Fabrication of skeletal muscle tissue constructs using coculture and microfabrication systems

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Submitted by

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Chapter 1

Introduction

1.1 Regenerative medicine

Regenerative medicine is the potential tool to heal or replace tissues and organs damaged by different disease or trauma or due to the congenital defects. Now a day, regenerative medicine is the common term among physicians and scientists for treating different chronic diseases and acute insults e.g. different types of dermal wounds, cardiovascular diseases, traumas and also treatment for certain types of cancers [1-3]. Current therapy of transplantation of organs and tissues to treat tissue and organ failures sometimes difficult due to limited donor supply. Also, after transplantation there is a risk of immune rejection of transplanted tissue or organ grafts. But, these obstacles may potentially be solved through the use of regenerative medicine strategies.

The process of regenerative medicine is given in Fig. 1-1. It requires two main strategies for generating patient-specific cells of a desired type. Pluripotent cells to be used for regenerative medicine can be either patient-derived (induced pluripotent stem cells (iPSCs)) or non-patient-derived (either embryonic stem cells (ESCs) or iPSCs). Pluripotent cells can be differentiated in vitro to a desired cell state (directed differentiation, right). Alternatively, primary cells derived from a patient can be used to generate a desired cell type directly reprogramming (left). Cells of a desired type obtained by either of these methods can then be studied in vitro (bottom) or used for transplantation into patients (top).

The field of regenerative medicine covers a wide range of areas including the use of materials and de novo generated cells, as well as various combinations thereof, to take the place of missing tissue, effectively replacing it both structurally and functionally or to contribute tissue healing [4]. Normally, body's innate immune system is involved to

promote regeneration, but adult humans possess limited regenerative capacity in comparison with lower vertebrates [5].

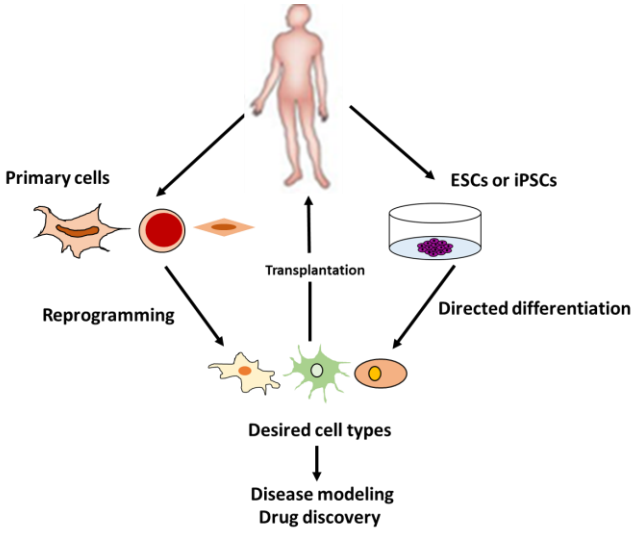

Fig. 1-1. Process of regenerative medicine

Since tissue engineering and regenerative medicine emerged as an industry about two decades ago, Food and drug administration (FDA) approved a number of therapies for commercial availability. Till today, the delivery of therapeutic cells that directly contribute to the structure and function of new tissues is a principle paradigm of regenerative medicine. Generally, autologous or allogenic and typically differentiated cells are used in these therapies those have proliferative capacity [6]. Besides cells, materials are often an important component of current regenerative medicine strategies because material can mimic the native extracellular matrix (ECM) of tissues and contribute to the structure and function of new tissues and locally present growth factors [7]. For example, 3D polymer scaffolds are used to promote expansion of chondrocytes and cartilage repair and provide scaffolds for fibroblasts in the treatment of venous ulcers [8]. Sometimes a material can alone provide cues for regeneration and graft or implant integration, as in the case of bioglass-based grafts that permit fusion with bone [9]. Incorporation of growth factors promote healing or regeneration into biomaterials can provide a local and sustained presentation of these factors, and this approach has been exploited to promote wound healing by delivery of platelet derived growth factors and bone formation via the delivery of bone morphogenetic protein (BMP-2) [10].

However, the study of regenerative medicine comprises a wide range of studies at both pre-clinical and clinical stages which includes: recapitulating organ and tissue

structure via scaffold fabrication, integrating grafts with the host via vascularization and innervation, and, altering the host environment to induce therapeutic responses through cell infusion and modulating the immune system [11]. Most regenerative medicine strategies rely on an ample cell source, but identifying and obtaining sufficient numbers of therapeutic cells is often a challenge. Stem, progenitor and differentiated cells derived from both adult and embryonic tissues are widely being explored in regenerative medicine although adult tissue-tissue derived cells are the dominant cell types used clinically to date due to both their availability and safety [6].

There is a great chance in obtaining a large number of stem cells from adult tissues and in identifying stem cell populations are suitable for therapeutic use in tissues [12]. Stem cells play a vital role in the field of regenerative medicine. For example, coculture of hematopoietic stem cells (HSCs) with cells implicated in the HSC niche and in microenvironments engineered to mimic bone marrow improve HSC stemness during expansion, enhancing stem cell numbers for transplantation such as direct contact of HSCs with mesenchymal stem cells (MSCs)grown in a 3D environment induces greater $CD34^{+}$ expansion than with MSCs grown in 2D substrate [13]. Another example is that culture of skeletal muscle stem cells on substrate with mechanical properties similar to normal muscle leads to greater stem cell expansion, rescue impaired proliferative ability in stem cells from aged animals [14]. Also, Embryonic stem (ES) cells are characterized potential infinite sources of cells for regeneration and can be used for clinical application [15]. ES cells are derived from blastocyst stage embryos and have been shown to be pluripotent, giving rise to tissues from all three germ layers [16].

1.2 Tissue Engineering

Tissue engineering is an interdisciplinary field which comprises the principle of engineering and life sciences toward the development of biological substitutes that restore, maintain, and improve tissue function or whole organ [17]. Development of biological substitutes by using tissue engineering techniques based on three key elements: living cells, biomaterials that provide the base for cells to attach, and bioactive factors that stimulate cells to make proliferation and differentiation.

Tissue engineering approach includes isolation and purification of a cell source, seeding of the cells on scaffold, stimulation of the cell -seeded scaffold to develop a tissue equivalent and implantation of the construct *in vivo.*

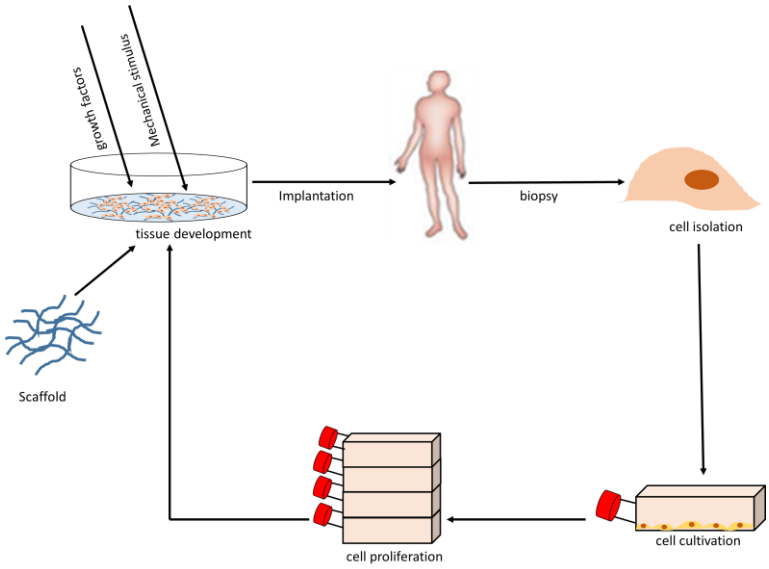

Fig.1.2. Strategies of tissue engineering

Tissue engineering strategies require interaction and integration through incorporation of appropriate physical and cellular signals. Therefore, inclusion of modifying factors such as biologically active proteins and DNA are critical to success [18]. Two main approaches are utilized in this area to produce engineered tissue. First, scaffolding can be used as a cell support device upon which cells are seeded *in vitro*, cells are then encouraged to lay down matrix to produce the foundations of a tissue for transplantation. The second approach involves using the scaffold as a growth factor/drug delivery device. This strategy involves the scaffold being combined with growth factors, so upon implantation cells from the body are recruited to the scaffold site and from tissue upon and throughout the matrices. These two approaches are not mutually exclusive and can be easily combined [19]. The assembly of cells into tissues is highly orchestrated set of events that requires time scale ranging from seconds to weeks and dimensions ranging from 0.0001 to 10 cm. Until today different engineered tissues have been approved by the Food and Drug Administration (FDA). But, still there are many technical challenges to overcome before the creation of the shelf tissues that represent the translation of scientific discoveries into treatments for millions of patients. The successful large- scale production

of engineered tissues requires an adequate source of healthy expandable cells, the optimization of scaffolds, and the creation of bioreactors which mimic the environment of the body and that are amenable to scale up. Another type of challenge includes the preservation of the product so that it has a long shelf-life and successful use of various approaches to prevent tissue rejection.

The important prerequisite for effective tissue engineering is the cellular environment that allows the cells to function as they do in native tissue. Sometimes environment mimics several critical aspects of the in vivo niche through proper control of the chemical and mechanical setting as well as the chemical stimuli. Cell scaffolds generally serves several purposes including: a). cell attachment and perhaps migration; b). retention and presentation of biochemical factors; c). porous environment for adequate diffusion of nutrients, expressed products and waste, and d). mechanical rigidity or flexibility [20].

The production of engineered tissues *in vitro* requires the use of cells to populate matrices and produce matrix resembling that of the native tissues. The source of the cells is also an important choice for scaffolds [21]. A range of cell types can now be combined with scaffolds to produce tissue engineered constructs. Major success in this field have been achieved from the use of patient specific primary cells to fabricate tissues for re-implantation. However, this strategy has some limitations because of the invasive nature of the cell collection and the potential for the cells to be in a diseased state [22]. Recently, different types of cell lines and stem cells including ES cells, bone-marrow derived mesenchymal stem cells (BM-MSCs) and umbilical- cord derived mesenchymal stem cells (UM-MSCs) are being extensively used for fabricating artificial tissues. Many researchers have chosen cell lines for tissue fabrication rather than primary cells as they are highly proliferative and easier to culture and transfect [23]. Cell lines applied in the field of tissue engineering includes: C2C12 cell lines for skeletal muscle tissue engineering, MC-3T3 cell lines for bone tissue engineering, human umbilical vein endothelial cord (HUVEC) cell lines for vascular tissue engineering, mouse fibroblast cell line (NIH-3T3) for skin tissue engineering etc. ES cells allow the production of type-matched tissues for each patient, either through stem cell banking or by the use of therapeutic cloning. ES cells have the ability to be maintained for long (theoretically indefinite) culture periods, therefore potentially providing large amounts of cells for tissues that could not be derived directly from a tissue source. Proof of the true pluripotent nature of ES cells is teratoma formation.

This property demonstrates the ability of stem cells to tissue-engineer multiple tissue types but also highlights the importance of using a terminally differentiated cell stock without latent stem cell-like properties. The use of stem cells will therefore require a method to ensure differentiation, either by demonstration of selection of only non-stem cells or by removal of all stem cells [24] and by *in vivo* demonstration of an absence of teratoma formation. On the other hand, mesenchymal stem cells (MSCs) specially bone marrow derived- MSCs (BM-MSCs) are very popular for engineering of bones and cartilages due to its highly osteogenic and chondrogenic potential [25].

Magnetic Force Based Tissue Engineering (Mag-TE)

In recent years, magnetic nanoparticles with a variety of nanometer and micrometer scales have applied in various biological and biomedical research fields, such as cell mechanical investigation, cell separation, cell targeting and cancer therapy [26-31]. Most of these studies have used two major characteristics of magnetic particles; large surface area for biochemical modification in accordance with targets, and magnetic attractiveness to high magnetic flux density. Thus, magnetic particle-labeled targets, for example cells, can be remotely controlled by an external magnetic field, which provide a useful physical manipulation tool compared with other techniques.

Previously, Ito *et al.* has proposed a novel tissue engineering methodology, named magnetic force-based tissue engineering (Mag-TE), based on the concept of constructing tissues using functionalized magnetic nanoparticles (Fig. 1-3) and magnetic force [32]. As functionalized magnetic nanoparticles, magnetic cationic liposomes (MCLs), which were prepared by encapsulating 10 nm magnetite nanoparticles into cationic liposomes were developed [33]. Because MCLs have positive surface charges, target cells can be labeled with MCLs via electrostatic interaction between MCLs and cell membrane, enabling the physical manipulation of MCL labeled cells by magnetic force. Sheet-like multilayered 3D constructs with a high cell density were successfully created by accumulating MCL-labeled cells in the presence of magnetic field without scaffold materials [34, 35]. Therefore, this thesis focused on the technological advances of magnetic tissue engineering and their application to the fabrication of 3D tissue constructs required for next-generation of regenerative medicine.

1.3 Heterotypic cellular communication

Proper functioning of biological tissues relies on the interaction of cells with other cell types and with their extracellular matrix [36]. These types of cellular communications can be unidirectional or bi-directional and may occur at both micro- and macro- scales. Cell-cell interactions control organ function in both tissue homeostasis and disease by regulating basic cellular functions such as survival/apoptosis, migration, proliferation, and differentiation [37]. Intercellular crosstalk is involved in both the innate and adaptive immune systems formation of new blood vessels, tumor growth and stem cell differentiation. During tissue regeneration, interactions between stem cells and other cell types within the "niche" environment are essential to preserve stem cell proliferative potential and multipotency [38-42].

Generally, co-culture models are used to anticipate the individual and collective effects of physical contact and soluble factors via paracrine signaling [43]. The simplest co-culture system requires physical contact between cells consists of a mixed monolayer of the cell types of interest [44]. Generally, monoculture systems provide us knowledge only about the cell growth environment, but not intercellular signaling factors. Cell-cell interactions are controlled by direct intercellular contact, as well as by signaling molecules secreted from cells [45]. Co-culture systems can be divided into direct and indirect systems depending on spatial arrangement in which the cells are cultured: direct co-culture system and indirect co-culture system. In direct co-culture systems, cells are mixed together in the culture environment and can make direct contact with each other. The force of cell-cell adhesion between different types of cells is strong and effective [46]. In direct co-culture system cells connect with each other in three different ways: gap junctions, tight junctions and desmosomes [47]. In indirect co-culture systems, two or more cell types are physically separated and cultured under the same conditions without direct cell-cell interaction. Paracine signaling is only the way of this type of indirect cell-cell interaction. Secretion of different proteins such as growth factors and cytokines control cell behavior, proliferation, maturation and differentiation [48, 49]. In case of indirect co-culture system, signaling between different cell types occur through paracrine effects via soluble factors. These biochemical interactions can regulate cell fate and promote metabolism without the need for physical contact between distinct cell types. Also, this type of indirect co-culture system

can able to assess the influence of co-culture on growth behavior of one or both cell types without pre-labeling of the cell population.

Indirect co-culture system or cell-cell interactions between two different types of cells are important for engineering of complex tissues. This type of co-culture system is widely used in case of multilinage tissue engineering.

1.4 Synaptogenesis between muscle and nerve

Synaptogenesis between muscle and nerve is notably a coordinated process that involves a multitude of muscle as well as nerve factors. Most researchers supported to the 'neurocentric model' of synaptogenesis which hypothesized that the nerve was principally responsible for inducing synaptogenesis. This was supported by the observation that nerves can induce ectopic NMJs on denervated muscle. When the peroneal nerve of a rat was transected and transposed onto a denervated gastrocnemius muscle at a site outside its old endplate, the implanted nerve formed a functional NMJ with the muscle in two weeks [32-34]. Clearly the nerve supplied important signals that not only stimulated post-synaptic membrane development but also determined the location of the synapse on the sarcolemma.

However, this traditional hypothesis has been challenged in light of recent findings on the ability of muscle to regulate NMJ formation. It is well known that success rate of re-innervation of muscle is difficult. Chronically, re-innervation of denervated muscle is difficult [30]. Furthermore, innervated muscles become susceptible to further innervation by another nerve. In one study the peroneal nerves that were exchanged onto gastrocnemius muscles with the original nerve supply left intact failed to form ectopic NMJ while synaptogenesis occurred in gastrocnemius muscles with their original nerve supply divided [35]. All these examples highlighted the importance of the target muscle in the regulation of receptivity to incoming neurites. However, the most fascinating evidence for the importance of myogenic factor in NMJ formation was the observation that muscles are capable of accumulating the post-synaptic apparatus independent of neurons. Transgenic mice that lack nerves develop aneural AChR clusters on the muscle membrane suggesting that the muscles are pre-programed to form neuromuscular junctions and the aneural AChRs may provide critical cues to inducing synaptogenesis [20].

1.5 Microfabrication of artificial tissues

Microscale technologies are effective tools to face some challenges in tissue engineering [50]. Microelectromechanical systems (MEMS) which are an extension of the semiconductor and microelectronics industries, can be used to control features at length scales from <1 μm to > 1 cm [51]. These techniques are now facilitating fabrication of cell-material composites that can be used for tissue engineering.

Recent years, microfabrication has been widely used in biomedical and biological applications because of the emergence of techniques such as soft lithography to fabricate microscale devices without the use of expensive clean rooms and photolithographic equipment [52]. Microfabrication techniques and engineered biomaterials are being used for tissue engineering in a variety of applications: for example, by fabricating scaffolds with control over features such as shape and pore architecture, as templates for microtissue formation, or as improved bioreactors. Moreover, microscale control of cellular environments has been used to probe the influence of the spatial and temporal effects of specific cell-cell, cell-ECM and cell-soluble factor interactions on cell fate. The ability of this techniques to simultaneously test many environmental factors on cell behavior has been used to optimize culture conditions and material-cell interactions.

Microfabrication technologies are very important for fundamental studies of muscle biology and also can be incorporated into drug screening assays. These technologies have also been widely used for generating muscle actuators in the context of microrobotics and in miniaturized biological pumps. An important area of recent study involves coculture with cell types that either activate muscle or facilitate its function [53].

Organ-on-chip

Normally an organ-on-a-chip is a microfluidic cell culture device created with microchip manufacturing methods that contains continuously perfused chambers possessed by living cells to mimic tissue and organ-level physiology [54]. The goal of this technique is not to build a whole living organ but rather to synthesize fundamental functional units that recapitulate tissue and organ level function. By using this technology multicellular architecture can be recapitulated and tissue-tissue interfaces, physicochemical microenvironments and vascular perfusion of the body can be understood better which cannot be possible by using conventional 2D or 3D culture system [55].

The simplest example of organ-on-a chip system is a single, perfused microfluidic chamber composed of one kind of cultured cell (e.g. hepatocytes or kidney tubular epithelial cells). On the other hand, complex designs are developed by connecting two or more microchannels by porous membranes, lined in opposite sides by different cell types to recreate interfaces between different tissues. The word chip on an organ-on-a-chip system is related with original fabrication method which is a modified form of photolithographic etching used to manufacture computer microchips, which allows control of surface feature shapes and sizes on the same scale (nm to μm) that living cell sense and respond to their natural tissue milieu [56]. Later, this system was modified by pouring liquid polymer, such as poly-dimethylsiloxane (PDMS) and allowed it to polymerize into an optically clear rubber like material by creating a rubber stamp [57].

Now a day, with the development of organ-on-a chip technology, researchers can now easily investigate the basic mechanism of organ physiology and diseases more easily [58]. Researches have already been developed different organ-on-a chip system including liver, kidney, intestine, lung, blood vessel etc. But, all of these systems cannot be designated as a perfect model of organ because only one type of cell was cultured in one microchannel. However, researchers are now trying to develop this system by incorporating two different or more types of cells in one microchannel. For example, formation of synapse at the neuromuscular junction was analyzed in chip containing mouse embryonic stem cell derived motor neurons and C2C12 myotubes. Moreover, myelination has also been studied on chip by co-culturing human embryonic stem cells derived schwann cells with human axons [59-66].

Till today, there are very few successes regarding organ-on-chip formation which can mimic specific organ level functions. Further improvement of this technology will open a new era of biological research specially to understand organ physiology, drug screening and toxicology research. Moreover, organs-on-chips developed from iPS cells isolated from different genetic subpopulations, disease subgroups or individual patients can facilitate drug discovery targeted to specific subpopulations or clinical trial design.

1.6 Drug screening based on 3D microtissues

As we have discussed previously that *in vitro* micro-technologies to engineer microscale versions of various organs and tissues are becoming popular among the researchers day by

day. These micro-tissues have been developed for transplantations of cornea, retina, bladder or heart to recapitulate organ level function for drug screening, drug discovery, personalized medicine and disease model [67-74]. Drug screening is a lengthy process including several stages from target identification which ultimately leads to discovery and optimization, preclinical validation and clinical trials. All of these stages are very important to get clinical approval for any drugs. For many years, animal testing by using different types of laboratory animals have been playing a crucial role in predicting pharmacokinetics as a preclinical test in drug discovery. But, number of issues are related regarding this type of experiments such as ethical consideration, species difference etc. To solve this problem in vitro drug screening based on 2D cell culture technology can be a good option. But, cultivated cells often don't retain their original organ functions and morphologies. However, with development of microfabrication technology, now a day, engineered micro tissues are very effective to investigate disease pathology and screen drugs against that disorder. An important step in this process is high-throughput screening (HTS) of small compound libraries for lead identification. At present, the majority of cell-based HTS is being carried out on cultured cells propagated in two dimensions (2D) on plastic surfaces optimized for tissue culture [75]. Thus, HTS based on 3D microtissues can be a powerful tool for future drug screening as it recapitulates most of the morphological features of the body.

In the past, cell based drug discovery highlighted chemical screens in well-characterized cell monolayers, and mostly in cancer drug discovery. But, in recent years, with the advancement of current microfabrication techniques, it is now possible to develop 3D tissues microenvironment which has made the drug screening process easier than before. To achieve more success in drug discovery process, 3D cell culture model will need to take into account that the response to a broad spectrum of drugs. Also, complex microtissues should be developed by co-culturing different types of cells in a single chamber which can mimic the physiological microenvironment more clearly thus will help to screen the drugs more accurately.

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Chapter 2

Background

2.1 Skeletal muscle tissue engineering

Skeletal muscle is one of the most abundant and dynamic and tissues of the human body. In humans, skeletal muscle comprises approximately 40% of total body weight, and contains 50–75% of all body proteins. Muscle is mainly composed of water (75%), protein (20%), and other substances including inorganic salts, minerals, fat, and carbohydrates (5%). In general, muscle mass depends on the balance between protein synthesis nutritional status, hormonal balance, physical activity/exercise, and injury or disease.

The various protein compartments (structural, contractile, and regulatory) have received significant scientific attention because of their huge contribution to mobility, exercise capacity, functioning, and health. The architecture of skeletal muscle is characterized by a very particular and well-described arrangement of muscle fibers which is also referred to as myofibers or muscle cells and associated connective tissue. At the whole muscle level, the size of a muscle is determined mostly by the number and size of individual muscle fibers although pathological infiltration by fat and connective tissue may alter this relationship [1, 2].

Satellite cells are the adult stem cells of skeletal muscle. These cells are located between the sarcolemma and the basal lamina and contribute to muscle growth, repair, and regeneration. Satellite cells are activated by myogenic factors and differentiate and proliferate into muscle fibers [3-5]. An individual muscle is surrounded by a layer of connective tissue known as the epimysium. Group of fibers within that muscle are arranged in bundles and surrounded by another layer of connective tissue known as the perimysium. A single muscle fiber (with approximate dimensions of 100 lm in diameter and 1 cm in length) is surrounded by a cell membrane or sarcolemma. Associated with the sarcolemma there is a complex of several proteins that is physically connected to the internal myofilament structure; particularly to the actin protein present in the thin filament.

However, the self-repair capacity of adult skeletal muscle is very week in its ability to

restore significant tissue loss caused by traumatic injury, congenital defects, prolonged denervation, or functional damage due to various types of myopathies. Conventional surgical treatments which includes both local or distant autologous muscle transposition yield a limited degree of success. However, transplantation of satellite and myoblast cells has been proposed to increase the regenerative capacity of skeletal muscle. Although, different intramuscular injection of allogenic myoblasts sometimes causes immunerejection [6-9].

With the development of tissue engineering technology, now it is possible to get successful therapeutic remedy in case of severe muscle loss. Now a day it is an appealing treatment option where engineered tissue substitutes could be used for the functional and aesthetic reconstruction of damaged skeletal muscle [8]. However, *in vitro* fabrication of artificial skeletal muscle constructs ensure following therapeutic benefits compared with traditional surgical transplantation or cellular replacement therapy: a) the ability to preengineer custom tissue architecture for precise structural repair at the site of injury , b) the ability to precondition tissue implants for specific mechanically or metabolically demanding host environments such as sarcopenic muscle in the elderly or site of traumatic injury and c) localized delivery of concentrated angiogenic and anti-apoptotic paracrine factors upon implantation [10]. However, to engineer dense skeletal muscle tissues with adequate dimensions, uniform cell alignment and reproducible architecture is still challenging. A biomimetic functional engineered skeletal muscle tissue should manifest native like structural properties and, specifically, contain densely packed and uniformly aligned myofibers throughout a relatively large tissue volume. High muscle cell density and alignment was previously achieved by constraining the cell growth within thin cylindrically shaped collagen gels [11] and self-organization of cells in scaffold- free myooids under passive tension [12]. Recently, one approach is very popular for engineering of biomimetic skeletal muscle tissue is the use of biocompatible hydrogels. But, the main problem for muscle tissue fabrication with this method is the difficulty in fabricating tissue constructs with arbitrary 3D shapes.

However, there are several methods for skeletal muscle tissue engineering, which includes cell alignment by topography, surface patterning, mechanical stimulation, magnetic or electrical field etc. The brief description is given bellow:

2.1.1 Cell alignment by topography

Cellular behaviors are significantly influenced by topographical features such as size and geometry on the cellular response. Among these topographical features, parallel grooves are among the most studied patterns to elongate muscle cells in one direction. Primary studies of this technique determined how the cells sense their environment and which caused the cells to undergo development. Thus, grooved patterns with different widths and depths were tested. Evans et al. generated micropatterened grooves with depths ranging from 40nm to 6mm and widths ranging from 5 to 100 mm on silicon substrates by etching with conventional photolithographic methods and studied myoblast alignment and direction. On the other hand, Clark et al. developed nanosized grooves with a width of 130nm and a depth of 210 nm also induced myoblast alignment [13-15].

2.1.2 Cell alignment by surface patterning

Surface patterning is a general term used to describe the modification of a biomaterial's surface by patterning techniques. This technique is based on the use of an elastomeric master that is easy to mold or emboss and can be used directly as substrate for biological applications or as mold. Among the elastomers used, PDMS is the most popular elastomer for biological applications, and the construction of a PDMS master is related to another mold prepared by conventional photolithography approaches [16]. Cell patterning has been mostly used to study cell behavior, such as cell migration, proliferation, cell–cell interactions, and drug screening, in a 2D environment. However, this approach is also appealing for the creation of 3D tissue-like constructs via cell-sheet-based tissue engineering. Indeed, various methods exist for the harvesting of prepatterned cell sheets. For example, Nagamine et al. used a fibrin gel to embed aligned myotubes into a 3D hydrogel system [17]. Similarly, Huang et al. transferred aligned myotubes from a parallel micropattern of poly (2-hydroxyethyl methacrylate) (pHEMA) to a type I collagen gel overlaid on the micropattern. After 3 days of culture, the collagen sheet was rolled around a biodegradable polymericmandrel to fabricate a tubular muscle-like construct with aligned myotubes.

By patterning hydrophilic polymer on thermo responsive surface and by using a plunger coated with gelatin to harvest the different cell sheets of human skeletal myoblasts, Takahashi et al. showed that an anisotropic cell sheet placed on the top of four random cell sheets stacked together induced the myoblasts and the ECM alignment in the whole

construct [18]. Recently, Guillaume-Gentil et al. described a method to fabricate and harvest micropatterned heterotypic cell sheets by local electrochemical dissolution of a polyelectrolyte coating [19]. Such methods introduce the possible creation of cocultured harvestable cell sheets and the growth of more complex tissue constructs via cell-sheet-based tissue engineering.

2.1.3 Cell alignment by mechanical stimulation

Lack of stimulation and mechanical force causes muscle degeneration which is seen in disabled individual or during skeletal muscle atrophy. Although the role of mechanical stimulation has been widely studied in gene regulation, endogenous protein regulation, accumulation, and metabolic products [20, 21]. it has been less studied as a tool in SMTE. However, it has been reported that under continuous uniaxial strain, avian myoblasts and L6 rat skeletal muscle cells cultured on an elastic substratum differentiated into myotubes oriented parallel to the direction of strain, whereas under stretching/ relaxation cycles, the myotubes were aligned perpendicular to the stretch direction [22-24]. Other studies of myoblasts encapsulated in a collagen hydrogel and treated by continuous uniaxial strain also showed the formation of myotubes parallel to the direction of the strain [25- 27].

2.1.4 Application of magnetic nanoparticles for skeletal muscle tissue engineering

As, we have discussed before that Mag-TE technique is very fruitful for fabrication of functional tissue constructs, it has huge amounts of applications for the fabrication of artificial skeletal muscle tissues. Previously, it was investigated that static magnetic field alone can induce the differentiation of myoblasts. Yamamoto *et al*. reported that C2C12 cells were elongated along the axis of a magnetic field after endocytosis of magnetic nanoparticles. Moreover, by using this method of Mag-TE, which promotes tissue organization under magnetic field, Akiyama *et al*. fabricated 3D tissue architecture whereas, Yamamoto *et al*. fabricated 200μm thick skeletal muscle tissues [28-31].

To fabricate a 1.9mm thick skeletal muscle tissue, Yamamoto *et al*. combined the application of a magnetic field to C2C12 cells loaded with magnetic nanoparticles to induce tissue organization with the use of cell culture in a perfused hollow fiber reactor that allowed the maintenance of high cell density by supplying oxygen and nutrients. Later, by applying Mag-TE technique, Ito *et al.* fabricated functional skeletal muscle tissue constructs by using electrically stimulated C2C12 cells. Also, with the help of Mag-TE technique, Ikeda

et al. developed an *in vitro* drug testing system based on the contractile force produced by 3D skeletal muscle tissue constructs [32-34].

2.1.5 Stem cells for skeletal muscle tissue engineering (SMTE)

Satellite cells are the native stem cell precursor to skeletal muscle and the most common starting source for engineering of skeletal muscle tissue. But, use of satellite cells require some difficulties including: laborious isolation procedure, time consuming and variable between research laboratories. Acquiring enough satellite cells from a small skeletal muscle biopsy to produce a sufficiently sized tissue is challenging, yet a large skeletal muscle biopsy would not be clinically feasible because of the potential irreversible injury at the biopsy site. Senescence of satellite cells as they are expanded in vivo causes them to lose proliferative capacity, which reduces their ability to contribute to myogenesis over time [35, 36].

Several studies were performed on satellite cell-based SMTE in VML animal models [37, 38]. Machingal *et al*. created and tested the first tissue-engineered muscle repair (TEMR) construct [39]. The researchers created the TEMR by performing an enzymatic dissociation isolation of rat muscle to obtain muscle progenitor cells (MPCs), which is a more general category of Pax7+ cells that includes satellite cells. They cultured the MPCs to obtain rat myoblasts, and seeded the myoblasts onto a porcine bladder acellular matrix (BAM) scaffold to produce the TEMR.

Adipose derived stem cells (ADSCs) are an abundant, adult, multipotent, mesenchymal stem cell (MSC) variety with myogenic potential. MSCs are a general category of nonhematopoietic stem cells with specific cluster of differentiation (CD) protein surface markers that reside in many tissue types, including adipose tissue. MSCs have the capacity to differentiate into tissues of mesodermal lineage, including cartilage, bone, adipose tissue, and skeletal muscle. First ADSCs derived engineered skeletal muscle construct was developed to treat VML injury in a murine model and compared it to MPC derived artificial skeletal muscle construct [40-43].

Another important candidate for skeletal muscle tissue engineering is induced pluripotent stem cell (iPSC). iPSCs are produced in the laboratory by culturing adult cells and forcing them to upregulate transcription factors that induce pluripotency. They are pluripotent and capable of unlimited self-renewal in culture, which is a major advantage

over satellite cells. Previously, Takahashi and Yamanaka determined four essential transcription factors which must be transduced into starting cells using a viral vector to reprogram the cells into iPSCs: KLF4, c-MYC, OCT4, and SOX2. However, application of iPSCs for skeletal muscle tissue engineering is quite new. Recently, functional human skeletal muscle tissue construct has already been engineered by using human pluripotent stem cells (hPSCs). Although, three dimensional (3D) human induced pluripotent stem cells (hiPSCs) derived artificial skeletal muscle tissues have also been engineered by incorporating hiPSCs derived vascular cells and motor neurons [44- 46].

So, it is seen that satellite cells, ADSCs and iPSCs are particularly promising candidates for SMTE. Each offers potential advantages while having high proliferative capacities and significant myogenic differentiation potentials. However, ensuring proper myogenic differentiation of alternative stem cells either in vitro or in vivo will be particularly crucial because unlike satellite cells, they are capable of differentiating into multiple tissue types.

2.2 Nerve-muscle coculture

Effective models of mammalian tissues must represent correct interactions between co-cultured cell types in order to produce culture microenvironments as like as *in vivo* system. In case of skeletal muscle, the formation of such a culture model, integrating multiple relevant cell types within a biomimetic scaffold, would be of significant benefit for investigations into the development, functional performance and pathophysiology of skeletal muscle tissue.

The strategy of nerve muscle coculture was very old. The first nerve-muscle co-culture system was developed over a century ago [47]. Conventional cell culture techniques have shown that motor neurons can elicite end-plate potentials in co-cultured myotubes. Although, co-culture between mouse embryonic stem cell (ESC) derived motor neurons and C2C12 myotubes shown that electrical activation of neurons will trigger post-synaptic potentials in associated muscle fibers in vitro [48]. Moreover, functional analysis of primary rat skeletal muscle myotubes on microscale cantilevers has demonstrated that activation of co-cultured rat embryonic ventral horn motor neurons through application of glutamate induced contractile activity in underlying myotubes and the contractile activity was blocked through addition of acetylcholine receptor inhibitor D-tubocurarine [49]. Southam *et al*. developed a nerve-muscle co-culture system in order to better model the

compartmentalized nature of the *in vivo* peripheral nervous system [50]. Also, microfluidic nerve-muscle coculture model have been developed where fluids are manipulated at the submillimeter scale. By using this approach, multicompartment culture chambers can be produced which not only enable neuronal processes and muscle cells to be fluidically isolated from cell bodies, but also allows the cells to be monitored by live cell imaging [51]. Nerve-muscle coculture system provides us valuable informations regarding the generation of *in vitro* functional synapses between motor neurons and muscle cell types and also to investigate acetylcholinesterase production and cycling at the NMJ [52].

Besides, 2D nerve-muscle coculture system, 3D muscle constructs by applying nerve-muscle coculture have also been developed by several research groups. 3D co-culture of fibrin based skeletal muscle tissue constructs with organotypic neuronal slices was previously been shown to promote up-regulation of important NMJ markers [53]. On the other hand, Larkin *et al*. developed 3D skeletal muscle tissue constructs by incorporating neuronal cells and found improved myosin heavy chain (MHC) expression patterns appeared to progress from neonatal isoforms towards a more developmentally mature phenotype [54]. Dhawan *et al*. also developed a fibrin based constructs seeded with muscle-derived cells (MDCs) and implanted in close proximity to the transected femoral nerve and found enhanced contractile function of cocultured constructs when compared with aneural constructs [55]. Recently, a new co-culture system was developed by supporting synaptic contact between aligned myotubes and motor neuron neurospheres derived from mouse neural stem cells which was able to improve contractile functions compared with muscle-only controls and spontaneous twitch activity was interrupted by the treatment with AchR blocker curare [56]. This type of 3D co-cultured skeletal muscle tissue constructs has considerable benefits for studying neuromuscular physiology and pathology.

Recently, work has been published demonstrating the production of a co-culture system supporting synaptic contact between aligned myotubes and motor neuron neurospheres derived from mouse neural stem cell [57]. These cultures were also able to promote improved contractile properties compared with muscle-only controls and spontaneous twitch activity that was interrupted by treatment with the AChR blocker curare. Innervated *in vitro* 3D skeletal muscle models are also very amenable to mechanical strain [58] and electrical stimulation [59] which are key signals likely governing the

development of the neuromuscular system and as such could be used to understand how these signals alter neuromuscular maturation. 3D skeletal muscle constructs with a neuronal input also accurately replicate the *in vivo* niche will be used to manipulate and further understand the basis of muscle plasticity.

2.3 Neuromuscular junction

The neuromuscular junction (NMJ)is a synapse formed between motoneurons and skeletal muscle fibers that is covered by schwann cells (SCs). It is essential for controlling skeletal muscle tissue contraction. After arrival of action potentials (AP), the motor neuron terminals release acetylcholine (Ach) which activates acetylcholine receptors of muscle fibers to depolarize the muscle cell and trigger calcium release from the sarcoplasmic reticulum to initiate muscle contraction. The formation of NMJ involves the differentiation of presynaptic nerve terminals, postsynaptic muscle membranes, and terminal Schwann cells (tSCs). After innervation of nerve terminals into the muscle fibers, they induce new clusters and disperse those in non- synaptic areas.

Generally, NMJs are formed in the middle region of muscle fibers. Initially, AchR clusters appear like oval plaques that are often innervated by more than one axon. In matured stage, the plaques become perforated and eventually appear as pretzel-shaped arrays or branches of AchR. Branches are filled with junctional folds that are usually perpendicular to the long axis of the arrays, and AChRs are concentrated at the shoulder areas of junctional fold crests.

NMJ development requires extensive communication among the three components of the tripartite synapse: presynaptic motoneurons, postsynaptic muscle fibers, and SCs. In fact, the synaptic cleft is filled with synaptic basal lamina that is enriched with many proteins from these cells for NMJ development and maintenance. NMJs occupy less than 0.01–0.1% of the entire muscle surface. How this small area of muscle membrane becomes differentiated to possess a high concentration of AChRs, a hallmark of the NMJ, has riveted neuroscientists over many generations. Nerve terminals secrete positive factors such as agrin to concentrate AChRs at the NMJ by promoting the transcription of genes of AChR subunits and other proteins for NMJ structure and function in synaptic nuclei, AChR transport to the postjunctional membrane, AChR clustering, or anchoring and AChR stability. They also release ACh, a negative signal that suppresses these machineries, to

eliminate supernumerary AChR clusters from extrasynaptic regions [60-63].

The development of NMJ can be categorized into two phases: a) the primary specialization phase (before birth) and b) the secondary specialization phase (after birth). In the primary specialization phase, varicose protrusions are developed in the nerve terminals which lined up with depressions in the muscle membrane to form primary synaptic clefts. ACh clusters are formed on the post-synaptic membrane and the pre-synaptic schwann cells which are capped off the synaptic cleft. On the other hand, during secondary synaptic specialization, rudimentary synapses further differentiate. The pre synaptic membrane on the nerve terminal and active zones develop on the membrane [64]. In the post synaptic membrane, secondary synaptic folds form and the AchR become concentrated at the crests. The pre and post synaptic apparatus become spatially aligned and the synaptic ECM further matures. Initially each post-synaptic membrane and its AchR clusters are innervated by multiple nerves from different motor units and each muscle fiber may contain multiple NMJ.

Another interesting point that, aneural AchR clusters play an important role for the regulation of NMJ formation. Aneural AchR clusters governs the competing axon towards itself to form new synapses. Moreover, dennervated muscle grafts with intact end plates are more amenable to forming NMJ after implantation compared to grafts with endplate zone excised, suggests that aneural AchR clusters on muscles potentially prime muscle for innervation by interacting with incoming neurites [65]. However, the final location of NMJ likely results from a dynamic interplay between the incoming neurite and muscle membrane and it can be said that the gross location of the synapse is likely set by the aneural AChR.

2.3.1 AChR subtype

AChRs are receptor gated sodium channels that are important in producing endplate potentials needed to stimulate an action potential in response to ACh. nicotinic AChR on muscle can be broadly classified into the embryonic isotype containing the **γ** subunit and the mature isotype containing the **ε** subunit [66]. At birth the **γ** AChR subunit is present at very high levels while ε AChR subunit is relatively low [67]. However, over the course of two to three weeks, the ᵞ subunit decreases to undetectable levels while the ε subunit rises to its peak [68]. It was found that approximately 30% of agrin induced AChR clusters

contained a combination of ε and ɣ subunits while 70% contained only ɣ subunits [69]. One study showed that both three dimensional myotube cultures and neural-muscle co-cultures expressed ε AChR subunit mRNA while two dimensional myotube cultures failed to show any ε AChR mRNA [70]. The absence of ε subunits in two dimensional cultures suggests that ε mRNA expression is also sensitive to the scaffold that the muscles are immobilized in.

2.3.2 Agrin

Agrin is an important biomolecule which have the capacity of AChR clustering and is believed to be the main mediator of the post synaptic organization and differentiation [71] [72]. It is a heparin sulfate proteoglycan that is widely present in schwann cells, muscle and motoneurons. There are many subtypes of agrin, among them neural agrin is secreted by motoneurons into the synaptic cleft where it is deposited with the extracellular matrix at the synaptic site [73]. It was found that deficiency of neural agrin resulted aberrant NMJ formation with dispersed AChRs and poor juxtaposition between nerve endings and AChRs [74]. Also it was found that, gene transfection of denervated muscle with neural agrin induced extensive AchR clusters at extra synaptic sites and assembly of key molecules of the post synaptic apparatus at the receptor cluster site [75].

Agrin clusters AChR through cytoskeletal remodeling. Agrin stabilizes AChR clusters by capturing cytoskeletal filaments at sub-synaptic sites and linking the filaments to the receptors. In addition to affecting post-synaptic development, agrin has also been found to regulate the electrophysiological behaviors of muscle cells and the maturation of the excitation-contraction (E-C) coupling mechanism. Agrin aggregates sodium channels at AChR clusters and upregulates the expression of mature isoforms of voltage gated sodium channels [76-78]. Agrin treated three dimensional muscle constructs display superior contractility and tetanic force generation in response to direct stimulation [79].

2.3.3 Role of neurotrophic factors and cytokines for the formation of NMJ

Large number of neurotrophic factors and cytokines e.g. bFGF, ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) etc. are involved for the retention of NMJ leading to polyinnervated muscle fibers. Some growth factors must be delivered during the synapse elimination phase in order to retain synapses such as CNTF [80]. On the post-synaptic membrane, BDNF and NT4 inhibit agrin induced

AChR clustering on muscle cells [81, 82]. Some neurotrophic factors such as GDNF also are capable of increasing AChR insertion into the muscle membrane [83]. On the other hand, it was found that NGF facilitates the expression of AchR. Previously, it was investigated that, PC12 cells treated with NGF promotes the expression of AchR.

2.4 Microfabrication of skeletal muscle tissues

Muscle cells are the most abundant cell types in the body, serving numerous critical physiological functions. At the same time, muscle is subject to a variety of incapacitating or fatal disorders such as Duchenne muscular dystrophy affecting skeletal muscle cells, respectively.

Recently, different types of microdevices are widely used for the fabrication of skeletal muscle tissues where microdevices allow for the controlled formation of 3D tissues and the ability to apply various well-defined cues, be they chemical, mechanical or electrical. Microdevices have already been used successfully as 2D culture platforms [84]. Made popular by George Whitesides in the late 90's, soft lithography makes possible the rapid fabrication of PDMS-based biocompatible microdevices for use in bioengineering and biology. And, at recent years, these devices have proven to be extremely valuable tools to investigate cell behavior in the context of chemotaxis, mechanotransduction or stem cell research [85-88].

For, fabrication of skeletal muscle microtissues, one of the most important cue is to control of muscle differentiation in microdevices. Differentiation of functional tissues from pluripotent stem cells (PSC) is an attractive strategy to investigate the underlying biology and physiology, generate patient-specific pathological cells or engineer replacement tissues. Previously, it was found that satellite cells (SCs) represent decreased proliferative capabilities and quickly lose their abilities to form myofibers *in vitro*. Moreover, embryonic stem cells (ESC) and mesenchymal stem cells showed great pluripotency but a risk of tumoregenicity. Recently, with the development of induced pluripotent stem cells (iPSCs) have opened a new dimension combining qualities such as accessibility, and their autologous and non-immunogenic nature. However, iPSCs require genetic manipulation and further analysis to exclude possible tumorigenic effects.

Previously, it was investigated that, different types of stimuli (biochemical, mechanical

or electrical) are involved to induce the differentiation of source cells into myocytes. Chemical cues can be provided to the cells in the form of soluble factors embedded in the extracellular matrix on which the cells are cultured. For example, it was found that differentiation of human embryonic stem cells (hESCs) into skeletal myoblasts was enhanced with strong expression of myogenic regulatory factors (MRFs) and MyoD due to the addition of retinoic acid. Similarly, a combination of myogenic factors, TGF-β and ascorbic acid embedded within viscoelastic hydrogels promoted myogenic induction of hMSC. On the other hand, using an injection based growth factors bio-printer, Ker and co-workers were able to control geometrical and biochemical cues in order to promote musculoskeletal alignment and differentiation of C2C12 cells [89-91].

As, it is known that bioelectricity and transmembrane potentials play important role in case of stem cell differentiation and development. It was found that myogenic potential of muscle precursor cells are improved by electrical excitation. Electrical stimulation influence stem cell conditioning and promote muscle differentiation with different efficiencies and specific gene regulations [92-94]. All of these factors are important for the differentiation of stem cells into muscle precursor cells.

The limitations associated with traditional culture assays of muscle tissue encompass the physiological relevance, most commonly plated in culture dishes evolve in two dimensional sheets, far from the well-aligned and organized structures they would assume in their native environment, with the risk to detach from the surface when acquiring contractile capabilities [95]. A number of efforts were made to guide the formation microfabricated aligned muscle tissue. One successful attempt was done by fabricating substrates with parallel linear microgrooves [96]. An alternative approach to microgrooves or micropatterning to produce cell alignment is the formation of 3D muscle tissues. These 3D constructs are inherently more physiological because 3D tissues formed between attachment points anchors better than adherent cultures and they spontaneously align along the long axis of the construct [97]. Further, Vandenburgh *et al.* developed a system where small muscular tissues termed as “miniature bioartificial muscles” or mBAM were fabricated in 96 well plates and each well containing a muscle construct [98]. In this method, the tissue was fabricated by seeding primary myoblasts suspended in a collagen-Matrigel mix in wells containing a set of two 4-5 mm long pillars, positioned 4 mm apart. The compliant pillars were made of PDMS. The tissues were functional and in case of force

production, the static tension (also called passive or resting tension) reached approximately 700 μN after 6 days in culture, and electrically triggered tetanic forces were measured to stabilize around 40μN after 8 days in culture. Moreover, the same researcher further monitored the gene expression profile of "miniature bioartificial muscles" in the context of drug screening. They designated this approach as High-content drug screening (HCS) and illustrated it in a subsequent study where the throughput of the platform was put to good use, by testing 31 compounds at 3 to 6 different concentrations on muscle tissues differentiated from mdx murine myoblasts, the genetic homolog of Duchene Muscular Dystrophy (DMD) and by merely measuring the output tetanic force [99]. However, mBAM doesnot represents all physiological properties as like as *in vivo* skeletal muscle tissues, rather it represents a "rapid and cost-effective" intermediate step, bridging the cell-based assays and animal studies.

Most of the existing 3D microfabricated skeletal muscle systems rely on some sort of self-aggregation of myoblasts seeded in a hydrogel, giving rise to a muscle construct whose final shape is reminiscent of the original geometry of the chamber and that of the supporting structures which lead to some inherent heterogenecity. To mitigate this problem, a very compatible 3D fascicle-like skeletal muscle tissue construct was developed [100], where PDMS wells containing a rod of desired dimensions, a sacrificial gelatin gel was cast, which, upon removal of the rod following gelation, retained the cylindrical lumen. C2C12 myoblasts suspended in a liquid fibrin gel were subsequently injected into the lumen, allowed to settle and as the temperature was raised, the gelatin melted, leaving behind a perfectly shaped muscle fascicle. This method of micro skeletal muscle tissue fabrication is thought to be extremely versatile and fruitful to investigate the influence of muscle size together with the gel composition on its differentiation efficiency.

In living system, muscle cells can never function itself independently without interacting fibroblasts, endothelial cells or neurons. All these cell types act synergistically provide support during homeostasis, development and repair. Several types of coculture systems were developed by using microdevices to generate neuronal and vascular networks into skeletal muscle tissues. The first microfluidic device for neuromuscular coculture was developed by Takeuchi *et al*. which consists of PDMS-based microchambers seeded with 4-8 superior ganglia (SCG) neurons, connected by minute grooves allowing axonal outgrowth and connection with neighboring compartments [101]. Moreover,

Morimoto *et al.* developed a neuromuscular device in which murine neural stem cell (NSC) derived motor neurons could innervate a 3D muscle strip differentiated from C2C12 myoblasts and determined the formation of neuromuscular junction by identifying the clustering of AChR and contraction of the muscle bundles upon glutamate-induced neuronal excitation [102].

For many decades, scientists are trying to develop proper vascular network inside the skeletal muscle tissue construct. However, spontaneous revascularization in "adult" or *in vitro* muscle tissue occurring before or after implantation, lacks proper organization and structure and therefore has a lower efficiency than normal in vivo vascular networks. The past few years have seen the emergence of microdevices whose objective was to guide the formation of well-controlled microvascular networks (MVN). A microfluidic device was developed in the George lab, which having a different design and requiring interstitial flow for the formation of vascular structures allowed for the spontaneous formation of a perfusable network in a 3D hydrogel [103]. In case of muscle vascularization, two important strategies are very important for generating perfusable muscle tissue. The first one, consisting of forming lumens in the muscle strip before lining it with endothelial cells and another one was demonstrated in a platform adapted from the design by Vandenburgh. Another important system was developed by using a reversed order, constructing the vasculature before forming the muscle tissue [104] where a PDMS substrate patterned with linear microgrooves and coated with a proangiogenic factor releasing hydrogel and vein explants on each side of the platform. But all of the investigations regarding vascularization of artificial skeletal muscle microtissues were not so successful. Still, researchers are trying to get success regarding these phenomena.

2.5 Skeletal muscle tissue engineering for drug screening

Now a day, researchers are focusing on *in vitro* drug screening based on the function of engineered 3D tissues. It is now an active area of translational research. Recent technological advancement regarding *in vitro* drug screening and also tissue engineering, high-throughput screening (HTS) and high-content screening (HCS) of drugs with engineered tissues are now possible which rapidly analyze hundreds of thousands of compounds affecting single biochemical reaction or gene expression and by altering tissue functions. These technologies regarding *in vitro* drug screening with engineered tissues are

rapid, cost effective and reduce the number of small animals required for conventional *in vivo* method of drug screening.

As we know that contractile skeletal muscle tissue engineered from precursor muscle cells (myoblasts) has a number of potential applications including tissue repair [105], therapeutic protein discovery [106], mechanotransduction mechanistic studies and also *in vitro* drug screening. Engineering of skeletal muscle tissues as a tool for in vitro drug–screening applications has been envisioned since the field's inception [107, 108]. and has contributed a lot for identification of new drugs for the treatment of different clinical disorders. Engineered skeletal muscle offers a physiologic approach to HTS, providing a holistic approach for determining a compound's effect on the multiple pathways regulating important physiological parameters such as muscle strength, fatigability and contraction-induced injury in both normal and diseased phenotypes. A significant advantage of drug screening with engineered tissues is the ability to utilize cells from either human with a neuromuscular disorder affecting muscle function or equivalent rodent models of the diseases. DMD is a progressive lethal, muscle-wasting disease resulting from a defect in the dystrophin gene [109]. A genetic murine homolog of DMD exists (mdx) and the myoblasts from the mdx mouse have been conditionally immortalized providing an ideal cell source to screen for compounds that might attenuate the progressive muscle weakness associated with the disease. Moreover, identification of factors that improve muscle function in patients with DMD could lead to an improved quality of life. Tissue engineered mdx mBAMs from mdx myoblasts are capable of generating active forces comparable to control murine mBAMs and this type of screening is very beneficial to identify proper therapy against DMD.

However, drug screening with engineered skeletal muscle tissues is still in early stages of development. Microfluidic bioreactors [110], microfluidic scaffolds [111], and microfluidic patterning all are contributing to a more *in vivo* like environment especially for pharmacokinetic studies. The screening of new potential drug therapies directly against a specific disease along with pharmacogenetics that is, individual patient tissue screens [112] is also an exciting future direction for the field. Finally, induced pluripotent stem (iPS) cells and embryonic stem cells [113-115] are potential future sources of cells for engineered tissue drug-screening applications.

2.6 Purpose of the study

Tissue engineered skeletal muscle construct is very effective as a regenerative medicine in case of different types of severe trauma or wounds where extensive amounts of muscle loss occurs. The most important parameter of a functional artificial skeletal muscle construct is its contractile force production capability which will make the artificial construct similar to the native skeletal muscle tissues. But, unfortunately the contractile force produced by artificial skeletal muscle construct is still far from its native counterpart. Force generation capacity of skeletal muscle tissue solely depends on electrical pulse stimulation (EPS) from the central nervous system (CNS) via the motor neurons. So, innervation of neurons into the engineered skeletal muscle tissue constructs is a reliable approach to improve its contractile force generation capacity. So, one of the purpose of my study is to engineer a muscle-neuron construct with improved contractile force generation by applying C2C12/PC12 coculture system with the help of Mag-TE technique.

On the other hand, application of microdevices for engineering of 3D skeletal micro tissues are very potential for drug screening against different muscular disorders also can work as muscle-on-a-chip system. Here, we fabricated skeletal muscle microtissues by Mag-TE technique to develop a high-throughput drug screening system.

2.7 Thesis components

In chapter 1, introduction**,** discussion about regenerative medicine, tissue engineering specially Mag-TE, heterotypic cellular communication, microfabrication of artificial tissues and organ-on-chip and drug screening based on engineered 3D tissues.

In chapter 2, background study which includes skeletal muscle tissue engineering (SMTE) such as different techniques of artificial skeletal muscle tissue fabrication with recent advances and application of Mag-TE for engineering of skeletal muscle tissue constructs, discussion about nerve-muscle coculture, neuromuscular junction, microfabrication of skeletal muscle tissue constructs, drug screening based on skeletal muscle microtissues and purpose of the study.

In chapter 3, a study for fabrication of muscle-neuron constructs by using C2C12/PC12 coculture system for enhancement of contractile force generation which improved the contractile force production of skeletal muscle tissue constructs due to innervation. Also, can act as a potential tool for screening of drugs against neuromuscular disorders.

In chapter 4, a studyof microfabrication of artificial skeletal muscle tissue constructs for developing high-throughput drug screening system where skeletal muscle microtissues were fabricated by using both C2C12 and hiPSCs with the help of Mag-TE with the capability of contractile force generation and investigated the effect of drugs on contractile force generation and tissue morphology.

In chapter 5, the contents of thesis and future perspectives are summarized.

2.8 References

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Chapter 3

Fabricating muscle-neuron constructs for enhancement of contractile force generation

3.1 Introduction

Tissue-engineered skeletal muscle constructs are considered an unique achievement in the field of regenerative medicine [1]. Muscle tissue replacement is often mandatory in cases of genetic and metabolic disorders, such as diabetes and muscular dystrophies, major accidents when functional muscle loss occurs and the patients have to undergo surgical treatment. In these cases, tissue-engineered skeletal muscle constructs can be a useful alternative to native skeletal muscle tissues as it represents many morphological and functional properties similar to native skeletal muscle tissues [2]. A functional tissue-engineered skeletal muscle constructs should consist of densely packed and highly differentiated myofibers, and be capable of generating contractile forces similar to natural skeletal muscle tissues [3]. Till today, different skeletal muscle myoblast cell lines and primary myoblasts have been used in models for engineering of skeletal muscle tissues. However, the most prominent cell line used for skeletal muscle tissue engineering is the mouse myoblast C2C12 cell line because of their infinite proliferation and differentiation capacity into multi-nucleated myotubes [4]. Moreover, development of *in vitro* tissue-engineered skeletal muscle constructs using cell culture systems would offer an alternative to conventional animal models for drug screening, leading to reduced pre-clinical studies [5, 6]. To date, many techniques have been applied for the fabrication of skeletal muscle tissue constructs, including myoblast alignment by topographical constraints, electrical field, mechanical stretching, chemical patterning and 3D bio-printing [5-11]. One successful approach for the fabrication of functional skeletal muscle tissue constructs is magnetic force-based tissue engineering (Mag-TE), whereby cells labeled with functionalized magnetic nanoparticles have been used to fabricate tissue constructs by applying a magnetic force. In our previous study, a Mag-TE technique enabled the formation of a ring-shaped tissue construct possessing high cell density and an oriented multilayered structure [12-14]. Furthermore, the skeletal muscle constructs fabricated by the Mag-TE technique

generated contractile forces in response to electrical stimulation [15]. However, the contractile force of these artificial skeletal muscle tissue constructs was considerably low compared with that of native skeletal muscle tissues.

Heterotypic cellular interactions are vital for biological functions, and coculture with different cell types have already been proved as a successful approach for establishing cellular communication *in vitro* [16]. It has been reported that coculture of C2C12 cells with fibroblasts can improve myogenic cell differentiation [17], and that coculture of C2C12 cells with endothelial cells can result in the development of prevascularized tissues with the formation of a capillary network [18]. The major property of skeletal muscle tissue maturation and functionality is the force generation capacity. Because the force generation capacity depends on electrical pulse stimulation (EPS) from the central nervous system (CNS) via the motor neurons, innervation of neurons into the skeletal muscle tissue constructs is a feasible approach. Moreover, neuronal input is intrinsic for optimal muscle development and function, and its absence results in the degeneration of myotubes [19]. Innervation of neurons into a muscle fiber forms a chemical synapse between neuron and muscle cells, called the neuromuscular junction (NMJ). In NMJs, synaptic transmission is achieved through the action of neuronally-released acetylcholine (Ach) and acetylcholine receptors (AchR) located in the post-synaptic membrane of the muscle fibers. In vertebrates, clustering of AchRs is the main feature of NMJ formation. During the interaction between neuron and muscle cells, the neuron secretes a 200 kDa proteoglycan, agrin, that causes AchR clustering on the surface of the cultured myotubes. Recently, Ostrovidov et al. have reported that 3D coculture of C2C12/PC12 cell is effective for the maturation of C2C12 myotubes and upregulation of neuromuscular markers [20-23]. However, the crosstalk between two different types of cells and the impact on enhancement of contractile force generation have yet to be elucidated. In the present study, we aimed to investigate whether coculture of C2C12 cells with PC12 neural cells leads to the formation of NMJs and improves the contractile force generation of skeletal muscle tissues constructed by Mag-TE.

3.2 Materials and Methods

3.2.1 Cell culture

Mouse myoblast C2C12 cells (American Type Culture Collection, Manassas, VA) were grown in low glucose Dulbecco's modified Eagle's medium (LG-DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G potassium, and 0.1 mg/mL streptomycin sulfate. To induce myogenic differentiation, the medium was changed to LG-DMEM supplemented with 2% calf serum (CS) for 2D cell culture or 0.4% Ultroser G (Pall, East Hills, NY) for 3D tissue culture, with antibiotics. Cells were cultured at 37°C in a 5% (v/v) CO_2 incubator.

Rat pheochromocytoma PC12 neural cells (Riken Bioresource Center, Ibaraki, Japan) were grown in DMEM (high glucose) supplemented with 10% FBS, 10% horse serum (HS) and antibiotics. Cultures were maintained for 3 days at 37°C in a 5% (v/v) CO_2 incubator until confluence and were then harvested for coculture. For neural differentiation, the medium was changed to high glucose DMEM supplemented with 1 ng/mL of NGF (R&D Systems, Minneapolis, MN), N2 supplement (R&D Systems) and antibiotics.

3.2.2 2D muscle-neuron coculture

For 2D muscle-neuron coculture, C2C12 cells (2×10^4 cells) were seeded into wells of a 24-well tissue culture plate (Thermo Fisher Scientific, Darmstadt, Germany) in LG-DMEM with 10% FBS. On day 2 of culture, PC12 cells (2×10^4 cells) were seeded onto the C2C12 cells (day 0). The coculture condition was maintained for 7 days by replacing the differentiation medium [LG-DMEM with 2% CS with or without the addition of 1 ng/mL NGF and N2-supplement] at 24 h intervals (Fig. 3-1).

3.2.3 Immunocytochemical analysis

Immunofluorescent staining was performed to show PC12 neurites and C2C12 myotubes. Samples of 2D monoculture and coculture on day 7 were stained with anti-MHC (sc20641; Santa Cruz Biotechnology, Santa Cruz, CA) and/or mouse anti-beta III tubulin (TuJ) antibodies (R&D systems). For determining the TuJ-positive area, cocultured samples from days 1, 4 and 7 were stained with anti-TuJ antibody (R&D systems) and the TuJ-positive area in each field (mm^2) was measured using BZ-Analyzer software and a BZ-9000 microscope (Keyence, Tokyo, Japan). Micrographs of five fields of view in three separate

wells were randomly captured and myogenic differentiation rate and myotube width were determined using previously described methods [23]. Briefly, cell samples from day 7 were double-stained with anti-α-actinin antibody (EA-53, Sigma-Aldrich, St. Louis, MO) and 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA). Micrographs were then randomly captured and α-actinin-positive myotube widths and numbers of DAPI-stained nuclei were measured using BZ-Analyzer software (Keyence). The differentiation rate was calculated using the following equation: Differentiation rate = (the number of DAPI-stained nuclei in α-actinin-positive myotubes in a field of view / the number of DAPI-stained nuclei in a field of view). To determine mean myotube width between samples, the five largest myotubes within the five fields of view for each of the three separate wells per sample were measured using BZ-Analyzer software (Keyence).

For qualitative assessment of the formation of AchR clusters, day 7 samples of 2D monocultures and cocultures were incubated with Alexa Fluor 488-conjugated alpha-bungarotoxin (α-BTx) (Thermo Fisher Scientific) dissolved in differentiation medium with or without NGF (1:1000) for 1 h at 37°C, before fixation. The samples were then stained with mouse anti-TuJ antibody (R&D systems). Finally, AchR clusters were observed under a FV10i confocal laser-scanning microscope (Olympus, Tokyo, Japan). For quantitative analysis, after incubation with Alexa Flour 488-conjugated α-BTx (Thermo Fisher Scientific), samples were stained with anti-α-actinin antibody (EA-53, Sigma-Aldrich). Then, the α-BTx-positive area (μm^2) within the α-actinin-positive myotubes in each field of view was measured using BZ-Analyzer software (Keyence). A standard cluster was classified as a α-BTx-positive area above 3 μm^2. Then, the total number of standard clusters in each field was counted. Micrographs of five fields of view in each of three separate wells were randomly captured.

3.2.4 Myotube striation

To investigate the striation patterns of myotubes, C2C12 cells (2.7×10^3 cells) and/or PC12 cells (2.7×10^3 cells) were cultured in 35-mm glass bottom dishes (Code 3970-035, Asahi Techno Glass, Tokyo, Japan). On day 7, the differentiated myotubes of both C2C12 monoculture and C2C12/PC12 coculture samples in the 35-mm glass bottom dishes were placed in a chamber for electrical pulse stimulation (C-dish, IonOptix, Milton, MA).

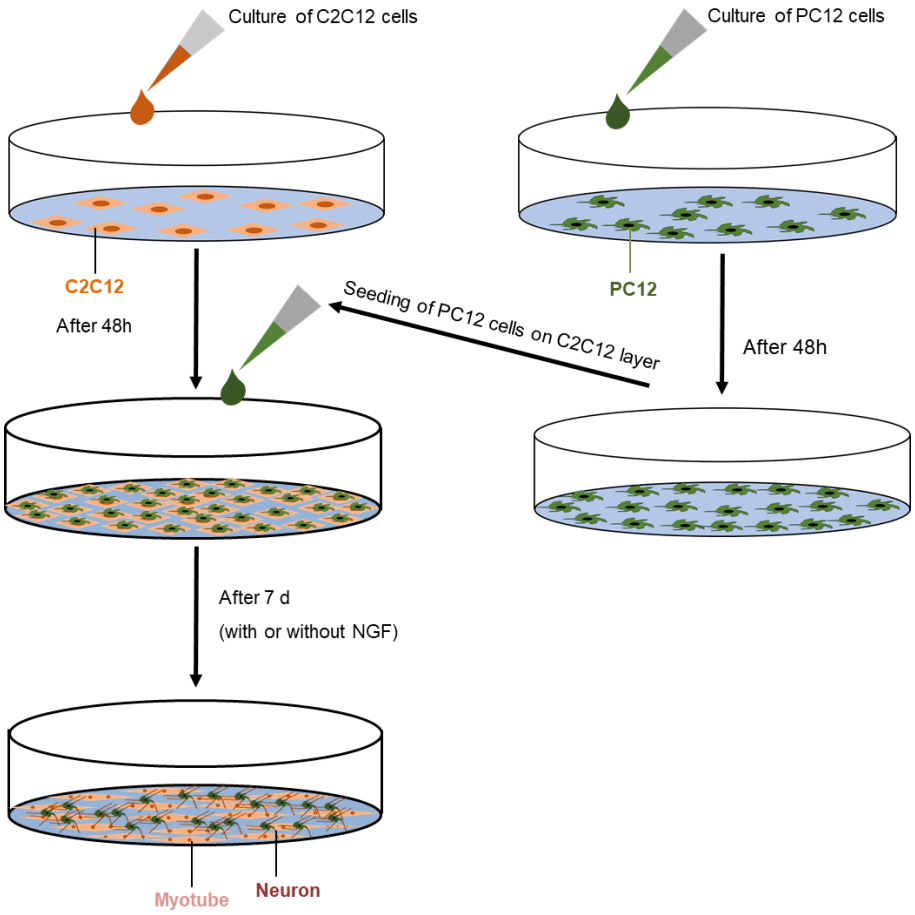

Fig. 3-1. Schematic diagram of 2D muscle-neuron coculture. C2C12 cells were seeded into wells of a 24-well tissue culture plate. On day 2 of culture, PC12 cells (were seeded onto the C2C12 cells (day 0). The coculture condition was maintained for 7 days by replacing the differentiation medium at 24 h intervals. At day 07, both C2C12 and PC12 cells were differentiated into neurons and myotubes and contact between neurons and myotubes was formed.

3.2.5 Myotube contractile activity

Contractile activity of myotubes of both monoculture and coculture samples in 35-mm cell culture dishes (Greiner Bio-one, Frickenhausen, Germany) was measured before and after incubation with 12.5 μM (+)-tubocurarine chloride pentahydrate (curare; Tokyo Chemical Industries, Tokyo Japan). On day 7, the dishes were placed in a chamber for electrical stimulation (C-dish). Electrical pulses were applied at 0.3 V/mm for 4 ms at 2 Hz and myotube movement was recorded at a rate of 15 frames/s for 20 s at three positions on the bottom surface in each of three separate dishes using a BZ-9000 microscope (Keyence). The dishes were then incubated with curare for 15 min at 37°C. The electric pulses were applied again and myotube movement was recorded using a BZ-9000 microscope (Keyence). To estimate the range of displacement, five myotubes displaying the highest contractile activity in each of three fields of view in three separate dishes were measured using the motion analyzer software (Keyence) [23, 24].

3.2.6 PC12-conditioned medium

PC12 culture medium was harvested every 24 h and filtered. C2C12 cells were cultured in medium containing a 1:1 ratio of PC12 culture medium to fresh culture medium. Cells were cultured to day 7 with medium replacement at 24 h intervals. On day 7, myotube contractile activity was determined [25].

3.2.7 Agrin expression analysis

Agrin expression level was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Ray Biotech, Norcross, GA). On day 7, cells were washed with phosphate buffered saline (PBS) and treated with radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific). Cells were then collected from the dish by gently scraping with a cell scraper before being transferred to microtubes. The collected cocultured cell samples were centrifuged at 600 ×g for 10 min at 4°C, and agrin-ELISA was performed in accordance with the manufacture's protocol. The limit concentration level of agrin detection using the agrin-ELISA kit was 0.06 ng/mL.

3.2.8 Magnetite cationic liposome (MCL) preparation

MCLs were prepared from colloidal magnetite and a lipid mixture consisting of N-(trimethylammonioacetyl)-didodecyl-D-glutamate chloride, dilauroylphosphatidylcholine, and dioleoylphosphatidylethanolamine, in a molar ratio of 1:2:2, as described previously [26]. The magnetite particles (Fe_3O_4; average particle size, 10 nm) were purchased from Dai-ichi High Frequency (Tokyo, Japan). The magnetic characteristics at 796 kA/m (room temperature) were as follows: 2.0 kA/m coercivity; 63.9 Am^2/kg saturation flux density; and 2.6 Am^2/kg remanent flux density.

3.2.9 Fabricating 3D neural cell- incorporated skeletal muscle tissue constructed by Mag-TE

For magnetic labeling, C2C12 cells (3×10^6 cells) were seeded in 100-mm tissue culture dishes (Thermo Scientific) containing 10 mL of culture medium in the presence of MCLs (net magnetite concentration, 100 pg/cell), and incubated for 6 h to allow for MCL uptake by the cells [23]. A collagen solution was prepared by mixing type I collagen solution (Nitta Gelatin, Osaka, Japan), DMEM and neutralization buffer (0.05 M NaOH) at a volume ratio of 8:1:1. A PC12 cell suspension (1×10^6 cells) was mixed with an extracellular matrix (ECM) precursor solution composed of collagen solution (70 μL; final concentration, 0.5 mg/mL), FBS (15 μL) and matrigel basement matrix (15 μL; BD Biosciences). The PC12 cells in the ECM solution (100 μL, final concentration) were mixed with the MCL-labeled C2C12 cell suspension (1×10^6 cells in 50 μL). The C2C12/PC12 mixture was then added to wells of a 24-well ultra-low attachment plate (150 μL/well, Corning, New York, NY) with a polycarbonate cylinder (diameter, 12 mm; height, 5 mm) fixed at the center of each well, and a neodymium magnet (diameter, 30 mm; magnetic induction, 0.4 T) was placed under the wells. Thereafter, growth medium was added to each well. One day after cell seeding, the cell layer shrank around the cylinder, resulting in the formation of a ring-shaped cellular construct. The cellular ring was removed from the cylinder and hooked around two stainless-steel pins (Shiga, Tokyo, Japan) that were positioned 10 mm apart from one another. For myogenic differentiation of tissue constructs, the cellular rings were cultured in differentiation medium consisting of LG-DMEM supplemented with 0.4% Ultroser G (Pall) with or without N2 supplement (Gibco) and 1 ng/mL NGF (R&D systems) in wells of 6-well culture plate for 7 days (Fig. 3-2).

3.2.10 Measurement of contractile force generation

Contractile force generation of skeletal muscle tissue constructs was measured as described previously [15]. Briefly, carbon electrodes were placed 10 mm apart at the opposite sides of a tissue culture plate. A muscle bundle was hooked around two stainless-steel minute pins (Shiga). One pin was attached to a force transducer (AE-801, SonorOne, Sausalito, CA), and the other was fixed to a silicon rubber sheet placed at the bottom of the culture plate. Electrical pulses were computer-controlled with specially designed LabView software (National Instruments, Austin, TX). For measuring twitch contractions, the tissue sample was stimulated with an electrical pulse of 20 V with a width of 10 ms. For measuring tetanic contractions, the tissue sample was stimulated with electrical pulses with the following properties: voltage, 20 V; width, 10 ms; frequency, 50 Hz; and duration, 2 s.

3.2.11 Immunohistochemical analysis

For immunostaining, tissue constructs were washed with PBS and fixed with 4% paraformaldehyde for 15 min. Tissue constructs were then immersed in PBS containing 0.2% Triton-X 100 for 15 min, washed three times with PBS, and blocked in PBS containing 1% (w/v) bovine serum albumin for 30 min. The specimens were stained with anti-MHC (sc20641; Santa Cruz Biotechnology) and anti-TuJ (R&D systems) antibo*d*ies. After washing three times with PBS, tissues were observed under a FV10i confocal laser-scanning microscope (Olympus).

3.2.12 Statistical analysis

Statistical comparisons were evaluated by the Mann-Whitney rank sum test, and values of $P < 0.05$ were considered significantly different.

3.3 Result

3.3.1 Effects of C2C12 cells on neural differentiation of PC12 cells in 2D coculture

Immunocytochemical analysis of C2C12/PC12 cocultured cells using anti-MHC (myogenic differentiation marker) and anti-TuJ (neuronal differentiation marker) antibodies revealed that both C2C12 and PC12 cells differentiated into myotubes and neuronal cells, respectively, in coculture conditions (Fig. 3-3).

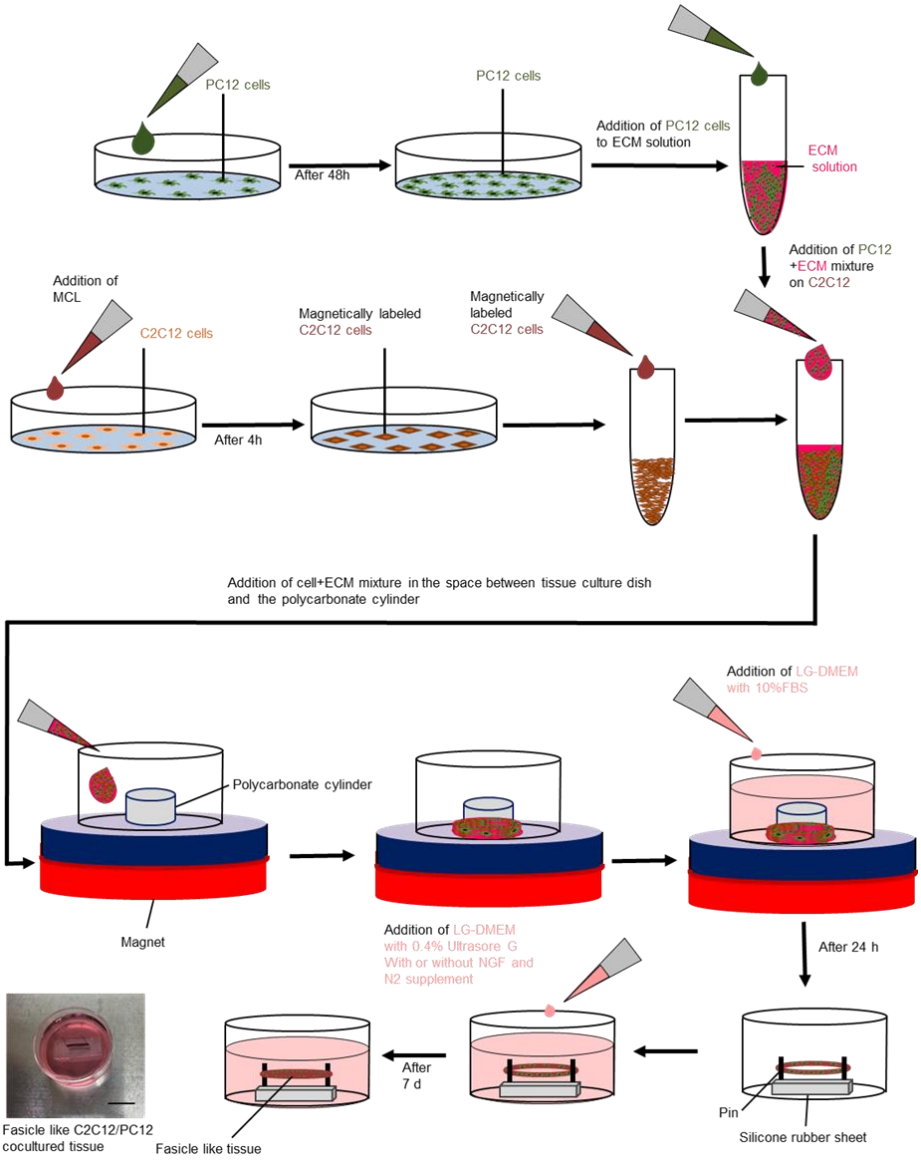

Fig. 3-2. Schematic diagram of fabrication of 3D innervated artificial skeletal muscle construct by C2C12/PC12 coculture system. Magnetic labeling of C2C12 cells were done by incubating with MCL for 6h. ECM precursor solution was prepared by using collagen solution and matrigel basement matrix. After that, PC12 cells were added to the ECM precursor solution. The PC12 cells in the ECM solution were mixed with the MCL-labeled C2C12 cell suspension. The C2C12/PC12 mixture was then added to wells of a 24-well ultra-low attachment plate with a polycarbonate cylinder fixed at the center of each well, and a neodymium magnet was placed under the wells. Thereafter, growth medium was added to each well. After 24h of cell seeding, the cell layer shrank around the cylinder, resulting in the formation of a ring-shaped cellular construct. The cellular ring was removed from the cylinder and hooked around two stainless-steel pins that were positioned 10 mm apart from one another. For myogenic differentiation of tissue constructs, the cellular rings were cultured in differentiation medium consisting of LG-DMEM supplemented with 0.4% Ultroser G with or without N2 supplement and NGF for 7 days. After 7d fascicle like tissues were formed. Scale bars, 10 mm.

Furthermore, addition of NGF resulted in extensive neuronal protrusions along the myotubes. To investigate the effects of coculture on the differentiation of PC12 cells, TuJ-positive neuronal area at different coculture-time intervals was evaluated (Fig. 3-4). PC12 cells started to differentiate on day 1 of coculture, and the TuJ-positive neuronal area increased over the 7-day culture period (Figs. 3-4 A and C). NGF increased the TuJ-positive area of monocultured PC12 cells (Figs. 3-4 B and D). Furthermore, cocultured C2C12 cells increased the TuJ-positive area of PC12 cells, and the addition of NGF to the coculture further increased this (Figs. 3-4 B and D).

3.3.2 Effects of PC12 cells on myogenic differentiation of C2C12 cells in 2D coculture

To investigate the impact of coculture on myogenic differentiation of C2C12 cells, myotubes on day 7 were immunostained with anti-α-actinin antibody (myogenic differentiation marker) (Fig. 3-5 A) and differentiation rate and myotube width were determined as an indicator of hypertrophy. Both differentiation rate and myogenic width did not differ between coculture and monoculture conditions (Figs. 3-5 B and C).

In contrast, larger numbers of sarcomeric α-actinin-positive striated myotubes were observed in cocultured myotubes, with NGF addition further increasing the number of myotubes with striation (Fig. 3-6). To explore the effects of coculture on myotube function, 2D contractile activity was analyzed by examining myotube movement in response to electrical pulses. Myotubes on day 7 displayed contractile responses to EPS of 0.3 V/mm for 4 ms at 2 Hz. As shown in Fig. 3-7, contractile activity of cocultured myotubes was higher than that of monocultured myotubes, and NGF addition slightly, but not significantly, enhanced myotube displacement.

3.3.3 Formation of neuromuscular junctions

To determine the formation of functional synaptic contact between neurons and myotubes, the neuromuscular blocking agent curare was added to the medium and the contractile activity of myotubes was subsequently monitored. As a result, curare attenuated the contractile activities of cocultured myotubes Figs. 3-7A and B, indicating that the enhanced activity of myotubes was attributed to the functional synaptic interaction based on NMJs.

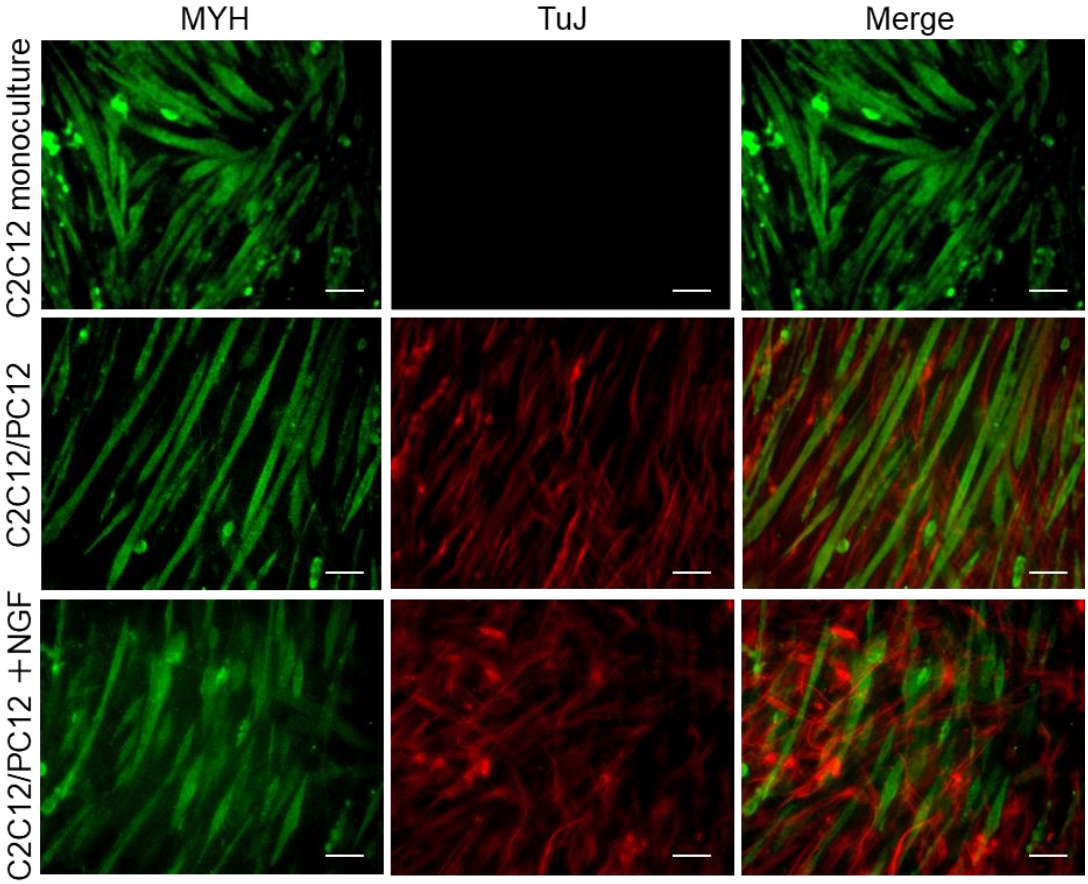

Fig. 3-3. Immunocytochemical analysis using anti-myosin heavy chain (MYH; green) and anti-TuJ (red) antibodies for C2C12/PC12 coculture. Scale bars, 50 μm.

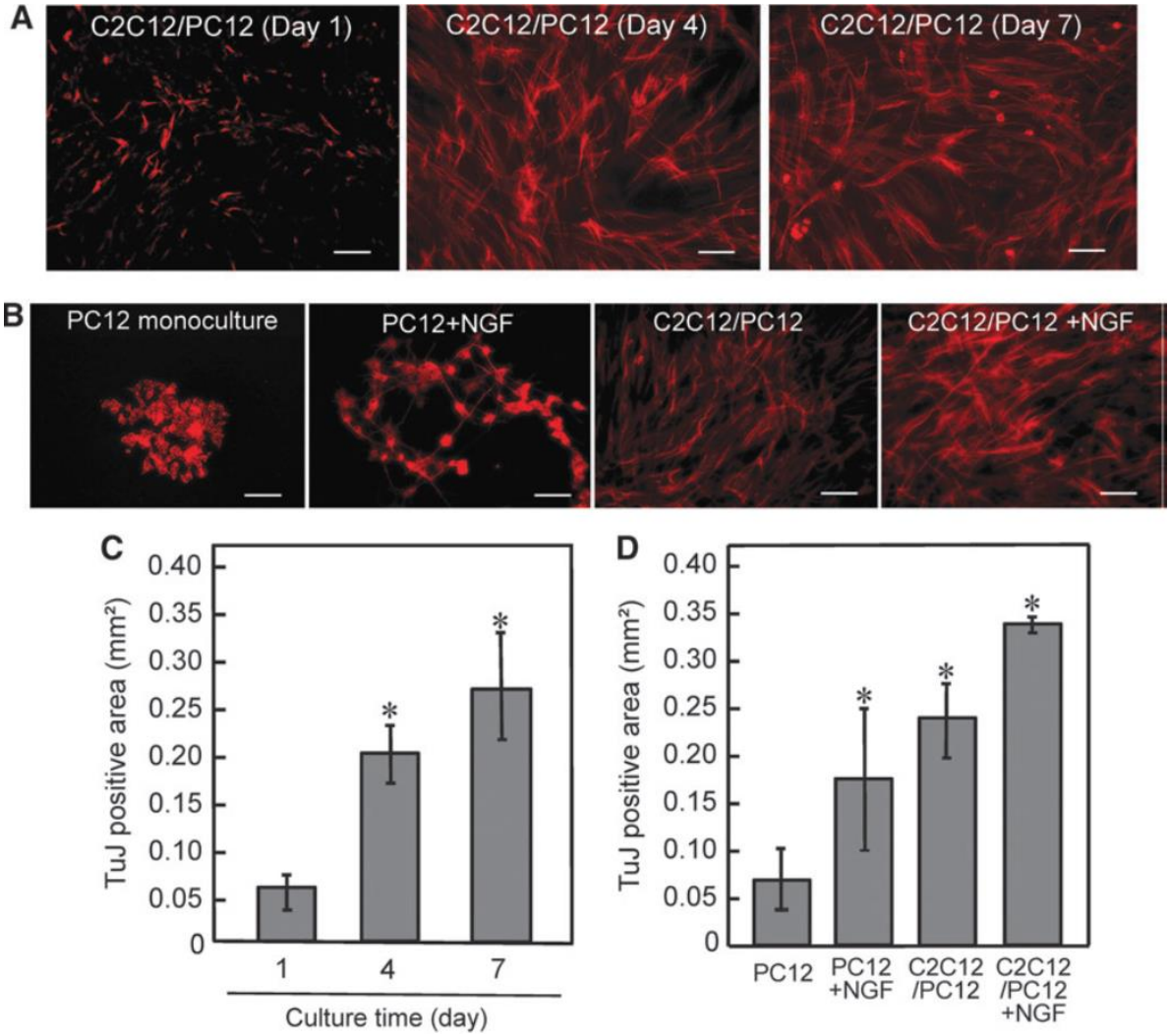

Fig. 3-4. Effects of coculture on PC12 differentiation. **(A)** Fluorescence microscopic images showing immunocytochemical analysis of anti-TuJ antibody (red) in C2C12/PC12 cocultures on days 1, 4 and 7. Scale bars, 50 μm. **(B)** Fluorescence microscopic images showing immunocytochemical analysis of anti-TuJ antibody (red) in PC12 monocultures and C2C12/PC12 cocultures with or without addition of NGF on day 7. Scale bars, 100 μm. **(C)** Quantitative image analysis of the TuJ-positive neuronal area in C2C12/PC12 cocultures on days 1, 4 and 7. **(D)** Quantitative image analysis of the TuJ-positive neuronal area of PC12 monocultures and C2C12/PC12 cocultures with or without addition of NGF on day 7. Data are expressed as means ± standard deviation (SD) (n = 3). * Indicates a statistically significant difference ($P < 0.05$).

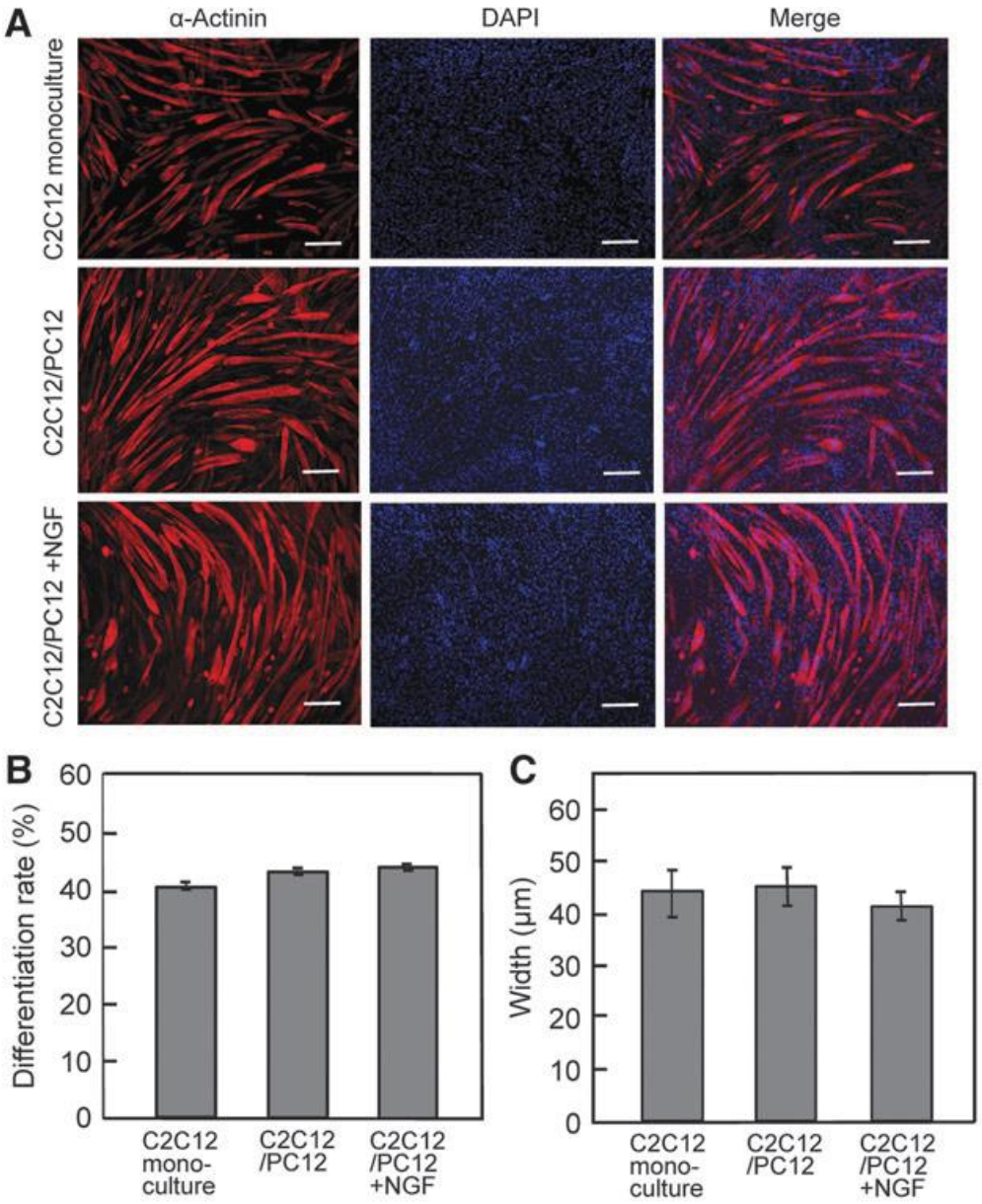

Fig. 3-5. Effects of coculture on C2C12 differentiation. **(A)** Immunocytochemical analysis using anti-α-actinin antibody (red) in monocultured and cocultured myotubes with or without NGF on day 7. DAPI (blue) was used for nuclei staining. Scale bars, 100 μm. **(B)** Quantitative image analysis for myogenic differentiation rate. **(C)** Quantitative image analysis for myotube width. Data are expressed as means ± SD (n = 3).

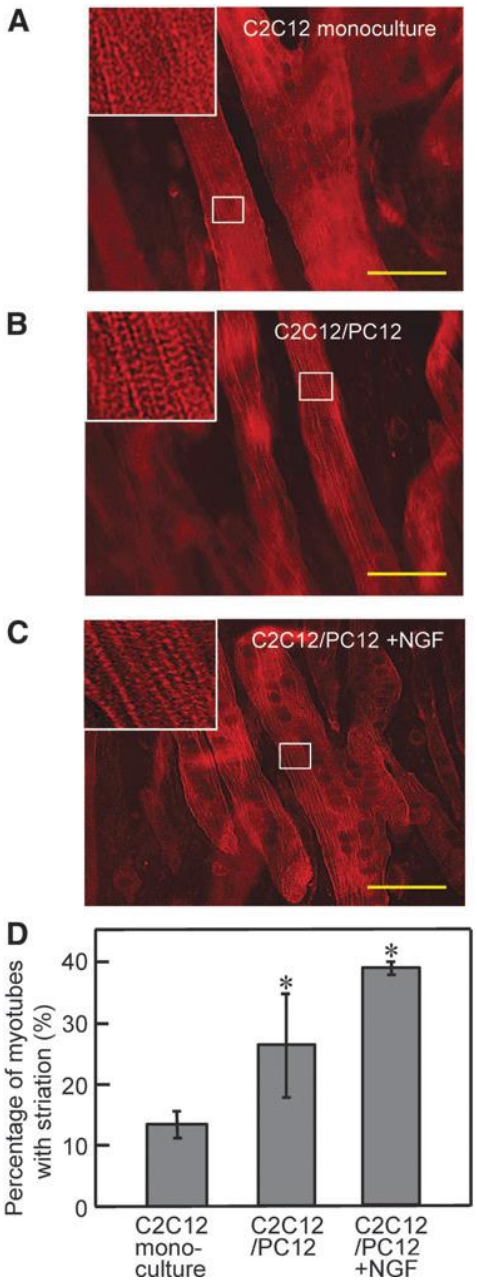

Fig. 3-6. Effect of coculture on striation formation in myotubes. **(A–C)** Fluorescence microscopy images of myotubes with striations of sarcomeric α-actinin (red) on day 7. Scale bars, 50 μm. Inset: Magnified images of area in white rectangle. Scale bars, 50 μm. **(D)** Quantitative image analysis to determine the percentage of myotubes with striations. Data are expressed as means ± SD (n=3). * Indicates a statistically significant difference ($P < 0.05$).

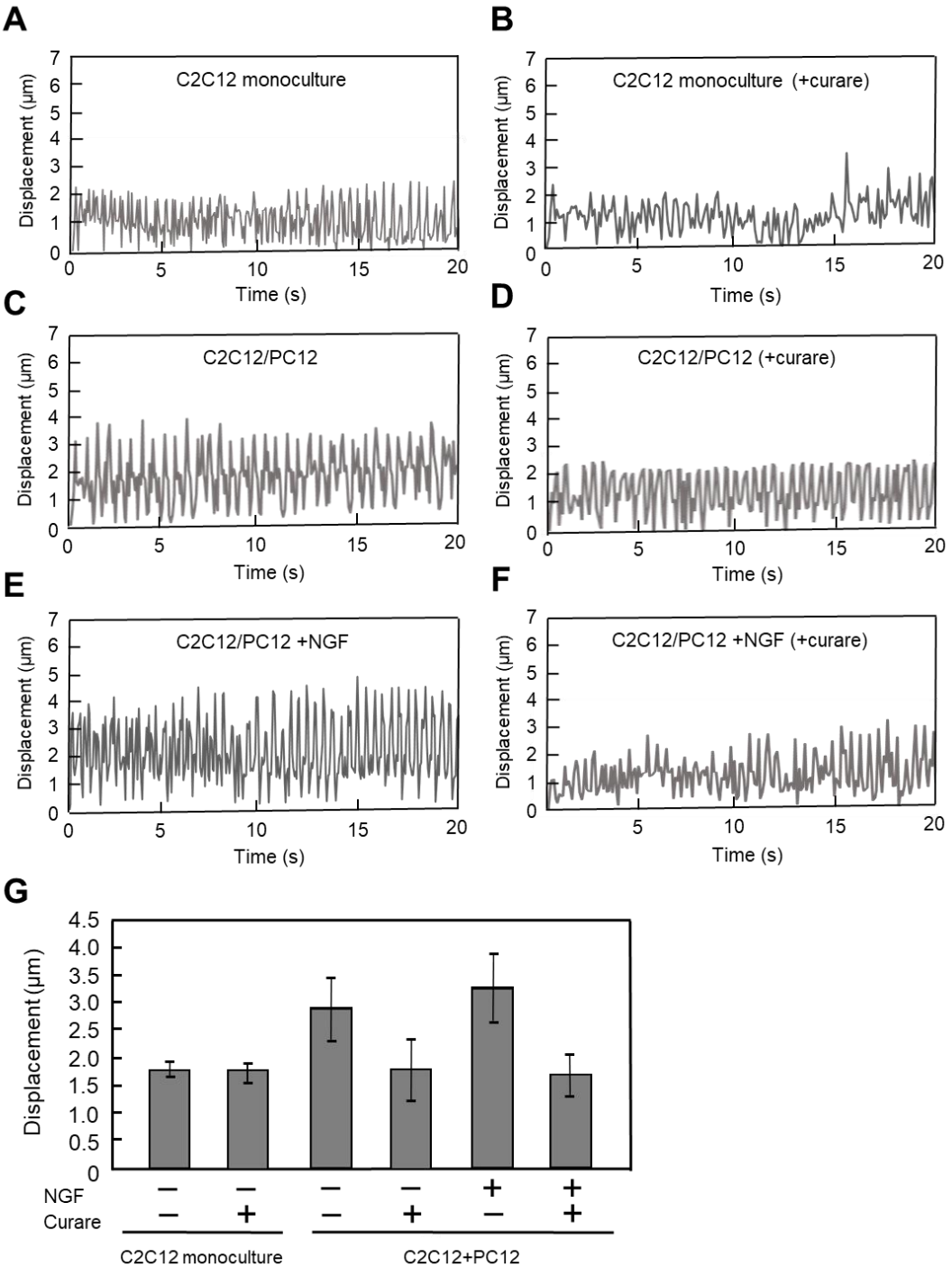

Fig. 3-7. Effects of coculture on the contractile activity of myotubes. **(A)** Contractile movements of myotubes under EPS (voltage, 0.3 V/mm, width, 10 ms, frequency, 1 Hz) before and after addition of curare on day 7. **(B)** Quantitative analysis of displacement. Data are expressed as means ± SD (n = 3). * Indicates a statistically significant difference (P<0.05).

AchR clustering between neurons and myotubes is another important feature to determine the formation of NMJs. Immunocytochemical analysis using α-BTx neurotoxin, which is known to bind to the AchR found at the NMJ, revealed that AchR clusters were found with α-actinin-positive myotubes (Fig .3-8 A) and with TuJ- positive neurites Fig. 3-9 in C2C12/PC12 cocultured cells. As shown in Fig. 3-9 B, cocultured cells significantly increased the number of AchR clusters with a greater than 3 μm^2 size compared with monocultured cells. When considering the mechanism of action, PC12-derived neurons may enhance the contractile activity of myotubes by secreting paracrine factors. To elucidate this, C2C12 cells were cultured in conditioned medium and the contractile activities of the myotubes was evaluated under EPS (Fig. 3-10). No significant differences in both contractile activity profiles (Fig. 3-10 A)and myotube displacement (Fig. 3-10 B) were observed for the cells cultured in conditioned medium, suggesting that the enhanced contractile activity of myotubes in coculture was attributable to cell–cell attachment between C2C12 and PC12 cells.

To further explore the mechanism of how PC12 cells enhanced myotube activity via NMJs, expression of agrin protein—known to be secreted by neurons and involved in the aggregation of AchR [22]—was investigated. A substantial level of agrin was detected in C2C12/PC12 cocultured cells (Fig. 3-10 C) and monocultured PC12 cells (data not shown), while no agrin was detected in the conditioned medium; suggesting that the level of agrin released from PC12 cells is extremely low and the close contact between C2C12 and PC12 cells is important for mutual interaction. Thus, these results indicated the formation of NMJs in the C2C12/PC12 coculture system.

3.3.4 Contractile force generation by artificial muscle-neuron constructs

The coculture system was applied for the fabrication of artificial muscle-neuron constructs using a Mag-TE technique. MCL-labeled C2C12 cells mixed with PC12 cells were uniformly accumulated at the bottom of the well by applying a magnetic force, and a sheet-like structure contracted to form a ring-like tissue construct surrounding the cylinder. The tissue ring coated with ECMs formed fascicle-like tissues on day 7 in the differentiation medium. The average diameter of the tissue-engineered skeletal muscle constructs was approximately 500 μm. Histological examination of the tissue constructs showed that C2C12 cells differentiated into multinucleated myotubes and the PC12 cells existed

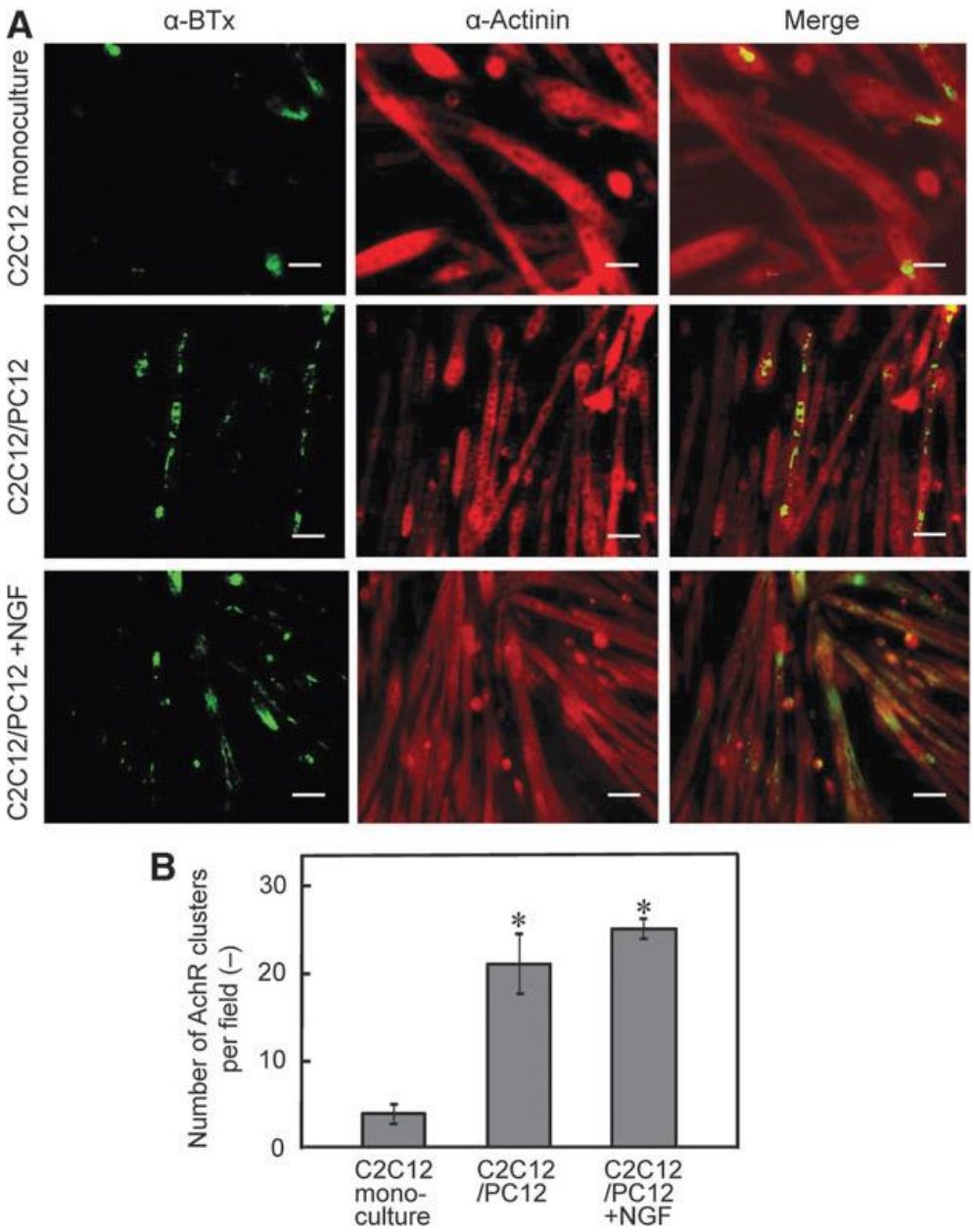

Fig. 3-8. Effects of coculture on the formation of AchR clusters on myotubes. **(A)** Immunocytochemical analysis using α-bungarotoxin (BTx; green) and α-actinin (red) for monoculture and cocultured cells (with or without NGF) on day 7. Scale bars, 50 μm. **(B)** Quantitative image analysis of the number of AchR clusters. Data are expressed as means ± SD (n = 3). * Indicates a statistically significant difference ($P < 0.05$).

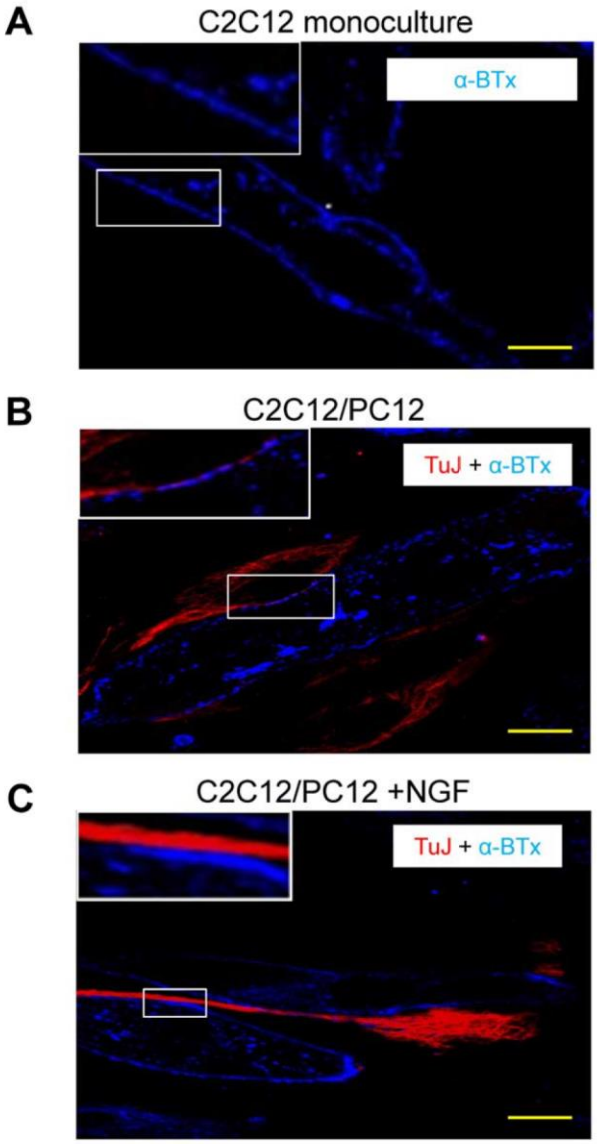

Fig. 3-9. Immunocytochemical analysis for anti-α-bungarotoxin (blue; α-BTx) and anti-TuJ (red) antibodies for C2C12 monoculture (**A**) and C2C12/PC12 coculture without (**B**) or with **(C)** NGF. Scale bars, 20 μm. Inset; magnified image of area in white rectangle.

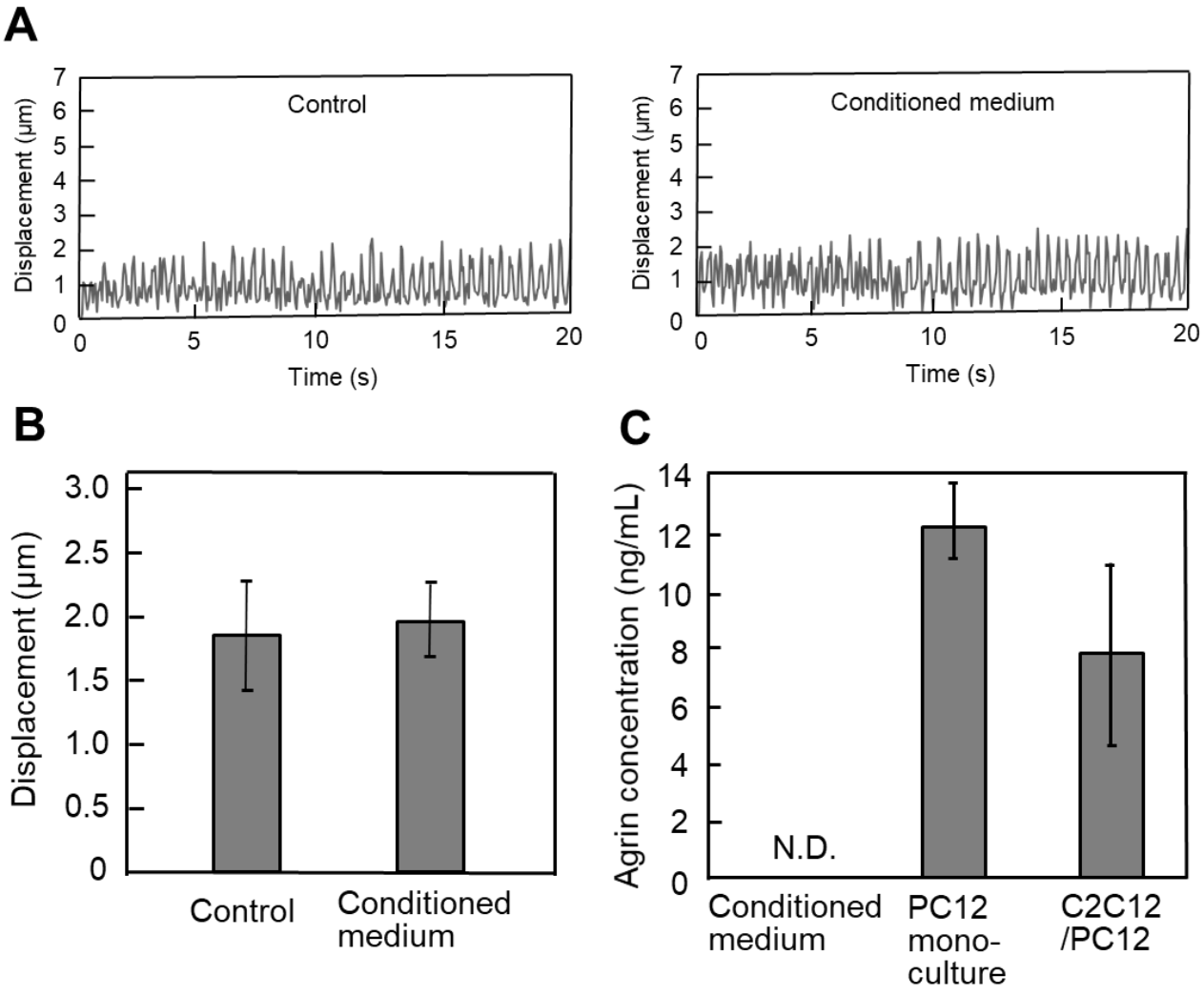

Fig. 3-10. Effects of PC12 cell-conditioned medium on contractile activity. C2C12 cells were cultured with PC12 cell-conditioned medium. Electrical pulse stimulation (voltage, 0.3 V/mm, width, 4 ms, frequency, 2 Hz) was applied, and the profiles of myotube displacement (**A**) and quantitative data **(B)** were analyzed. **(C)** Quantitative analysis of agrin expression by ELISA. N.D., not detected. Data are expressed as means ± SD (n = 3).

alongside the myotubes (Fig. 3-11). To evaluate contractile force generation, both innervated (C2C12/PC12 cocultured tissue) and aneural (C2C12 cell tissue) constructs were stimulated with electrical pulses. The force generation profiles in response to electrical pulses are shown in (Figs. 3-12 A and B). Twitch contractions were observed after low frequency stimulation (Fig. 3-12 A), while tetanus was observed by repeated electrical stimulation at higher frequency (Fig. 3-12 B). The innervated muscle tissue constructs generated higher physical forces by twitch (Fig. 3-12 C) and tetanus contractions (Fig. 3-12 D) compared with those of aneural muscle bundles, and NGF addition further enhanced force generation. These results clearly indicate that coculture with PC12 neuronal cells had a positive effect on the contractile force generation of the C2C12 skeletal muscle constructs.

3.4 Discussion

In vitro 3D models of muscle-neuron constructs with NMJs between cocultured myotubes and neurons would be a considerable benefit for biological study, drug testing and regenerative medicine. Muscle/neuron coculture systems using primary neuron and muscle cells have been reported [27, 28]. Although primary cells including autologous and patient-derived cells are considered to be a first choice for regenerative medicine, muscle/neuron coculture systems based on primary cells have drawbacks with biological studies and for drug testing, including batch-to-batch variation of cell-type ratio. Moreover, primary coculture systems often require a cocktail of multiple myogenic and neurotrophic factors that have not been thoroughly studied [29]. In the present study, we established a C2C12/PC12 coculture system, analyzed its efficacy on the morphology and contractility of myotubes in 2D culture, determined the formation of NMJs, and finally applied this system for the fabrication of 3D muscle-neuron constructs to improve contractile force generation.
Cell lines including C2C12 and PC12 cells have the capability to proliferate indefinitely. In addition, use of cell lines overcomes problems of cell-type heterogeneity associated with primary cell cultures. Thus, we used C2C12 and PC12 cell lines to allow for the study of experimental parameters under a controlled and reproducible environment, which is more amenable to analysis. In the present study, we showed that both C2C12 and PC12 cells underwent clear differentiation in the coculture condition.

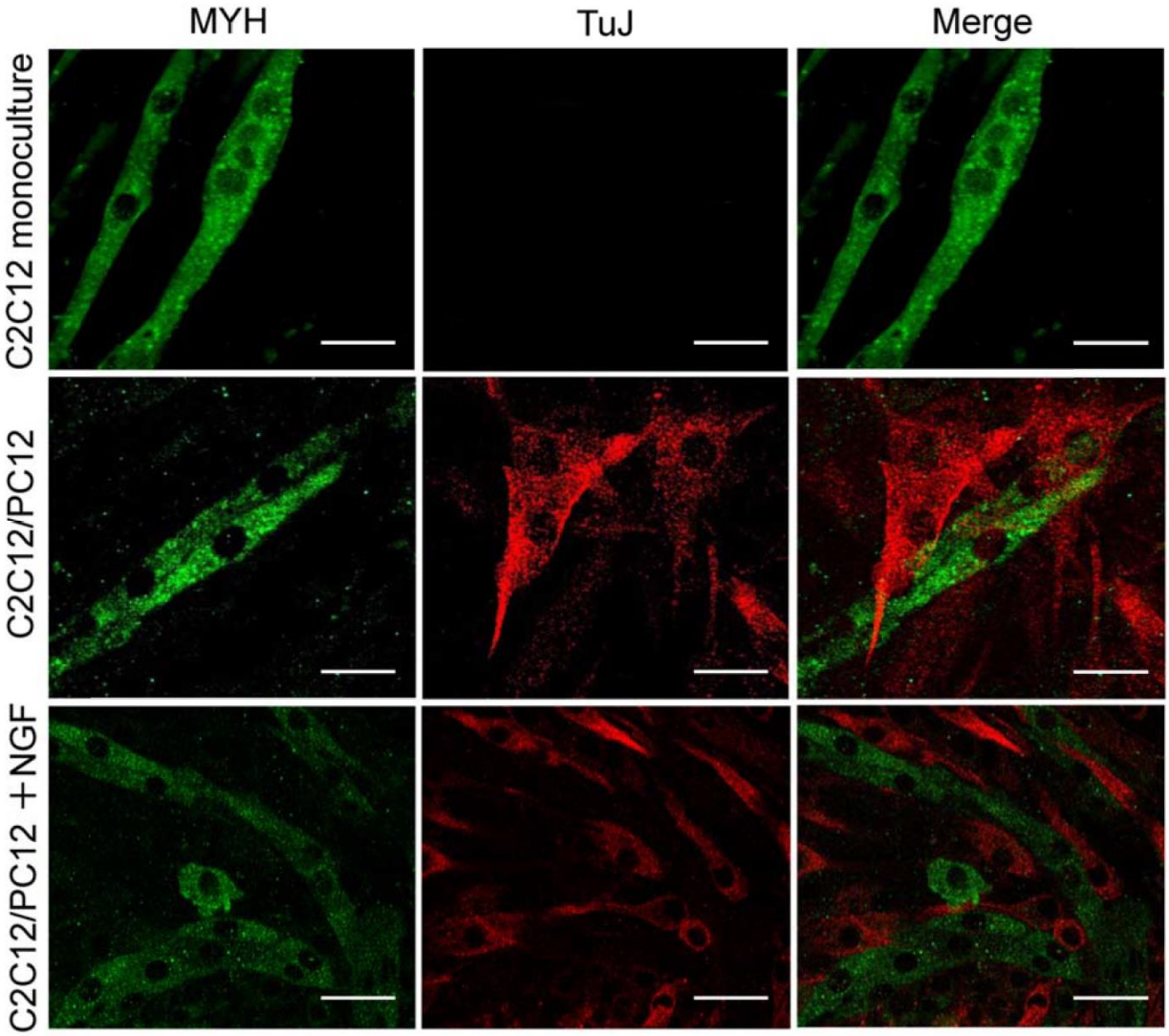

Fig. 3-11. Histology and contractile properties of C2C12/PC12 cocultured tissue constructs. Immunocytochemical analysis using myosin heavy chain (MYH; green) and TuJ (red) for skeletal muscle tissue constructs. Scale bars, 20 μm.

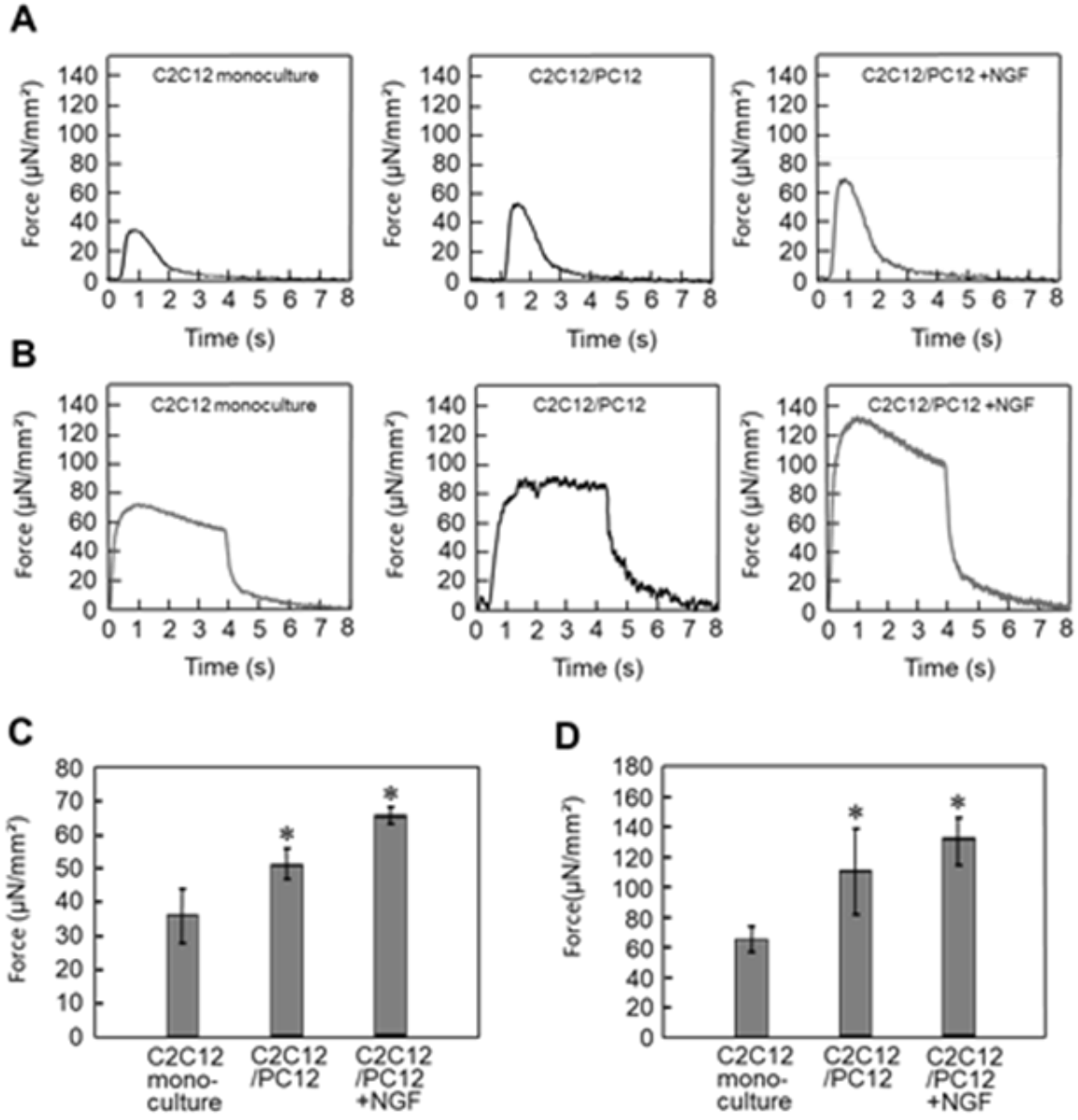

Fig. 3-12. (A) The twitch force generated by skeletal muscle tissue constructs on day 7. EPS condition; voltage, 15 V; width, 10 ms. **(B)** Fusion of the tetanus of skeletal muscle tissue constructs on day 7. EPS condition; Voltage, 15 V; width, 10 ms; frequency, 50 Hz; duration, 2 s. **(C)** Quantitative analysis of peak twitch forces of skeletal muscle tissue constructs. **(D)** Quantitative analysis of tetanus contraction forces of skeletal muscle tissue constructs. Data are expressed as means ± SD (n = 3). **P* < 0.05 versus C2C12 monoculture skeletal muscle tissue constructs.

It has been previously reported that neurotrophins, which are involved in neurite outgrowth [30] and include NGF, brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), are secreted from C2C12 cells [31]. Thus, the neurotrophins secreted from C2C12 cells may induce PC12 neural differentiation within the coculture system, and addition of external NGF further augmented this function (Fig. 3-4). Interestingly, the C2C12/PC12 coculture system had neither any impact on myogenic differentiation rate nor myotube hypertrophy. Similarly, Martin et al. reported that the presence of motor neurons had no significant effects on myogenic cell fusion and myotube hypertrophy [32]. In contrast, Ostrovidov et al. reported that C2C12/PC12 coculture improved the myogenic differentiation rate [33]. The differentiation rate in the present study (around 40%) was much higher than that reported by Ostrovidov et al. (10–20%). In addition, Martin et al. used primary muscle-derived cells and constructed highly matured 3D skeletal muscle tissues with striated myotubes [32]. The level of muscle striation is recognized as a surrogate marker of muscle maturity. In the present study, the number of myotubes displaying striation was increased by coculture (Fig. 3-6 D). We therefore speculate that the degree of myogenic differentiation (the differentiation and maturation level) could explain differences between studies.

We previously demonstrated that morphological analysis using myogenic differentiation markers, including MHC and α-actinin, is insufficient for evaluating the function of skeletal muscle tissues. This is because the level of myogenic differentiation rate is not always consistent with that of contractile force generation of 3D tissue-engineered skeletal muscle constructs [23]. Contractile force generation capability should be the most important feature of tissue-engineered skeletal muscle constructs. This is because it reflects not only the level of differentiation—including the expression of muscle regulatory factors, but also the niche—including the ECM, as well as the architecture—including sarcomere formation. In the present study, the number of myotubes with striation of sarcomeric α-actinin was increased in coculture conditions (Fig. 3-6 D), along with enhanced 2D contractile activity (Fig. 3-7 D), and these parameters were highly correlated with contractile force generation of 3D tissue-engineered skeletal muscle constructs (Fig. 3-12). These results indicate that the evaluation of sarcomere formation and/or contractile activity of myotubes is an indispensable indicator for drug testing of neuromuscular diseases.

The goal of this study was to fabricate innervated 3D skeletal muscle tissue

constructs with contractile force generation capability using the C2C12/PC12 coculture system. The Mag-TE technique has been demonstrated as a powerful tool for fabricating contractile force-producing skeletal muscle tissue constructs [15], [23], [24]. In this study, cocultured muscle tissues were successfully fabricated using the Mag-TE technique, and the C2C12/PC12 cocultured tissues showed significant improvement of physical force generation (Fig. 3-12). Electrical impulse stimulation from motor neurons is the most important cue for skeletal muscle development and maturation [12], [33], therefore we hypothesized that coculture with PC12 neural cells can mimic the *in vivo* niche to engineer *in vitro* functional C2C12 skeletal muscle tissues. Neuronal innervation leads to phenotypical changes of both AchR and sodium channel subtype, as well as MHC expression [34], and it is believed that these types of changes in molecular expression result in enhancement of contractile activity. In the present study, we showed NMJ formation evidenced by AchR clustering (Fig. 3-8) with agrin expression (Fig. 3-10 C), and inhibition of myotubes contraction by a neuromuscular blocking agent, curare (Fig. 3-7). A possible mechanism for the enhanced contractile activity in this study may be the release of Ach from PC12 cells, because Ach can activate myotubes at the NMJ. To elucidate this mechanism, we added the AchR antagonist curare to both monocultured and cocultured cells. Curare significantly attenuated the contractile activity of cocultured myotubes, but had no effect on the monocultured myotubes. These results suggest that the enhanced contractile activity of cocultured myotubes was caused by Ach-mediated neural induction.

3.5 Conclusion

Innervated skeletal muscle tissue constructs were successfully fabricated by using a C2C12/PC12 coculture system and Mag-TE. These innervated skeletal muscle tissue constructs generated significantly higher contractile forces compared with aneural (monoculture) skeletal muscle tissue constructs. These innervated skeletal muscle tissue constructs may be a useful tool for drug testing and biological research for neuromuscular diseases.

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Chapter 4

Microfabrication of artificial skeletal muscle constructs

4.1 Introduction

Development of biological relevant models of human tissues and organs is very important for disease modeling and drug discovery. Recent advancements of tissue engineering have led to the fabrication of *in vitro* biomimetic tissues which have made the drug screening researches easier rather than conventional *in vivo* animal models. Tissue engineered organoids can successfully recapitulate the structural and biological features of native tissues which has already been proved very fruitful to study tissue morphogenesis and function. However, development of an effective technological platforms that generate 3D organoids is an important prerequisite for 3D tissue fabrication. Many researchers have performed studies for successful application of these tools in case of drug screening [1-5]. Further, such technologies may expedite the development of patient specific disease model and advanced personalized treatment regimens.

At present days, application of microdevices are becoming popular for engineering of 3D tissues compared with existing conventional assay platforms. Microfluidic devices have already been covered the 2D platforms [6]. Recently, this technology has been emphasizing the 3D culture systems. Previously, George *et al*, developed PDMS-based biocompatible microdevices by using soft lithography [7] which have been used in bioengineering research. Besides their small dimensions reducing the amount of medium and cells per experiment and their optical clarity amenable to live cell imaging. Microdevices allow for the controlled formation of 3D tissues and the ability to apply various well-defined cues [8]. Such types of microscale functional units of tissues or organs also termed as Organs- on- chips that mimic specific organ functions. Previously, the chips mimicking liver, lung, heart, blood-brain barrier, gut and kidney processes were developed [9-14].

Engineered skeletal muscle tissue models can be used as a potential regenerative medicine and also for screening drugs against different muscular disorders. Skeletal

muscle is the most abundant tissue of the human body which is responsible for movement, posture, temperature control and various other metabolic functions. It is composed of long, cylindrical, multinucleated muscle fibers surrounded by connective tissue [15,16]. Regeneration of damaged muscles occur in case of significant trauma and different types of incurable or inherited disorders such as muscular dystrophies and diabetes [17,18]. But sometimes in case of severe trauma where massive amounts of functional muscle loss occur, leading to the formation of fibrous scar tissues [19]. In that case, tissue engineered skeletal muscle constructs can be used as a potential regenerative therapy. Skeletal muscle tissue engineering (SMTE) intended to replicate the structure and function of skeletal muscle tissue *in vitro* and *in vivo* to generate a functional construct for using as an implantable therapeutic device [20]. For, *in vitro* fabrication of artificial skeletal muscle constructs, both primary myoblast cells and different skeletal myoblast cell lines are used. The most widely used cell lines for SMTE is C2C12 mouse myoblast cell lines due to its indefinite proliferation and differentiation into multinucleated myotubes [21].

Fewer studies have reported methods to engineer skeletal muscle tissue by using human cells [22, 23]. But, most of the studies regarding SMTE based on primary human cells from invasive muscle biopsies faced different challenges such as poor cell availability, limited cell expansion potential and exhaustion of differentiation availability. To mitigate this phenomena, human pluripotent stem cells (hPSCs) can be used as an effective tool for SMTE as it exhibits attractive cell sources for engineering biomimetic skeletal muscle due to unlimited proliferative potential and their ability to differentiate into myogenic cells [24-29]. Recently, functional human skeletal muscle tissue construct has already been engineered by using human pluripotent stem cells (hPSCs) [17]. Although, three dimensional (3D) human induced pluripotent stem cells (hiPSCs) derived artificial skeletal muscle tissues have also been engineered by incorporating hiPSCs derived vascular cells and motor neurons [30].

Thus, with the development of tissue engineering and micro-electromechanical systems (MEMS), different techniques have been applied for the fabrication of 3D skeletal muscle microtissues. At first, Vandenbourgh *et al*. developed a microdevice consists of two silicone rubber posts, fabricated contractile force producing 3D muscle tissues on that device by using primary rodent myoblasts [31]. Further, they developed an automated drug screening system by fabricating skeletal muscle microtissues on that

device by using primary myoblasts of mdx murine model of Duchene muscular dystrophy (DMD) [32]. Furthermore, a novel microfluidic device was designed to engineer skeletal muscle microtissues capable of generating contractile force and also developed a drug screening system for muscle atrophy based on the contractile force [33, 34]. Recently, Agarwal *et al*. developed a skeletal muscle-on-a-chip system by using 3D photo-patterning technology, measured the passive tension generated by muscle constructs and also assessed the subsequent effect of cardiotoxin on engineered muscle tissue architecture and on passive tension [35].

One promising approach for the fabrication of functional muscle tissue constructs is magnetic force-based tissue engineering (Mag-TE), whereby cells labeled with functionalized magnetic nanoparticles have been used to fabricate tissue constructs by applying a magnetic force [36, 37]. In our previous study, a Mag-TE technique enabled the formation of a ring-shaped tissue construct possessing high cell density and an oriented multilayered structure [38]. Furthermore, the skeletal muscle constructs fabricated by the Mag-TE technique generated contractile forces in response to electrical stimulation [39]. Nevertheless, the previous Mag-TE based muscle tissue fabricating method couldn't construct numerous tissues at a same time. Consequently, this research has concentrated on a novel Mag-TE based tissue engineering method that can develop a drug screening platform including a big number of artificial skeletal muscle tissues. Herein, a new device was designed where cells were seeded. Underneath the device a specifically shaped iron sheet was set between device and magnet. This iron sheet was for sake limit magnetic force into a particular area alike the shape of itself, so that MCLs labeled cells could be attracted and concentrate in a high density. Subsequently during differentiation, skeletal muscle tissues would be formed and shrink to spontaneously move up to the top of pillars

4.2 Materials & methods

4.2.1 Microdevice design and fabrication

The microdevices used for engineering skeletal muscle tissues and evaluating their contractile forces were developed as previously reported with slight modifications [21]. All the microdevices used in this study were made and delivered by Dr. Kazunori Shimizu (Dept. of biotechnology, School of engineering, Nagoya university, Japan). Devices were fabricated by molding polydimethylsiloxane (PDMS; SILPOT 184, Dow

Corning Toray, Tokyo, Japan) using a Teflon mold. The Teflon mold was immersed in a 10:1 mixture of pre-cured PDMS, placed in a vacuum chamber to remove air bubbles, and baked at 70°C for 60 min. Subsequently, the mold was removed and the cured PDMS was cut into individual devices. Each device had a 6.0 mm diameter base and contained two vertical 4.0 mm tall by 0.8 mm diameter flexible PDMS microposts spaced 3.0 mm apart. A circular PDMS thin membrane 1.5 mm in diameter was attached to the tip of the micro-post using pre-cured PDMS as an adherent, and the pre-cured PDMS was cured at 70°C for 60 min.

4.2.2 C2C12 cell culture

Mouse myoblast C2C12 cells (American Type Culture Collection, Manassas, VA) were grown in low glucose Dulbecco's modified Eagle's medium (LG-DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G potassium and 0.1 mg/ml streptomycin sulfate. To induce myogenic differentiation for 3D tissue culture, the medium was changed to LG-DMEM supplemented with 0.4% Ultrasore G (Pall, East Hills, NY) with antibiotics. Cells were cultured at 37°C in a 5% (v/v) CO_2 incubator.

4.2.3 Culture and myogenic differentiation of hiPSCs

MyoD-hiPS cell line was generated [22] and gifted by Dr. Hidetoshi Sakurai (Center for iPS cell research and application, Kyoto University, Japan). MyoD-hiPSCs were cultured in human iPS medium composed of primate ES medium (ReproCELL, USA) supplemented with 1μg/mL recombinant human basic fibroblast growth factor (bFGF) (Wako, Japan) and maintained according the previous method [23].

For myogenic differentiation, MyoD-hiPSCs were seeded onto collagen I coated dishes (Iwaki, Japan) without feeder cells. Matrigel was diluted with the ratio of 1:50 with primate ES medium. MyoD-hiPSCs were trypsinized and dissociated into single cells. The cell number plated ranged from 2.0×10^5 to 1.0×10^6 per 10 cm^2. Culture medium was replaced to human iPS medium (StemFit) (Takara Bio, Japan) without bFGF and with 10 μM Y-27632 (Nacalai Tesque, Japan). After 24 h, 1 μg/mL doxycycline (Sigma Aldrich) was added to the culture medium. After 24 h of doxycycline addition, culture medium was changed to differentiation medium composed of alpha minimal essential medium (αMEM) (Invitrogen, USA) with 5% knockout serum (KSR), 50 mU/L penicillin, 50μg/mL streptomycin and 100 μM 2-marceptoethanol (2-ME) and retinoic

acid (Wako, Japan). After 5 days, culture medium was changed to DMEM with 5% horse serum (HS) 50 mU/L penicillin, 50μg/mL streptomycin and 10 ng/mL recombinant human insulin-like growth factor 1, 2mM L-glutamine and 100 μM 2-ME. After 48 hours, myogenic differentiation was analyzed.

4.2.4 Magnetic cationic liposome (MCL) preparation

MCLs were prepared from colloidal magnetite and a lipid mixture consisting of N-(trimethylaminoacetyl)-didodecyl-D-glutamate chloride, dilauroylphosphatidylcholine and dioleoylphosphatidylethanolamine, in a molar ratio of 1:2:2, as like as previous method [40]. The magnetite particles (Fe_3O_4; average particle size, 10 nm) were purchased from Dai-ichi High Frequency (Tokyo, Japan). The magnetic characteristics at 796 kA/m (room temperature) were as follows: 2.0 kA/m coercivity; 63.9 Am_2/kg remnant flux density.

4.2.5 Fabrication of skeletal muscle microtissues on PDMS device

To select an optimum condition for fabrication of skeletal muscle microtissues by Mag-TE, we optimized the total process under three groups designated group-(a), (b) and (c) by altering the number of cells. In case of C2C12 cells, the number of cells in group-(a), (b) and (c) are 0.75×10^5 cells/well, 1.5×10^5 cells/well and 3×10^5 cells/well respectively. On the other hand, in case of MyoD hiPSCs, the number of cells in group-(a), (b) and (c) are 0.1×10^6 cells/well, 1.0×10^6 cells/well and 2.0×10^6 cells/well respectively. For magnetic labeling, C2C12 cells (3×10^6 cells) were seeded in 100 mm tissue culture dishes (Thermo Scientific) containing 10 ml of culture medium in the presence of MCLs (net magnetite concentration, 100 pg/cell), and incubated 6h to allow for MCL uptake by the cells [41]. MyoD hiPSCs were also labeled with magnet as same way. A collagen solution was prepared by mixing type I collagen solution (Nitta Gelatin, Osaka, Japan), DMEM and neutralization buffer (0.05 M NaOH) at a volume ratio of 8:1:1. The MCL-labeled cell suspension (both C2C12 and MyoD hiPSCs) (13.2 μL) as mixed with an extracellular matrix precursor solution composed of collagen solution (6.4 μL) and matrigel basement matrix (0.4 μL).

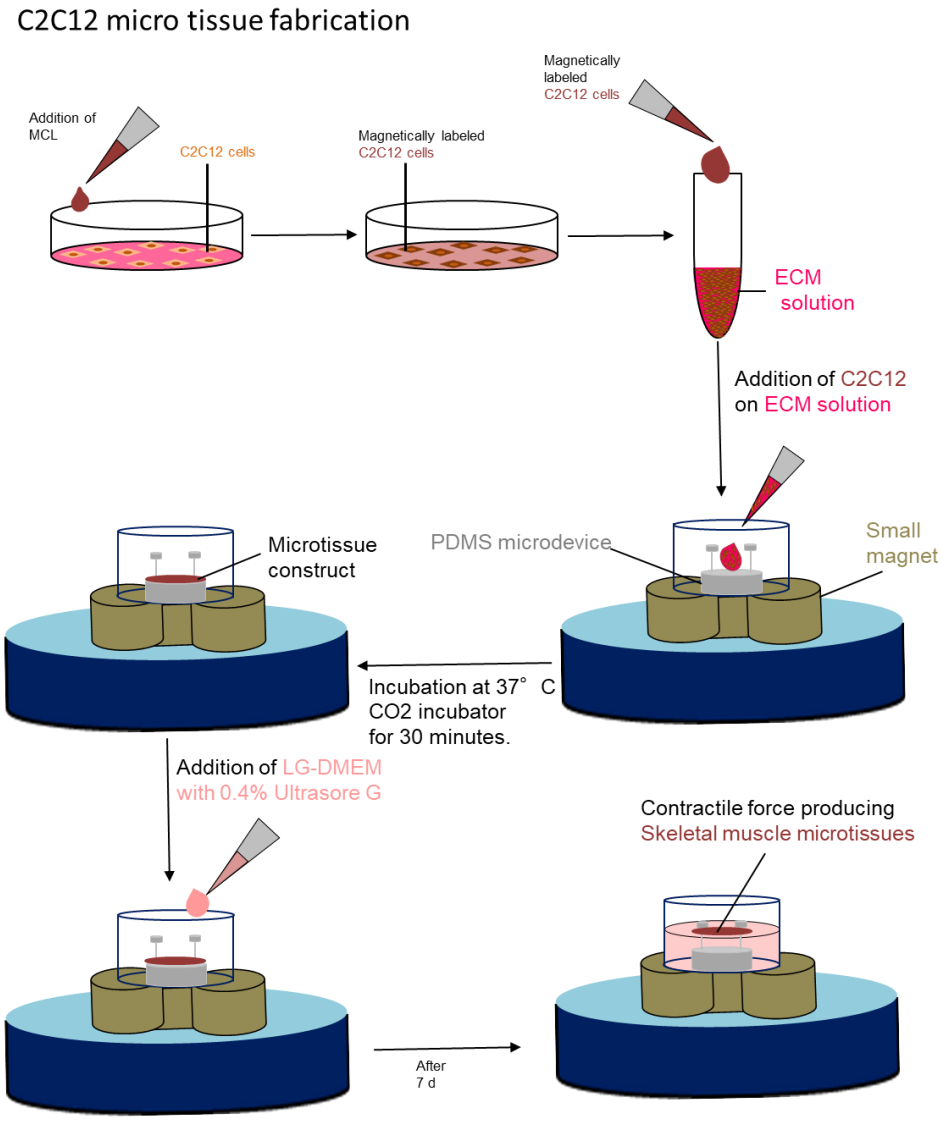

Fig. 4-1. Schematic diagram for the fabrication of 3D artificial skeletal muscle microtissues by using C2C12 cells with help of Mag-TE. Magnetic labeling of C2C12 cells were done by incubating with MCL for 6h. ECM precursor solution was prepared by using collagen solution and matrigel basement matrix. Then C2C12 cells were added to the ECM precursor solution. Then, the mixture was seeded on PDMS microdevices placed on 96-microwell plates and incubated at 37°C on a small magnetic plate in a 5% (v/v) CO_2 incubator for 30 minutes. After 30 minutes of incubation, the cell layer shrank and resulting in the formation of small shaped cellular construct. After that, for myogenic differentiation, the micro tissues were cultured in differentiation medium consisting of LG-DMEM supplemented with 0.4% Ultroser G (Pall) in wells of 96-well culture plate for 7 days. After 7 days, fascicle shaped microtissues were formed.

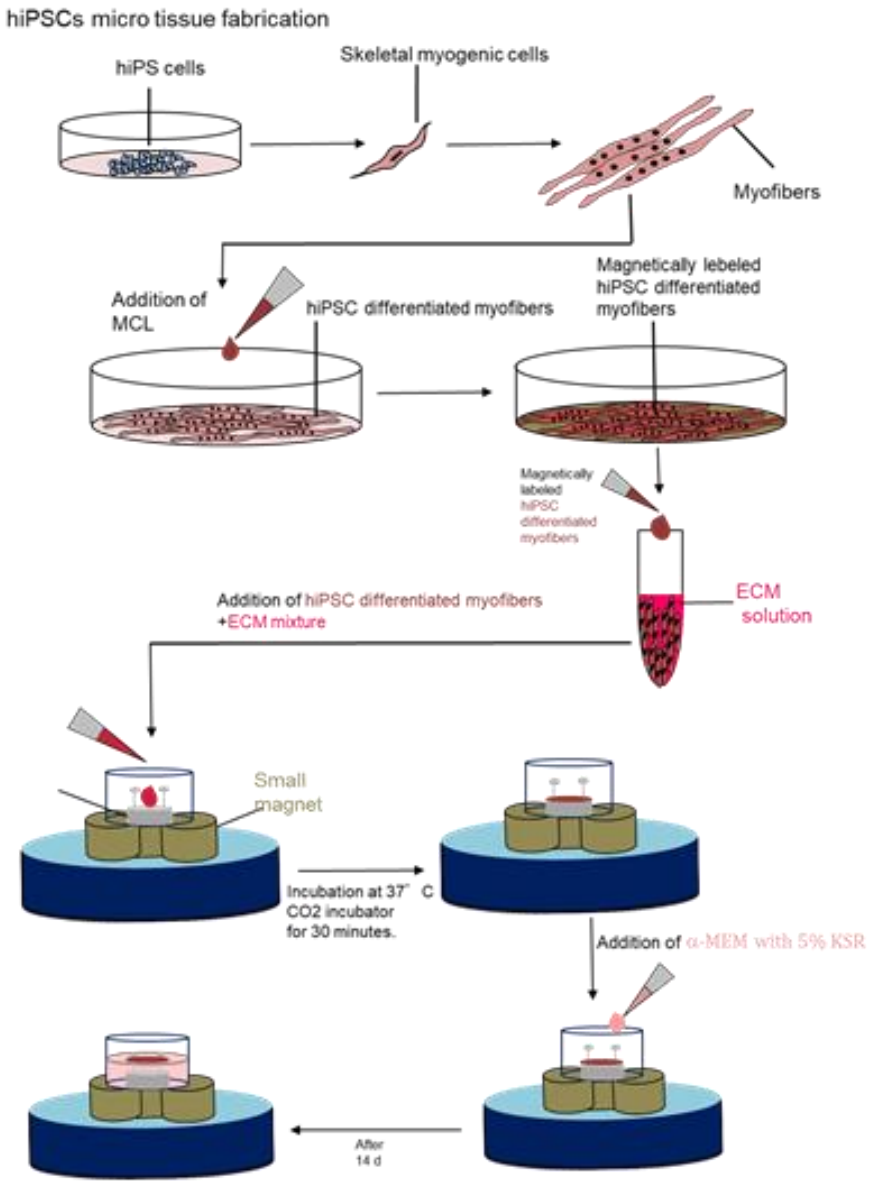

Fig. 4-2. Schematic diagram for the fabrication of 3D artificial skeletal muscle microtissues by using MyoD hiPSCs. After EPS treatment, magnetic labeling of hiPSCs differentiated myofibers were done by incubating with MCL for 6h. ECM precursor solution was prepared by using collagen solution and matrigel basement matrix. Then MCL labeled hiPSCs differentiated myofibers were added to the ECM precursor solution. Then, the mixture was seeded PDMS microdevices placed on 96-microwell plates and incubated at 37°C on a small magnetic plate in a 5% (v/v) CO_2 incubator for 30 minutes. After 30 minutes of incubation, the cell layer shrank and resulting in the formation of small shaped cellular construct. For myogenic differentiation, the micro tissues were cultured in differentiation medium consisting of LG-DMEM supplemented with 0.4% Ultroser G (Pall) in wells of 96-well culture plate for 14 days. After 14 days fascicle shaped microtissues were formed.

Then, the mixture of cell suspension and ECM (both C2C12 and hiPSCs differentiated myofibers) (total 20 μL) mixture was then seeded on PDMS microdevices placed on 96-microwell plates. A small magnetic plate was placed under the 96-microwell plate and was incubated at 37°C in a 5% (v/v) CO_2 incubator for 30 minutes. After 30 minutes of incubation, the cell layer shrank and resulting in the formation of small shaped cellular construct. For myogenic differentiation, the micro tissues fabricated with C2C12 cells were cultured in differentiation medium consisting of LG-DMEM supplemented with 0.4% Ultroser G (Pall) in wells of 96-well culture plate for 7 days. On the other hand, in case of MyoD hiPSCs, after fabrication of microtissues, they were cultured in differentiation medium consisting of α-MEM (Invitrogen) supplemented with 0.4% Ultrasore G (Pall) in wells of 96-well culture plate for 15 days with or without EPS.

To check the effect of drugs on micro tissue constructs, L-carnosine was added at different concentration (0.01 μM, 0.1 μM, and 1 μM) in differentiation medium during myogenic differentiation of C2C12 microtissues (Fig. 4-1 and 4-2).

4.2.6 Measuremnet of contractile force

Contractile forces of the tissues were calculated with the formula below Contractile forces were measured at 7 days after seeding cells on devices. Electrical pulse stimulation (30V; 17A) was loaded on both Mag-TE and non Mag-TE skeletal
muscle tissues on 1Hz by using an electrical pulse stimulation chamber (C-dish). Under electric pulse stimulation, muscle tissues contracted to produce displacement of pillars. Tissues contractions were recorded by microscope (BZ9000 micro, Keyence, Tokyo, Japan), the displacement of each pillar was measured. Contractile forces of the tissues were calculated with the formula below

$$F=\frac{(a-b)}{2}\cdot k \qquad k=3.91$$

4.2.7 Histological study

Immunohistochemical analysis was done to investigate the morphology of the tissues. For immunostaining, tissue constructs were washed with PBS and fixed with 4% paraformaldehyde for 15 min. Tissue constructs were then immersed in PBS containing 0.2% Triton-X 100 for 15 min, washed three times with PBS, and blocked in PBS

containing 1% (w/v) bovine serum albumin for 30 min. The specimens were stained with anti-α-actinin (EA-53, Sigma-Aldrich, St. Louis, MO), anti-MHC (sc20641; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies and 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA). After washing three times in PBS, tissues were observed under a FV10i confocal laser scanning microscope (Olympus, Tokyo, Japan).

4.2.8 Statistical analysis

Statistical comparisons were evaluated by the Mann-Whitney rank sum test, and values of $P < 0.05$ were considered significantly different.

4.3 Result

4.3.1 Optimization of skeletal muscle microtissue fabrication process

To elucidate a perfect condition of skeletal muscle microtissue fabrication, at first the microfabrication method was optimized in three different groups by altering the number of C2C12 and MyoD hiPSCs. Total 14 tissues were fabricated in each three different groups. In all groups, the tissues were shifted to the upper parts of the posts on the first day of growth culture and formed ribbon-shaped tissues by self-organization (Fig. 4-3 A). After day 7, 2 tissues were broken down in group-a). But, all 14 tissues were remained intact in group-(b) and group-(c) (Fig. 4-3 B and C). On the other hand, the width of group-(a) tissues were higher than that of group-(b) and group-(c) (Fig. 4-3 C). Immunohistochemical analysis of micro tissue constructs with anti-α-actinin antibody (myogenic differentiation marker) showed that C2C12 cells were differentiated into myotubes (Fig. 4-4 A). Finally, contractile forces generated by skeletal muscle microtissues of all three different groups were measured and found that, contractile forces of group-a) tissues were significantly higher compared with group-(b) and group-(c) but no significant differences were observed between the contractile forces generated by group-(b) and group-(c) (Fig. 4-4 B). Take a whole consideration, protocol of group-(b) was selected an optimized one for fabricating skeletal muscle microtissues by using Mag-TE by using C2C12 cells.

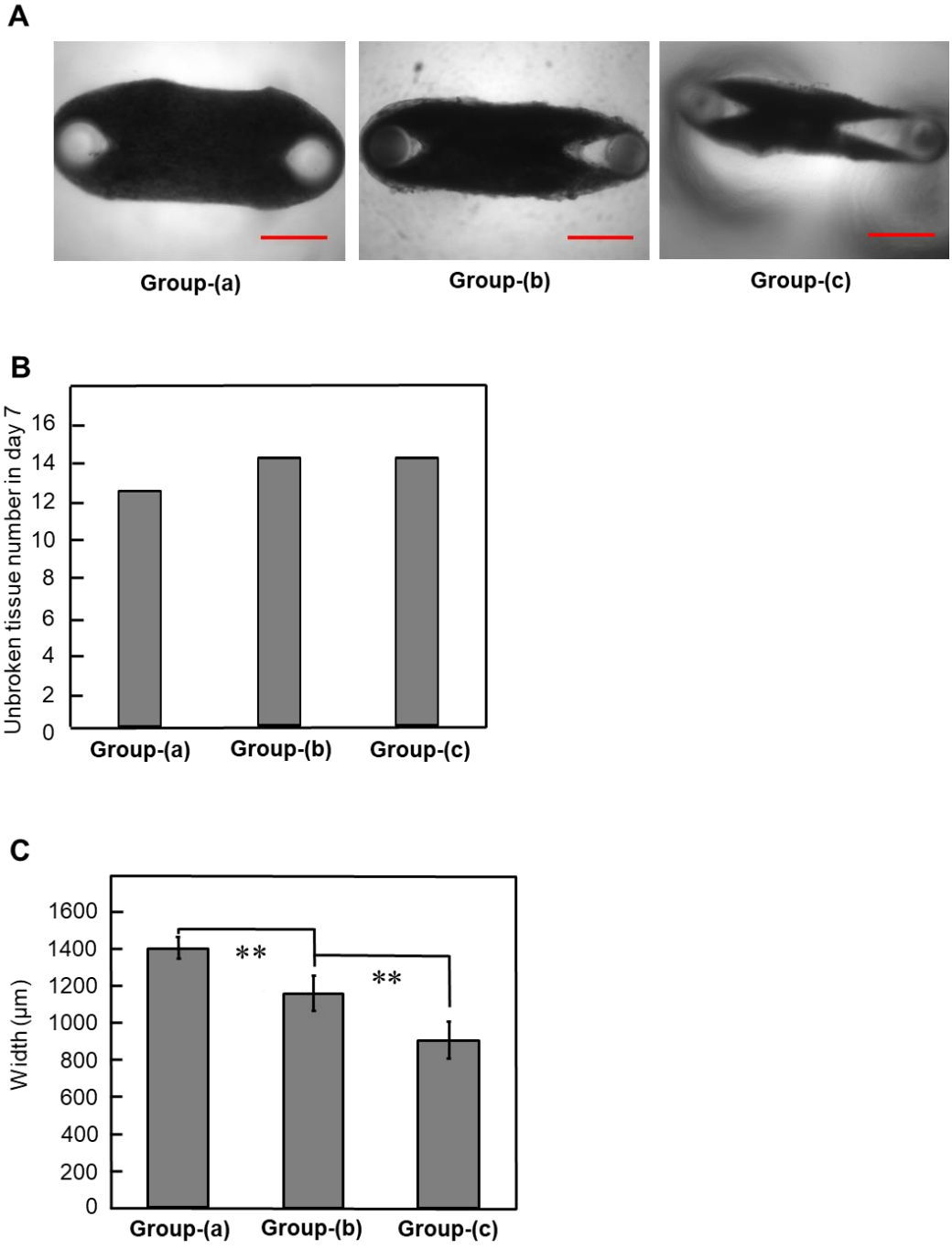

Fig .4-3. Optimization of the microfabrication process of skeletal muscle tissues by using C2C12 cells on the basis of tissue yield and width. **(A)** Phase contrast view of C2C12 cell derived muscle microtissues of three groups. Scale bars, 50 μm. **(B)** Quantitative analysis of the yield of skeletal muscle microtissues of each three groups. **(C)** Quantitative analysis of the widths of microtissues in each three groups. Data are expressed as means ± SD (n = 3).

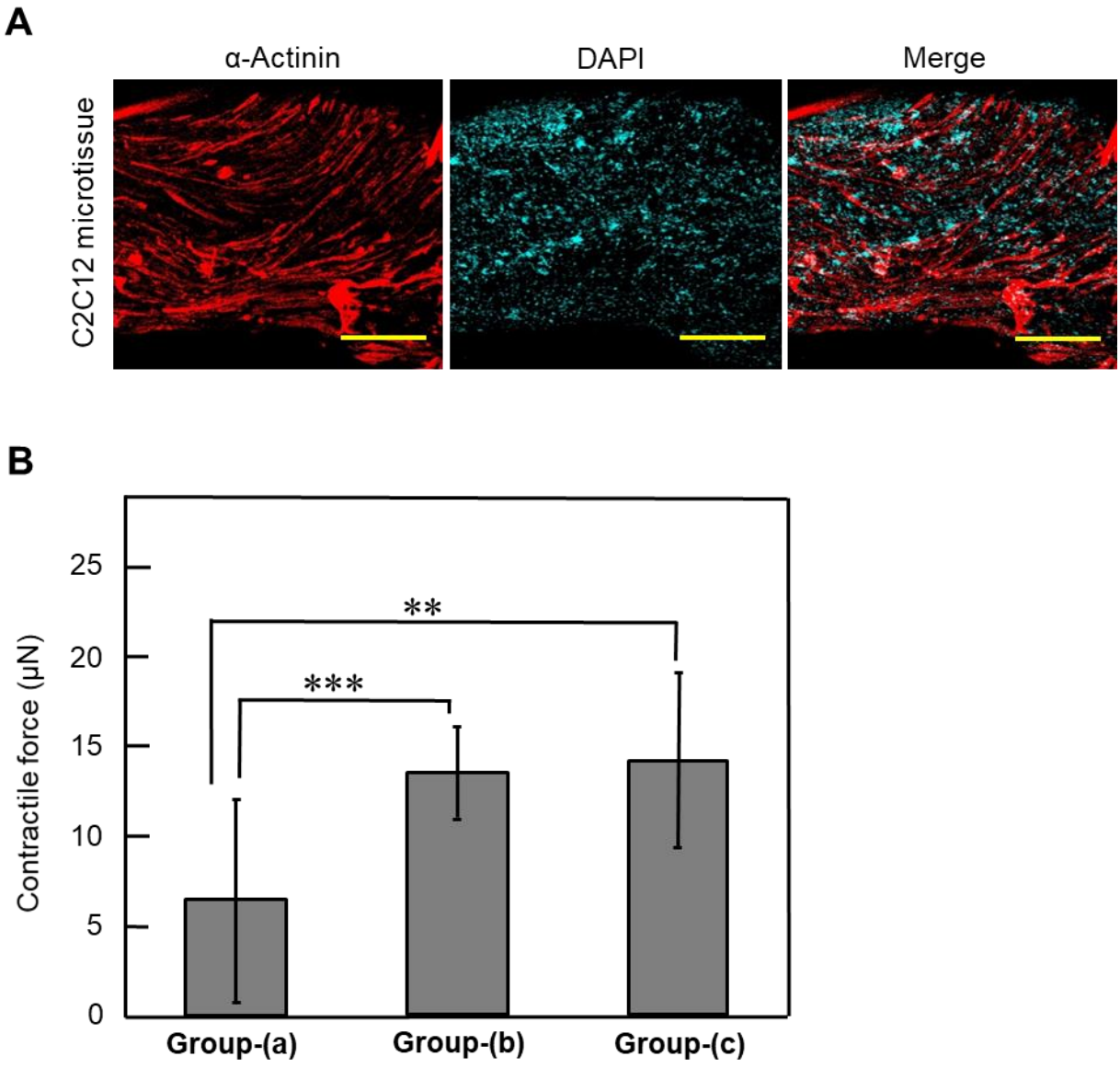

Fig .4-4. Optimization of the microfabrication process of skeletal muscle tissues by using C2C12 cells on the basis of histology and contractile force generation capability. **(A)** Immunohistochemical analysis of skeletal muscle microtissues by using anti-α-actinin antibody. Scale bars, 50 μm. **(B)** Quantitative analysis of the contractile properties of tissue constructs. Data are expressed as means ± SD (n = 3).

4.3.2 Microfabrication of skeletal muscle tissues by Mag-TE

Mag-TE method was applied for the microfabrication of skeletal muscle tissues and a comparison was made between Mag-TE and non-Mag-TE microtissue constructs. MCL-labeled C2C12 cells were mixed with ECM solution were uniformly accumulated at the bottom of the PDMS microposts by applying a magnetic force and a very small sheet like structure contracted to form a ribbon shaped tissue construct between these two PDMS microposts. This small size ribbon shaped tissues coated with ECMs formed fascicle like tissues on day 7 in the differentiation medium. At day 7, the width of non-Mag-TE tissues were significantly higher compared with the width of Mag-TE tissues. Furthermore, tissues fabricated by Mag-TE showed a more particular shape that was alike iron sheets (Fig.4-5 A and C). Interestingly, totally opposite phenomena were observed in case of hiPSC-microtissues (Fig. 4-5 B and D). In that case the width of Mag-TE microtissues are higher compared to non-Mag-TE. Immunohistochemical analysis of both Mag-TE and non-Mag-TE microtissues with anti-α-actinin (myogenic differentiation marker) and anti-MHC (myogenic differentiation marker) antibodies showed that, in both cases C2C12 cells were differentiated into multinucleated myotubes (Fig. 4-6 A and B). Tissues fabricated with magnet generated significantly higher contractile forces compared with the tissues fabricated without magnet (Fig. 4-6 C). Similar result was also observed in case of hiPSC-microtissues (Fig. 4-7 A). On the other hand, no contractile forces were generated by MyoD hiPSCs microtissues fabricated without magnet (Fig. 4-7 B). Also, no contractile forces were generated by MyoD hiPSCs microtissues fabricated with magnet but without the treatment of EPS. Contractile forces are generated only by Mag-TE tissues of group-(b) and (c) treated with EPS. There were no significant differences between the contractile force generation between hiPSCs microtissues of group-(b) and (c) (Fig 4-7 B).

4.3.3 Effect of L-carnosine on functionality and morphology of skeletal muscle microtissues

We fabricated 3D skeletal muscle microtissue constructs of C2C12 cells by using Mag-TE technique to investigate the effects of L-carnosine (0.01 μM, 0.1 μM and 1 μM) on contractile force generation and morphology of skeletal muscle microtissues. It was found that, L-carnosine improved the width of microtissues compared to control (Fig. 4-8 A and B). On the other hand, myogenic hypertrophy of tissue constructs was also

significantly improved due to the addition of L-carnosine (Fig. 4-8 C and D). Interestingly, contractile forces generated of skeletal muscle microtissues were improved slightly but not significantly due to the addition of L-carnosine (Fig. 4-7).

4.4 Discussion

Engineered skeletal muscle microtissues with contractile force generation capability can be a useful asset in case of *in vitro* drug screening against different muscular disorders. Previously, skeletal muscle microtissues were fabricated by using primary mouse myoblasts [31, 32]. Generally, primary cells including autologous and patient derived cells are considered to be a first choice of regenerative medicine, but it includes some drawbacks with biological studies and also for drug screening such as batch –to-batch variation of cell-type ratio [42]. Furthermore, recent invention of hiPS cells has made it possible to develop *in vitro* patient specific 3D microenvironment which have already been proved very fruitful for drug screening. So, artificial skeletal muscle tissue constructs fabricated by using hiPSC-differentiated myogenic cells can be an impressive tool for drug screening and also to understand muscle biology.

In the present study, microtissue fabrication process was optimized by altering the number of C2C12 myoblasts and investigated their influence on tissue width and contractile force generation capacity. Since it has been well known that higher ratio of cell number to ECM volume could result in better functionality of skeletal muscle tissue, we expected to seek a composition with high ratio of cell number to ECM volume. Conversely, ECM was essential to maintain an intact structure without rapture resulted from the whole construction shrinking during cells fusing into myotubes. The higher ratio of cell number to ECM could lead to thinner tissue width. In this study in case of C2C12 myoblasts, the yield of skeletal muscle tissues of group-(a) (0.75×10^5cells/well) and group –(b) (1.5×10^5cells/well) were higher compared to group-(c) (3×10^5cells/well). Interestingly, the width of tissues in each group showed an obvious difference as I expected. Group –(a) to (c) resulted in tissue width from high to low orderly.

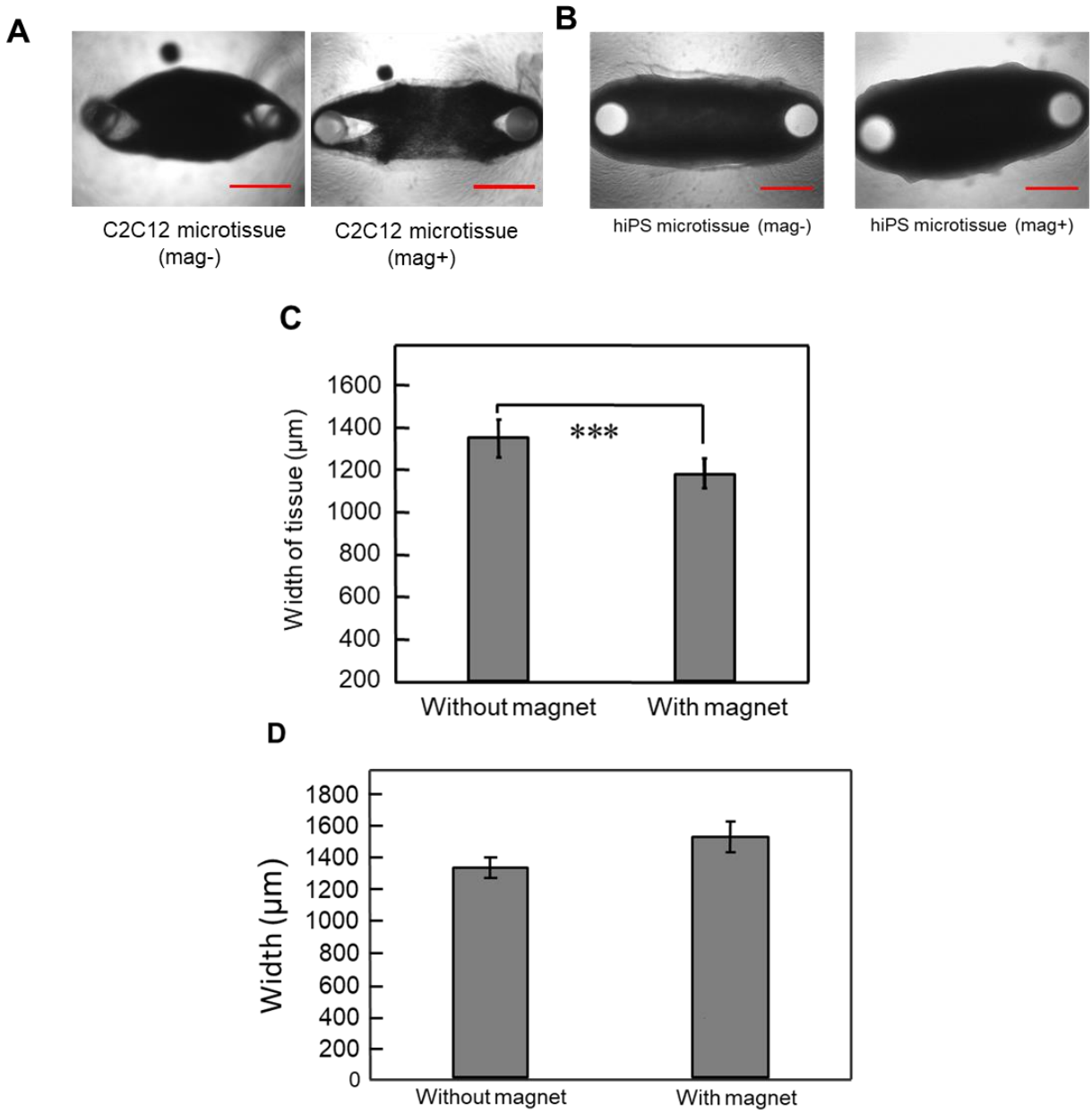

Fig .4-5. Microfabrication of skeletal muscle microtissues by using C2C12 cells and hiPSCs differentiated myofibers fabricated with or without magnets **(A)**Phase contrast images of C2C12 microtissues fabricated with or without magnets. Scale bars, 50 μm. **(B)** Phase contrast images of hiPSCs microtissues fabricated with or without magnets. Scale bars, 50 μm. **(C)** Quantitative analysis of width of C2C12 microtissues fabricated with or without magnet. Data are expressed as means ± SD (n = 3). **(D)** Quantitative analysis of hiPSC of the widths of hiPSC microtissues fabricated with or without magnets. Data are expressed as means ± SD (n = 3).

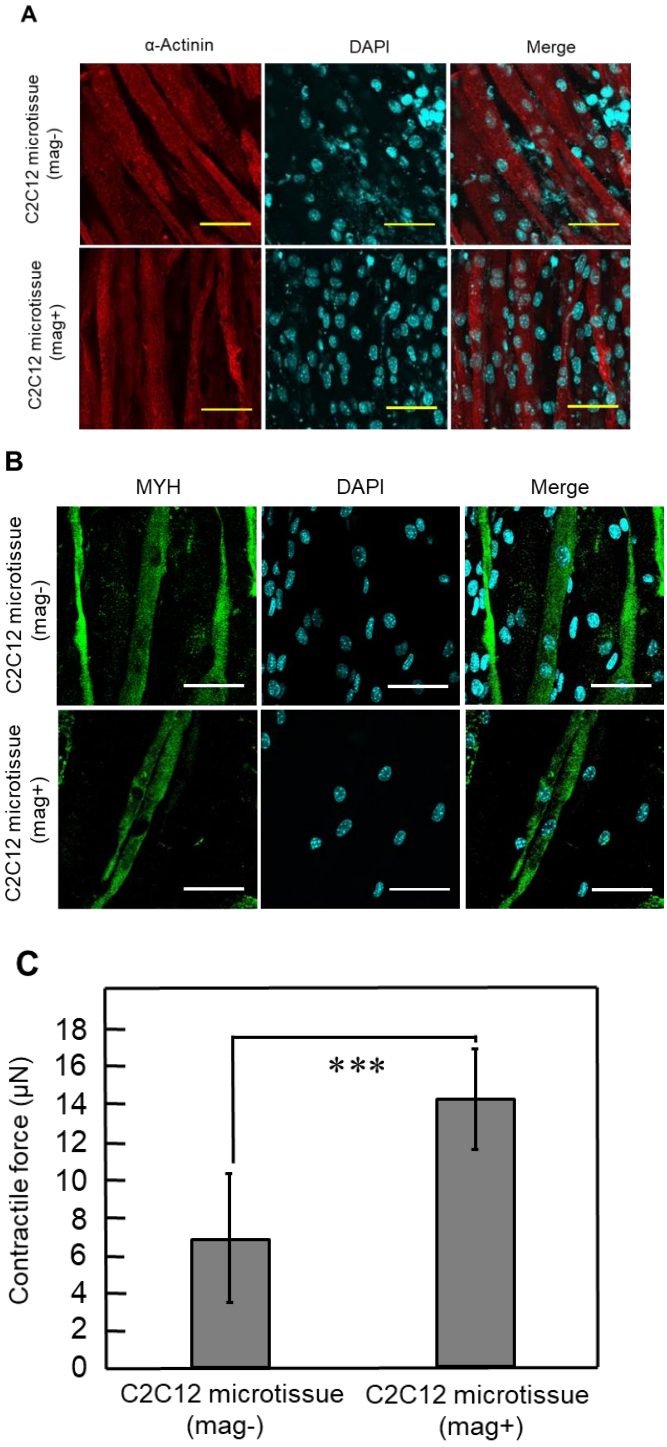

Fig .4-6. Histological and contractile properties of C2C12 microtissues fabricated with or without magnets **(A)** Immunohistochemical analysis of C2C12 microtissues fabricated with or without magnets by using anti-α-actinin antibody. Scale bars, 50 μm. **(B)** Immunocytochemical analysis of C2C12 microtissues fabricated with or without magnets by using anti-MYH antibody. Scale bars, 50 μm. **(C)** Quantitative analysis of the contractile forces generated by C2C12 microtissues fabricated with or without magnets. Data are expressed as means ± SD (n = 3).

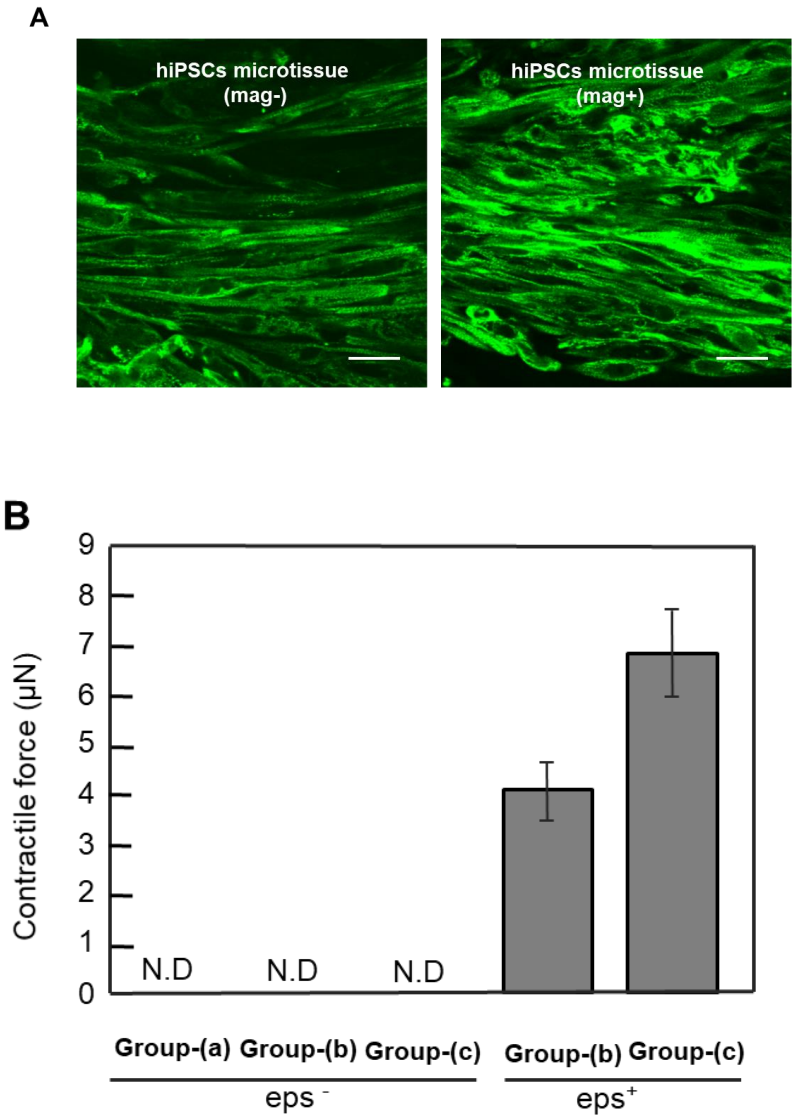

Fig. 4-7. Histological and contractile properties of hiPSC microtissues. **(A)** Immunohistochemical analysis of hiPSC microtissues fabricated with or without magnets by using anti-α-actinin antibody. Scale bars, 50 μm. **(B)** Quantitative analysis of the contractile force generated by hiPSC microtissues fabricated with or without EPS. Data are expressed as means ± SD (n = 3).

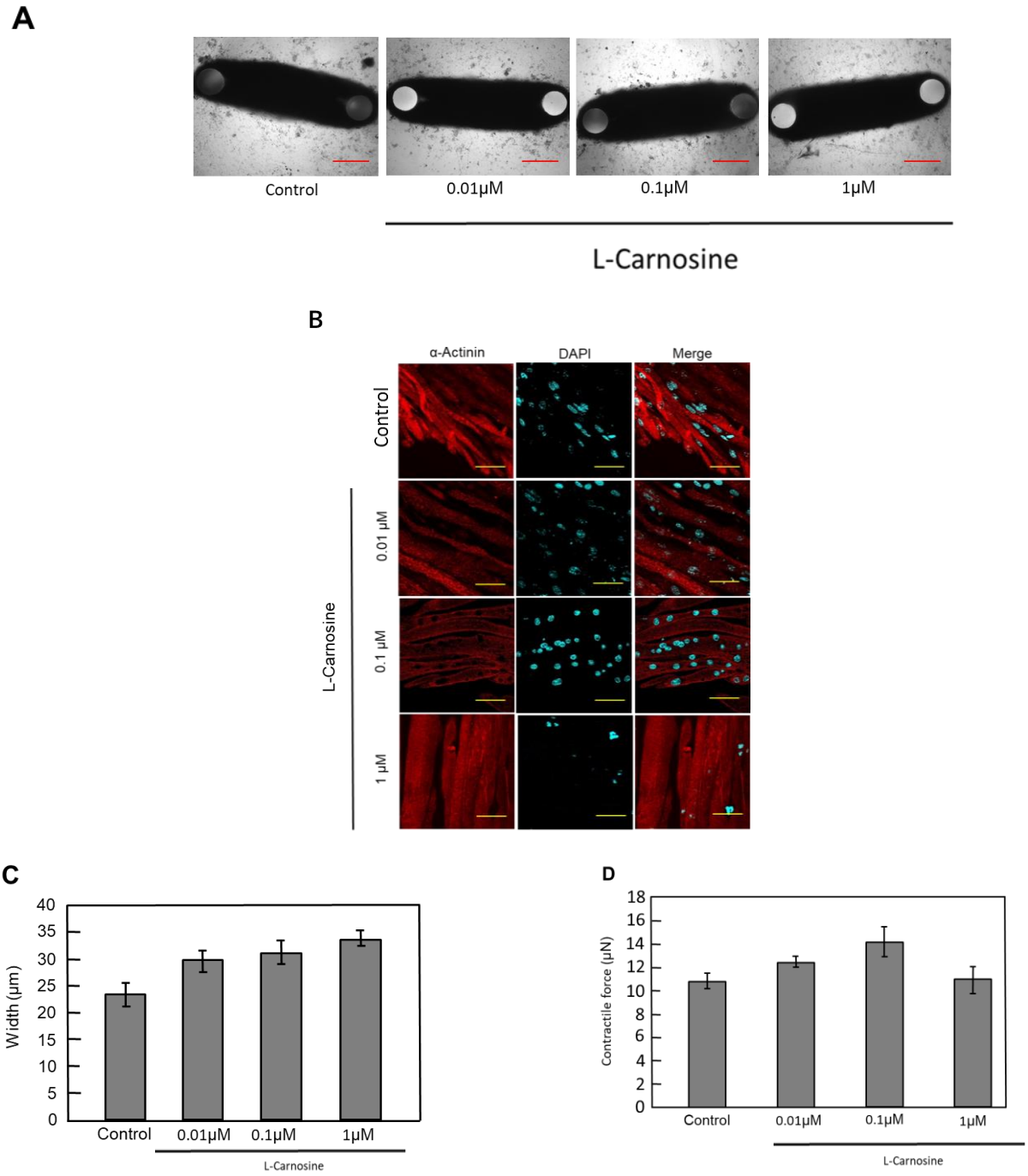

Fig. 4-8. Effect of L-carnosine on morphological and contractile properties of C2C12 microtissues. (A) Phase contrast images of C2C12 microtissues with or without treatment of L-carnosine. Scale bars, 50 μm. **(B)** Immunohistochemical analysis of C2C12 microtissues with or without treatment of L-carnosine by using anti-α-actinin antibody. **(C)** Quantitative analysis of the myogenic hypertrophy of tissue constructs with or without treatment of L-carnosine. Scale bars, 50μm. **(D)** Quantitative analysis of the contractile force generated by tissue constructs with or without treatment of L-carnosine. Data are expressed as means ± SD (n = 3).

On the other hand, the contractile forces of tissues in group-(a) were significantly weaker compared to group-(b) and (c). No significant difference was observed between group-(b) and (c). Moreover, immunohistochemical analysis of group-(b) tissue constructs showed highly aligned and oriented myofibers. Finally, group-(b) (1.5×10^5cells/well) was selected as optimized one. Previously, Vandenbourgh *et al*. fabricated skeletal muscle microtissues by using 2×10^5/well primary mouse myoblasts [31]. Also, Agarwal *et al*. developed skeletal muscle-on-a chip by using 1.25×10^6 C2C12 cells per ml.

Previously in our laboratory, we fabricated functional and mature skeletal muscle tissue constructs with the help of Mag-TE [38, 39]. In this study, we applied Mag-TE technique for further improvement of the functionality of skeletal muscle microtissues. It has been supposed that magnetic force could concentrate cells in a higher density and contribute to contractile force furtherly. Based on the hypothesis, the tissue width would be higher and the contractile forces would be weaker in the group without magnet. It was found that the width of tissues fabricated without magnet were significantly wider compared with tissues fabricated with magnet. Moreover, Immunohistochemical analysis also showed the highly aligned myofibers with strong expression of α-actinin and, MHC. It is known that, morphological analysis using myogenic differentiation markers, including MHC and α-actinin, is insufficient for evaluating the function of skeletal muscle tissues. This is because the level of myogenic differentiation rate is not always consistent with that of contractile force generation of 3D tissue-engineered skeletal muscle constructs [42]. Contractile force generation capability should be the most important feature of tissue-engineered skeletal muscle constructs. This is because it reflects not only the level of differentiation—including the expression of muscle regulatory factors, but also the niche—including the ECM, as well as the architecture. In our study, tissues in group with magnet showed significantly stronger contractile forces compared with tissues fabricated without magnet. This followed our hypothesis that magnetic force led to concentrated cell densities and furtherly enhanced functionality of muscle tissues. On the other hand, in case of skeletal muscle microtissue fabrication by using hiPSCs differentiated myofibers, we found that, microtissues fabricated with Mag-TE generated stronger contractile forces were no contractile forces were generated by microtissues fabricated without magnet. Also, no

contractile forces were generated by hiPSCs microtissues without EPS treatment. It is known that EPS mimics the electrical cues in the *in vivo* niche, allowing the creation of functional skeletal muscle tissues *in vitro* [42]. This result has proved again that, to fabricate functional skeletal muscle tissues in vitro, recapitulation of the in vivo niche, including physical stimuli, is crucial. Thus, all of these results further demonstrates that the contractile maturity of skeletal muscle microtissues fabricated with magnet which is an indispensable indicator for drug screening against different muscular disorders. EPS mimics the electrical cues in the *in vivo* niche, allowing the creation of functional skeletal muscle tissues *in vitro*.

One of the potential application of skeletal muscle microtissues is to use it as a candidate for drug screening. In this study, the effect of L-carnosine at different concentration on the morphology and functionality of microtissue constructs were checked. Carnosine is a naturally occurring intramuscular dipeptide which is involved to attenuate fatigue during high intensity exercise. Previously, it was investigated that, enhancing carnosine level has positive effects on contractile apparatus of skeletal muscle tissue **[43, 44].** We found that, skeletal muscle microtissues fabricated with the treatment of L-carnosine are wider compared with tissues fabricated without the treatment of L-carnosine. Similarly, immunohistochemical analysis showed the improved myogenic hypertrophy of microtissues fabricated with the treatment of L-carnosine. Finally, it was found that, contractile forces produced by microtissues fabricated with the treatment of L-carnosine are stronger compared with its non-treated counterpart. These results indicate that, L-carnosine treatment is essential for attaining morphological and contractile maturity of *in vitro* fabricated artificial muscle tissue constructs. Also, this assessment has established this microfabrication system based on Mag-TE as an excellent drug screening platform.

4.5 Conclusion

In this research, a novel method for fabricating skeletal muscle tissues based on Mag-TE has been developed. The micro size of PDMS devices provide a high efficiency of fabricating tissues because the devices can be put inside wells of a 96 well plate. Meanwhile, functionality of tissues was promoted by applying magnetic force for concentrating magnetically labeled myoblasts into a high density during formation in differentiation medium. Considerable contractile force generation was observed for the tissues.

Microfabrication of functional skeletal muscle tissues using a Mag-TE technique developed in this research have also shown a promising approach of drug screening. By applying this technique, it is possible to develop high-throughput drug screening system with the help of robotic technology and large number of tissues can be fabricated to screen different drug candidates at a same time.

4.6 References

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Chapter 5

Conclusion & future perspectives

5.1 Summary of the thesis

The aim of skeletal muscle tissue engineering is to engineer artificial skeletal muscle tissue construct by mimicking the structure and function of native skeletal muscle tissue *in vitro* and *in vivo* to make it effective for using as an implantable therapeutic device [1]. This field is becoming wider day by day. Moreover, this discipline is more challenging than other branches of tissue engineering and has huge amount of clinical applications [2]. Most of the SMTE constructs that are currently being developed rely on small mammals as animal models to study their effects on muscle repair are available and provide comparable result between different laboratories also suffer from sustainable limitations in the translation clinic. In the translation to the clinic, TE devices are now facing some new challenges due to their being artificial constructs made of synthetic and biologic material, the latter being of autologous, syngeneic or even xenogeneic origin [3]. The only regenerative medicine devices to have reached clinical application are cellular compounds derived from animal ECM used *in loco* to improve the motility and strength of skeletal muscles subjected to traumatic injury or cell therapy strategies that are injected locally and systematically to improve syndromes stemming from genetic diseases [4].

Previously, skeletal muscle tissue constructs were successfully engineered by Mag-TE which are capable of generating contractile force. However, our previous studies were limited only for fabricating homotypic tissue constructs. But, in living system, the skeletal muscle tissue composed of neuronal and vascular network. Among them, neuronal networks inside skeletal muscle tissues are involved in muscle contraction process. So, to mimic natural skeletal muscle tissue and also to improve contractile force generation capability, we fabricated innervated skeletal muscle tissue constructs in this study. On the other hand, microfabricated artificial skeletal muscle tissue constructs are being used now for drug screening against different types of muscular

diseases. So, we tried to develop a high-throughput drug screening system by fabricating skeletal muscle microtissues by using Mag-TE.

In chapter 1, introduction which gives us informations about tissue engineering and regenerative medicine, heterotypic cellular communication, microfabrication technique and drug screening method with engineered tissues. And these research statuses reflexed breakthroughs of the study in this thesis.

In chapter 2, background which discusses about SMTE, nerve-muscle coculture and neuromuscular junction, microfabrication of skeletal muscle tissue constructs and its application for drug screening. This part of the thesis gives informations about the research themes of this thesis.

In chapter 3, fabrication of muscle-neuron constructs by using C2C12/PC12 coculture system for enhancement of contractile force generation. In this study, 2D C2C12/PC12 coculture system was developed and the effect of coculture on morphology and contractile activitiy of myotubes were analyzed. We also investigated the formation of NMJ in coculture system and proved that C2C12/PC12 coculture is beneficial for improving contractile maturity of myotubes. Finally, this system was applied for fabricating 3D muscle-neuron constructs and found that neuronal innervation successfully improved the contractile force generation capability of skeletal muscle tissue constructs. This type of muscle-neuron constructs is very useful as a model of *in vitro* drug screening against different neuromuscular disorders.

In chapter 4, microfabrication of artificial skeletal muscle tissue constructs for developing high-throughput drug screening system. In this study, contractile force producing skeletal muscle microtissues by using C2C12 myoblasts and hiPSCs was developed by using Mag-TE technique. Also, the effect of L-carnosine on contractile force production capability and morphology of C2C12 microtissues were checked and finally found that L-carnosine improved the myotubes hypertrophy of C2C12 microtissues and slightly but not significantly improved the contractile force generation capability of C2C12 microtissues. This, microfabrication system to fabricate skeletal

muscle tissue constructs has a unique application for high-throughput drug screening system against different muscular disorders.

In chapter 5, the thesis study is summarized and prospects of future applications are described.

5.2 Prospects

Engineered skeletal muscle construct is an appealing tool in the field of regenerative medicine. But, it is still in its earliest stages. Establishment of NMJ in these constructs has brought us closer to creating muscle with controlled contractile activity. This represents a powerful tool in regenerative medicine with huge scope for applications in biotechnology. Also, muscle-neuron constructs with improved contractile force generation capability will open a new dimension for designing bioactuator. Furthermore, high-throughput drug screening system based on contractile force generated by microfabricated skeletal muscle tissue constructs also will help us to develop newer therapeutics against different muscular disorders such as DMD. Also, skeletal muscle tissue fabrication by using hiPSCs differentiated myoblasts will help to develop model of respective muscular disorders which will be very beneficial to screen accurate drugs against that disorder. Moreover, this high-throughput technique has made possible to screen different types of drugs together at the same time and also can be an ideal technique for pharmaceutical companies to develop new drugs.

5.3 References

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