (DMEM, Sigma-aldrich). After removing the pins from the gelatin-solidified chips, the cell and gel mixture was seeded in the holes of the gelatin sacrificial mold.

Forming fibrin matrix is also important phenomena in the fascicle-like device. The seeded chip was heated to 37°C inside of the incubator, and the gelatin solution was melted. The thrombin of the gelatin solution in liquid state started to diffuse into the cell mixture, and it converted fibrinogen into gel-forming fibrin. As a result, fibrin became main ECM component together with matrigel for the muscle cells. Remaining gelatin was diluted by medium, and it was removed by changing medium every day.

For two days after seeding, the cells were cultured in growth medium (GM) with 1 mg/ml aminocaproic acid (AA). AA was added to mitigate ECM degradation by proteinase secreted from surrounding cells. After that, we changed the media into differentiation media (DM) with 1 mg/ml AA. DM is the same as GM except for 10% horse serum instead of the fetal bovine serum (FBS). The medium was changed every day for totally 2 weeks.

2.3 Materials and Methods

2.3.1 Fabrication of Static Stretching System

There are four functional requirements of the static stretching device: 1) Muscle strips in the PDMS chip can be easily elongated as much as 20%. 2) Material of the stretching system should be non-toxic, and keep clean. 3) The system is small enough to put in the incubator with muscle tissue. 4) The system should not disturb imaging of the muscle. In order to fulfill the requirements, I designed the stretching system with two bars for squeezing the PDMS chip to elongate the muscle strips. The muscle strips in the PDMS chip is anchored at both ends to the

cylinder-shaped holes. The cross section of the holes changed into ellipses by the squeezing the chips, so the muscle strips in the hole are elongated following the poisson's ratio (Fig.2.2). Screws can change the distance between the two bars. These bars were constructed by 3D printer (Dimension SST 1200es, Stratasys) and assembled the devices to apply 12 % strain to the muscle tissues (Fig.2.3). By measuring the length changes from images, the average of actual applied strain was 13.32±1.58 %.

The fabricated system also satisfied other functional requirements. I put the printed body into 70% ethanol for overnight and printed new body every two weeks to keep clean. Since the static stretching system was consisted of only two 1 cm thick and 6 cm long bars with two screws, it was small enough to put in an incubator. Also, it does not disturb the muscle tissue imaging at all. In the process of assembly, even tiny physical impact could damage the muscle strips. Therefore, the stretching had applied at least 1 hour after the seeding when thrombin diffused to form fibrin, and the strip formation was stabilized.

2.3.2 Culture of C2C12 Myoblasts

The C2C12 mouse myoblast cell line, used for study, was transfected with Channelrhodopsin-2 DNA to enable control and contraction via controlled light (470~490 nm, Fig. 2.4) using by pAAV-Cag-Chr2-GFP-2A-Puro with LipofectamineTM 2000 (Invitrogen). We gifted by Professor Kamm's lab at MIT.

The myoblasts were maintained in growth media (GM), which is Dulbecco's modified Eagle medium (DMEM, ATCC) supplemented with 10% Fetal Bovine Serum (FBS, Sigma), 1% Penicillin-Streptomycin (PS, Sigma), and 0.2% Normocin (InvivoGen). The cells were passaged when 70~80% confluent. The growing media was changed every 2 days.

2.3.3 Immunostaining

Immunostaining technique is used to visualize actin, nucleus, and alpha-actinin, which is located in the Z band/disc and cross-links with F-actin in skeletal muscle cell. We used alpha-actinin and striated actin as markers for developed muscles. After fixation of the cell using 6% Paraformaldehyde (Sigma), the cells were treated 1% Triton-X (Invitrogen) for permiablization, and 1% bovine serum albumin (BSA, Sigma) for nonspecific binding of antibody. Incubation with primary antibody (Invitrogen) of alpha-actinin was performed for overnight at 4°C, and second antibody (Invitrogen) was treated for 1 hour. Hoechst (Invitrogen) and rhodamine

phalloidin (Invitrogen) were treated together for 30 min to visualize nucleus and actin, respectively.

2.4 Results and Discussion

Moderate static stretching is applied to the fascicle-like muscle tissues to understand a role of force to muscle development. To achieve this goal, we applied a tension to the muscle tissue with approximately 12% strain in the axial direction.

2.4.1 Maturation of Intercellular Structure

Muscle contractility and controllability are important features for muscle as an actuator, and a good marker for muscle maturation. When we stimulated the muscle tissues with blue light in an impulse function, emitting the light for approximately 0.35 s, muscle started to contract after 0.18 s and peak time was 0.27 s (Fig.2.5). The maximum contraction was happened after turning off the light, and the tissue was relaxed for 0.18s.This short, and sudden muscle contraction is called twitch.

Furthermore, when the light in step function stimulated the muscle tissue, emitting for approximately 4 s, the muscle tissue kept the contraction with the light for a relatively long time (Fig.2.6). In other words, we induced the prolonged muscle contraction that maintained for number of seconds, which is called tetanus. The contraction was delayed about 0.1 s from the light. There was overshoot at the initial stage, and they kept about 60% of maximum contraction for about 2 s.

These results showed that we could control contraction of the stretch fascicle-like muscle tissue by exposure time of blue light. We induced both twitch and tetanus which means that we made the engineered muscle tissue that is more mature, and to have higher potential of muscle contraction.

The fascicle-like muscle tissues were stretched from 1 hour after seeding, and fixed at day 6 and day 14. We fixed the cells at these days because the tissues usually started to contract from the day 6 and degraded after 2 weeks. We wanted to check an effect of the external stretching to muscle development in time-related manner.

When the muscle tissue got stretched for the first 6 days, they developed alpha-actinin as more filamentous and tube structures than control tissues (Fig.2.7). Alpha-actinin is a

cytoskeletal actin-binding protein, and it cross-links actin filaments in order to form a lattice-like structure with forming adjacent sarcomeres. This formation with alpha-actinin helps to stabilize the muscle contractile apparatus [35, 36]. However, in this experiment, F-actin was not formed well in both conditions yet.

Based on the results, we could suppose that forming filamentous structure of alpha-actinin is earlier step than actin's even though alpha-actinin is actin binding protein. Also, external stretching could accelerate a formation of stable contraction apparatus by affecting the alpha-actinin related signaling pathways.

When the muscle tissues were stretched for 14 days, stretched tissue showed well-developed alpha-actinin in filamentous and tube structures (Fig.2.8). The amount of alpha-actinin expression was compared by Western blot. There was more amount of alpha-actinin in the stretched tissues than control (Fig.2.9). Also, F-actin was formed in both stretched and control tissues (Fig.2.8).

To investigate the effect of stretching in early stage of muscle development, we started to give external stretching from 9 days from seeding, which the tissues usually contract well with stimulating by the blue light (Fig.2.10). In the result, expressions of alpha-actin in the stretched and control tissues were similar, but the stretched tissue showed more striated actin, which is one of the evidences of stable striated muscle (Fig.2.9, Fig.2.10). We infer from this result that applying stretching for the first few days affects tube shape formation of alpha-actinin, and stretching at later development stage accelerates formation of the mature actin structure.

2.4.2 Changes from Focal Complexes to Adhesions in 3-D

Fraley et al. claimed that focal adhesions were not observed in 3-D. They said if focal adhesions existed in 3-D, then their size was smaller than 0.3 μm or their lifetime is shorter than 1s. In general, focal adhesions of cells on the 2-D plastic dish are as large as 15 μm and exist more than 15 minutes [37]. However, in Cukierman et al.'s paper, 3-D cell-matrix adhesion also contains the same proteins with 2-D focal adhesion, which are paxillin, vinculin, and integrin. [38]. Currently, the most reliable hypothesis for 3-D cell adhesion is that cells form 'focal complex', which is a nascent adhesion, in 3D matrix. Because cells in soft 3-D matrix get smaller tension from outside, the 3-D cultured cells form weaker adhesion than on 2D dish. Spanjaard et al. said the focal complex is matured into focal adhesion by increasing tension [39].

Based on the previous studies, the 3-D muscle strips that were applied the static stretching could have stronger cell-matrix adhesion than non-stretched ones. C2C12 myoblasts were seeded on the plastic dish in growth medium, and stained vinculin, actin, and nucleus of the cells (Fig. 2.12). On the 2-D dish, the cells formed clear vinculin dots for connecting to the bottom, which were focal adhesions. 2-D cultured myotubes also formed strong focal adhesions at the ends of the myotubes and some small ones at the boundary of the tube (Fig. 2.13). In the 3-D fascicle-like device, it was hard to see aggregated form of vinculin in the non-stretched myotube. On the other hand, there were aggregated vinculin dots in the stretched muscle strips (Fig. 2.14). According to the previous researches, the 3-D non-stretched myotubes might have only focal complexes, which are too small to image by normal fluorescence microscope. However, the small focal complexes might be matured into stronger focal adhesion in the stretched myotubes to make balance with the external tension. This result was obtained from two stretched strips and three controls from one experiment, so it should be repeated by more experiments.

2.5 CONCLUSIONS

In this chapter, we sought to understand effects of moderate external stretching to the fascicle-like muscle tissues development by mechanical and biological tests.

We induced both twitch and tetanus of the stretched muscle tissues by changing stimulation time of blue light. In impulse response, the muscle tissue showed the maximum contraction after turning off the light. Channelrhodopsin-2 inside of the muscle tissue absorbs blue light (~470 nm) and open the pore in the cell membrane. Since the channelrhodopsin is nonspecific cation channels, it allows the flow of Ca2+, K+, Na+, and H+, which affect muscle contraction. Without the light, the pore is closed, and the flow of ions is stopped, but it takes about milliseconds. Therefore, we have to consider the time delay caused from a nature of channelrhodopsin-2 when we control the muscle tissue as an actuator.

In addition, applying external stretching at early muscle development stage and at the later stage respectively affect expressions of alpha-actinin and F-actin, which play critical roles in muscle contraction. Applying the moderate stretching conditions induced alpha-actinin in tube shape and striated actin, related with stable muscle contraction apparatus.

External stretching in the axial direction of the tissue could cause optimal alignment of the muscle cells itself and intercellular structures such as cytoskeletons. The stretched muscle

tissue could develop stronger cytoskeletons and binding proteins to make force equilibrium with the external stretching. Because most of these proteins are also related to muscle contraction, it could affect signaling pathways to induce more muscle contraction and maturation.

From the results, stretching the tissue helps the muscle to have more mature intercellular structure and to have higher performance. Applying the moderate static stretching induced α-actinin in tube shape and striated actin, related with stable muscle contraction apparatus. The stretched muscle tissue could develop stronger cytoskeletons and binding proteins such as stable focal adhesion to make force equilibrium with the external stretching. In addition, optogenetic control of the well-developed muscle tissue enables a new type of actuator and lots of engineered muscle tissue applications in the future.

2.6 Figures and Captions

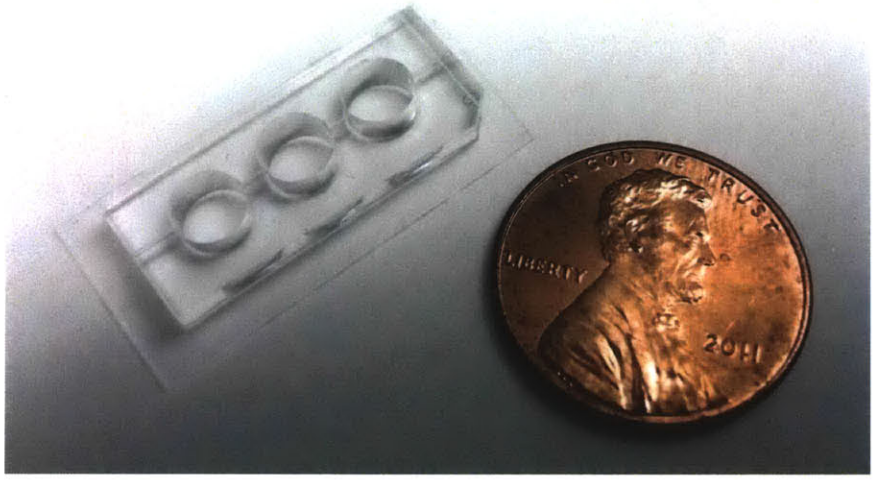


Fig. 2.1 | The fascicle-like device made by PDMS. Muscle cells and gel mixture were seeded after autoclave.

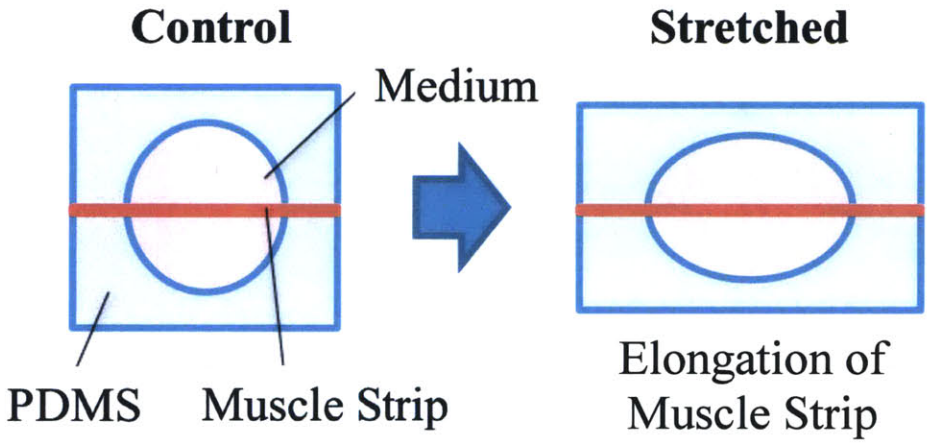


Fig. 2.2 | Elongation of muscle strip. Squeezing the fascicle-like muscle device stretched the muscle strips.

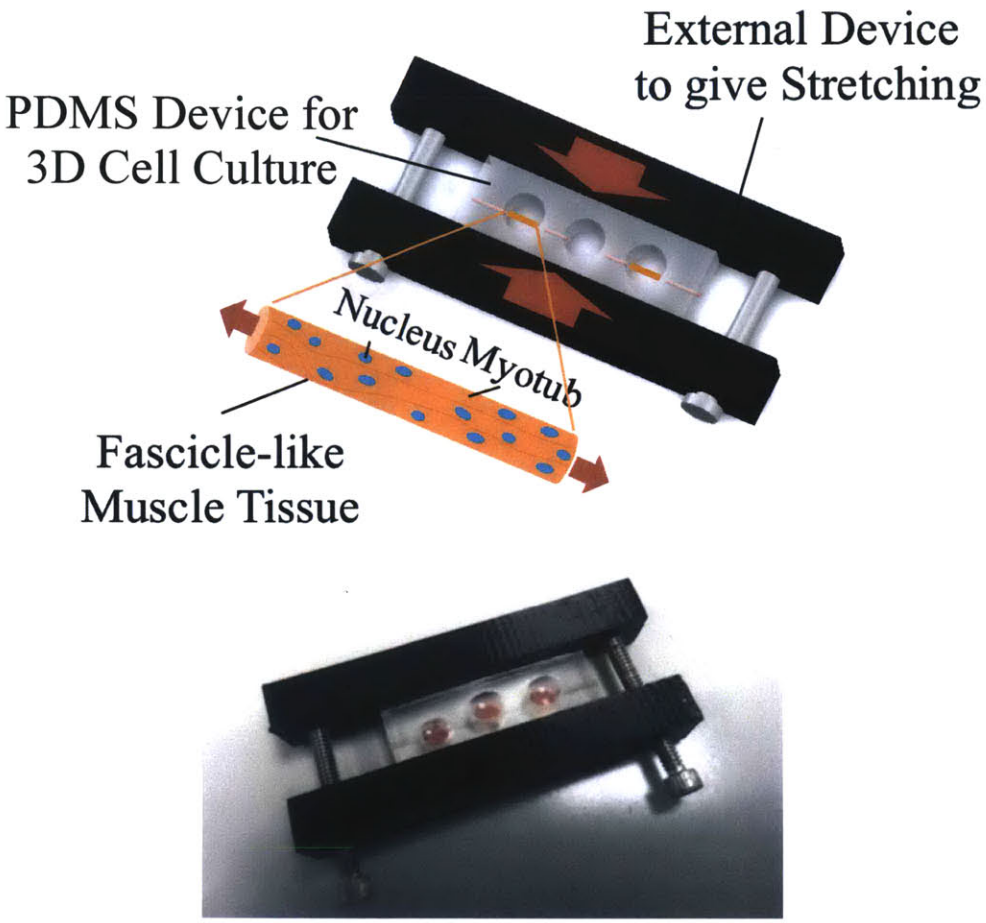


Fig. 2.3 | Device for applying static stretching to the fascicle-like muscle tissues. The static stretching device was printed by 3-D printer in order to squeeze the fascicle-like device.

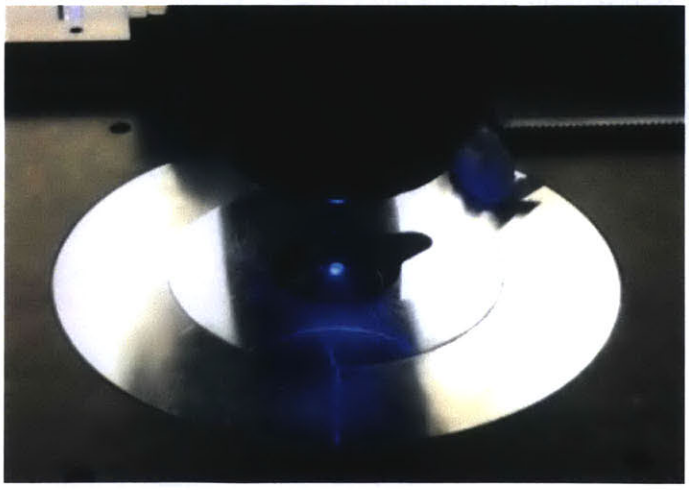


Fig. 2.4 | Emitting blue light (~470 nm) using Zeiss microscope. This blue light induces muscle contraction of ChR2-transfected muscle cells.

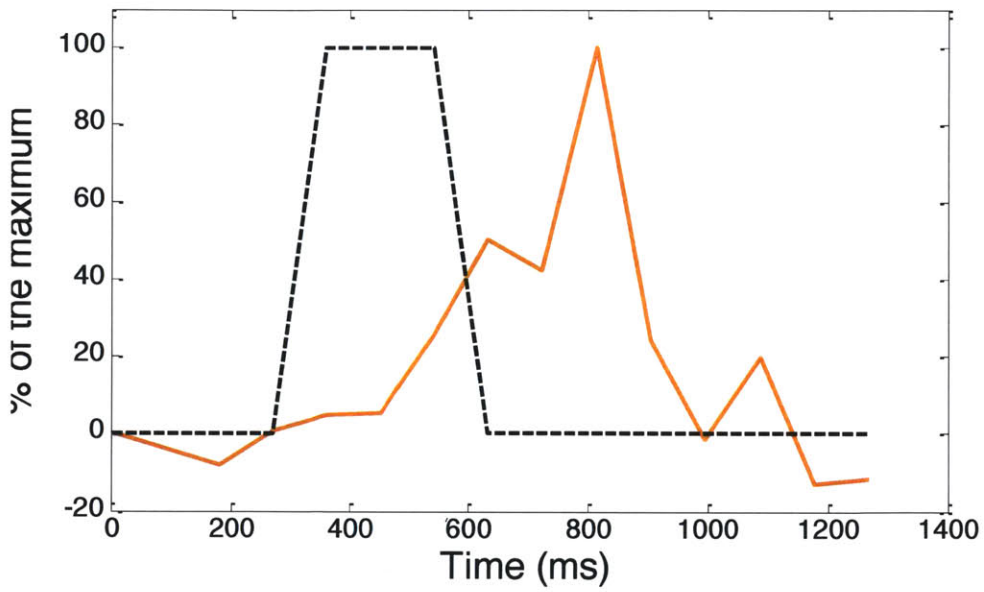


Fig. 2.5 | Impulse response of muscle (twitch). Muscle strips were stimulated by blue light (~470nm), and dotted line indicates emitting of the blue light. Muscle length is normalized by initial muscle length.

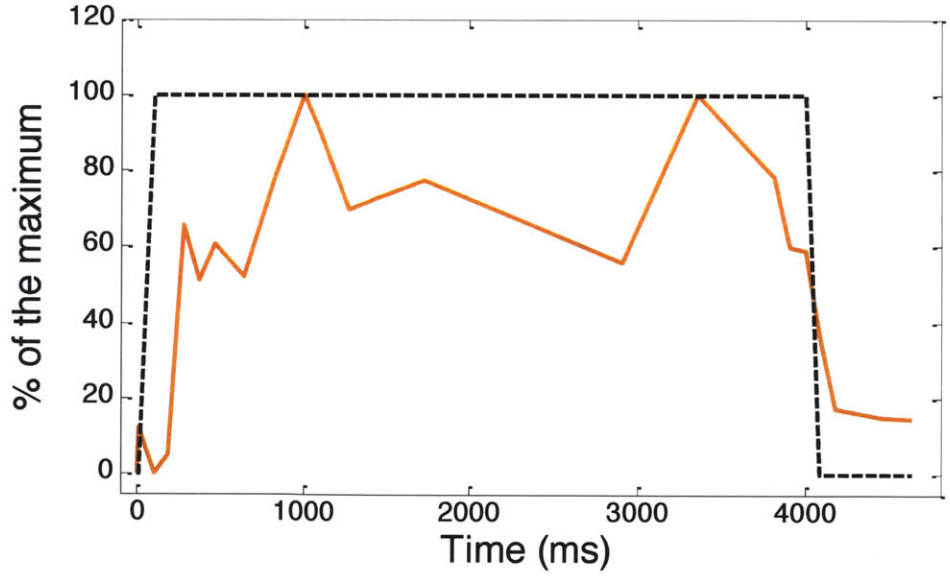


Fig. 2.6 | Step response of muscle (tetanus). Muscle strips were stimulated by blue light (~470nm), and dotted line indicates emitting of the blue light. Muscle length is normalized by initial muscle length.

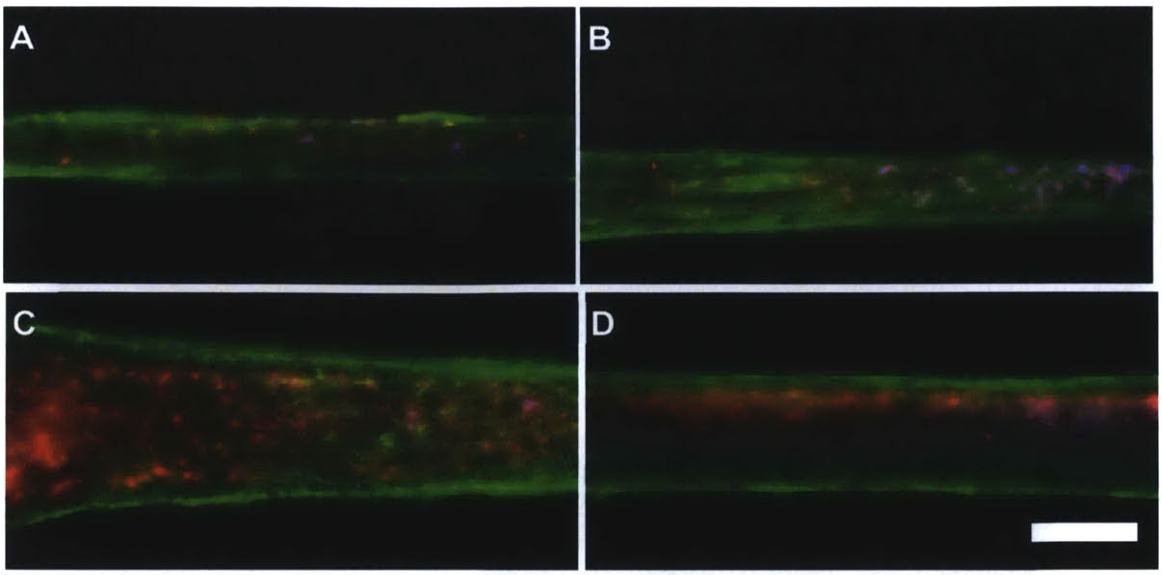


Fig. 2.7 | Visualizing of actin, nucleus, and α-actinin by staining. Red, blue, green indicates actin, nucleus, α-actinin, respectively. Muscle strips were fixed at 6 days from seeding, A, B: the stretched muscle tissues, C, D: control, Scale bar = 100 um.

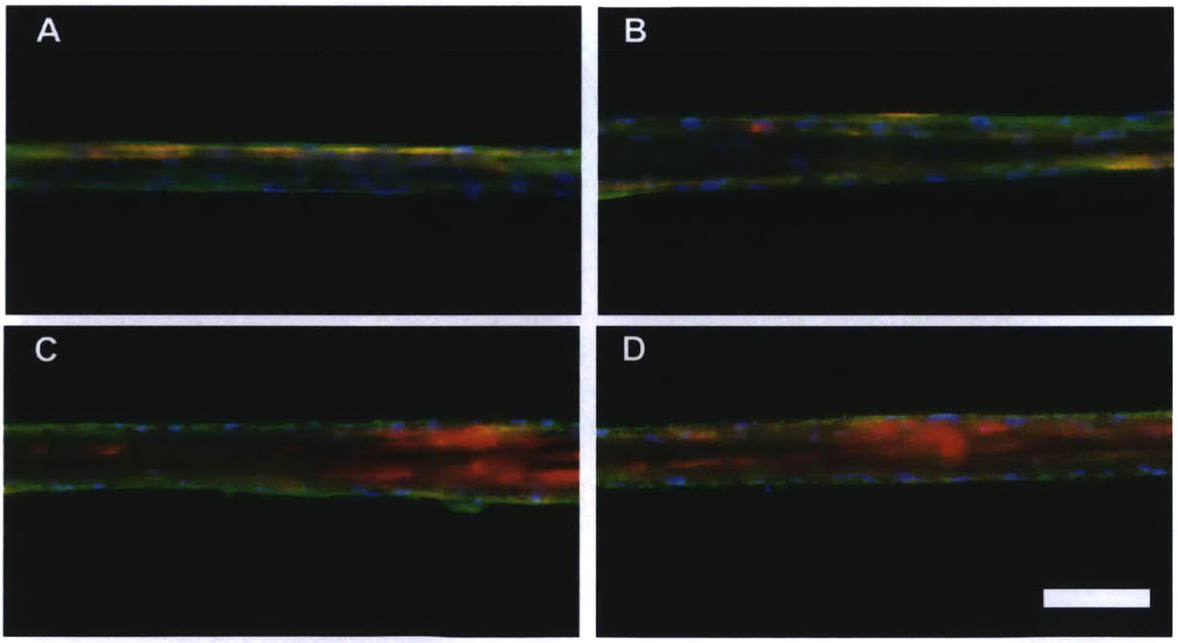


Fig. 2.8 | Visualizing of actin, nucleus, and α-actinin by staining. Red, blue, green indicates actin, nucleus, α-actinin, respectively. Muscle strips were fixed at 14 days from seeding, A, B: the stretched muscle tissues, C, D: control, Scale bar = 100um.

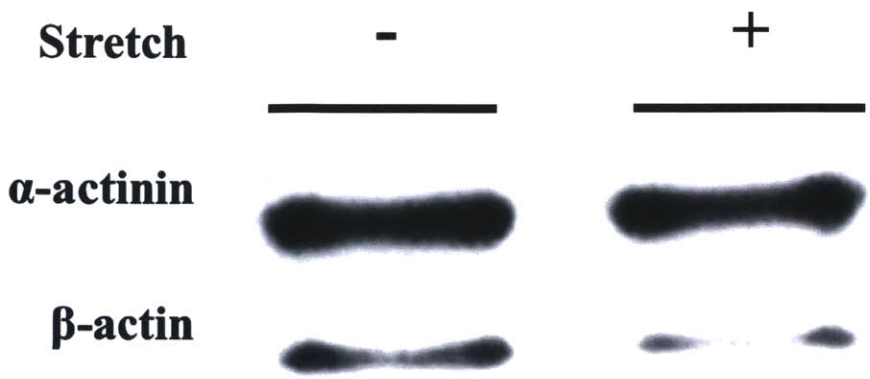
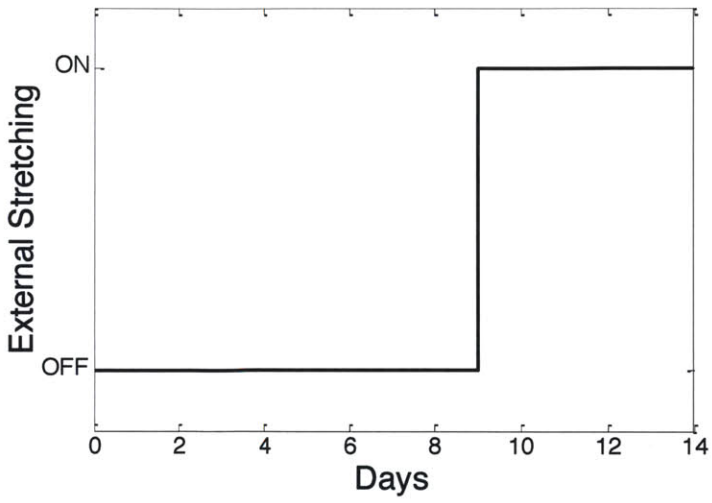


Fig. 2.9 | Western blot result of α-actinin. Muscle strips were stretched for 14 days from seeding, and harvested the muscle cells from the tissue.



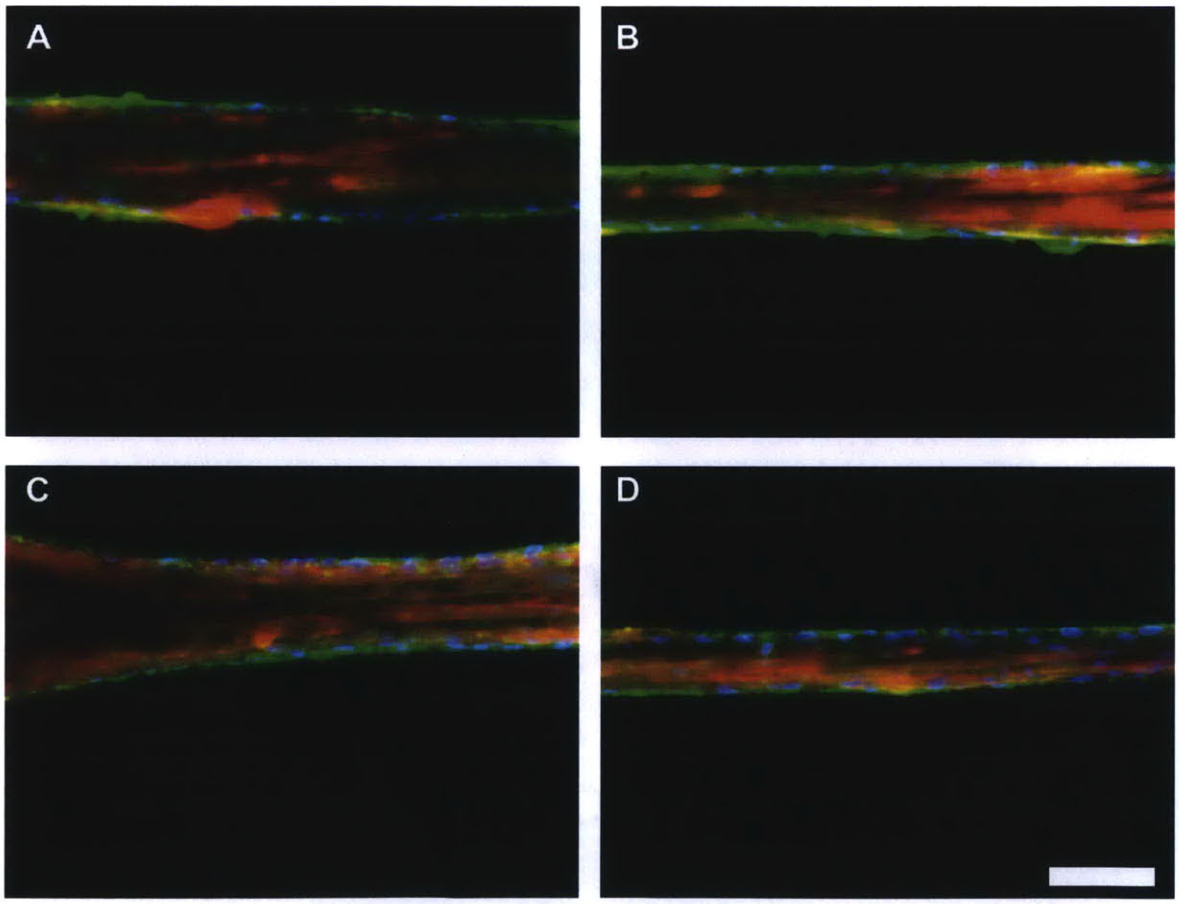


Fig. 2.10 | Visualizing of actin, nucleus, and α-actinin by staining. Red, blue, green indicates actin, nucleus, α-actinin, respectively. We started to give stretching from 9 days to 14 days from seeding, A, B: the stretched muscle tissues, C, D: control, Scale bar = 100um.

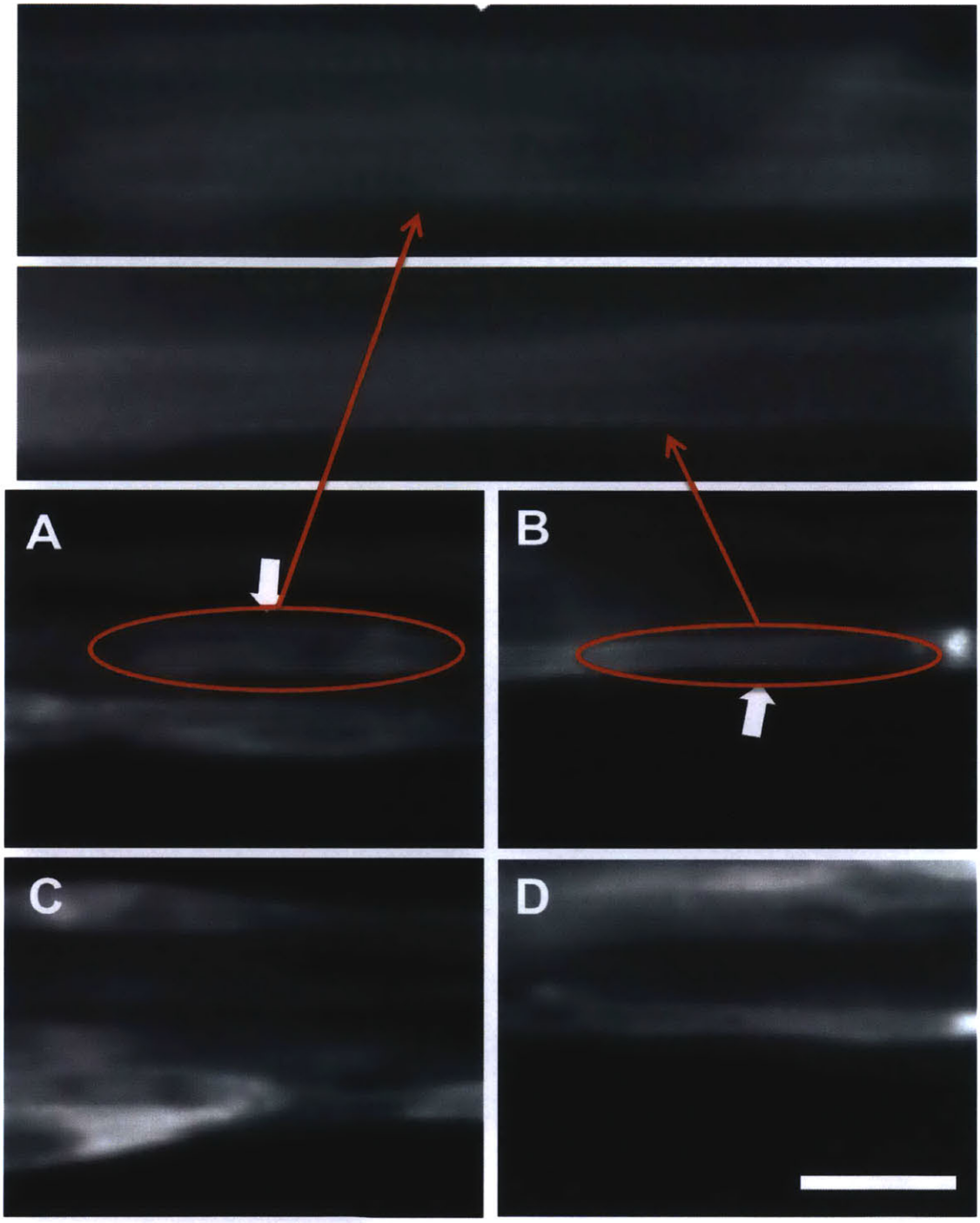


Fig. 2.11 | Visualizing of actin. We started to give stretching from 9 days and fixed at 14 days from seeding, white arrows mean striated actin fibers, A, B: the stretched muscle tissues, C, D: control, Scale bar = 50um.

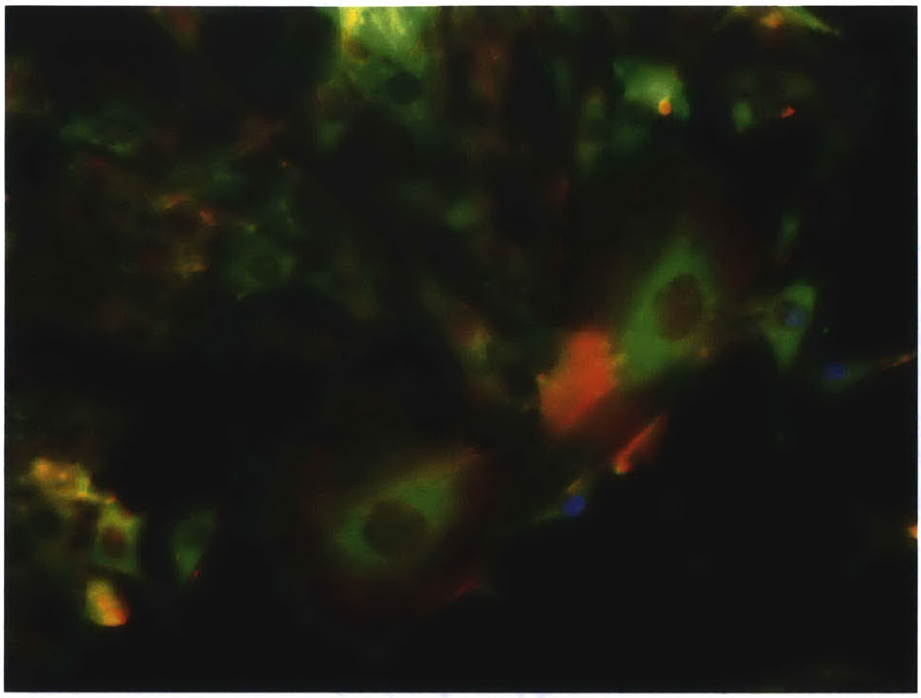


Fig. 2.12 | Immunostaining of 2-D cultured myoblasts. C2C12 were cultures on the plastic dish in growth media. Green, red, and blue indicates vinculin, actin, and nucleus, respectively.

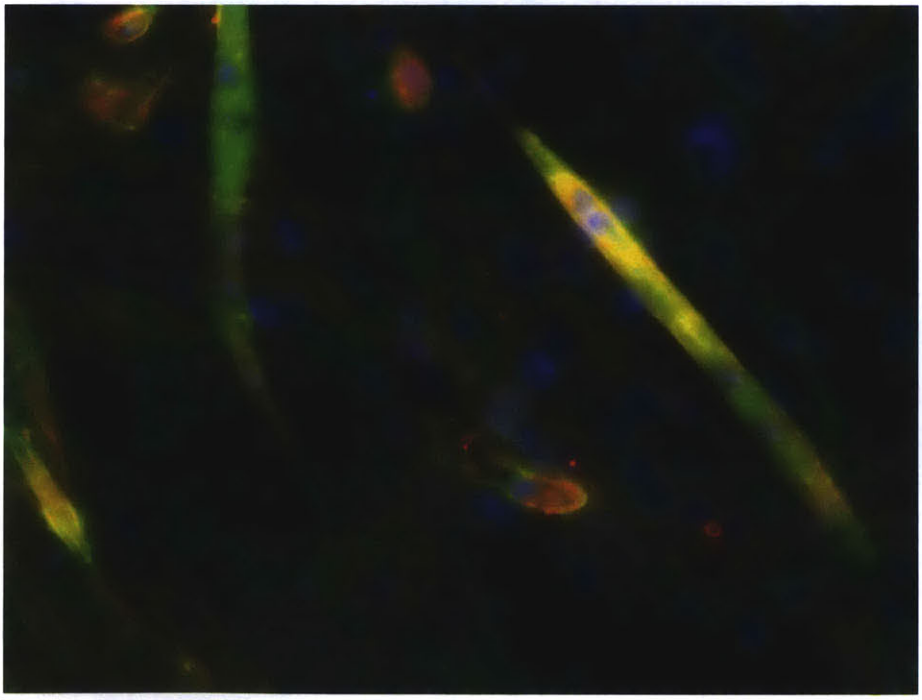


Fig. 2.13 | Immunostaining of 2-D cultured myotubes. C2C12 were fused into myotubes on the plastic dish in differentiate media. Green, red, and blue indicates vinculin, actin, and nucleus, respectively.

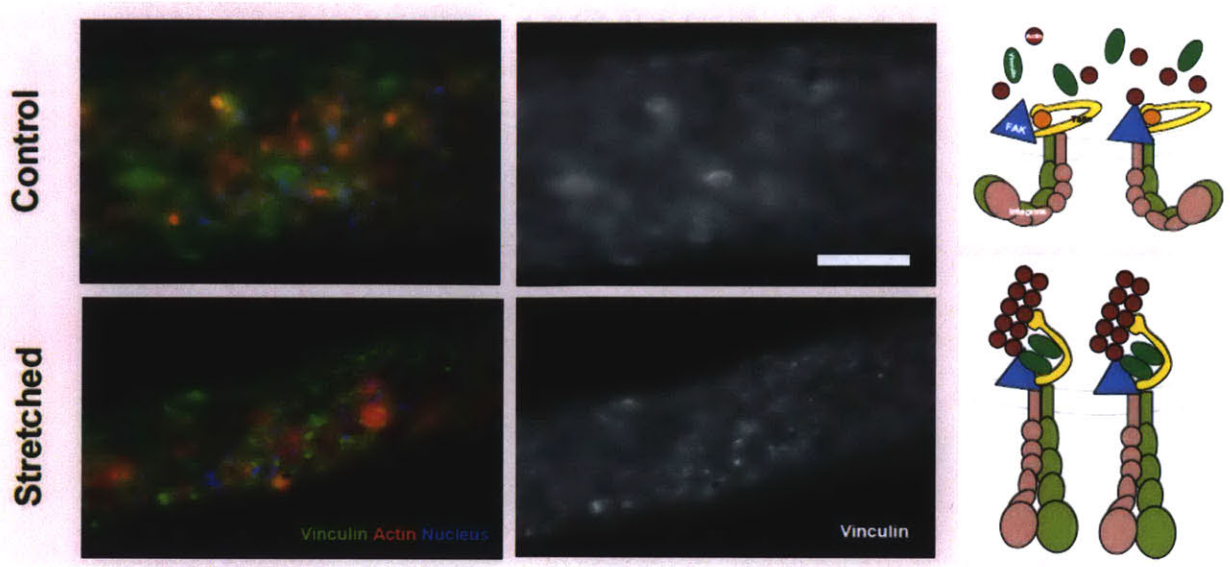


Fig. 2.14 | Visualizing of focal complexes and adhesions. The muscle tissues were stretched for 4 hours at day 6, Green, red and blue are vinculin, actin, and nucleus, respectively. Scale bar = 50 um. Upper diagram explains the focal complexes (nascent adhesions) of the control tissue, and the lower diagram indicates the focal adhesions of the stretched tissue.

CHAPTER 3: CHARACTERIZATION OF CYCLICALLY STRETCHED 3-D SKELETAL MUSCLE TISSUES

3.1 INTRODUCTION

The engineered muscle in vitro needs appropriate mechanical cues for development instead of in vivo environment. Therefore, we applied static stretching in chapter 2, and obtained the more developed muscle tissues which had more α-actinin, striated actin, and stronger cell-matrix adhesions. However, the static stretching was not enough to produce fully developed muscles. Instead of static stretching, cyclic stretching which mimics an exercise might be the more effective mechanical cue, because dynamic exercise enhances muscle development and performance than static stretching in vivo [40]. Thus, we hypothesize that moderate cyclic stretching produces more developed muscles in vitro, which have stronger intercellular structures.

A number of research efforts have been reported on cyclic stretching for mimicking exercise. During cyclic stretching in 2-D, the myotube size increased with induction of Akt signaling [14]. Furthermore, nitric oxide, bFGF, and cellular protein concentration were produced [15, 16], and they activated proliferation [17]. These results show parts of the mechanism of muscle growth by exercise. In 3-D stretching tests, scientists have observed activation of proliferation of cardiac cells via p38-MAP Kinase Phosphorylation [18], and denser myofibril [19]. They said stretching might enhances nutrient diffusion into the tissue and reduces collagen cross-linking [19]. These earlier studies showed that cyclic stretching is helpful for muscle development and proliferation, analogous to exercise in vivo [40, 41]. Even though there were previous studies on the cyclically stretched muscles, they are still limited to 2-D culture, low cell density tissue in 3-D, and cardiac cells. To improve those limitations, we exerted cyclic stretching to the fascicle-like skeletal muscles, which are closer to in vivo and have high cell density in 3-D. Furthermore, no hard contact in 3-D environment leads to direct delivery of stretching to the muscles. We also developed systems that trained the muscle tissues by the cyclic stretching, and observed cell-ECM adhesions in 3-D, which are related to mechano-transduction and development.

3.2 Materials and Methods

3.2.1 Culture of C2C12 Myoblasts

The C2C12 mouse myoblast cell line, used for study, was transfected with Channelrhodopsin-2 DNA to enable control and contraction via controlled light (~470 nm) using by pAAV-Cag-Chr2-GFP-2A-Puro with LipofectamineTM 2000 (Invitrogen). The myoblasts were maintained in growth media (GM), which is Dulbecco's modified Eagle medium (DMEM, ATCC) supplemented with 10% Fetal Bovine Serum (FBS, Sigma), 1% Penicillin-Streptomycin (PS, Sigma), and 0.2% Normocin (InvivoGen). The cells were passaged when 70~80% confluent. The growing media was changed every 2 days.

3.2.2 Immunostaining

Immunostaining technique is used to visualize actin, nucleus, and alpha-actinin, which is located in the Z band/disc and cross-links with F-actin in skeletal muscle cell. After fixation of the cell using 6% Paraformaldehyde (Sigma), the cells were treated 1% Triton-X (Invitrogen) for permiablization, and 1% bovine serum albumin (BSA, Sigma) for nonspecific binding of antibody. Incubation with primary antibody (Invitrogen) of alpha-actinin was performed for overnight at 4°C, and second antibody (Invitrogen) was treated for 1 hour. Hoechst (Invitrogen) and rhodamine phalloidin (Invitrogen) were treated together for 30 min to visualize nucleus and actin, respectively.

3.3 Results and Discussion

3.3.1 Intercellular Structure Changes

The cyclic stretch was applied to the muscle cells from 12 hours after the seeding (Fig. 3.1). The systems were printed by 3-D printer (Dimension SST 1200es, Stratasys). The muscle cells were stimulated in 12% strain, 0.33Hz for 4 hours. After this stimulation, we fixed the cells to stain them to see the intercellular structural changes by the stretching. In order to visualize the cell-matrix adhesions, we stained a vinculin, which is one of the important components of focal adhesions [42]. The stretched muscle cells had larger aggregated vinculin dots than the dots in non-stretched cells (Fig. 3.2, 3.3). Usually, the stretching induces stronger cytoskeleton to make force balance between inside and outside of the cells. However, the cyclic stretching made the

actin filament less aligned structure like the honeycomb (Fig. 3.4, 3.5). In the fascicle-like tissue, the gel was compacted by the time especially at the first few days. During the gel compaction, the muscle cells got tension in longitude direction of the cylinder, and it induced cell alignment and stronger actin filament. The strong cytoskeletal structure seems to be weakened by compression stage during the cyclic stretching.

Cells in the gel attach to the ECM via integrins, and they spread with forming focal adhesions. Those adhesions contain vinculin and are connected to actin stress fiber [43]. These structures of cells generate tension for cell migration or morphological changes, and the force is transmitted to the nucleus [44]. The focal adhesion complex helps to make a force balance between a cell-generated traction force that pulls ECM and external tension from the ECM fibers [43]. The size of vinculin recruitment at the cell-matrix adhesion is regulated by the amount of tension [45]. According to those previous studies, the larger vinculin dot of cyclically stretched muscle is substantial evidence of cell signaling changes by the external force (Fig. 3.2, 3.3). We used the normal fluorescent microscope for the imaging, but we might get clearer image of the focal adhesion in 3-D tissue by confocal or two-photon microscopes. Also, this result was obtained from two stretched strips and two controls from one experiment, so it should be repeated by more experiments.

3.4 CONCLUSIONS

In this chapter, we figured out the changes of the focal adhesions and actin fibers by the cyclic stretching in the 3-D artificial muscle. To determine the role of the stretching in intercellular structures of skeletal muscle, we developed system for applying cyclic stretching to the fascicle-like muscle tissues, which are 3-D artificial muscle tissue having optimized environment for muscle development. The vinculin had larger aggregation in the stretched muscle cells, which meant that stretched muscle had stronger adhesion to the cell matrix. Along with the adhesion maturation, usually actin stress fibers become thicker to keep the force balance with outside of cells. However, the muscle cells, which got the 4 hours cyclic stretching, had not stronger actin fibers with less alignment. We suggest that those structural changes by the cyclic training are related with cell signaling regulated by cyclic stretching. Thus, the result in this chapter will help to understand the exact mechanism of muscle changes by exercise. Also, more

research about this will contribute to make the 3-D artificial muscle that has high performance by the training.

3.5 Figures and Captions

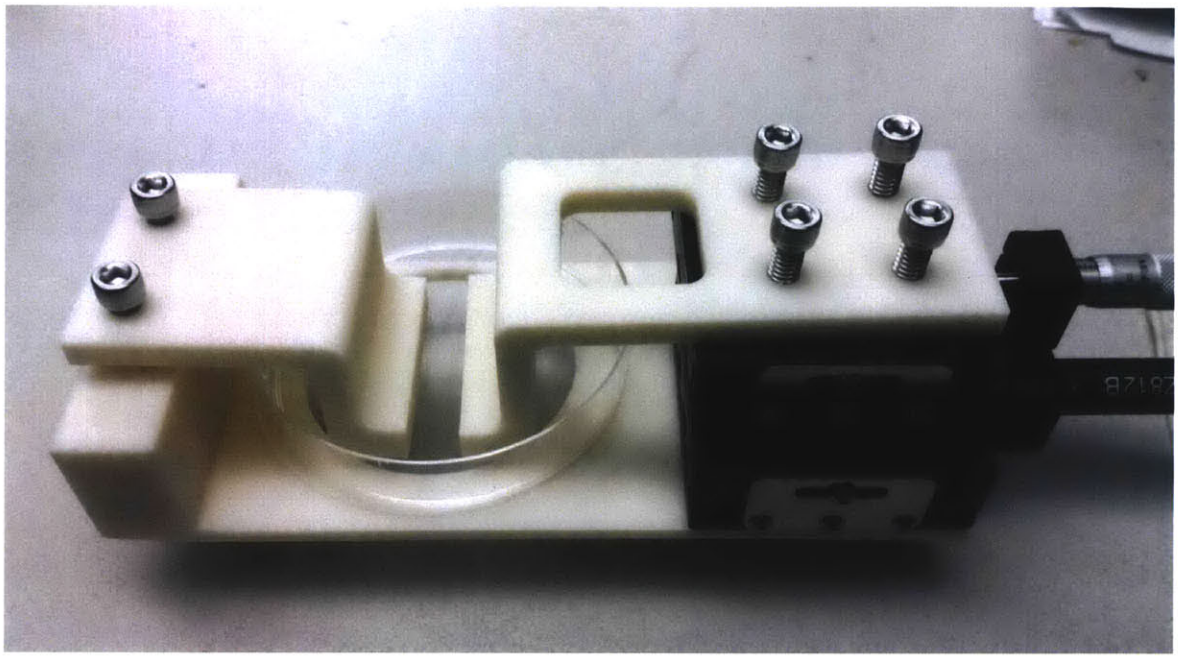


Fig. 3.1 | Device for applying the cyclic stretching to the fascicle-like muscle tissues. This system is able to put in the incubator, and imaging friendly. There is no sharp edge to avoid stress concentration.

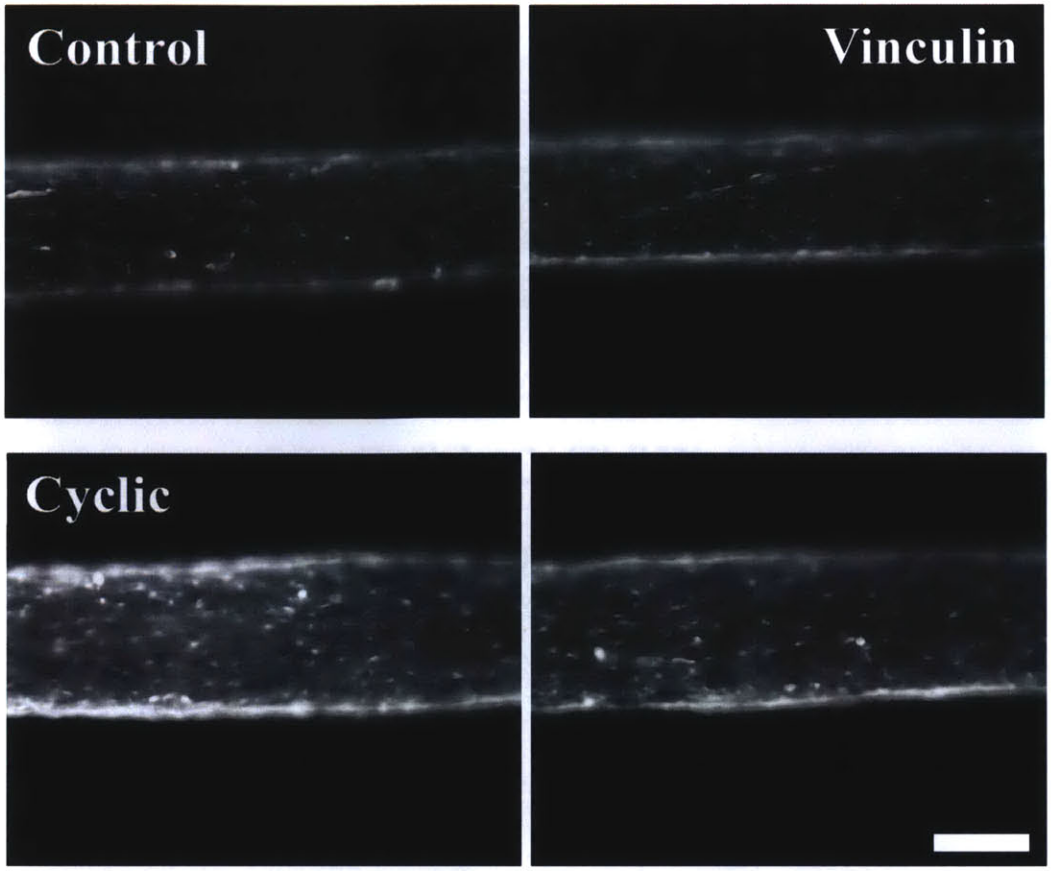


Fig. 3.2 | Visualizing of vinculin. The muscle tissues were cyclically stretched for 4 hours after 12 hours after seeding. Scale bar = 50 um.

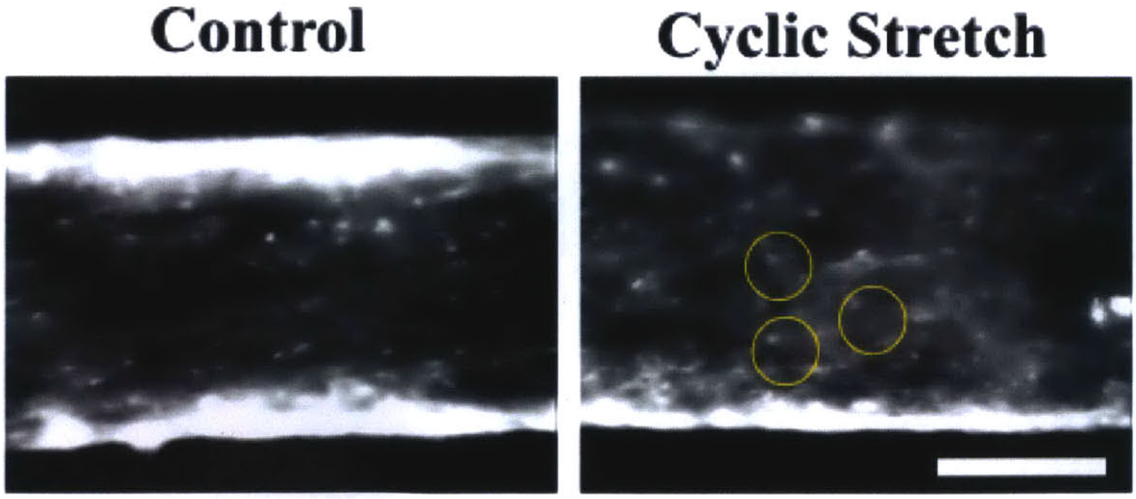


Fig. 3.3 | Visualizing of vinculin. The muscle tissues were cyclically stretched for 4 hours after 12 hours after seeding. Scale bar = 50 um.

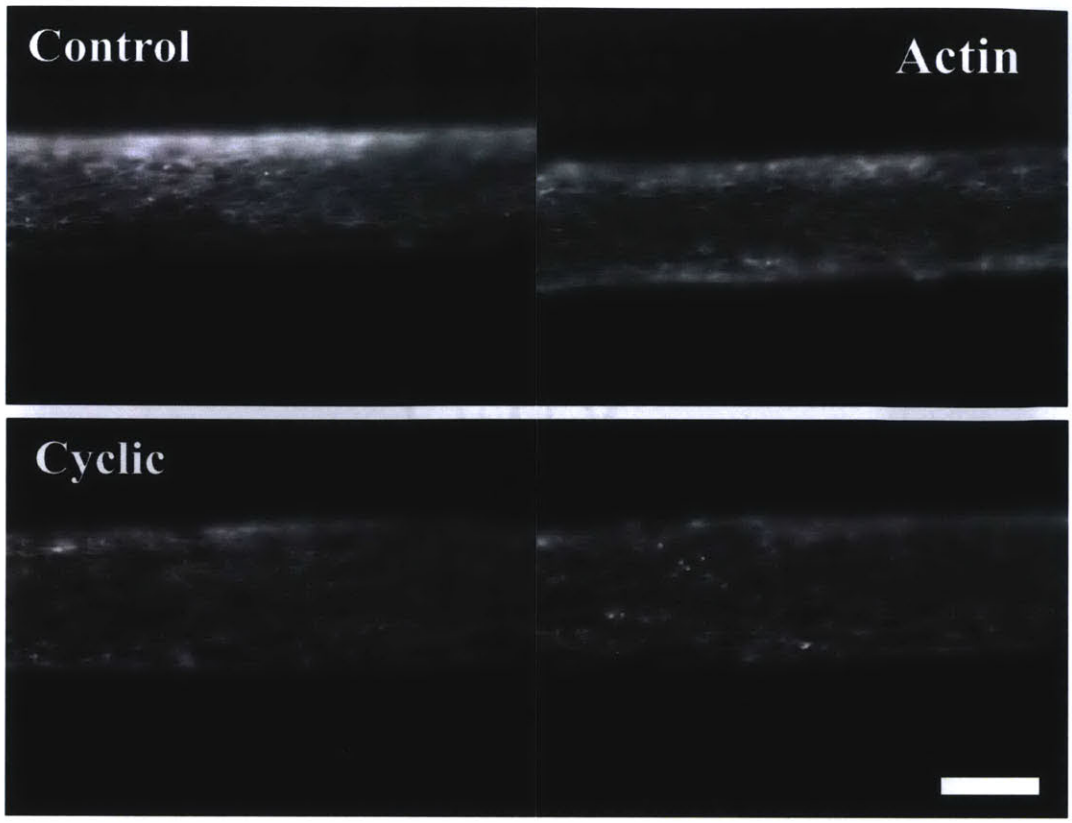


Fig. 3.4 | Visualizing of actin. The muscle tissues were cyclically stretched for 4 hours after 12 hours after seeding. Scale bar = 50 um.

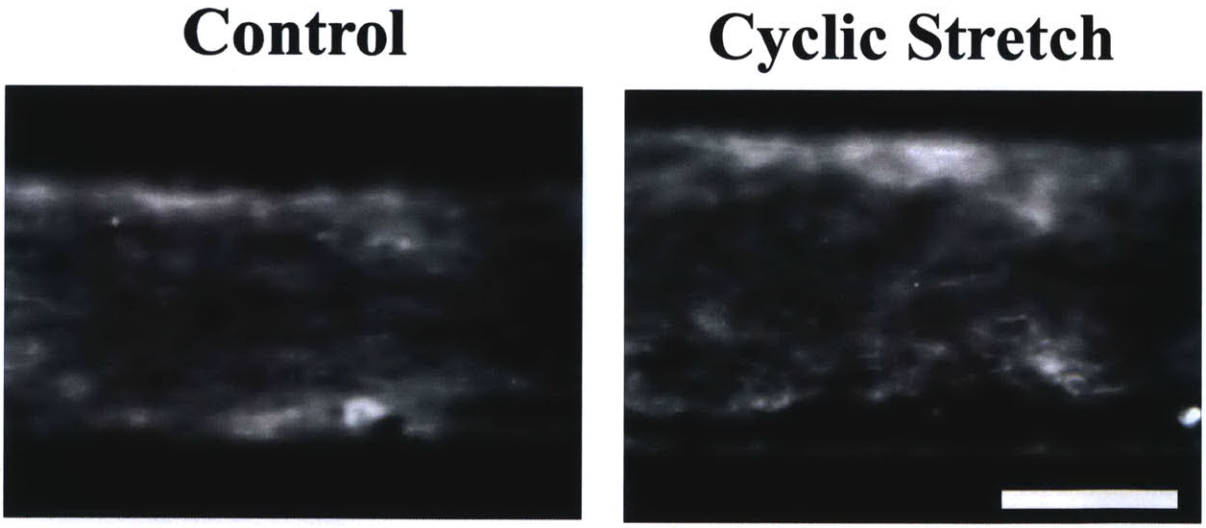


Fig. 3.5 | Visualizing of actin. The muscle tissues were cyclically stretched for 4 hours after 12 hours after seeding. Scale bar = 50 um.

CHAPTER 4: CHARACTERIZATION OF CO-STIMULATED SKELETAL MUSCLE TISSUES

4.1 INTRODUCTION

In this study, we tried to find optimal training and stimulating protocol to produce higher performance of artificial muscles. In order to make strong muscles in vitro, it is substantial to give optimal cues for muscle development instead of in vivo environment. We have been produced more developed artificial muscles which have stronger intercellular structure and cell-ECM adhesions by stretching in chapter 2 and 3. However, the stretched artificial muscles are still inferior to real muscle in terms of maturation and contractibility. We supposed that the artificial muscle might need optimal way to stimulate them more effectively. Muscles are usually exposed to electrical stimulation from neurons in combination with mechanical stimulation by contraction of surrounding muscles. However, most of studies have used electrical stimulation or light alone to stimulate muscle contraction. Thus, we hypothesize that the combination of electrical and mechanical stimulation might produce stronger contraction of muscles than electrical stimulation alone. Previous studies have shown that applying electrical stimulation to skeletal muscles promoted the transition from primary myotubes to secondary myotubes during development [46], and activated glucose metabolism with increasing the mitochondria protein activities [47]. However, if some parameter of the stimulation is wrong, than it can induce electrochemical damage to the muscle [48]. Besides the electrical stimulation, sizes of the myotubes which got mechanical stimulation such as stretching were increased with induction of Akt signaling [14], bFGF [15], and nitric oxide [16]. Based on these researches, moderate electrical or mechanical cues could affect to the muscle to generate development or maturation. Therefore, we believe that electrical and mechanical stimulations can make synergetic effect for stronger muscle contraction and more development than electrical stimulation.

In order to know how the different stimuli affect the fascicle-like muscles, we applied not only mechanical or electrical signals alone, but also combination of the two different stimuli at the same time. We called this new condition as 'co-stimulation'. We would like to produce stronger contraction of the artificial muscle tissues by the co-stimulation, which is closer to physiological condition.

We suppose that there is a synergistic effect if electric and mechanical stimuli are provided in a coordinated manner. Both electrical and mechanical stimulation affect to calcium ion concentration in the muscles, and the calcium concentration affects the exitent of muscle contraction. A calcium ion influx is induced when electrical stimulation depolarizes a cell membrane. Then, the signal is transmitted to the sarcoplasmic reticulum, and plenty of the stored calcium ions come out to the cytoplasm. Mechanical stimulation, especially the stretching, also induces calcium ion flux by the stretch activated channel (SAC). SACs are non-specific cation ion channels at membrane, which open their pores in response to stretching. Skeletal muscle cells also have some SACs, such as TRPV1 and TRPCs [49, 50], and they might allow higher calcium ion concentration in the cell by the stretching. Muscle contraction is regulated by the calcium ion concentration because calcium ions binding to troponin-tropomyosin initiates muscle contraction. Since both electrical and mechanical stimulation make change in calcium ions, it is important to apply them at right timing to produce the synergistic results. Here, we tried in-phase and out-of-phase conditions (Fig. 4.4). The in-phase condition was applying electrical stimulation only when the muscles were maximally stretched. The out-of-phase condition was giving the electrical stimulation only when the muscle tissues restored to the original position. We would like to see the difference of muscle performance by the phase shift of the two stimuli. We also compared with the electrical stimulation alone to check whether the co-stimulation induces stronger contraction than electrical stimulation. To achieve this goal, we developed the device to apply the co-stimulation, and compared the performance by electrical stimulation.

4.2 Materials and Methods

4.2.1 Fabrication of Multi-stimulation System

The multi-stimulation system is printed by 3-D printer (Dimension 1200es) to give electrical and mechanical stimulation at the same time (Fig. 4.1). Electrical stimulation is given by two platinum wires that put in the medium beside tissues. They allow to give uniform electric field, 5 V/mm, to the muscle tissues. The copper wire cantilever applies mechanical stimulation by pushing the muscle. Tip of the cantilever is located at the center of the muscle strips, and the tip movement induces muscle elongation (Fig. 4.2). The cantilever is compliant, and this could be used for quantifying the muscle contractions. When we stretch the muscles in 5% strain by pushing the cantilever tip, the tension in the muscle and a restoring force of cantilever balances.

When we apply the electrical stimulation additionally, the tip is displaced by the muscles contractions towards muscle's original position. We use the same cantilever for all the results here, so the large tip displacement by contraction means larger contraction force by muscles (Fig. 4.3). We track the displacement of the tip by the tracker program (comPADRE).

4.2.2 Culture of C2C12 Myoblasts

The C2C12 mouse myoblast cell line, used for study, was transfected with Channelrhodopsin-2 DNA to enable control and contraction via controlled light (~470 nm) using by pAAV-Cag-Chr2-GFP-2A-Puro with LipofectamineTM 2000 (Invitrogen). The myoblasts were maintained in growth media, which is Dulbecco's modified Eagle medium (DMEM, ATCC) supplemented with 10% Fetal Bovine Serum (FBS, Sigma), 1% Penicillin-Streptomycin (PS, Sigma), and 0.2% Normocin (InvivoGen). The cells were passaged when 70~80% confluent. The growing media was changed every 2 days.

4.2.3 Fascicle-like Muscle Tissue

The PDMS chips were autoclaved before seeding to avoid contamination. Gelatin solution was poured in the PDMS chips as a sacrificial gel. The 5% gelatin solution melted by 37°C growth medium was solidified at 4°C for 30 minutes. The solution also contained thrombin to use fibrin as the extracellular matrix (ECM), and 0.05 M NaOH solution for pH adjustment. Then, the trypsinized C2C12 mouse myoblasts were centrifuged, and the cell pellet was mixed with growth factor reduced matrigel (356231, BD Matrigel), fibrinogen, and cold growth medium. After removing the pins from the gelatin-solidified chips, the cell and gel mixture is seeded in the holes of the chips. The seeded chip was heated to 37°C in the incubator, and the gelatin solution was melted. Thus, the thrombin of gelatin solution in liquid state diffused into the cell and gel mixture, and it converted fibrinogen into gel-forming fibrin. As a result, fibrin became main ECM together with matrigel for the muscle cells. Remaining gelatin was diluted by medium and it was removed by changing medium every day.

For two days after seeding, the cells are cultured in growth medium (GM) with 1 mg/ml aminocaproic acid (AA). AA was added to mitigate ECM degradation by proteinase secreted from surrounding cells. After that, we changed the media into differentiation media (DM) with 1 mg/ml AA. DM is the same as GM except for 4% horse serum instead of the fetal bovine serum (FBS). The media was changed every day up to 10 days.

4.3 Results and Discussion

4.3.1 Improvement in Muscle Contraction

Our goal was comparison of the muscle performances changed by different kinds of stimuli: Muscles were stimulated with 1) the in-phase and 2) the out-of-phase co-stimulation. Also, we compared those co-stimulation results with 3) contraction by the electrical stimulation. We applied 5% strain stretching in 0.33Hz. Also, the condition for electrical stimulation was 5 V/mm, 1 ms impulse. After seeding the C2C12 cells in the fascicle-like PDMS device, we waited for 10 days, when the artificial muscles tissues had maximum performance by maturation. After the 10 days, muscle started degradation. Because each muscle strip had different performance, we used the same muscle strip within one experiment for comparison. The muscles got stimulation for 3 minutes, and measured stimulation for about 1.5 minutes, and took the rest for 3 minutes between the conditions.

As a result, the muscle contraction by the in-phase and the out-of-phase co-stimulation were 2 times and 1.2 times higher than only electrical stimulation (Fig. 4.5). In addition, the in-phase co-stimulation induced higher performance than the out-of-phase co-stimulation (p value=0.0001), even the only difference between the two stimuli were phase shift. We tested four experiments with totally 26 and 37 contractions for out-of-phase and in-phase conditions. We successfully gained consisted results with Fig. 4.5 from the three out of the four experiments. The inconsistent result had strong contractions in both out-of-phase and in-phase conditions (p value=0.0023).

The order of the stimulation could be important because we used the one strip per one experiment for a fair comparison. As time goes on, the muscle performance can be decreased by being exposed to non-physiological condition outside of incubator. Also, the previous stimulation that the muscle had received could affect the subsequent results if rest time was too short. Therefore, we measured the muscle performances with different orders. In the previous experiment, we tested in the order of electrical, out-of-phase and in-phase co-stimulation. By changing the order, we applied in-phase co-stimulation first, out-of phase one second, and repeated the in-phase one again. After that, we measured the contraction by electrical stimulation as the fourth step. As a result, muscle performance from the first and third in-phase co-stimulation were almost same, and out-of-phase condition had lower performance than in-phase

one (Fig. 4.6). This result means the in-phase co-stimulation induces higher muscle contraction than the contraction by the out-of-phase one regardless of order.

On the other hand, the electrically stimulated muscle contractions depended on the experimental order. Before we tested the fourth only electrical test, the muscle tissue got twice of in-phase and one out-of-phase co-stimulation with every 3 minutes of the rest time between the tests. Then, the fourth ordered electrical stimulation produced the strong muscle contraction as much as the third ordered in-phase result unlike the previous result (Fig. 4.7). However, the first applied electrical stimulation induced lower contractions than the following co-stimulation (Fig. 4.6). These results indicate that there could be permanent change in contractile structure or calcium influx by previous co-stimulation. Fig. 4.6 and 4.7 were obtained by at least 6 contractions per each condition from one strip. These results should be repeated by more experiments.

4.4 CONCLUSIONS

We sought to compare the muscle performances stimulated by different kinds of stimuli. The muscle contraction by the co-stimulation, especially by the in-phase condition, was larger than the contraction by the electrical stimulation. The in-phase co-stimulation induced the higher muscle performance than the out-of-phase one, even though we switched the experiment order. We applied the each stimulation for 3 minutes, and it was too short time to change developmental stage or protein expression levels. Also, when we tested co-stimulation first and then tested the electrical stimulation, the electrical stimulation caused high performance as much as the co-stimulation. Thus, the performance improvement could be produced by instant and permanent structural changes in complex contraction structures. The fascicle-like muscle tissues at day 10 had lower performance than an actual muscle. This means that the tissues had less developed contractile structure. Therefore, the structure could be irreversibly improved or enhanced by the co-stimulation.

Another possible reason is that the muscle tissues are already matured, but not optimally stimulated. In vivo, skeletal muscles usually get electrical and mechanical stimulation at the same time. Because the co-stimulation is closer to physiological condition than only electrical stimulation, the co-stimulation could induce larger contraction. This might be allowed by additional calcium ions influx from the stretch activated ion channel (SAC). Thus, the

mechanical stretching of the co-stimulation could allow distinct calcium ion behavior in the muscle, and this might induce the larger contractions. This also might explain why the in-phase condition produced higher contraction than out-of-phase condition. The in-phase condition could have higher calcium ion concentration than out-of-phase one by the adding more calcium through SACs at the timing that the concentration reached maximum by electrical stimulation. More investigation is needed to figure out the exact mechanism of the co-stimulation. This result will contribute to an optimal protocol for making high-performance artificial muscles.

4.5 Figures and Captions

Fig. 4.1 | Device for applying co-stimulation to the fascicle-like muscle tissues. The co-stimulation system was printed by 3-D printer. There are two electrodes for electrical stimulation, and one cantilever for mechanical stimulation and measuring contraction.

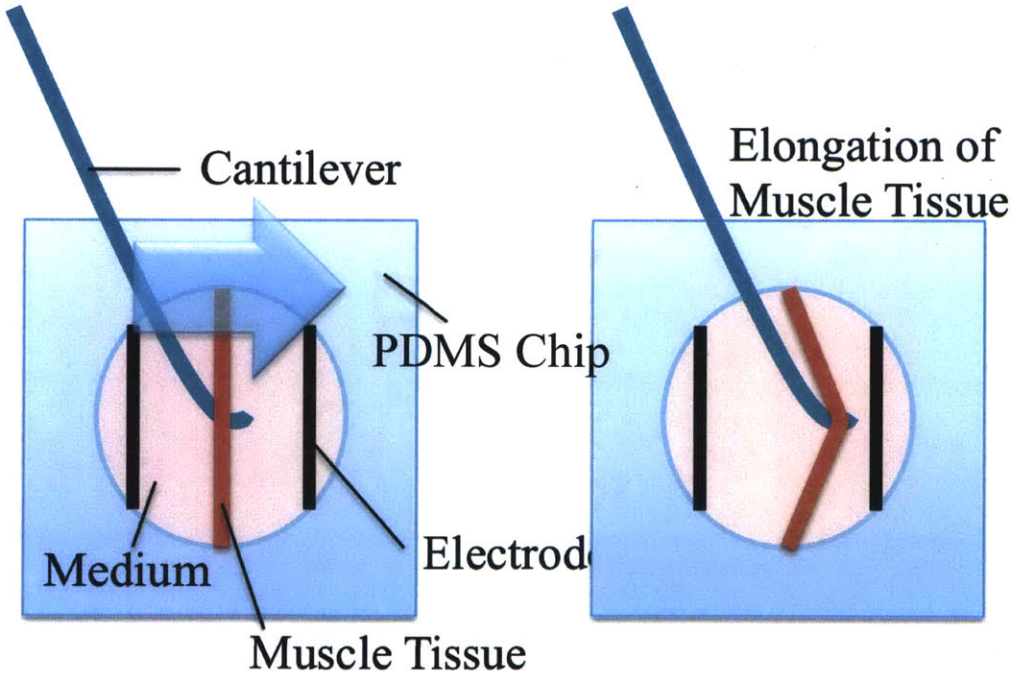


Fig. 4.2 | Elongation of the fascicle-like muscle tissues using cantilever. Tip of the cantilever was put beside the center of the muscle tissue, and pushed the tissue to stretch them.

Fig. 4.3 | Measurement of the muscle contraction. Tip of the cantilever was put beside the center of the muscle tissue, and the muscle is stretched as much as 5% strain. Muscle contraction by electrical stimulation pushed the tip toward muscle's original position. Red dot was original point, and the red dot moved to the yellow point by muscle contraction. The distance between the two dots is measured into contraction distance.

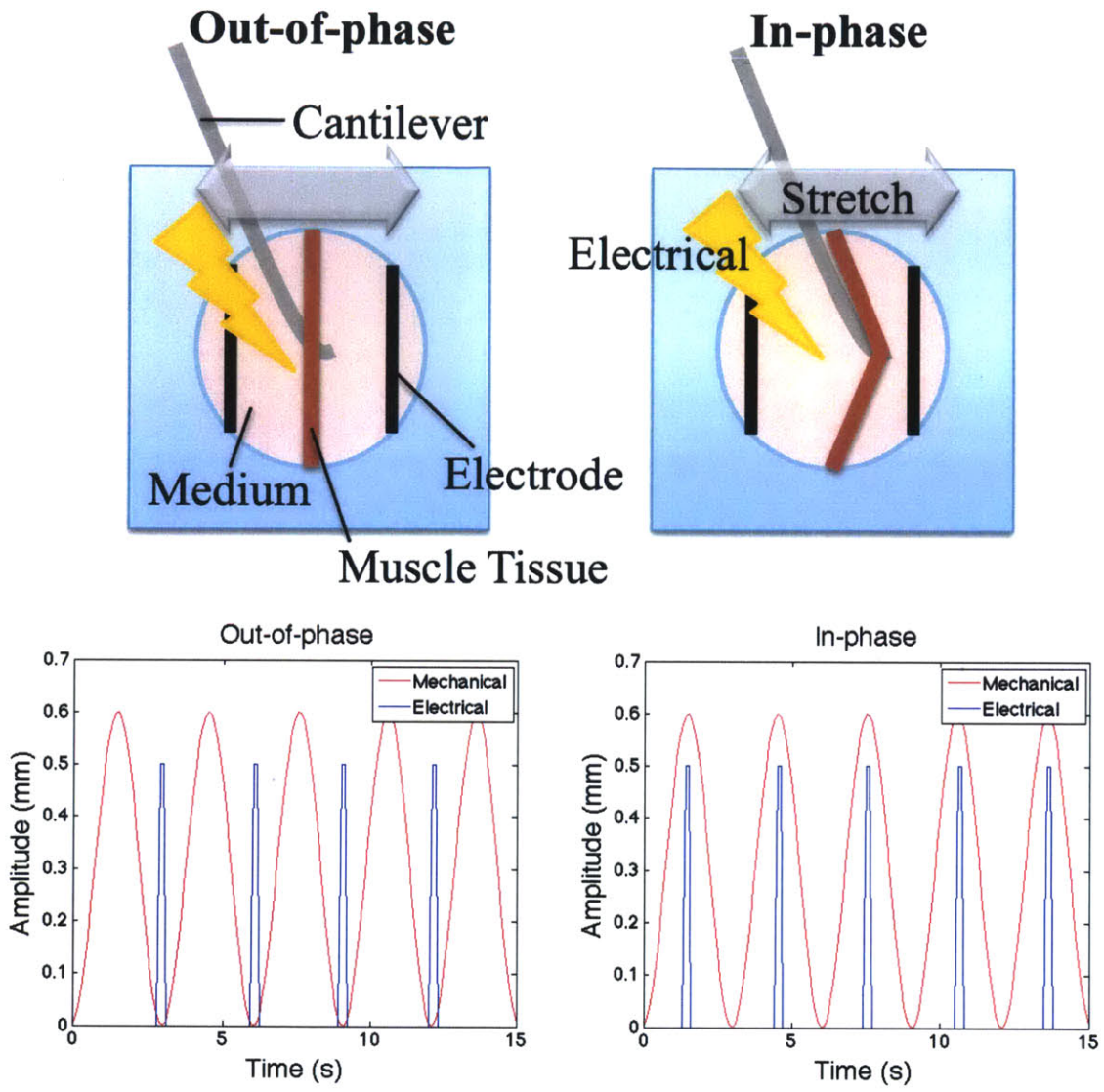


Fig. 4.4 | Two kinds of the co-stimulation: out-of-phase and in-phase. During the cyclic stretching, out-phase is giving electrical when the tissue is relaxed, and in-phase is electrically stimulating when the muscle is maximally stretched.

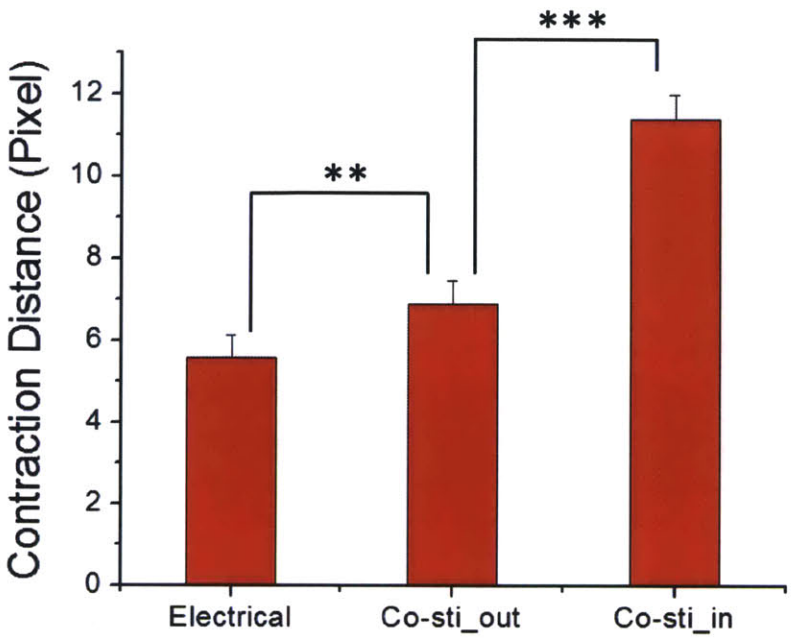


Fig. 4.5 | Comparison of muscle performance by the co-stimulation and electrical stimulation. The co-stimulation were applying stretching and electrical field at the same time with in-phase and out-of-phase. SD, n=6, 5, 8, respectively. **p<0.01 (Very significant), ***p<0.001 (Extremely significant).

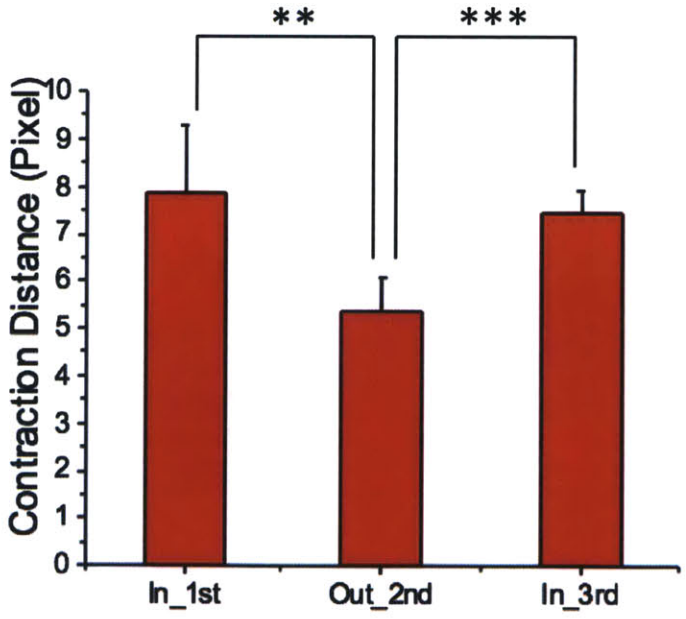


Fig. 4.6 | Comparison of muscle performance by co-stimulation with changing the testing order. The co-stimulation was applying stretching and electrical field at the same time with in-phase and out-of-phase. SD, n=8, 7, 6, respectively. **p<0.01 (Very significant), ***p<0.001 (Extremely significant).

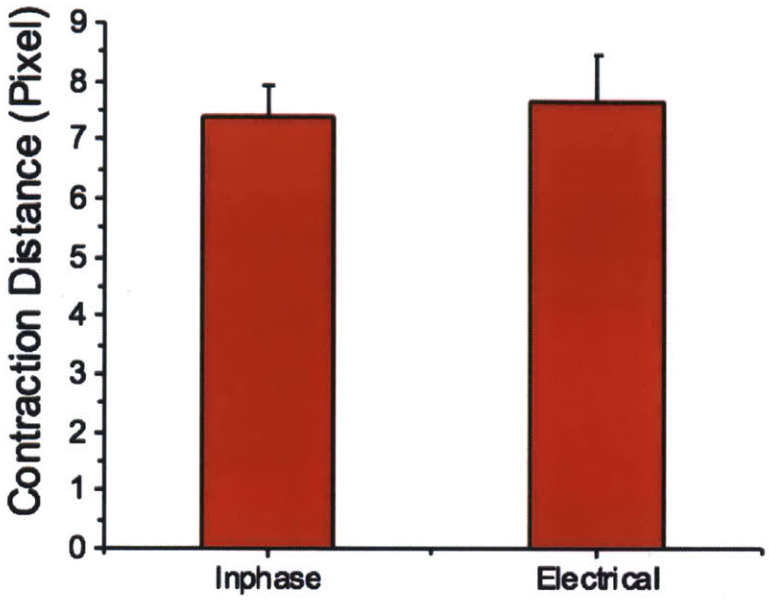


Fig. 4.7 | Comparison of muscle performance with changing the testing order. The co-stimulation were applying stretching and electrical field at the same time with in-phase and out-of-phase. SD, n=6. $p>0.05$ (Not significant)

CHAPTER 5: CONCLUSION AND FUTURE DIRECTIONS

5.1 CONCLUSION

We realized that the fascicle-like muscle tissue, which is the densely populated 3-D tissue, could have stronger performance and more matured structure by appropriate stimulation. In this study, we applied static (Chapter 2) and cyclic (Chapter 3) stretching, electrical stimulation and the co-stimulation (Chapter 4). Both the static and cyclic stretching caused larger aggregated vinculin dots in soft gel, which mean stronger cell-ECM adhesions by external force. This result also showed that 3-D cultured cells have not only small focal complex but also strong focal adhesions even in 3-D matrix, but the component of the adhesion could be different with 2-D cultured cells. The static tension at day 9 induced striated actin of the muscles (Fig. 2.10), but the cyclic tension at day 1 caused weakening of actin with less alignment (Fig. 3.4, 3.5). Similarly, the stretching can affect other proteins, especially related to muscle development. As a result, the effect of the stretching to the muscle tissue is highly time-dependent, and it is important to find optimal start timing for efficient training.

The co-stimulation system allows to compare muscle performance with many different conditions of stimulation, and to quantify the muscle contractions. Using this system, we have shown that the co-stimulation induces higher performance of the muscles than the electrical stimulation (Fig. 4.5). Although conditions of the mechanical and electrical stimulation were same, the performance could be changed only by phase shift between the two stimuli (Fig. 4.5, 4.6). In addition, the performance was order-dependent between the electrical and co-stimulation conditions (Fig. 4.7). This means that there could be permanent structural change instantly by the co-stimulation, which could be improvement or adjustment of the contractile structures. Another possible reason is calcium ions influx by stretch activated ion channel such as TRPV1 and TRPCs. The mechanical stimulation of the co-stimulation might produce more calcium ion influx in the muscle, and this might induce the stronger contractions. In conclusion, we still do not know exact mechanism, but the co-stimulation can be used to produce higher performance of artificial muscle in drug testing or moving bio-robots.

5.2 FUTURE DIRECTIONS

This study will contribute to make optimal protocol for high performance artificial muscle tissue. To achieve this goal, we should try more number of conditions with changing the start timing of the stretching and frequencies to effectively train the muscle. In order to see the exact structure changes by the co-stimulation, we are going to take confocal images of the stimulated muscle tissue. Also, the calcium imaging of the muscle contraction by the co-stimulation will also help to figure out what happens inside of the muscles. Based on the knowledge about a nature of the muscle, we would like to induce maximum performance for many applications. The possible applications of the artificial muscle tissues are an actuator for micro-robot, which has high degree of freedom motion. If we success to bundle each well-developed fascicle-like tissue, it can be possible to make a large-scale actuator like the muscles in animals. In addition to use the artificial muscle tissue as an actuator of bio-bot, it can be used for drug screening platform for muscle diseases such as Duchenne muscular dystrophy [51], transplant to patient, meat production in vitro [52], and understanding nature of muscle development. Further researches about co-culture with blood vessel cells or neurons might be needed for the successful transplantation.

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CHAPTER 6: REFERENCES

1. Koch, Ute, Rajwinder Lehal, and Freddy Radtke. "Stem cells living with a Notch." Development 140.4 (2013): 689-704.

2. Li, Zhenlin, et al. "Desmin is essential for the tensile strength and integrity of myofibrils but not for myogenic commitment, differentiation, and fusion of skeletal muscle." The Journal of cell biology 139.1 (1997): 129-144.

3. Sanger, Joseph W., et al. "Myofibrillogenesis in skeletal muscle cells." Clinical orthopaedics and related research 403 (2002): S153-S162.

4. Nawroth, Janna C., et al. "A tissue-engineered jellyfish with biomimetic propulsion." Nature biotechnology 30.8 (2012): 792-797.

5. Chan, Vincent, et al. "Development of miniaturized walking biological machines." Scientific reports 2 (2012).

6. Williams, Glenn N., Michael J. Higgins, and Michael D. Lewek. "Aging skeletal muscle: physiologic changes and the effects of training." Physical Therapy 82.1 (2002): 62-68.

7. Vandenburgh, Herman H., Patricia Karlisch, and Lynne Farr. "Maintenance of highly contractile tissue-cultured avian skeletal myotubes in collagen gel." In vitro cellular & developmental biology 24.3 (1988): 166-174.

8. Strohman, Richard C., et al. "Myogenesis and histogenesis of skeletal muscle on flexible membranes in vitro." In Vitro Cellular & Developmental Biology 26.2 (1990): 201-208.

9. Dennis, Robert G., et al. "Excitability and contractility of skeletal muscle engineered from primary cultures and cell lines." American Journal of Physiology-Cell Physiology 280.2 (2001): C288-C295.

10. Kosnik, Paul E., John A. Faulkner, and Robert G. Dennis. "Functional development of engineered skeletal muscle from adult and neonatal rats."Tissue engineering 7.5 (2001): 573-584.

11. Huang, Yen-Chih, et al. "Rapid formation of functional muscle in vitro using fibrin gels." Journal of Applied Physiology 98.2 (2005): 706-713.

12. Neal, Devin, et al. "Formation of elongated fascicle-inspired 3D tissues consisting of high-density, aligned cells using sacrificial outer molding." Lab on a Chip 14.11 (2014): 1907-1916.

13. Shimizu, Kazunori, Hideaki Fujita, and Eiji Nagamori. "Alignment of skeletal muscle myoblasts and myotubes using linear micropatterned surfaces ground with abrasives." Biotechnology and bioengineering 103.3 (2009): 631-638.

14. Soltow, Quinlyn A., et al. "Cessation of cyclic stretch induces atrophy of C2C12 myotubes." Biochemical and biophysical research communications 434.2 (2013): 316-321.

15. Clarke, M. S., and DANIEL L. Feeback. "Mechanical load induces sarcoplasmic wounding and FGF release in differentiated human skeletal muscle cultures." The FASEB journal 10.4 (1996): 502-509.

16. Soltow, Quinlyn A., et al. "Nitric oxide regulates stretch-induced proliferation in C2C12 myoblasts." Journal of muscle research and cell motility 31.3 (2010): 215-225.

17. Goto, K., et al. "Effects of heat stress and mechanical stretch on protein expression in cultured skeletal muscle cells." Pflügers Archiv 447.2 (2003): 247-253.

18. Zimmermann, W-H., et al. "Tissue engineering of a differentiated cardiac muscle construct." Circulation research 90.2 (2002): 223-230.

19. Clause, Kelly C., et al. "Engineered early embryonic cardiac tissue increases cardiomyocyte proliferation by cyclic mechanical stretch via p38-MAP kinase phosphorylation." Tissue Engineering Part A 15.6 (2009): 1373-1380.

20. Cheng, Wel, et al. "Stretch-induced programmed myocyte cell death." Journal of Clinical Investigation 96.5 (1995): 2247.

21. Powell, Courtney A., et al. "Mechanical stimulation improves tissue-engineered human skeletal muscle." American Journal of Physiology-Cell Physiology283.5 (2002): C1557-C1565.

22. Deisseroth, Karl. "Controlling the brain with light." Scientific American 303.5 (2010): 48-55.

23. Deisseroth, Karl. "Optogenetics." Nature methods 8.1 (2011): 26-29.

24. http://www.physioweb.org/muscular/sk_muscle.html.

25. Adam F., Alex F., et al. 2007, "Muscular Thin Films for Building Actuators and Powering Devices". Science, 37, September, pp. 1366-1370.

26. Weining B., Nenad B., 2009, "Engineered Sekeltal Muscle Tissue Networks with Controllable Architecture", Biomaterials, 30, pp. 1401-1412.

27. Altomare, L., Riehle, M., Gadegaard, N., 2010, "Microcontact printing of fibronectin on a biodegradable polymeric surface for skeletal muscle cell orientation", Int J Artif Organs, 33, pp. 535.

28. Jianzhong X., Jacob S., Carlo M., et al., 2005, "Self-assembled microdevices driven by muscle", Nature Materials, 4, February, pp. 180-184.

29. Rossi A., Flaibani, M., Blaauw, B., et al., 2011, "In vivo tissue engineering of functional skeletal muscle by freshly isolated satellite cells embedded in a photopolymerizable hydrogel", FASEB J, 25, pp. 2296.

30. Stern-Straeter J., Bach D., Stangenberg L., et al. 2005, "Impact of electrical stimulation on three-dimensional myoblast cultures – a real-time RT-PCR study", J Cell Mol Med, 4, pp. 883-892.

31. Wehrle U., Dusterhoft S., Pette D., 1994, "Effects of chronic electrical stimulation on myosin heavy chain expression in satellite cell cultures derived from rat muscles of different fibertype composition", Differentiation, 1, pp. 37-46.

32. Bursac N., Papadaki M., Cohen R,, et al., 1999, "Cardiac muscle tissue engineering: toward an in vitro model for electrophysiological studies", Heart, 277(2), August, pp. H433-H444.

33. Frank E., Yixin Z., 2010, "Reversible photoswitching of protein function", Mol. BioSyst., June, pp. 2103-2109.

34. Mahmut S., Devin N., et al., 2012, "Formation and optogenetic control of engineered 3D skeletal muscle bioactuators", Lab Chip, pp. 4976-4985.

35. Squire, J. M, et al. 1997 "Architecture and Function in the Muscle Sarcomere". Curr. Opin. Struct. Biol., 7, pp. 247–257.

36. Davison, D., Critchley, R., 1988, "Alpha-Actinins and the DMD Protein Contain Spectrin-like Repeats". Cell, 52, pp. 159–160.

37. Fraley, Stephanie I., et al., 2010, "A distinctive role for focal adhesion proteins in three-dimensional cell motility." *Nature cell biology* 12.6, pp. 598-604.

38. Cukierman, Edna, Roumen Pankov, and Kenneth M. Yamada., 2002, "Cell interactions with three-dimensional matrices." *Current opinion in cell biology*14.5, pp. 633-640.

39. Spanjaard, Emma, and Johan de Rooij. 2013, "Mechanotransduction: vinculin provides stability when tension rises." *Current Biology* 23.4 pp. R159-R161.

40. Ramsey, Robert W., and Sibyl F. Street. "The isometric length - tension diagram of isolated skeletal muscle fibers of the frog." Journal of Cellular and Comparative Physiology 15.1 (1940): 11-34.

41. Curtin, Nancy A., and R. E. Davies. "Chemical and mechanical changes during stretching of activated frog skeletal muscle." Cold Spring Harbor Symposia on Quantitative Biology. Vol. 37. Cold Spring Harbor Laboratory Press, 1973.

42. Wehrle-Haller, Bernhard. "Structure and function of focal adhesions." Current opinion in cell biology 24.1 (2012): 116-124.

43. Brammer, Karla S., Christine J. Frandsen, and Sungho Jin. "TiO2 nanotubes for bone regeneration." Trends in biotechnology 30.6 (2012): 315-322.

44. Goldmann, Wolfgang H. "Mechanotransduction and focal adhesions." Cell biology international 36.7 (2012): 649-652.

45. Parsons, J. Thomas, Alan Rick Horwitz, and Martin A. Schwartz. "Cell adhesion: integrating cytoskeletal dynamics and cellular tension." *Nature reviews Molecular cell biology* 11.9 (2010): 633-643.

46. Wilson, Suzanne J., and A. John Harris. "Formation of myotubes in aneural rat muscles." Developmental biology 156.2 (1993): 509-518.

47. Khodabukus, Alastair, and Keith Baar. "Defined electrical stimulation emphasizing excitability for the development and testing of engineered skeletal muscle." Tissue Engineering Part C: Methods 18.5 (2011): 349-357.

48. Nikolić, Nataša, et al. "Electrical pulse stimulation of cultured human skeletal muscle cells as an in vitro model of exercise." PLoS One 7.3 (2012): e33203.

49. Luo, Zhidan, et al. "TRPV1 activation improves exercise endurance and energy metabolism through PGC-1α upregulation in mice." Cell research 22.3 (2011): 551-564.

50. Vandebrouck, Clarisse, et al. "Involvement of TRPC in the abnormal calcium influx observed in dystrophic (mdx) mouse skeletal muscle fibers." The Journal of cell biology 158.6 (2002): 1089-1096.

51. Vandenburgh, Herman, et al. "Automated drug screening with contractile muscle tissue engineered from dystrophic myoblasts." The FASEB Journal 23.10 (2009): 3325-3334.

52. Langelaan, Marloes LP, et al. "Meet the new meat: tissue engineered skeletal muscle." Trends in food science & technology 21.2 (2010): 59-66.