Formation and Characterization of

*in vitro* Bioengineered Neuromuscular Junction Models

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ABSTRACT

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_In vitro_ models of the neuromuscular junction (NMJ) are emerging as a valuable tool to study synaptogenesis, synaptic maintenance, and pathogenesis of neurodegenerative diseases. Many models have previously been developed using a variety of cell sources for muscle and motoneurons, but the models can be further improved by integrating beneficial features to better mimic the native milieu of NMJ development. We created a functional _in vitro_ model of NMJ by bioreactor cultivation of C2C12 myoblasts, transdifferentiated myocytes and stem cell-derived motoneurons with electrical stimulation. Proper coculture medium and electrical stimulation led to improved functional coupling between the emerging motoneurons and myocytes, as evidenced by mature cellular structures, increased expression of neuronal and muscular genes, clusterization of acetylcholine receptors (AChRs) in the vicinity of motoneurons, and the response of the coculture to glutamate stimulation. To validate the models and demonstrate utility for pharmacological testing, we analyzed the potency of the drugs that affect key pathways during NMJ signal transduction, including acetylcholine (ACh) synthesis, ACh vesicular storage, ACh synaptic release, AChR activation, and ACh inactivation in the synaptic cleft. The models properly responded to the drugs in a concentration-dependent manner. The proposed _in vitro_ NMJ model could thus be used in pharmacological screening and controlled studies of neuromuscular diseases.
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Everyday.
I Introduction
1 Background

This dissertation amalgamates the results from doctoral research studies attempting to develop, optimize and characterize coculture systems of skeletal muscle and motoneurons for applications in neuromuscular interaction analysis and pharmacological testing. The Introduction will emphasize on the physiology and development of skeletal muscle, motoneurons and neuromuscular junction (NMJ) in order to provide a fundamental understanding of how these structures develop and function in living systems. The NMJ-related diseases and the current advancement of NMJ \textit{in vitro} models will also be discussed to highlight the necessity for more biomimetic culture models in future research.

1.1 Skeletal Muscle

Skeletal muscle consists of highly organized, unbranched, contractile fibers facilitating the voluntary unidirectional contraction of the tissue. Each muscle fiber is a syncytium with nuclei and organelles located in the cell periphery because most of the intracellular space is occupied by the myofibrils. Myofibrils are comprised of elongated repeating contractile units called sarcomeres. Each sarcomere consists of a network of longitudinal filaments arranged in a regular pattern that gives the fiber its striated pattern. Different bands visible in the muscle fiber are due to variations in the refractive index of its myofibrils and the separation of different filament types [1] as shown in Figure 1. The isotropic bands mainly consist of thin filaments of actin that run spirally along a
tropomyosin protein. One end of an actin filament is bound to a z disc and the other end extends into the anisotropic band where they interlock with thick filaments of myosin.

Figure 1 Skeletal muscle structure

Sarcomere shortening, which leads to muscle contraction, is initiated by an increase in cytosolic calcium ions (Ca$^{2+}$), secreted from sarcoplasmic reticulum, upon the arrival of an action potential (AP) at the NMJ and the subsequent depolarization of the postsynaptic muscle membrane. Transverse tubules (T-tubules), which are invaginations of the sarcolemma, run perpendicular to the length of the fiber. These structures allow depolarization of the muscle membrane to penetrate rapidly to the center of the cell. In addition, T-tubules are heavily populated with L-type Ca$^{2+}$ channels to ensure synchronous
activation of sarcomere shortening across all the myofibrils in the muscle. Influx of $\text{Ca}^{2+}$ binds to troponin complex and causes the conformational change of the actin-tropomyosin structure, allowing the myosin heads to bind to active sites on actin filament and pull two $z$ discs closer together. When cytosolic $\text{Ca}^{2+}$ ions are pumped back into the sarcoplasmic reticulum, the actin-tropomyosin structure returns to its original conformation, blocking myosin head binding and therefore relaxing the muscle.

Figure 2 Molecular mechanisms during sarcomere shortening
Regarding to the embryonic development as shown in Figure 3, the mammalian skeletal musculature originally derives from the somites, paraxial mesoderm segments that form on either side of the neural tube [2]. Correct development of skeletal muscle is critically dependent on a family of basic helix-loop-helix transcription factors, Myf5,
MyoD, Mrf4 and myogenin, which are collectively termed the myogenic regulatory factors (MRFs), as well as the paired box (Pax) transcription factors Pax 3 and Pax 7 [2-4]. Initially, delaminating cells from the edges of the dermomyotome, the Pax 3/7 positive dorsal region of the somite, downregulate Pax 3, becoming myoblasts through Myf5 and Mrf4 activation [5]. Mrf4, MyoD and myogenin activity then promote differentiation of myoblasts into myocytes, which subsequently fuse into multinucleated myotubes. These myotubes mature into muscle fibers, forming a continuous layer within the embryo called myotome. Once formed, the myotome is post-mitotic since the muscle fibers present within it are multinuclear [5]. Further growth of the skeletal muscle tissue is thus facilitated by Pax 3/7 positive cells migrating out from the center of the dermomyotome as it loses its epithelial structure during somite maturation [6]. A substantial subpopulation of these proliferating cells gives rise to myogenic cells positive for Myf5 and MyoD, which are then capable of fusing with existing fibers as well as with each other, creating new fibers, and therefore facilitating further growth of the skeletal musculature [2, 5, 6].

1.2 Motoneuron

Motoneuron is characterized by the presence of an axon and dendritic tree. Its cell body is located in the spinal cord with an axon projecting outside the spinal cord to directly or indirectly control effector organs such as muscles and glands [1]. Incoming APs arrive via the dendrites and are propagated to the target tissue via the axon. The axon splits into many branches at its axon terminal, forming synaptic contacts with other cells.

There are two main types of motoneurons: autonomic and somatic motoneurons. Autonomic motoneuron innervates smooth muscle or glands only by the intermediary of a
postganglionic motoneuron located in an autonomic ganglion whereas somatic motoneuron directly synapses with striated muscle fibers by motor endplates. Therefore, only somatic motoneurons play a significant role in NMJ function. Somatic motoneurons are subdivided into alpha (α)-, beta (β)- and gamma (ϒ)-motoneurons. Individual muscles are composed of a mixture of fiber types, which can be grouped into two major categories: extrafusal and intrafusal. Extrafusal fibers are primarily responsible for skeletal movement whereas intrafusal fibers modulate the sensitivity of muscle to stretch [7]. α-motoneurons are the most abundant of these subtypes and they innervate the extrafusal muscle fibers responsible for triggering muscle contraction [8]. ϒ-motoneurons involved in motor control via the intrafusal fiber innervation whereas β-motoneurons innervate both fiber types and have a poorly understood role in regulating muscle contraction [8]. The collection of motoneurons responsible for innervating an individual muscle is termed a

Figure 4 Types of motoneurons
motor pool. The motor pools are collectively located within the ventral horn of the spinal cord, arranged topologically to match the muscular layout of the organism [8].

Developmentally, motoneurons are derived from the neuroepithelium and their embryonic differentiation is controlled by the notochord via Sonic hedgehog (Shh) activity [9]. Expression patterns of LIM homeodomain class transcription factors are believed to govern the developing motoneuron subtype identity which in turn dictates axonal trajectory from the neural tube. Axonal growth is controlled by a complex interplay of chemo-attractants and repellents and allows for the targeted movement of the developing growth cone towards its specific muscle group [10]. Motoneurons facilitate the contraction of skeletal muscle by conveying efferent signals from the central nervous system to the NMJ. Chemical transmission across this synapse triggers contraction of the muscle fiber and is discussed in more detail in the next section.

1.3 Neuromuscular Junction

NMJ is the synaptic connection between motoneurons and skeletal muscle. Motoneurons reside in the motor columns of the ventral horn and each individual muscle fiber is innervated by the terminal branch of a single motoneuron. Initially, however, each muscle fiber develops synapses with multiple motoneuron axons. The elimination process occurs during the neonatal period, leaving each fiber innervated by a single nerve [11]. The elimination of poly-neuronal innervation is achieved in an activity-dependent and competitive manner and is believed to involve the activation of protein kinase C (PKC), mediated by the serine protease thrombin [12].
During development, NMJ forms in a series of elaborate signal interactions between axon terminals of motoneurons and excitable plasma membrane of muscle, requiring intricate communications of cytokines, growth factors and cellular structures [13, 14]. The molecular mechanisms controlling NMJ formation are not completely characterized, but the process is believed to involve the activity of the muscle membrane bound receptor tyrosine kinase muscle-specific kinase (MuSK) [15, 16]. The MuSK gene promoter contains a sequence targeted by myogenin, thereby ensuring its upregulation during myoblast differentiation [17].

Before innervation, MuSK initially binds to Wnt signalling proteins, which play a role in early AChR clustering [18, 19]. Wnts, secreted by the neuron growth cone, bind to MuSK and are thought to promote the activation of the cytoplasmic protein Dishevelled (Dvl) [20]. Dvl acts as a bridge molecule between MuSK and the p21 kinase PAK, allowing MuSK to regulate actin dynamics and thereby promote cytoskeletal changes to bring about AChR clustering [18]. These early clustering events do not dictate the location of NMJ formation because incoming spinal motoneuron axons tend to ignore most preexisting AChR clusters, instead forming synapses at new locations [21]. These primitive, Wnt-MuSK mediated, aneural AChR clusters form around the central region of the fiber and, while not the exact target of the developing neurite, are believed to play a role in axon guidance, ensuring the growth cone is directed to the center of the muscle fiber. Eventually, however, aneural AChR clusters have completely disappeared and all remaining AChR clusters are apposed by nerve terminals [22].

Important molecular pathways linked to NMJ stabilization and formation include the agrin–muscle-specific kinase (MuSK), neuregulin (NGR-1)-erythroblastosis oncogene
B (ErbB), and ACh-AChR. Agrin, a polypeptide secreted by the motor axon terminal, binds to a preformed MuSK–lipoprotein receptor-related protein 4 (Lrp4) complex to promote MuSK transphosphorylation and cluster AChRs through the cytoplasmic linker protein rapsyn [17, 23-25]. It is an important inducer and a stabilizer of AChR clustering in the postsynaptic membrane [26, 27]. NGR-1, released by motoneurons, activates ErbB in the muscle membrane to potentiate AChR transcription in synaptic [14]. Both pathways comprise positive signals for NMJ assembly induction. In addition, ACh plays an important role in NMJ formation and stabilization. As motoneuron terminal makes synaptic connection with the plasma membrane of skeletal muscle, ACh released by the motoneuron induces a postsynaptic depolarizing potential, stabilizing previous AChR
clusters in the synaptic area and preventing AChR aggregation in extra-synaptic regions. Global AChR transcription is suppressed by ACh through myogenin inhibition [28, 29].

Figure 6 Important components of NMJ and neurotransmission process

Neuromuscular transmission is triggered when an AP propagates to an axon terminal, causing an influx of Ca^{2+} through voltage-gated calcium channels and the release of an excitatory neurotransmitter acetylcholine (ACh) from synaptic vesicles. ACh diffuses though synaptic cleft and activates nicotinic acetylcholine receptors (AChRs) on the postsynaptic plasma membrane of skeletal muscle, allowing the influx of sodium ions (Na^{+}) and the outflow of potassium ions (K^{+}). This leads to miniature endplate potentials and greater depolarization of the muscle plasma membrane [1]. When the depolarization reaches a specific threshold, AP will be initiated. Propagation of the APs along the muscle membrane and through the T-tubules triggers the release of Ca^{2+} from the sarcoplasmic reticulum into the cytosol, leading to sarcomeric shortening as discussed previously.
1.4 NMJ-Related Diseases

NMJ formation and stabilization are tightly controlled via intricate molecular pathways. Mutation in the genes that participate in neurotransmission activities can lead to several neurodegenerative disorders that selectively affect motoneurons and skeletal muscle. Examples of NMJ-related diseases are spinal muscular atrophy (SMA), amyotrophic lateral sclerosis (ALS), hereditary spastic paraplegia (HSP), progressive bulbar palsy (PBP) and myasthenia gravis (MG). Common symptoms for patients with motoneuron diseases are compromised control of voluntary muscles of the body, muscle weakness and muscle wasting.

In particular, SMA is an autosomal recessive neuromuscular disorder characterized by deterioration of lower motoneurons causing progressive muscle atrophy. The disease typically manifests early in life and it is the leading genetic cause of death in infants. Approximately 1 in 6,000 to 1 in 10,000 infants are affected with SMA, and about 1 in 40 people are genetic carriers of the SMA gene [30]. In its acute form, SMA is fatal before the age of two [31]. SMA has historically been considered a pure motoneuron disorder and majority of researchers still believe that motoneurons play a critical role in the development of SMA. For example, a study showed that anti-smn1 miRNA expression in motoneurons, but not in muscle cells, was sufficient to cause SMA pathologic hallmarks, including defective motoneuron development, reduced motor function and early death [32]. Other studies have shown nerve and muscle specific events in SMA, including atrophy of muscle fibers and accumulation of neurofilaments at NMJ [33], loss of motoneuron cell bodies [34] and denervation of NMJ caused by loss of presynaptic inputs [35]. But there are emerging numbers of studies showing the potential role of muscle in the pathogenesis
of SMA. For instance, an experiment using coculture of rat motoneurons and human SMA myoblasts pointed to an active role of muscle in the degeneration [36]. Clearly, NMJ model provides myriad insight and useful information for SMA research and in vitro models can be used to further elucidate the potential roles of motoneurons and muscle in the progress of SMA. For instance, stem cell or iPSC techniques allow the derivation of skeletal muscle or motoneurons from diseased/healthy patients [37-42]. The coculture of diseased motoneurons with healthy muscle cells or vice versa allows the study of relative roles of each cell component on progression of SMA.

Currently, there are many treatments under investigation to treat SMA. For instance, gene therapy focuses on SMN1 gene replacement to restore its function by inserting nucleotide sequence of SMN1 transgene into target motoneurons, which could potentially lead to increased production of SMN protein required to alleviate SMA progression [43, 44]. SMN2 alternative splicing modulation is also currently explored to increase the likelihood of full-length SMN protein synthesis from SMN2 gene, which normally only leads to short SMN protein [45]. In addition, some treatment targets muscle function restoration instead of motoneurons in hope to counter the effect of SMA such as fast skeletal muscle troponin activator (FSTA) to potentially slow the rate of calcium release from the regulatory troponin complex of fast skeletal muscle fibers and may improve muscle function and physical performance [38, 46, 47].

Currently, there is one approved treatment for SMA. Nusinersen is the first approved drug to treat patients with SMA and it was approved by the U.S. Food and Drug Administration in late December 2016 and by the European Medicines Agency in May 2017 after initial clinical trials showing that nusinersen was safe, well tolerated and
Nusinersen is an antisense oligonucleotide intended to treat SMA caused by chromosome 5q mutations which lead to deficiency in SMN protein synthesis [48]. It modulates alternate splicing of the SMN2 gene by selectively binding to mRNAs carrying information needed for SMN protein synthesis and has the potential to enhance the amount of functional SMN protein in infants and children with SMA [49-51]. Other neuromuscular disorders such as ALS and Duchene muscular dystrophy are also being extensively investigated [52-54]. With advancement in cell and molecular biology, gene therapy and increased awareness of the devastating neurodegenerative diseases, substantial resources has been put to the research of SMA and NMJ-related diseases, steering the development of the treatment, or even cure, in an encouraging direction.

1.5 Current Effort and Applications of NMJ in vitro Models

NMJ is widely used as a model to investigate synaptogenesis and pathophysiological development of neurodegenerative diseases [55-58] because of its relatively large size and accessibility compared to central synapses [11, 59]. The ability for dissociated primary muscle cells and motoneurons to generate functional neuromuscular transmission in vitro was first reported using chick cells in 1970 [60]. In such cultures, electrical stimulation led to action potentials recorded in the neuron which were followed by a brief depolarizing potential in the associated myotube membrane [61]. Early work on neuromuscular interaction in vitro focused on the chick and Xenopus model systems due to the ease and rapidity associated with using these cell. Studies utilizing rodent cells were later developed and seen as advantageous [62]. First, NMJ development in vivo has been studied in more detail in the rodent than in any other mammalian species and a greater
number of antibodies and gene expression probes are available for rodents than for avian or amphibian species. Moreover, established techniques for culturing rodent cells allowed for the development of cultures derived from transgenic or knock-out mice, even in cases where the mutation is lethal in late fetal development. Relatively recently, many research groups have created in vitro coculture systems using combinations of cell lines, primary cells, and stem cell-derived cells. For example, NMJ models were established in a serum-free culture of rat primary cells [63], in a coculture of human stem cell-derived motoneurons and rat skeletal muscle [64], in a low-density coculture of mouse stem cell-derived motoneurons and muscle cells [65], and in a coculture of motoneurons and myotubes generated from the same line of human induced pluripotent stem cells [66].

NMJ in vitro models along with embryonic stem cell (ESC), induced pluripotent stem cell (iPSC) and direct reprogramming technology have provided effective approaches to study synaptogenesis and the underlying disease mechanisms. For instance, in vitro models generated from patient-specific tissues with genetic defects allow researchers to investigate the relative roles of muscle and motoneurons in neurodegenerative diseases [57, 67-70]. Gene editing technique also provides the ability to repair underlying gene mutations in patient-specific cells, allowing the possibility of autologous cell transplantations [71, 72]. Therefore, in vitro models can significantly contribute to the analysis of the pathophysiology of neurodegenerative diseases, help screening for therapeutic candidates and closing the gap between research work and clinical applications.
2 Objective

The goal of the project is to develop a stable and functional model of NMJ to understand the synaptogenesis, regeneration, and maintenance of NMJ. Most of the current NMJ models [61, 62, 73, 74] are simply the coculture of muscle fibers and motoneurons. However, due to the complexity of the system and the motility properties of contracting muscle, the development of a practical NMJ model still remains a challenge. Further improvements are necessary to enhance the physiological relevance of the NMJ development system. An ideal NMJ model should provide a biomimetic niche for motoneurons and muscle cells, and enable precise control of the factors of interest such as the application patterns of electrical and mechanical stimuli. The model should also be simple, universal and easy to analyze.

2.1 Hypothesis

We hypothesized that the provision of a biomimetic environment guiding the organized growth of motoneurons and muscle cells will enhance functional coupling of the cells and enable quantitative studies of regulatory factors and underlying mechanisms of NMJ formation. Optimized coculture medium and electrical stimulation will support proper development of motoneurons, muscle fibers and NMJ formation.

2.2 Specific Aims

This dissertation aims to study extracellular signals as a modulator of NMJ development and function, with potential applications of the in vitro NMJ models as a therapeutic screening
platform and NMJ-related disease model. The biomimetic approach will be utilized to create a functional coculture system. Advantageous techniques in tissue engineering will be integrated to the model, including media optimization and electrical stimulation. Before the synergistic effects of these features can be investigated, their individual effects on muscle, motoneurons and NMJ should be well understood first. The study used mouse cells to create a relatively simple system.

2.2.1 Optimize culture medium and protocol to support proper development of skeletal muscle, motoneurons and NMJ

The cellular niche consists of the cell in coordination with the surrounding cells, the extracellular matrix, soluble factors, and biophysical signals [75]. Extracellular cues within this niche are incredibly important for promoting NMJ formation and stabilizing synaptic connection. Culture medium is a pivotal component for proper development of skeletal muscle, motoneurons and NMJ. Specifically, the culture medium must be able to support the growth of myocytes and maturation of motoneurons, induce multinucleated myotube formation, and enhance functional coupling of NMJ. We hypothesized that culture medium supplemented with growth factors and cytokines based on the distribution of their cognate receptors during muscle, motoneurons and NMJ development should lead to improved growth and functional coupling of NMJ.

2.2.2 Determine the effects of electrical stimulation on skeletal muscle, motoneuron and neuromuscular junction formation

The neuromuscular niche is an environment ideal for synaptic transmission and functional coupling contraction. Because muscle and motoneurons are both electrically excitable and the development is closely linked to their electrical activity, an integration of electrical stimulation to the coculture system of NMJ model should be advantageous. The goal is the
optimization of electrical stimulation parameters that can lead to proper cell development. The optimal electrical stimulation should attain a desired physiological response with minimal damage to the stimulated tissue and it should be designed to mimic the signal existing in nature under the conditions of interest, in this case, nerve signal. There are many variables in the electrical stimulation that can be regulated, for example, amplitude, frequency, stimulation duration, and waveform shape. Initiation and stimulation period also need to be considered.

The efficiency of stimulation regime can be assessed by evaluating the following cell characteristics, including proliferation, maturation, differentiation, and functional integration of NMJ. Specifically, for C2C12, the effects of electrical stimulation on important synaptic proteins such as brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), Neurotrophin-3/4 (NT-3/4), ciliary neurotrophic factor (CNTF), and AChRs are examined. As for motoneurons, the study should focus on expression level of presynaptic factors, such as MuSK and agrin. The well-defined characterization of stimulation effect will provide improved options for directing and fine-tuning functional engineered NMJ model.

2.2.3 Validate the functionality of in vitro models using pharmacological testing

The ultimate objective of the research is to use the established NMJ model in real-world applications such as diagnostic platforms for drug screening, cytotoxicity study and investigation of the pathogenesis of neuromuscular disorders. For the dissertation, the validation and functionality of the established in vitro models will be demonstrated by using drugs that affect mechanisms involved in synaptic transmission. Pharmacological potency of each drug will be quantified by the drug-induced contraction frequency of the coculture. The goal is to show that this simple, yet effective, coculture system can be used for pharmacological screening and to enhance the knowledge of pathogenesis of neurodegenerative diseases.
II Optimization of Culture

Protocols for Proper Development

of Skeletal Muscles and

Motoneurons
3 Effects of Different Culture Media on Mouse Myoblasts and Myotube Development

3.1 Introduction

Myoblasts are primordial muscle cells with the potentiality of developing into myocytes and subsequently, myotubes. Cultured myoblasts retain a hypertrophic ability in vitro and are able to fuse, forming multinucleated myotubes capable of spontaneous contractile activity given the correct stimuli [76]. The formation of these multinuclear cells is fundamental to the in vitro study of skeletal muscle. Such cultures allow for investigation of the metabolic and functional activity of muscle fibers and muscle precursor cells without interference from other tissues as normally occurs in vivo. Skeletal muscle is heavily influenced by external stimuli and interactions with other cell types [77-79]. In vitro culture removes such external or systemic cues, therefore allowing a clearer investigation of the specific effect of chemical, physical and pathological challenges on the physiology and metabolism of skeletal muscle cells.

For the simplicity of in vitro models, an immortalized mouse myoblast cell line C2C12 was chosen. C2C12 can capture the essential characteristics of skeletal muscle and has gained utility in the biomedical research for decades. The C2C12 cell line is a subclone of myoblasts established from normal adult C3H mouse leg muscle [80]. These cells are capable of rapid proliferation under high serum condition and they can later fuse to form multinucleated myotubes under low serum conditions or starvation, the precursors to contractile skeletal muscle
cells in the process of myogenesis [81]. Culture medium, hence, has significant effect on C2C12 differentiation. Studies have shown that low level of serum or no serum at all can lead to improved differentiation and some has claimed that serum level has no effect on differentiation [82-85].

The goal of the study is to determine the optimal culture medium that can effectively support the growth and differentiate C2C12 to myotubes. In addition, because C2C12 will later be cultured in the coculture medium (CM) in NMJ experiments, this study will compare the capacity of CM to other differentiation media in supporting proper development of C2C12. Three media will be examined: serum-free CM, differentiation medium (DM) and the combination of the two (Combined). The efficiency of the media will be assessed using the microscopic structure of muscle cells, cell viability in the culture, important gene expressions and protein levels, and functionality of myotubes derived from C2C12.

3.2 Materials and Methods

3.2.1 Cell Culture

![Figure 7 Experimental procedures: effects of different culture media on C2C12 differentiation](image)
C2C12 was plated on a plastic tissue culture plate at the density of 250 cells/mm². The number of cells in solution was calculated using a hemocytometer. 10 µL of the cell suspension was added to 10 µL 0.4% Trypan Blue solution (Sigma-Aldrich) to help distinguish between dead and living cells. 10 µL of the cell/Trypan Blue solution was injected at each end of the hemocytometer to fill the 2 chambers by capillary action. Under a light microscope at 10X magnification, the number of cells present in each chamber was counted. Then the number was adjusted to obtain an estimation of the number of cells per mL, taken into accounting the dilution factor, squares counted and square volume. C2C12 was culture in growth medium (GM) for 2-3 days until reaching about 80% confluent. Before changing to differentiation medium, cells were washed with phosphate buffered saline (PBS) (Thermo Fisher Scientific). There were three study groups using different media to differentiate C2C12 myoblasts into myotubes as shown in Table 1. Cells were culture in differentiation media for 9 days.

<table>
<thead>
<tr>
<th>Growth Medium (GM)</th>
<th>Differentiation Medium (DM)</th>
<th>Coculture Medium (CM)</th>
<th>Combined Medium (Combined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM, high glucose 12.5 mM HEPES 10% FBS 1% P/S</td>
<td>DMEM, high glucose 12.5 mM HEPES 2% horse serum 1% P/S 1X ITS 20 ng/mL IGF-1</td>
<td>Advanced DMEM/F12 Neurobasal medium 1% P/S 1X B-27 1X GlutaMAX 12.5 mM HEPES 100 µM IBMX 10 µM forskolin 10 ng/mL BDNF 10 ng/mL GDNF 10 ng/mL CNTF 20 ng/mL NT-3 20 ng/mL NT-4 2 µg/mL laminin</td>
<td>DMEM, high glucose Advanced DMEM/F12 Neurobasal Medium 1% P/S 2% horse serum 1X ITS 1X B-27 1X GlutaMAX 12.5 mM HEPES 100 µM IBMX 10 µM Forskolin 10 ng/mL IGF-1 10 ng/mL BDNF 10 ng/mL GDNF 10 ng/mL CNTF 20 ng/mL NT-3 20 ng/mL NT-4 2 µg/mL Laminin</td>
</tr>
</tbody>
</table>

Table 1 Culture media for C2C12 differentiation
3.2.2 Cell Viability Test

Culture medium was aspirated and cells were washed with PBS to remove serum esterase activity generally present in serum-supplemented media. Serum esterase could cause an increase in extracellular fluorescence by hydrolyzing calcein AM. LIVE/DEAD™ Viability/Cytotoxicity Kit (Thermo Fisher Scientific) for mammalian cells was used. The assay solution was prepared by adding 20 uL of the supplied EthD-1 stock solution and 5 uL of the supplied calcein AM stock solution to 10 mL of sterile PBS. The resulting solution was vortexed to ensure thorough mixing and added to culture plates. Cells were incubated for 30-40 minutes at room temperature before analysis under fluorescence microscope.

3.2.3 Immunostaining

For acetylcholine receptor (AChR) staining, cells were incubated with $5 \times 10^{-8}$ M of α-bungarotoxin (Life Technologies) for 1.5 hours at 37 C prior to washing and fixing. Cells were then washed with PBS, fixed in PBS containing 4% paraformaldehyde (Sigma-Aldrich) for 15 min, permeabilized using 0.1% Triton-X (Sigma-Aldrich) for 15 min, and blocked with 5% goat serum (Sigma-Aldrich) in PBS for 20 minutes. Cells were stained with primary antibodies for 2-3 hours using monoclonal anti-α-actinin (sarcomeric) antibody (1:400, Sigma-Aldrich). After 3 washes with PBS, cells were stained for one hour with the following secondary antibodies: goat anti-mouse Alexa Fluor 488 (1:500, Life Technologies), anti-GFP antibody (1:500, Abcam), Alexa Fluor 546 phalloidin (1:500, Life Technologies) for actin and 4',6-Diamidino-2-Phenylindole (DAPI) (1:1000, Life Technologies) for nuclear staining.

3.2.4 Quantitative Polymerase Chain Reaction

Culture wells were aspirated, washed twice with PBS and treated with 500 µL TRIzol (Life Technologies). A scraper was used to scrape the surface of the well before transferring the
solution to an RNase-free 1.5 mL Eppendorf tube. Samples were treated with 100 µL chloroform (Sigma-Aldrich) and were shaken vigorously for 2 minutes. Following this, the samples were spun for 15 minutes at 4 °C at a speed of 12,000 g. The upper aqueous phase from the resulting solution was transferred to fresh 1.5 mL Eppendorf tubes and treated with 250 µL isopropyl alcohol (Sigma-Aldrich). Then the samples were incubated at room temperature for 10 minutes before being spun for 10 minutes at 4 °C at a speed of 12,000 g. The supernatant was removed and discarded. The samples were subsequently treated with 75% ethanol, incubated at room temperature for 5 minutes and spun for 5 minutes at 4 °C at a speed of 10,000 g. The supernatant was then discarded and the samples left to dry for 15 minutes. The purified RNA was finally resuspended in 20 µL nuclease-free water. RNA yield and purity were quantified using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific).

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actn2</td>
<td>Alpha actinin</td>
<td>Mm00473657_m1</td>
</tr>
<tr>
<td>Agrn</td>
<td>Agrin</td>
<td>Mm01545840_m1</td>
</tr>
<tr>
<td>Bdnf</td>
<td>Brain derived neurotrophic factor</td>
<td>Mm01334047_m1</td>
</tr>
<tr>
<td>Chrb1</td>
<td>Nicotinic cholinergic receptor</td>
<td>Mm00680412_m1</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Mm99999915_g1</td>
</tr>
<tr>
<td>Gdnf</td>
<td>Glial derived neurotrophic factor</td>
<td>Mm00599849_m1</td>
</tr>
<tr>
<td>Musk</td>
<td>Muscle associated receptor tyrosine kinase</td>
<td>Mm01346929_m1</td>
</tr>
<tr>
<td>Myh15</td>
<td>Myosin heavy chain</td>
<td>Mm01170207_m1</td>
</tr>
<tr>
<td>Myog</td>
<td>Myogenin</td>
<td>Mm00446195_g1</td>
</tr>
<tr>
<td>Ntf5</td>
<td>Neurotrophin 5</td>
<td>Mm01701591_m1</td>
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<tr>
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<td>Neurotrophic tyrosine kinase type 2</td>
<td>Mm00435422_m1</td>
</tr>
<tr>
<td>Syn2</td>
<td>Synapsin</td>
<td>Mm00449780_m1</td>
</tr>
<tr>
<td>TNNT3</td>
<td>Troponin</td>
<td>Mm01268863_m1</td>
</tr>
</tbody>
</table>

**Table 2 TaqMan Gene Expression Assays**

A high capacity cDNA reverse transcription kit (Applied Biosystems) was used to reverse transcribe mRNA to cDNA. The following reaction mix was prepared: 2 µL 10x RT buffer, 0.8 µL dNTPs (100 mM), 2 µL 10X RT random primers, 1 µL multismcribe reverse transcriptase (50
U/µL) and 4.2 µL nuclease-free water. Each sample was prepared in an Eppendorf tube on ice and then transferred to a thermal cycler (Thermo Fisher Scientific). The samples were then incubated at 25 C for 10 minutes, 37 C for 120 minutes and then 85 C for 5 seconds. After this stage, samples could be stored at -80 C or continued to quantitative PCR analysis.

Quantitative PCR was performed using the StepOnePlus Real-Time PCR system (Applied Biosystems). The PCR reaction mixture (12.5 µL TaqMan universal PCR Master Mix, 2.5 µL cDNA, 1.25 µL probe and 8.75 µL nuclease-free water) was prepared for each gene to be analyzed in triplicate wells in a 96-well plate (Applied Biosystems). The plate was incubated at 50 C for 2 minutes and 95 C for 10 minutes before being cycled 40 times at 95 C for 15 seconds and then 60 C for 60 seconds. The TaqMan Gene Expression Assays shown in Table 2 were used for detection of important muscular and motoneuron transcription factors. Gene expression values were normalized to GAPDH expression and presented as an expression relative to the control group by the $2^{-\Delta\Delta C_t}$ method [86].

3.2.5 Western Blot

Culture medium was aspirated and cells were rinsed with PBS 3 times to collect protein samples. CelLytic M (Sigma-Aldrich) supplemented with protease and phosphatase inhibitor cocktails (Roche) was added to lyse cells and solubilize proteins. Culture plate was placed on a shaker for 15 minutes at about 100 RPM before the solution was transferred to an Eppendorf tube and centrifuged at 12,000g for 10 minutes at 4 C. The supernatant was collected and protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Concentrated protein samples were diluted and 20 µL of the resulting solution was then mixed 1:1 with a laemmli buffer stock solution (Sigma-Aldrich). β-mercaptoethanol (Sigma-Aldrich) was also added to the laemmli buffer prior to mixing with the samples. The diluted samples were
incubated at 100 °C for 5 minutes and 40 µL of each was then loaded into separate wells of Mini-
PROTEAN TGX Precast Gels (Bio-Rad Laboratories). 5 µL of Precision Plus Protein Western C
standards (Bio-Rad Laboratories) was loaded into a single well in order to provide a reference
marker for determining molecular weight of the separated proteins. Loaded gels were clamped
into an electrode block and submerged in a running buffer consisting of 100 mL stock running
buffer (Tris-Glycine-SDS) diluted in 900 mL dH2O. Electrodes were attached to the block and
the samples subjected to a 120 V force for 75 minutes or until the samples had reached the
bottom of the gel. Afterwards, the gel was removed from the apparatus and placed on top of a
nitrocellulose membrane soaked in an ice cold transfer buffer consisting of 100 mL stock Tris-
Glycine transfer buffer (Bio-Rad Laboratories), 200 mL methanol and 700 mL dH2O. The gel
and membrane were clamped into an electrode block positioned so that the nitrocellulose
membrane was between the gel and the positive electrode to facilitate the transfer of negatively
charged protein from the gel onto the membrane. The block was immersed in cold transfer buffer
and subjected to a 100 V force for 120 minutes. After the run time, successful transfer of protein
to the membrane was visualized and confirmed using Ponceau solution (Sigma-Aldrich).

Presence of target proteins on the nitrocellulose membranes was confirmed through
probing with specific antibodies for myogenin, tropomyosin, myosin heavy chain, actinin and
GAPDH. Membranes were first washed 5 minutes 3 times in PBS to remove the Ponceau
solution before being immersed in a blocking solution consisting of 0.1% TWEEN (Sigma-
Aldrich) and 5% milk fat protein (Premier International Foods) in PBS for 1 hour. The
membranes were again washed 5 minutes 3 times with PBS and during this time primary
antibodies were diluted in blocking solution (1:1000 dilution). The primary antibody solution
was then added and the membrane incubated at 4 °C overnight. Following this incubation step,
the membranes were washed 5 minutes 3 times in PBS while secondary antibody solutions were prepared. Secondary antibodies conjugated with alkaline phosphatase were diluted 1 in 1000 in blocking solution. The membranes were incubated with the secondary antibody solution for 2 hours at room temperature and afterwards, washed 5 minutes 3 times. NBT/BCIP Substrate Solution (Thermo Fisher Scientific) was added to the membranes and produced purple-black precipitate, colocalized with the targeted protein bands. When the bands became easily visible, membranes were washed with water to stop the reaction and ready to be photographed.

3.2.6 Statistical Analysis

All experiments were repeated at least 3 times using cultures prepared on different days. Quantitative data were presented as mean ± standard deviation. qRT-PCR and western blot results were presented as a fold change relative to GAPDH. Statistical significance was evaluated with the one-way analysis of variance (ANOVA) with p = 0.05 (n = 5 biological replicates for qRT-PCR and n = 3 biological replicates for western blot).

3.3 Results

3.3.1 All groups of culture media can induce differentiation of myoblasts to form myotubes

C2C12 cultured in GM proliferated well and reached 80-90% confluent within 2-3 days (Figure 8A). After 9 days of culture in differentiation media, all study groups can induce C2C12 to fuse and form elongated, multinucleated myotubes as shown by brightfield images in Figure 1B, but cell fusion was more ubiquitous in DM and Combined groups. Fluorescence images showed the colocalization of actin and α-actinin, which indicates the formation of sarcomere structure. All study groups were stained positive for both proteins, but the colocalization was
Figure 8 CM, DM and Combined media can differentiate C2C12 to form multinucleated myotubes

(A) C2C12 cells were cultured in GM for 2-3 days until reaching 80-90% confluent. (B) After 9 days in differentiation media, C2C12 fused to form elongated multinuclear myotubes in all study groups. (C) Fluorescence images showed the colocalization of actin (red) and α-actinin (green) found in myotubes that were cultured in CM, DM and Combined media. (D) Fluorescence images showed the early AChR aggregation, a sign of muscle maturation, indicated by white arrows. Scale bar: 100 µm.
more noticeable in DM and Combined groups (Figure 8C). Immunostaining of AChRs also revealed the early aggregation of the AChRs in myotubes cultured in DM and Combined media.

### 3.3.2 Serum supplement supports the long-term culture of myotubes

After 9 days of culture in differentiation media, myotubes from different study groups were stained with LIVE/DEAD cytotoxicity kit. The extent of dead cells, indicated by red dye, was found to be more pronounced in the CM group, compared to the DM and Combined groups (Figure 9A). The results were more apparent when cell viability was assessed on myotubes that were cultured in differentiation media for 15 days (Figure 9B). Dead cells were commonly found in the culture using CM as a differentiation medium. The major difference between the CM and the other differentiation media is the lack of serum supplement. Therefore, the results support to the notion that serum supplement in differentiation medium is important for the long-term culture of myotubes.

![Figure 9](image.png)

**Figure 9** Serum supplement in differentiation medium supports the long-term culture of myotubes

Fluorescence images of LIVE/DEAD stains showed cell viability of myotubes that were cultured in different differentiation media after (A) 9 days and (B) 15 days. Scale bar: 100 µm.
Figure 10 Gene expression and protein levels of important muscular proteins found in myotubes cultured in different differentiation media

(A) qRT-PCR analysis showed normalized expression levels of important muscular genes of myotubes found in CM, DM and Combined culture media. The expression levels of actinin and Tnnt3 found in the Combined group were significantly increased compared to those of the CM group. (B) Western blot showed increased muscular protein levels of the Combined group, compared to CM and DM groups. Specifically, the protein levels of myogenin, tropomyosin and actinin were significantly higher in the Combined group compared to those of the CM group. Quantitative data presented as mean ± SD. Statistical significance was evaluated with ANOVA (*p < 0.05, n = 5 biological replicates for qRT-PCR and n = 3 biological replicates for western blot).
3.3.3 Myotubes cultured in the Combined medium show an increase in important muscular gene expressions and protein levels

qRT-PCR showed that expression levels of muscular genes were generally higher in myotubes that were cultured in the Combined medium, compared to those cultured in the CM and DM media (Figure 10A). Particularly, the expression levels of α-actinin and troponin from myotubes cultured in the Combined medium were more than 3 times of the expression levels of myotubes cultured in the CM medium and the differences were also statistically significant. In addition, the results from western blots confirmed that myotubes cultured in the Combined medium expressed higher levels of muscular proteins compared to the other groups (Figure 10B). Specifically, the protein levels of myogenin, tropomyosin and α-actinin found in the combined group were significantly higher than those of the CM group. Therefore, based on the results from qRT-PCR and western blot, the Combined medium could be a superior differentiation medium compared to CM and DM.

3.4 Discussion

C2C12 is an immortalized myoblast cell line that can proliferate indefinitely and with proper condition, it can develop into a muscle fiber through myogenesis. The aim of the study was to determine the optimal culture medium that could effectively orchestrate terminal differentiation process of C2C12 myoblasts, during which the proliferating mononuclear myoblasts undergo cell cycle arrest and fuse together to form multinucleated myotubes. Three differentiation media were evaluated: CM, DM and Combined. CM is a serum-free medium normally used for motoneuron culture while DM is a horse serum-supplemented medium commonly used to differentiate myoblasts to myotubes. Combined medium is simply the combination of the two media with a few extra supplements (For detailed composition, see Table
1). The efficiency of differentiation media was assessed by evaluating the following cell characteristics, including structure, maturation, proliferation, differentiation and contractility.

Structurally, all differentiation media could induce C2C12 to fuse and form elongated, multinucleated myotubes as shown in Figure 8B. Immunostaining was performed to examine the existence and the localization of interested proteins in myotubes. α-actinin, a protein in the z line, and actin filaments were used to identify sarcomere structure, which indicates the maturity of myotubes. Myotubes from all the groups showed the colocalization of actin and α-actinin, suggesting the formation of sarcomere structure because α-actinin is an actin crosslinking protein involved in the attachment of the actin cytoskeletal framework to the plasma membrane [87]. The degree of fusion was also more pronounced in the DM and Combined groups. Moreover, myotubes cultured in the DM and Combined media showed an early aggregation of AChRs, signifying an initial sign of maturity.

LIVE/DEAD stains revealed cell viability at the end of myotube culture. DM and Combined media could efficiently support the long-term culture of myotubes while CM medium led to significant cell death after 15 days of culture. One possible explanation was the lack of serum in the CM medium. Even though serum brought in the variability to the culture as the components were not defined, it contained high levels of nutrients and combination of growth factors. As a result, the capability to support a long-term culture was a justified trade-off for unaccountable inconsistency brought by the use of serum because coculture experiments would last longer than 9 days and it would provide more flexibility for the experiments.

Other than morphological analysis and immunostaining, the maturity of myotubes was assessed by gene expression and protein level analysis. Results from qRT-PCR and western blots showed that myotubes cultured in the Combined medium exhibited generally higher gene
expressions and protein levels for important muscular proteins, especially $\alpha$-actinin, myogenin, tropomyosin and troponin. In addition, spontaneous contraction was found in myotubes that were cultured in the DM and Combined media towards the end of the 9-day experiment. Contraction was more noticeable after 12 days in the differentiation media for all the groups, but it was relatively weak for myotubes cultured in the CM medium (Supplementary Video S1).

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>Myotube Formation</th>
<th>Cell Viability</th>
<th>Muscular Gene Expression Level</th>
<th>Specific Proteins</th>
<th>Contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM</td>
<td>+</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>DM</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>Combined</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 3 Summary table for effects of different differentiation media on myotube development

In summary, Combined medium is the best differentiation medium out of the three media because it could effectively induce myotube differentiation, support long-term culture, enhance important muscular gene expressions and protein levels, and lead to stronger spontaneous contraction as summarized in Table 3. Therefore, low level of serum and additional supplements were sufficient to differentiate C2C12 to form myotubes with proper morphological and functional development.
4 Effects of Electrical Stimulation on Skeletal Muscle Derived from Mouse Myoblasts

4.1 Introduction

Biomimetic niche is an extracellular environment that replicates the native milieu of tissues in the body. It consists of cells in coordination with the surrounding cells, the extracellular matrix, cytokines and biophysical signals [56]. Extracellular cues within this niche are incredibly important for promoting proper cell development. Because muscle is electrically excitable and the development is closely linked to the electrical activity, an integration of electrical stimulation to the culture system should be advantageous. Many studies have shown that electrical stimulation can accelerate sarcomere assembly [88, 89], promote appearance of myotubes, and subsequently produce spontaneously contracting muscle fibers [90-92].

Electrical stimulation needs to be applied such that a desired physiological response can be achieved with only minimal damage to the cells. During electrical stimulation, charge is carried by electrons in the electrode phase [93]. At the electrode/medium interface, charge is transferred and carried by ions in the culture medium. The ion movement determines the momentary presence of current in the medium and also the amount of electric stimulation that cells experience. To attain an appropriate electrical stimulation, many factors need to be considered. For example, electrodes must be biocompatible to avoid toxic or immune responses in the adjacent tissue or medium. They should also efficiently transfer charge from the electrode
material to the medium. Particularly, during charge transfer, nonreversible Faradaic reactions should be minimized because it is associated with electrode degradation and harmful byproducts. In many studies, carbon electrode is chosen due to its biocompatibility, high availability, superior charge injection characteristics, and high resistance to chemical reactions and corrosion [94-96].

The goal is the optimization of electrical stimulation parameters that can lead to proper cell development. The optimal electrical stimulation should attain a desired physiological response with minimal damage to the stimulated tissue and it should be designed to mimic the signal existing in nature under the conditions of interest, in this case, nerve signal. There are many variables in the electrical stimulation that can be regulated, for example, amplitude, frequency, stimulation duration, and waveform shape. Initiation and stimulation period also need to be considered.

To this end, C2C12 are electrically stimulated 6 hours per day with a frequency of 1 Hz and a pulse duration of 2 milliseconds, which is adequately long to excite skeletal muscle cells and also sufficient for the double layers on stimulation electrodes to dissipate between subsequent pulses [94]. Monophasic square-wave pulses are chosen due to the simplicity and compatibility with carbon rod electrodes [95]. Three stimulation voltages within the physiologically significant range of endogenous gradients of electrical fields will be examined: 3 V/cm, 5 V/cm and 7 V/cm, with an unstimulated control. The efficiency of stimulation regime can be assessed by evaluating the following cell characteristics, including proliferation, maturation, differentiation, and contractile function. Specifically, the effects of electrical stimulation on important muscular proteins such as actinin, GDNF, NT 5, MuSK, Myogenin, troponin and AChRs are examined. The well-defined characterization of stimulation effect will
provide improved options for directing and fine-tuning skeletal muscle development and function.

4.2 Materials and Methods

Figure 11 Experiment timeline and an electrical stimulation bioreactor culture plate

4.2.1 Bioreactor Culture with Electrical Stimulation

Polydimethylsiloxane (PDMS) (Dow Corning) was used to fabricate bioreactor cassettes. Each bioreactor cassette had 5 rows of 6 wells, 1 cm x 1 cm in size. Carbon rods of 0.120 inch in diameter (Ladd Research) spanned the two ends of each row, such that its six wells received the
same stimulation. The bioreactor was sealed to a 10 cm x 15 cm glass (Thermo Fisher Scientific) using PDMS. Before seeding cells, stimulation plates were coated with poly-L-ornithine (Thermo Fisher Scientific) and laminin (Thermo Fisher Scientific). Poly-L-ornithine solution was added to cover the whole culture surface and the plates were incubated for 2 hours or overnight at room temperature. The plats were rinsed with sterile water before adding the diluted laminin solution (20 µg/mL with sterile PBS) and incubating 2 hours or overnight at room temperature. Before use, laminin solution was aspirated and the plates were washed with PBS.

C2C12 was plated in the bioreactor culture plate at the density of 250 cells/mm² and was cultured in GM for 2 days until reaching about 80%-90% confluent. Cells were washed with PBS before changing to differentiation medium, Bare Minimum medium (BM) in this case. BM medium was composed of high glucose DMEM (Thermo Fisher Scientific), 2% house serum (Thermo Fisher Scientific) and 1% penicillin-streptomycin (Thermo Fisher Scientific). After a day of pre-culture in BM, electrical stimulation was initiated. Carbon rods of bioreactors were connected to leads from an S88X Dual Output Square Pulse Stimulator (Grass Technologies) generating continuous pulses at 1 Hz and 2 millisecond duration. Three voltages were evaluated: 3 V, 5 V and 7 V, with an unstimulated control, and cells were stimulated 6 hours per day for 8 days before samples were collected for analysis. For an additional control group, cells received electrical stimulation but 0.5 µM of tetrodotoxin (TTX) was added to the culture medium. We provided more description on bioreactor cleaning steps: After each experiment, the bioreactor cassettes were thoroughly cleaned. Specifically, to avoid protein accumulation on the carbon electrodes, the bioreactor culture surface and electrodes were scrubbed with a soft brush and then washed with a water jet. Afterwards, the plates were soaked in 70% ethanol for 1-2 hours, dried in the hood and autoclaved.
4.2.2 Calcium Imaging

Myotubes were assessed for response to electrical stimulation using calcium imaging. Cells were stained with 5 µg/mL Fluo-4 AM (Thermo Fisher Scientific) diluted in pre-warmed Tyrodes Solution (Sigma-Aldrich) for 30 minutes and washed twice with Tyrodes solution prior to imaging. Cells were electrically stimulated every 10 seconds by 2 stimuli of 2 millisecond duration and a gap of 300 milliseconds between the two stimuli. A concentric electrode (FHC, Inc) was positioned about 100 µm away from an area of interest, and the amplitude of the electrical signal was set to 0.5 V. Concentration of TTX was 0.5 µM. Videos were taken on the Olympus IX81 using the Pike camera at 100 frames per second via the SPLASSH software [78]. Videos were imported into a MATLAB R2016b software that allowed users to measure average fluorescence intensity in an area of interest for each frame, yielding calcium intensity over time.

4.2.3 Statistical Analysis

All experiments were repeated at least 3 times using cultures prepared on different days. Quantitative data were presented as mean ± standard deviation. LIVE/DEAD images were quantified using ImageJ and Prism 7. RT-PCR results were presented as a fold change relative to GAPDH. Statistical significance was evaluated with the ANOVA with p = 0.05 (n = 6 biological replicates for qRT-PCR and n = 6 biological replicates for LIVE/DEAD).

4.3 Results

4.3.1 Optimal electrical stimulation gradients do not cause significant cell death in bioreactor culture

After 8 days of electrical stimulation in bioreactor culture, myotubes experiencing different stimulation voltages were evaluated for cell viability (Figure 12). Optimal electrical
stimulation should not inflict too much damage on cells and thus, minimize cell death. Electric field strength of 5 V/cm and 7 V/cm led to substantial cell death as shown by red dye in figure 12A. Quantitatively, fraction of live cells dropped from 0.93 in control group to 0.62 and 0.36 in 5 V and 7 V stimulation groups, respectively. On the other hand, electric field stimulation of 3 V/cm did not cause significant cell death as the fraction of live cells was only reduced to 0.86 as demonstrated in Figure 12B. 3 V/cm was, thereby, chosen for the future stimulation experiments.

**Figure 12** Myotube viability after bioreactor electrical stimulation using different voltages

(A) Fluorescence images of LIVE/DEAD stains showed cell viability of myotubes that were electrically stimulated at different voltages for 8 days, 6 hours per day. Scale bar: 100 µm. (B) Fraction of live cells after 8 days of electrical stimulation in bioreactor culture. Quantitative data presented as mean ± SD. Statistical significance was evaluated with ANOVA (*p < 0.05, n = 6 biological replicates).
4.3.2 Electrical stimulation structurally matures myotubes

Myoblasts from both control and stimulation groups fused to form elongated, multinuclear myotubes with colocalization of actin and α-actinin. However, striated sarcomeres, indicators of skeletal muscle organization and maturation, were routinely found only in myotubes cultured with electric field stimulation of 3 V/cm, suggesting that proper electrical stimulation could benefit myotube development by accelerating striation formation.

![Control Group](image1.png) ![Stimulation Group](image2.png)

**Figure 13 Electrical stimulation structurally matures myocytes as evidenced by striated sarcomeres**

Florescence images showed the morphology of myotubes in the control and stimulation groups. Striated sarcomeres indicating mature myocytes were commonly found in myotubes from stimulation group using electric field strength of 3 V/cm. Red, green and blue represent actin, actinin and DAPI, respectively. Scale bar: 100 μm.
Figure 14 Effects of electrical stimulation on skeletal myotubes

(A) qRT-PCR analysis showed normalized expression levels of important muscular genes of myotubes found in control, electrical stimulation with TTX in culture medium and electrical stimulation groups. (B) Calcium transients during electrical stimulation. The trace consisted of 3 X ~60 seconds recording spliced together. Two electrical stimuli of 0.2 seconds apart were delivered every 10 seconds. The first section was the control, second section was in the presence of TTX and the last section was after 5-minute wash. (C) LIVE/DEAD florescence images showed that cell death was minimal in all the study groups even if more dead cells were observed in Stimulation+TTX group. Scale bar: 100 µm. Quantitative data presented as mean ± SD. Statistical significance was evaluated with ANOVA (*p < 0.05, n = 6 biological replicates for qRT-PCR).
4.3.3 Electrical stimulation upregulates important skeletal muscular genes and TTX suppresses the response to electrical stimulation and abolishes the effect of electrical stimulation

qRT-PCR results showed that expression levels of important skeletal muscular genes were generally upregulated in myotubes receiving electrical stimulation, compared to those found in myotubes receiving no stimulation and myotubes that received electrical stimulation, but TTX was added to the culture medium (Figure 14A). Particularly, α-actinin expression level of stimulated myotubes was almost 3 times of the expression level of myotubes receiving no stimulation. Moreover, the expression levels of AChR (Actn2), myogenin (Myog) and troponin (Tnnt3) were more than 3 times compared to those of unstimulated myotubes. Expression levels were also statistically significant when they were compared to those of control group and stimulation group with TTX, showing that TTX could abolish the effects of electrical stimulation on important skeletal muscular gene expression.

Calcium imaging revealed calcium transients during electrical stimulation in different conditions (Figure 14B). Myotubes responded well to electrical stimulation as evidenced by calcium fluxes synchronizing with electrical stimulation. After adding TTX to the culture, calcium activities due to electrical stimulation were gradually blocked and they were completely suppressed after about 40 seconds. Following the washout of TTX and medium replacement, myotubes responded to electrical stimulation at the pre-TTX treatment level. The result indicated that TTX could suppress the response of myotubes to electrical stimulation.

LIVE/DEAD fluorescence images compared viability of myotubes cultured in all the study groups (Figure 14C). Dead cells were not significant across all three conditions even
though more dead cells were found in the culture receiving electrical stimulation with TTX added to the culture medium.

4.4 Discussion

The goal of the study was to investigate the effects of electrical stimulation on the development of myoblasts into myotubes. Electrical stimulation was introduced to the bioreactor culture because skeletal muscle is electrically excitable and its development is interconnected with electrical activities. It was hypothesized that the implementation of electrical stimulation would be beneficial for functional coupling of myotubes. The first objective was to determine the stimulation voltage that would not inflict major damage and lead to significant cell death. In this study, stimulation regime was monophasic square-wave pulse with the frequency of 1 Hz, 2-millisecond duration and a relatively long time between pulses using carbon electrodes. Monophasic pulse was selected due to its simplicity. It also avoided the possible undesirable effect of biphasic pulse inhibiting signal propagation because the secondary hyperpolarizing pulse could interfere with the initiation of APs in motoneurons [97]. Moreover, carbon electrodes were chosen because they have high capacitance and high resistance to corrosion, resulting in better charge transfer characteristics and biocompatibility [95]. They were, consequently, nontoxic to cells as shown by Figure 12 that fraction of live cells was more than 90% when myotubes were cultured in the medium exposed to carbon electrodes. Importantly, muscle cells were maintained in the BM medium because the objective was to examine the effects of electrical stimulation on the development of myotubes, rather than the effects of enriched culture medium. Using the BM medium allowed the effects of electrical stimulation to be more discernible.
The results in Figure 12 suggested that electric stimulation of 3 V/cm did not impose too much stress on myotubes and thereby, did not cause significant cell death. Electrical stimulation also supported myotube maturation as demonstrated by the early appearance of striated sarcomeres when compared to unstimulated myotubes. In addition to improved cellular structures, electrical stimulation led to upregulation of muscle-specific genes. Particularly, gene expression levels of α-actinin, AChRs (Actn2), myogenin (Myog) and troponin (Tnnt3) were significantly increased. We hypothesized that the benefits of electrical stimulation stemmed from an increase in calcium transients and contractions that enhanced activity-dependent development of skeletal muscle. Even though the stimulation regimen used in the study could not directly induce contraction, an increase in cell depolarization due to electrical stimulation could lead to more contractions. The increase in calcium activities and contractions could, therefore, contribute to the upregulation of important genes found in the studies. In addition, when TTX, a voltage-gated sodium channel antagonist, was applied to the coculture, induced calcium transients as a result of electrical stimulation and contraction activities were significantly suppressed. The reduction of spontaneous contraction activities and calcium transients by TTX can potentially explain why the benefits of electrical stimulation were abrogated. Moreover, studies showed that denervation of muscle from motoneurons leads to muscle atrophy as denervation deprives muscle from synaptic activities and physiological Ca\(^{2+}\) transients [98, 99]. These results, therefore, further substantiate our hypothesis that the observed advantageous changes found in myotubes were induced by electrical simulation and that electrical stimulation might affect myotube development via calcium signaling.

TTX has been known to cause paralysis and inhibit muscle contraction via Na\(^{+}\) channel blockade [100]. TTX changes Na\(^{+}\) permeability normally associated with excitation, preventing
Na$^+$ inflow and therefore, preventing depolarization and contraction. Notably, muscle at different stages respond to TTX differently, for instance, myoblasts are resistant to TTX while myotubes are more amenable [101]; the sensitivity of the muscle action potential changes from 10 µM to 10-500 nM during development. In addition, it was shown that most spontaneous contractions of rat myotubes in culture were blocked by 100 nM TTX and that 1 µM TTX abolished contractile activity in both neural and aneural muscle cultures. This suggests that Na$^+$ play a major role in the dihydropyridine receptor (DHPR) voltage sensor activation at the level of the T-tubules, and consequently the calcium release from the sarcoplasmic reticulum and the contraction [102]. As a result, the concentration of TTX used in the present study should be sufficient to suppress most of the spontaneous activities. To further confirm the validity of using TTX to suppress contraction and calcium activities, the study conducted by Lorenzon, et al. showed that 1 µM TTX could effectively block global calcium activities of C2C12 [103], which could be a potential mechanism underlying the benefits of electrical stimulation. Therefore, suppression of spontaneous contraction activities and calcium transients by TTX can potentially explain why the benefits of electrical stimulation such as an upregulation of important muscular genes were abolished when C2C12 were electrically stimulated in the culture medium supplemented with TTX. However, some calcium activities, including Ca$^{2+}$ oscillations and localized Ca$^{2+}$ spikes, can still be found even when using high concentration of TTX [103]. To avoid ambiguity and improve the current study design, calcium channel blockers could be used instead of TTX to clarify the underlying mechanism of electrical stimulation on muscle cells.

In summary, the study suggested that proper electrical stimulation could benefit myotube development by improving skeletal muscle morphology, accelerating striation formation and enhancing important muscular gene expressions without imposing significant stress and damage.
on cells. Therefore, electrical stimulation should be incorporated in the bioreactor culture in the subsequent experiments.
5 Transdifferentiation of Mouse Embryonic Fibroblasts to Myocytes and the Effects of Electrical Stimulation

5.1 Introduction

Transdifferentiation, also known as lineage reprogramming, is a procedure that transforms mature, fully differentiated somatic cells into other somatic cell types through the introduction of defined transcription factors without undergoing an intermediate pluripotent state [104]. The technique is a promising cellular and tissue generating approach because it circumvents important concerns from the use of ESCs and iPSCs. First, isolation and expansion of human ESCs from unfertilized oocytes of human volunteers can pose a significant challenge due to limited availability and therefore, lack of steady cell sources [105]. Although the use of iPSCs to derive a desired cell type may overcome the low availability and the dependency on volunteer donation found in ESC method, a limitation of induced pluripotency is the time it takes to reprogram the cells and then subsequently direct them to the preferred fate [106-108]. Moreover, the efficiency can be low because the protocols to generate iPSCs include a number of stages [109]. Importantly, both ESC and iPSC approaches possess elevated risks of teratoma formation [110].
Transdifferentiation bypasses the pluripotent stage of ESCs and iPSCs. Studies have demonstrated that fibroblasts could be transdifferentiated to several lineages including cardiomyocytes [111, 112], hepatocytes [113], neurons [114] and skeletal muscle [115, 116] by ectopic expression of multiple lineage-specific transcription factors. Particularly, overexpression of myogenic differentiation factor (MyoD) has been shown to successfully convert many somatic cell types to skeletal muscle [90-92]. These transcription factors are introduced into cells via transfection or transduction. For this study, transduction using lentivirus, whose pathogenic and replicative properties have been removed to only keep the gene transport capability, is chosen as a gene transfer method. Compared to transfection, transduction is more versatile because the technique works in immortalized cells, primary cells and stem cells. In addition, its effect is dose-dependent and significantly less toxic to cells [117].

The tetracycline inducible system is a powerful tool that allows temporal and dose-dependent regulation of target transgene expression once lentiviral vector integrates its DNA into the host genome [118]. The transcription is reversible as it can be on/off in the presence of tetracycline or its derivatives such as doxycycline (Dox) [119]. Reverse tetracycline transactivator (rtTA-M2) is created by fusing tetracycline repressor (TetR) found in *Escherichia coli* bacteria with the VP16 activation domain of another protein found in the herpes simplex virus [120]. The resulting rtTA-M2 protein is capable of binding to DNA at specific tetracycline operator (TetO), placed upstream of a minimal promoter such as the CMV promoter, only if it is bound to tetracycline or one of its derivatives. Introduction of Dox to the system will, therefore, increase specific gene expression downstream of its promoter.
In the current study, the efficiency of lentiviral transduction and tetracycline inducible system to transdifferentiate MEFs into myocytes by overexpression of MyoD will be assessed via morphology, important gene expression levels and functionality. In addition, effects of electrical stimulation on transdifferentiation and myocyte development will be explored.

5.2 Materials and Methods

5.2.1 Lentivirus Production

Human embryonic kidney cells 293 (HEK 293) were cultured in GM in T75 flask until reaching 90% confluency. Then 7 mL of high glucose DMEM medium supplemented with 10% FBS, 2-mercaptoethanol (Sigma-Aldrich), gentamycin (Thermo Fisher Scientific), sodium pyruvate (Thermo Fisher Scientific), MEM non-essential amino acids solution (Thermo Fisher Scientific) and glutamax (Thermo Fisher Scientific) was added to the culture 30 – 60 minutes before transfection. Separately, 9.5 µg of gene of interest, MyoD1 in this case, 3.3 µg of pMD2.G (Addgene) and 6.2 µg of psPAX2 (Addgene) were added to 625 µL of high glucose DMEM medium without supplements. 58.5 µL of calfectin (SignaGen Laboratories) was diluted in 625 µL of high glucose DMEM medium and the solution was added to the solution prepared earlier and incubated for 15 – 20 minutes at room temperature. Afterwards, the transfecting solution was added to the culture flask. After 5 hours of incubation, 13 mL of fresh medium was added. At 28th, 52nd and 76th hours from the start of transfection, 12 mL of culture medium was collected each time and 12 mL of fresh medium was added back. In total, 36 mL of medium was collected. To precipitate HEK and cell debris, the virus supernatant was centrifuged at 1000 rpm for 5 minutes at 4 C. Then the supernatant was filtered using 0.45 µm filter and added to Amicon
tube (50kDa) (Millipore). The tube was spun at 3400 rpm for 20 min at 4 C, making 50X viral concentration. The solution was homogenized by pipetting and was stored in -80 C freezer.

5.2.2 Transdifferentiation of Skeletal Muscle from Mouse Embryonic Fibroblasts

MEFs were initially cultured with high-glucose DMEM supplemented with 10% FBS until reaching 70% - 90% confluent. Lentiviral gene delivery system was used to stably transduce cells with rtTA-M2 and MyoD gene (about 10 MOI). To improve transduction, hexadimethrine bromide (Sigma-Aldrich) was added to the medium at a concentration of 8 µg/mL. After transduction, Dox (Sigma-Aldrich) at a concentration of 3 µg/mL was added to the Combined differentiation medium. The culture medium was changed every two days and cells were cultured for 12 days before analysis. For electrical stimulation study, the stimulation was initiated one day after switching to the Combined medium with Dox. The stimulation regime was 3 V/cm with the frequency of 1 Hz and the duration of 2 milliseconds for 6 hours per day.

5.2.3 Statistical Analysis

Quantitative data were presented as mean ± standard deviation. RT-PCR results were presented as a fold change relative to GAPDH. Statistical significance was evaluated with the ANOVA with p = 0.05 and n = 5 biological replicates.
5.3 Results

5.3.1 MyoD overexpression via lentiviral transduction and tetracycline inducible system effectively transdifferentiates MEFs to functional myotubes

MyoD is a key regulatory factor in skeletal muscle and its ectopic overexpression induces transdifferentiation of MEFs into myocytes [121, 122]. MEFs were transduced with a Tet operator/CMV promoter-MyoD lentiviral construct (TetO/CMV-MyoD) and a lentivirus containing rtTA-M2. The introduction of Dox into the culture activated rtTA-M2 to bind to TetO and initiate transcription of MyoD (Figure 15A).

At the end of differentiation process, the morphology, protein and gene expressions levels of MEFs were compared to those of MEF-transdifferentiated myocytes. MEFs had round shape (Figure 15B) and stained positive for actin (Figure 15C), while MEF-transdifferentiated myocytes fused to form elongated and multinucleated myotubes (Figure 15D) that showed the colocalization of actin and α-actinin, indicating the formation of sarcomere structure (Figure 15E). In addition, transdifferentiation led to significantly higher expression levels of important muscular genes, including α-actinin, MyoD and Myogenin (Figure 15F), suggesting the effectiveness of the tetracycline inducible system and transdifferentiation. Gene expression levels in reprogrammed myocytes were comparable to those of C2C12 mouse myoblasts, while the expression of the same genes in the control MEFs was barely detectable (Figure 15F).

To demonstrate the functionality and maturity of MEF-transdifferentiated myocytes, muscle cells were electrically stimulated every 10 seconds using a concentric electrode with 2 stimuli of 0.5 V, 2 millisecond duration and 300 milliseconds between the two stimuli, and calcium transients were recorded. The spikes of intracellular calcium flux in transdifferentiated myocytes assumed the frequency of electrical stimulation, indicating that myocytes derived from
Figure 15 Derivation and characterization of transdifferentiation of MEFs into skeletal myocytes.

(A) The diagram showing rtTA-M2 lentiviral construct and a lentiviral construct containing TetO/CMV-MyoD. A tetracycline inducible system via rtTA-M2 was activated by doxycycline and initiated MyoD transcription. Overexpression of MyoD induced transduction of MEFs into skeletal myocytes. (B), (C) Brightfield and fluorescence images of MEFs after 12 days in Combined medium. Actinin was not found in MEFs. (D), (E) Brightfield and fluorescence images of MEF-transdifferentiated myocytes showed multinucleated myotubes and the presence of actinin protein after 12 days in Combined differentiation medium. Red, green and blue represent actin, actinin and DAPI, respectively. Scale bar: 100 µm. (F) qRT-PCR analysis showed relative expressions of important muscular genes found in MEFs, transdifferentiated myocytes and C2C12 myoblasts. Expression levels of each gene were normalized by the expression levels found in MEFs. Quantitative data presented as mean ± SD. Statistical significance was evaluated with ANOVA (*p < 0.05, n = 5 biological replicates) (G) A diagram showing calcium transients of MEF-transdifferentiated myocytes being electrically stimulated every 10 seconds as shown by red arrows. Myocytes showed significant increase of fluorescence intensity corresponding to electrical stimulation.
MEFs were excitable and functional (Figure 15G, Supplementary Video S2). The myotubes formed from transdifferentiated myocytes also responded to chemical stimulation using nicotine (Supplementary Video S3). These results demonstrated that myocytes transdifferentiated from MEFs were structurally and functionally mature. Additionally, some myocytes and myotubes were found in the culture of transduced MEFs in the Combined medium that was not supplemented with Dox (Figure 16).

Figure 16 Brightfield and fluorescence images of transduced MEFs and untreated MEFs
Myocytes and myotubes were commonly found in the culture of transduced MEFs in Dox-supplemented Combined medium. Some myotubes were present in the culture of transduced MEFs using Combined medium without Dox. There was no myocytes in the control MEFs. Scale bar: 100 µm.

5.3.2 Electrical stimulation upregulates the expression of muscular genes and improves the skeletal muscle morphology

Electrical stimulation upregulated important muscular genes in myotubes, including AChR, α-actinin, myogenin and troponin (Figure 17A). Expression levels of these genes were more than twice as high as in the unstimulated myotubes. Electrical stimulation also did not
Figure 17 Effects of electrical stimulation on transdifferentiated myotubes
(A) qRT-PCR analysis presented elevated relative expressions of important muscular genes of stimulated myocytes compared to those of control group after 12 days in culture. The expression levels of AChR, actinin, myogenin and troponin found in stimulated myocytes were significantly increased compared to those of the control group. (B), (C) LIVE/DEAD stains of control and stimulated myotubes. (D), (E) Fluorescence images showed the morphology of myocytes in both groups. Striated sarcomeres indicating mature myocytes were commonly found in myotubes from stimulation group. Scale bar: 100 µm. Quantitative data presented as mean ± SD. Statistical significance was evaluated with the Student’s t-test (*p < 0.05, n = 5 biological replicates).
cause significant cell death in the culture as shown in Figure 17C compared to the unstimulated myotubes in Figure 17B. In addition, striated sarcomeres, indicators of skeletal muscle organization and maturation, were routinely found in myotubes formed from electrically stimulated myocytes after 10 days of culture (Figure 17E). Spontaneous contractions were observed by day 9 of culture for electrically stimulated myotubes, and only after more than 2 weeks of culture for unstimulated myotubes (Supplementary Videos S4, S5).

![Figure 18 Striation found earlier in stimulation group compared to control group](image)

(A) Striation (showed by white arrows) was commonly found in myotubes receiving electrical stimulation as early as day 10 in the culture. (B) Striation of myotubes in control group was observed at day 19.

### 5.4 Discussion

Transdifferentiation of MEFs into functional myocytes via a tetracycline inducible system proved to be an effective derivation process. Ectopic overexpression of MyoD was sufficient to push MEFs toward myogenic differentiation. Transdifferentiated myocytes could form multinucleated myotubes with colocalization of actin and α-actinin, indicating mature sarcomere structure (Figure 15E). The expression levels of important muscular genes were also comparable to those of myotubes formed from C2C12 mouse myoblasts (Figure 15F), suggesting high efficiency of transduction and tetracycline inducible system. A possible explanation for
slight discrepancy between gene expression levels of transdifferentiated myocytes and C2C12 groups was that there were some undifferentiated MEFs after the transdifferentiation process. In addition to significant increase in muscular gene expressions, myotubes derived from MEFs were properly functional as they responded well to electrical stimulation and chemical stimulation using nicotine. Although Dox was not added to the culture of transduced MEFs, some cells still underwent transdifferentiation and converted to myocytes and myotubes (Figure 16) because horse serum used in the Combined medium contained traces of Dox. Therefore, when using the tetracycline inducible system in cell culture, it is important to confirm that each batch of serum has no or low level of contaminating tetracycline that cannot interfere with inducible system.

An advantage of transdifferentiation is that the technique not only allows autologous cell generation, but also bypasses the pluripotent ESC/iPSC stage, minimizing the risk of teratoma. The transdifferentiation process also takes shorter time compared to iPSC generation [123]. Moreover, the tetracycline inducible system has very tight control on expression, leading to low degree of leakiness. Nonetheless, this approach has some limitations. First, transdifferentiation has limited scalability because its lack of a proliferative precursor stage compromises the cell-generating capacity of the system. To solve the shortcoming, fibroblasts were chosen as a cell source because they are abundantly available and easy to obtain. Another drawback of this approach was the introduction of viruses into cells. The mechanisms of integrating viral vectors remain unclear and such usage could cause mutations when inserted into the genome. Transfection is an alternative approach, but transduction has been proven to be more effective and versatile as it works in immortalized cells, primary cells and stem cells. In addition, its effect is dose-dependent and significantly less toxic to cells. Regardless of a few downsides,
transdifferentiation offers a promising option for cell derivation in regenerative and personalized medicine applications.

It was hypothesized that the application of electrical stimulation will be advantageous for functional coupling of myocytes to myotubes because myocytes is electrically excitable and the development is closely connected to their electrical activity. Studies have shown that electrical stimulation can accelerate sarcomere assembly [88, 89], promote appearance of myotubes, and subsequently produce spontaneously contracting muscle fibers [90-92]. The results in Figure 17 proved that electrical stimulation led to a desired physiological response with minimal damage to the cells; 3 V/cm did not impose too much stress on myotubes and thereby, did not cause significant cell death. The stimulation also supported myotube maturation as demonstrated by the early appearance of striated sarcomeres when compared to unstimulated myotubes. In addition to improved cellular structures, electrical stimulation led to upregulation of muscle-specific genes. Particularly, gene expression levels of α-actinin, AChRs, myogenin and troponin were significantly increased. The results from transdifferentiated myocytes correspond with the results found in myotubes developing from C2C12. Notably, myotubes started to contract after 9 days of electrical stimulation, compared to up to 3 weeks for unstimulated cells [121]. Consistently, striations appeared sooner in stimulated myotubes than in the control group 10 days vs. 19 days, Figure 18).

In summary, the study demonstrated that transdifferentiation is a viable approach to derive functional myocytes from MEFs. It offers an alternative method to obtain target cells with inherent advantages compared to ESC or iPSC approaches. Furthermore, electrical stimulation can support the development and transdifferentiation of myocytes by improving skeletal muscle
morphology, accelerating striation formation and enhancing important muscular gene expressions without imposing significant stress and damage on cells.
Motoneuron Derivation and the Effects of Electrical Stimulation on Motoneuron Development

6.1 Introduction

Motoneuron, particularly lower motoneuron, is another main component in NMJ that propagates signals to transfer information from spinal cord to effector organs. Motoneurons are widely studied to investigate the development, synaptogenesis, coupling function and cell repair, both in monoculture and with other cell types such as muscle. Many research groups have studied cell interactions between motoneurons and muscle in in vitro NMJ models by utilizing primary motoneurons in the experiment. An advantage of using primary motoneurons is that the cell extraction process is relative simpler and required less time. However, disadvantages of using primary cell source are that cells had to go through harsh condition during enzymatic dissociation, which could cause major cell damage, and especially for motoneurons, heterogeneous mix of cells would likely be obtained from enzymatic separation. Stem cells are, therefore, preferable due to the superior characteristics, such as self-renewability and multipotency, making them an ideal candidate for in vitro models for disease study and drug screening. The ESC population is also far more reliable, originating from single clones instead of cells from the spinal cords of many litter mates. Moreover, the utilization of stem cells avoids the
problem of tissue source limitation and facilitates sufficient cell supply for repetitive or large-scale experiments. Importantly, stem cells allow genetic diversity in developing in vitro models as they can be genetically modified into specific disease models.

**Figure 19 Development and specification of motoneurons**

During gastrulation of early development, cell specification of the inner cell mass results in three germ layers such as ectoderm, endoderm and mesoderm. The dorsal region of the ectoderm is specified into the neuroectoderm via activin/bone morphogenetic protein (BMP) signaling inhibition and fibroblast growth factor (FGF) and Wnt signaling activation [124]. Then neural plate and neural folds are generated during neuralization process and they fuse to form
neural tube. Rostral neural progenitors develop into motoneurons in response to caudalizing signals, from molecules such as retinoic acid (RA), followed by the ventralizing activity of Sonic hedgehog (Shh) [125]. Understanding this developmental pathway has allowed the development of a protocol to promote motoneuron differentiation from ESCs through timed exposure to RA and Shh. Cells differentiated in such a manner express the LIM homeodomain proteins Isl1, Lhx3 and Lim1, which are normally associated with spinal motoneurons, as well as the motoneuron specific homeobox transcription factor HB9, indicating the development of a specific spinal motoneuron phenotype from within this population [125]. As demonstrated earlier, mouse ESC-derived motoneurons express cholineacetyl transferase (ChAT) and vesicular acetylcholine transporter (VACHT), indicating that these cells are able to develop a cholinergic transmitter phenotype [125, 126]. These cells also display passive membrane potentials comparable to those of E18 rat phrenic motoneurons and their electrophysiological responses to the neurotransmitters GABA, glycine and glutamate are likewise characteristic of embryonic motoneurons[126].

ESC-derived motoneurons can be purified and available in relatively vast quantities. A yield of 10 million motoneurons is not unusual using stem cell derivation technique while preparations from rat ventral horns usually yields just 2 to 3 million unsorted cells [9]. In this thesis work, magnetic-activated cell sorting (MACS) was chosen as a purifying method and it was performed using Miltenyi Biotec's MACS Technology, which used superparamagnetic nanoparticles (~ 100 nm) and columns. Motoneurons used in the study were differentiated from an ES cell line (HBG3) derived from a transgenic mouse line (mHB9-Gfp1b), in which enhanced green fluorescent protein (eGFP) and cluster of differentiation 2 (CD2) were expressed under the control of the mouse HB9 promoter. Antibody conjugated with magnetic nanoparticles was
designed to target CD2 on cell surface of derived motoneurons to capture them inside the column. The tagged cells were captured as the magnetic particle-cell complex passed through the column, which was placed between permanent magnets. Successful establishment and characterization of ESC-derived motoneurons of this culture model will allow smoother transition from animal model into developing human motoneuron-myotube cultures. This would greatly expand the use and relevance of this model for future research.

Because motoneuron is electrically excitable and the development is closely linked to their electrical activity, an integration of electrical stimulation to the culture should be advantageous. Many studies have shown that electrical stimulation can benefit neural development by guiding axonal growth [127], improving synaptic connectivity [128], and enhancing neural regeneration and reinnervation [129]. The goal is the optimization of electrical stimulation parameters that can lead to proper motoneuron development. The optimal electrical stimulation should attain a desired physiological response with minimal damage to the stimulated tissue and it should be designed to mimic the signal existing in nature under the conditions of interest, in this case, nerve signal. There are many variables in the electrical stimulation that can be regulated, for example, amplitude, frequency, stimulation duration, and waveform shape. Initiation and stimulation period also need to be considered. The efficiency of stimulation regime can be assessed by evaluating expression level of presynaptic factors, such as neuregulin and agrin. The well-defined characterization of stimulation effect will provide improved options for directing and fine-tuning functional engineered NMJ model.
6.2 Materials and Methods

6.2.1 Motoneuron Derivation

<table>
<thead>
<tr>
<th>ES cell medium</th>
<th>DFK5 medium</th>
<th>L15 medium</th>
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<tbody>
<tr>
<td>DMEM, high glucose</td>
<td>Advanced DMEM/F12</td>
<td>L15 solution</td>
</tr>
<tr>
<td>1X nonessential amino acids</td>
<td>1X nonessential amino acids</td>
<td>50 µM EDTA</td>
</tr>
<tr>
<td>1X nucleosides</td>
<td>1X nucleosides</td>
<td>4% BSA</td>
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<tr>
<td>0.1 mM 2-mercaptoethanol</td>
<td>0.1 mM 2-mercaptoethanol</td>
<td>25 mM glucose</td>
</tr>
<tr>
<td>2 mM L-glutamine</td>
<td>2 mM L-glutamine</td>
<td>2% Horse Serum</td>
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<tr>
<td>1% P/S</td>
<td>1% P/S</td>
<td>40 µg/mL DNase</td>
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<td>15% FBS</td>
<td>60 µM Putrescine</td>
<td>500 µg/mL Insulin</td>
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<tr>
<td>1000 u/mL LIF</td>
<td>20 nM Progesterone</td>
<td>0.01M Putrecine</td>
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<td></td>
<td>1X ITS</td>
<td>10 mg/mL Conalbumin</td>
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<td></td>
<td>10% Knockout Serum Replacement</td>
<td>30 µM Sodium Selenite</td>
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Table 4 Medium compositions for motoneuron derivation

Motoneurons used in the study were differentiated from an ES cell line (HBG3) derived from a transgenic mouse line (mHB9-Gfp1b), in which enhanced green fluorescent protein (eGFP) is expressed under the control of the mouse HB9 promoter. ES cell-derived motoneurons were obtained using methods described previously [125]. Briefly, HBG3 ES cells were grown on MEFs in ES cell medium. (Detailed compositions of each medium are provided in Table 4) After 2 days, ES cell colonies were partially dissociated and cultured in DFK5 medium. Medium was replaced after 2 days and supplemented with 1 µM RA (Sigma-Aldrich), 300 nM Shh (R&D Systems), and 1 µM hedgehog agonist (R&D Systems). Embryoid bodies (EBs) were cultured for 4–5 days before being dissociated and sorted using magnetic-activated cell sorting by cluster of differentiation 2 (CD2), which was engineered to express under the HB9 promoter. Upon dissociation and wash with L15 medium, cell suspensions were incubated with 80 µL of L15 medium and 10 µL of anti-rat CD2 antibody (Invitrogen; per 15 million dissociated cells) for 20
minutes at 4 °C. After washing again with L15 medium, cells were then incubated with anti-mouse magnetic microbeads (Miltenyi Biotec) for 20 minutes at 4 °C. Finally, cells were passed through a magnetic column to separate CD2-GFP-positive cells from the other types. Subsequently, dissociated cells were seeded in 24-well plate double-coated with poly-L-ornithine (Sigma-Aldrich) and laminin (Sigma-Aldrich) at a density of 750 cells/mm² and cultured in CM medium.

6.2.2 Electrical Stimulation
Stimulation plates were constructed from 12-well plate inserted with 4 pairs of carbon rods such that three wells in the same row received the same stimulation and carbon electrodes were spaced 1 cm apart for both bioreactors (Figure 20). Before plating cells, both stimulation plates were coated with poly-L-ornithine and laminin. After 2 days of pre-culture of motoneurons, carbon rods of bioreactors were connected to leads from an S88X Dual Output Square Pulse Stimulator (Grass Technologies) generating continuous pulses of 3 V, 1 Hz, and 2 millisecond duration corresponding to a field strength of 3 V/cm. Motoneurons were stimulated 6 hours per day for 6 days before samples were collected for analysis. The control groups in the bioreactors did not receive electrical stimulation. For additional study group, motoneurons received electrical stimulation, but 0.5 µM of TTX was added to the culture medium.

6.2.3 Current Clamp for Action Potential Recording

Motoneurons were seeded on glass coverslips double-coated with poly-L-ornithine and laminin. Astrocytes were also added to promote motoneuron survival and development. A few days after cell plating, motoneurons were identified based on GFP expression and action potentials recorded using conventional whole-cell current-clamp technique. Patch pipettes were fabricated from 1.5 mm outer diameter, 1.28 mm inner diameter filamented capillary glass (World Precision Instruments) with 2-5 MΩ resistance when filled with the pipette solution described below. Current protocol generation and data acquisition were performed using pClamp10 software and a Digidata 1550 digital to analog converter (Molecular Devices). Traces were filtered at 10 kHz using a low-pass Bessel filter, and digitized at 10 kHz. All recordings were performed at room temperature and are corrected for a calculated liquid junction potential of -14 mV. Internal solution contained the following: 130 mM potassium methanesulfonate, 10 mM sodium methanesulfonate, 10 mM HEPES, 10 mM EGTA, 1 mM CaCl₂, 5 mM MgATP,
and 0.3 mM NaGTP, and pH was adjusted to 7.4 using KOH. External solution was Ringer’s solution containing the following: 145 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM glucose, 2 mM CaCl₂, 2 mM MgCl₂. pH was adjusted to 7.4 using NaOH and osmolality adjusted to 325 mOsm with sucrose.

6.2.4 Statistical Analysis

Quantitative data were presented as mean ± standard deviation. RT-PCR results were presented as a fold change relative to GAPDH. Statistical significance was evaluated with the ANOVA with p = 0.05 and n = 5 biological replicates.

6.3 Results

6.3.1 Functional motoneurons are successfully derived from mouse embryonic stem cells

Motoneurons used in the study were derived from HBG3 ESC line through the differentiation pathway shown in Figure 21A. Because GFP and CD2 were genetically engineered to express under the control of the motoneuron specific promoter HB9, they allowed simple identification of motoneurons in the culture and the use of MACS to isolate the cells. The functional maturity of motoneurons was assessed by whole-cell current clamp in coculture with astrocytes (Figure 21B). Motoneurons were able to fire APs as early as on day 2 of the culture (Figure 21C), but these initial signals were weak and cells could not produce continuous trains of APs. By day 4, motoneurons could consistently generate a robust firing of APs, indicating mature functional development of motoneurons.
6.3.2 Electrical stimulation escalates important neuronal gene expressions and its effect is abolished by the presence of tetrodotoxin in the culture

After 6 days of electrical stimulation, expression of neuronal genes in stimulated motoneurons was compared to the control group receiving no stimulation and to the motoneurons receiving stimulation in the presence of tetrodotoxin (TTX) in culture medium. qRT-PCR showed significant increases in the levels of brain-derived neurotrophic factor (BDNF) and tropomyosin receptor kinase B (Ntrk2) genes in the stimulated group relative to the
Figure 22 Effects of electrical stimulation on motoneurons

(A) qRT-PCR analysis showed normalized expression levels of important neuronal genes of motoneurons found in control, electrical stimulation with TTX in culture medium and electrical stimulation groups. (B) Calcium transients during electrical stimulation. The trace consisted of 3 x ~60 seconds recording spliced together. Two electrical stimuli of 0.2 seconds apart were delivered every 10 seconds. The first section was the control, second section was in the presence of TTX and the last section was after 5 minute wash. (C) LIVE/DEAD fluorescence images showed the viability and morphology of motoneurons in culture. Scale bar: 100 µm. Quantitative data presented as mean ± SD. Statistical significance was evaluated with the ANOVA (*p < 0.05, n = 5 biological replicates).
control group of motoneurons. The fold change expressions of those genes were more than twice as high as expressions measured for the control group (Figure 22A). The levels of synapsin, a gene involved in the regulation of neurotransmitter release and neuromuscular synapse maturation [130], also showed a trend of increase over levels measured for unstimulated neurons, but the difference was not statistically significant. The presence of TTX in the culture suppressed the effect of electrical stimulation on these genes.

Calcium imaging revealed calcium transients during electrical stimulation in different conditions (Figure 22B). Motoneurons responded well to electrical stimulation as evidenced by calcium fluxes synchronizing with electrical stimulation. After adding TTX to the culture, calcium activities due to electrical stimulation were gradually blocked and they were completely suppressed after about 40 seconds. Following the washout of TTX and medium replacement, motoneurons responded to electrical stimulation at the pre-TTX treatment level. LIVE/DEAD fluorescence images showed viability of motoneurons cultured in different conditions (Figure 22C). Even though more dead cells were found in the electrically stimulated motoneurons and those that were stimulated and cultured in TTX, the extent of dead cells was not substantial. Motoneurons from these groups showed comparable degrees of growth and axonal spreading.

6.4 Discussion

Motoneurons derived from HBG3 ESC line were genetically engineered to express GFP for easy cell tracking and CD2 to allow the use of MACS for cell purification. CD2, a surface antigen of the T-lymphocytes, was chosen because it is a well-described epitope that is immunologically tractable. CD2 is not trypsin sensitive, which is important since trypsin was used during dissociation of EBs. Moreover, CD2 is not expressed in neural cells [131], which were the target cells to purify. Importantly, cells expressing CD2 have not showed any detectable
phenotypes when compared to parental lines [132]. Under the conditions established in this study, motoneurons were functionally mature after only 4 days of culture, as indicated by robust firing of APs (Figure 20C). The conditions and duration of neural maturation were appropriate, because motoneurons would be mature by the middle of the coculture experiment, allowing sufficient time for functional coupling of skeletal muscle and motoneurons during NMJ formation.

Because motoneurons are electrically excitable and the development is closely linked to their electrical activity, it was hypothesized that the implementation of electrical stimulation will be beneficial for functional coupling of motoneurons. We confirmed that electrical stimulation leads to upregulation of motoneuron-specific genes, such as Bdnf, Ntrk2 and Syn2. Adding TTX, a voltage-gated sodium channel antagonist, to the culture medium of motoneurons diminished calcium transients induced by electrical stimulation and abolished the effects of electrical stimulation on important neuronal gene expressions, confirming that the upregulation of neuronal genes was in fact triggered by electrical stimulation.

In summary, the results demonstrated that motoneurons derived from ESC using MACS were structurally mature and functional within 4 days. This information helped with design and duration of the coculture experiment. Additionally, the electrical stimulation regime used to stimulate skeletal muscle was safe and beneficial to motoneuron culture and development, and therefore, should be incorporated into the coculture bioreactor system.
Establish a Stable and Functional Coculture Protocol and Characterization of In Vitro Neuromuscular Junction Models
7 Effects of Different Culture Media on Neuromuscular Junction Development

7.1 Introduction

NMJ model is a useful system that has facilitated researchers’ investigation on the development and cell-cell interactions of NMJ, specifically synaptogenesis, function, maintenance and remodeling. NMJ has been widely studied to elucidate homeostasis and pathology of not only peripheral, but also central nervous systems thanks to its large size and easy access. Currently, there are many in vitro NMJ models, but limited success has been achieved in developing a stable, well-characterized and functional in vitro system for NMJ. Many research groups have created in vitro coculture systems using combinations of cell lines, primary cells, and stem cell-derived neural and muscle cells. In particular, NMJ models were established in a serum-free culture of rat primary cells [63], coculture of human stem cell-derived motoneurons and rat skeletal muscle [64], low-density coculture of mouse stem cell-derived motoneurons and muscle cells [65], and coculture of motoneurons and myotubes generated from the same line of human induced pluripotent stem cells [66]. Because coculture medium must support both motoneurons and muscle cells in the coculture, proper medium should be able to maintain both cell types so they are healthy and able to develop into functional NMJs. Some of the criteria are: (1) myocytes must be maintained in a low level of serum to induce myotube formation and maintain long-term culture; (2) the culture medium must be
supplemented with necessary growth factors and cytokines to improve finicky MN survival; and (3) the medium should be able to support NMJ development and functional coupling between motoneurons and muscle. The efficiency of coculture media will be assessed by evaluating the following cell characteristics, including maturation, differentiation, and functional integration of NMJ. The well-defined coculture medium will offer significant improvement on functional engineered NMJ model.

7.2 Materials and Methods

7.2.1 Cell Culture for Coculture Medium Study

![Figure 23 Timeline for coculture medium experiment and the components of each coculture medium]
C2C12 was first seeded at 250 cells/mm² in GM and cultured for 2 days. Then motoneurons were added to the coculture at 750 cells/mm². Three coculture media, including CM, Combined and CMHS, were used in the experiment to assess their ability to sustain cell survival and support the growth and formation of NMJ. The culture was maintained for 8 days in each medium before sample collection for subsequent studies such as immunostaining, contraction study, PCR and western blot. The coculture medium was changed every other day.

7.2.2 Statistical Analysis

Gene expression levels and pharmacological potency were analyzed using GraphPad Prism 7. Results were represented as mean ± standard deviation. Statistical significance levels were set to p = 0.05.

7.3 Results

7.3.1 Proper medium supplements support better morphological development and function of neuromuscular junction

Motoneurons and myocytes were cultured in the coculture media for 8 days and we found more myotube formation in the Combined and CMHS groups compared to the CM group. In addition, the colocalization of AChR clusterization and motoneurons and their axons were more distinct in the coculture that was maintained in CMHS medium. Noted that the moderate degree of clusterization was also observed in the Combined group. Importantly, muscle contraction was more widespread and stronger in the CMHS and Combined groups compared to the CM group (Supplementary Video S6). The contraction was observed as early as day 4, compared to day 7-9 in the monoculture of C2C12 (Supplementary Video S7).
7.3.2 Optimal medium composition upregulates important gene and protein expressions

PCR results showed the expression levels of important muscular and neuronal genes found in cells cultured in different coculture media. Among the three coculture media, CMHS generally led to the significant upregulation of gene expression levels, including AChR, MuSK, Myogenin, NT5 and TNNT3, followed by Combined and CM. Noted, the expression levels found in CMHS groups are more than two folds of those found in CM. In addition, combined medium increased the expression of level of GDGF to more than 4 times of the expression of the
CM group, followed by the CMHS group. The results from western blot further supported the outcomes found in PCR. CMHS and Combined media led to the significantly higher protein expressions of MuSK, myogenin, tropomyosin and MHC, compared to CM medium. CMHS also upregulated the expression level of AChR, but the increase was not statistically significant. Overall, the results from PCR and western blot might suggest the favorable effects of CMHS and Combined media in the development of NMJ.

Figure 25 Expression levels of important neuromuscular genes found in cells cultured in different medium

Figure 26 Expression of important neuromuscular proteins found in cells cultured in different medium
7.4 Discussion

Table 5: Summary table shows effects on different culture medium on the coculture

<table>
<thead>
<tr>
<th>Group</th>
<th>Morphology</th>
<th>Gene Expression Level</th>
<th>Specific Proteins</th>
<th>Contraction</th>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MIX</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CMHS</td>
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Table 5 summarizes the results found in the experiment. CMHS seems to be the optimal coculture medium out of the three media because it could improve the morphology of NMJ as shown by the formation of myotubes and the colocalization of AChRs and the axons of motoneurons. CMHS also led to early and strong contraction of the coculture and importantly, resulted in the significant upregulation of important neuromuscular genes and proteins as demonstrated by the PCR and western blot. Therefore, this study provides an excellent basic protocol for the culture system of an in vitro NMJ model and CMHS will be used for the subsequent coculture experiments in this thesis.

A potential explanation for the observed beneficial effects of CMHS might be attribute to the supplemented serum and other growth factors. Serum was added to the coculture medium to support myocyte differentiation into myotube and long-term culture. The coculture medium was also supplemented with many growth factors because motoneurons are notoriously difficult to maintain in vitro [9, 108]. Evidence suggests that motoneurons in vivo need to make contact with their target muscle fiber as well as with Schwann cells, glial cells and interneurons in order to receive the necessary positive signals to survive [109]. Conventional in vitro cultures obviously
lack this complexity and as such, cultured motoneurons do not receive the necessary secreted factors to promote their survival. Therefore, culture medium heavily supplemented with growth factors and supplements based on the distribution of the cognate receptors during muscle development and neuromuscular junction formation is more likely to better support the proper development of NMJ. Moreover, we aimed to culture motoneurons for at least 6-7 days, allowing cells to be fully mature and able to function based on the previous experiment on motoneuron monoculture where motoneurons could consistently fire APs in as early as 4 days, indicating its maturity and functionality.

In addition, we tried to use two medium recipes for the coculture system, in which the first medium contained growth factors such as BDNF, GDNF, NT 3/4 and CNTF, and the second medium was similar to the first medium, but without growth factors. The rationale was that switching of media from enriched medium 1, which ensured the survival and growth of motoneurons and muscle cells, to medium 2 enabled the reciprocal induction between motoneurons and myotubes that naturally occurs in NMJ development [64]. Mature muscle cells should be able to secrete BDNF, GDNF, and NT 3/4 to support motoneuron survival and attract neurite outgrowth, as well as provide distinct signals to organize the formation, maturation, and maintenance of motor nerve terminals. Moreover, motor axons should secrete neuregulin and agrin to increase local AChR synthesis and aggregation, and ACh to stabilize and refine the synapses. However, the two medium recipes for the coculture system could not sustain survival and growth of myotube and motoneurons, unlike CMHS medium. A possible explanation might be that the secreted growth factors from cells in the culture might not be sufficient to maintain the coculture.
8 Characterization of *In Vitro*

Neuromuscular Junction Models and an Application in Pharmacological Study

8.1 Introduction

*In vitro* NMJ model is a powerful cell biology tool to study NMJ formation and the role of different growth factors, hormones and cellular structures involving in synaptogenesis. The creation of a validated model will provide not only myriad insight about formation, regeneration, and maintenance of NMJ, but also useful information of synaptogenesis in general, allowing the NMJ to serve as a model for smaller and less-accessible central synapses. In this study, the established *in vitro* NMJ models will be validated by assessing their responses to therapeutic agents known to affect particular mechanisms during neurotransmission.

During synaptic transmission, a transferase enzyme, choline acetyltransferase (ChAT) synthesizes ACh from acetyl CoA and choline, which is brought into the cytoplasm from the synaptic cleft and extracellular space via choline transporter. ACh is packaged into neurotransmitter vesicles by ACh transporter. Neurotransmitters are situated in the readily releasable pools of vesicles near the presynaptic terminal of motoneurons. During synaptic vesicle fusion, neurotransmitter vesicles from reserve pools are tethered to the membrane. Then the v-SNARE and t-SNARE proteins transiently associate in a calcium-independent manner to
Figure 27 Mechanisms involved during neurotransmission

dock the vesicles, which are later primed and complexins stabilize the primed SNARE-complex, rendering the vesicles ready for rapid exocytosis. When the membrane at the synapse is depolarized by an AP, voltage-gated calcium channels around active zones will open, allowing an influx of Ca$^{2+}$ down its electrochemical gradient. Ca$^{2+}$ binds to synaptotagmin 1, dislodging complexin protein and allows the vesicle to fuse with the presynaptic membrane to release ACh neurotransmitter. ACh then diffuses across synaptic cleft to bind to nicotinic AChRs, ligand-gated ion channels, on the postsynaptic muscular membrane. When ACh binds to AChR, it induces a conformational structural change, which forces the pore open, allowing for the flow of positive ions (Na$^+$, K$^+$ and Ca$^{2+}$) down their respective concentration gradients in a non-specific
manner. This eventually triggers APs as well as contraction in the muscle. The effect of ACh in the cleft is terminated by an enzymatic hydrolysis via acetylcholinesterase (AChE), reverting the receptor to its resting state, blocking further ion flow. Choline is later recycled back to the motoneuron’s axon terminal for ACh synthesis.

In the experiment, five therapeutic agents were used to validate the established in vitro NMJ models. These drugs target different pathways during neurotransmission and they are useful in validating the function as well as the sensitivity of the established models. These agents include:

1. Acetyllethylcholine mustard hydrochloride, a precursor for ethylcholine mustard aziridinium ion, inhibits the synthesis of ACh by irreversibly inhibiting ChAT. In case of NMJ, the upstream blockade leads to the inhibition of neurotransmission and motoneuron’s inability to induce muscle contraction.
2. Vesamicol is an experimental drug, which is categorized as a cholinergic physiological antagonist since it decreased the activity of cholinergic neurons. The drug reversibly blocks the intracellular vesicular acetylcholine transporter (ChAT) responsible for transporting ACh into neurotransmitter vesicles in the axon terminal of motoneurons. Inhibiting ACh loading results in empty vesicles fusing with motoneuron presynaptic membranes, leading to reduced release of ACh into the synaptic cleft.

3. Botulinum toxin A (BoT-A) is a neurotoxin produced by *Clostridium botulinum* and it is one of the most lethal toxins on earth. The active form of the toxin protein composed of a heavy chain polypeptide joined via disulfide bond to a zinc metalloprotease light chain polypeptide
BoT-A enters an axon terminal and vesicles of motoneurons by binding to the presynaptic nerve terminal using its heavy chain. As the vesicle moves farther into the cell, the light chain is activated, pushing itself across the vesicle membrane into the cytoplasm to cleave SNAP-25, a member of the SNARE protein family, which is responsible for fusion, preventing the cell from releasing vesicles of neurotransmitter and thus, stopping neuromuscular transmission [134]. The effect of BoT-A will be gradually reversed as the toxin loses activity and the affected motoneurons slowly regenerate the SNARE proteins.

4. Pancuronium, an aminosteroid muscle relaxant, is a non-depolarizing curare-mimetic agent that blocks neurotransmission by competitively and reversibly binding to the nicotinic AChRs at the NMJ to inhibit the receptor activation by ACh.

5. Neostigmine is a medication used to improve muscle function in patients with myasthenia gravis and to reverse the effects of non-depolarizing muscle relaxants at the end of an operation. Neostigmine increases neuromuscular activities by binding to the anionic and esteric sites of AChE and therefore, inhibiting the breakdown of acetylcholine in the synaptic cleft. More ACh can bind to thepostsynaptic receptors and the depolarizing effect will also last longer.

If the NMJ models respond to the drugs in a dose-dependent manner, the potency such as half maximal inhibitory concentration (IC\textsubscript{50}) and half maximal effective concentration (EC\textsubscript{50}) can be evaluated and compared to the values in the literature to further validate and improve the usefulness and physiological relevance of the \textit{in vitro} models. This study is the first to validate the \textit{in vitro} models using therapeutic agents that affect neuromuscular transmission. Two models will be evaluated: skeletal muscle derived from C2C12 mouse myoblasts and ESC-derived motoneurons, and skeletal muscle transdifferentiated from MEFs and ESC-derived motoneurons.
We hypothesize that the established model can capture the important characteristics of NMJ and will respond to the neuromuscular drugs in the expected and dose-dependent manners. If successful, we will be the first group to demonstrate a potential application of the in vitro NMJ models in drug potency study.

8.2 Materials and Methods

8.2.1 Cell Culture

Figure 30 Timeline for coculture of ESC-derived motoneurons and MEF-derived myocytes

Reprogrammed myocytes from MEFs were obtained as described previously with slight modification. 2 days after transduction, cells cultured in differentiation media supplemented with Dox were electrically stimulated with continuous pulses of 3 V, 1 Hz and 2 ms duration for 3 days, 6 hours per day. After 3 days, motoneurons were added to the culture and cells were cultured in the CMHS coculture media supplement with Dox for 1 day before initiating another electrical stimulation. The coculture was stimulated with the same stimulation regime 6 hours per day for 6 days before analysis.

8.2.2 Glutamate-Induced Contraction

Figure 31 Timeline for glutamate-induced contraction experiment
Glutamic acid (Sigma-Aldrich) was prepared by dissolving in water with the help of vortexing. The pH was later adjusted to 7.4 to be compatible with cell culture. Contraction of the coculture was stimulated by adding glutamate solution with final concentration of 0.75 mM and was usually visible after a few minutes.

### 8.2.3 Pharmacological Potency Testing

Pharmacological testing was performed at the end of the coculture. Drugs used in the study were prepared as stock drug solutions in water or 100% dimethylsulphoxide (DMSO) and sonicated if necessary to dissolve the sample. For the assays, the stock solutions were further diluted to the appropriate concentration using coculture medium. The DMSO concentration in the culture wells with the highest drug concentration did not exceed 1% to avoid cytotoxicity. Coculture was exposed to neostigmine (Sigma-Aldrich) and pancuronium (Sigma-Aldrich) for 10 minutes and to acetyethylcholine mustard hydrochloride (Sigma-Aldrich), BoT-A (List Biological Laboratories) and vesamicol (Sigma-Aldrich) for 12 hours before being stimulated by glutamate (Sigma-Aldrich) with final concentration of 0.75 mM. After 5 minutes, coculture activities were recorded for contraction analysis.

### 8.2.4 Contraction Frequency Measurement

Contraction in coculture was recorded after drug exposure on the Olympus IX81 using the Pike camera at 75 frames per second via the SPLASSH software. The duration of a single recording was 30 seconds. At the beginning of each recording session, contrast and brightness of
the video image were optimized and the heated stage was kept at 37 °C during microscopic video recording. Videos were processed by a custom MATLAB software that correlated the changes of brightness between subsequent video frames in the region of interest with the frequency of muscle contractions [135].

8.2.5 Statistical Analysis

Contraction frequency of the coculture was quantified using MATLAB R2016b. Gene expression levels and pharmacological potency were analyzed using GraphPad Prism 7. Results were represented as mean ± standard deviation. Statistical significance levels were set to p = 0.05.

8.3 Results

8.3.1 Clusterization of AChRs in the vicinity of motoneurons and glutamate-induced contractions indicate the formation of functional NMJ

After 8 days of coculture, motoneurons extended their axons to myotubes to make synaptic contacts with skeletal plasma membrane (Figure 33A). The muscle/motoneuron interactions resulted in the formation of NMJ and in muscle contractions (Supplementary Video S8). In addition, cells were immunolabeled to examine cellular and protein colocalization in the coculture. Myocytes were stained with α-actinin and α-bungarotoxin for actinin and AChRs, respectively. Motoneurons formed neuronal networks in coculture and AChRs aggregated near motoneurons, indicating maturation of AChR clusters and NMJ formation (Figure 33B). To prove that the contractions were caused by synaptic transmission, the cocultured cells were stimulated using glutamate. Muscle contractions were observed shortly after adding glutamate to the system, suggesting that functional NMJ was formed (Supplementary Video S9). Contraction
NMJ formation and function. (A) A screenshot from a contraction video (See Supplementary Video S8) showed the interaction between myotubes and axons of motoneurons via NMJ after 8 days of coculture. An asterisk indicated a location of NMJ formation between axons of motoneurons and plasma membrane of myotubes. (B) Fluorescence image showed the localization of motoneurons and myotubes in coculture. Motoneurons formed a neuronal network and induced the clusterization of AChRs (showed by white arrows) in their vicinity, indicating properly developed myotubes and formation of NMJ. Red, green and magenta represent AChRs, motoneurons and actinin, respectively. Scale bar: 100 µm. (C) Normalized nicotine and glutamate-evoked calcium traces from calcium imaging on myotubes and motoneurons (See Supplementary Videos S9, S10). Nicotine could stimulate only myotubes while glutamate could trigger calcium flux only in motoneurons. Quantitative data presented as mean ± SD (n = 399 ROIs for myotubes and n = 105 ROIs for motoneurons).

Figure 33 NMJ formation and function.
of myotubes could only have resulted from neurotransmitter release at the NMJ due to motoneuron activation by glutamate. Glutamate could not directly stimulate myotubes as shown by calcium traces (Figure 33C), calcium transient recordings (Supplementary Videos S10, S11), and contraction videos (Supplementary Videos S12, S13).

8.3.2 Induced contraction by glutamate yields less variability in contraction frequency

The frequency distribution of spontaneous contraction and glutamate-induced contraction of the coculture were compared. Spontaneous contraction has an average contraction frequency of 0.56 Hz with standard deviation of 0.40 Hz while glutamate-induced contraction has an average contraction frequency of 1.15 Hz with the standard deviations of 0.25 Hz. It demonstrated that glutamate-induced contraction was less varied as shown in the following figure. The induced contraction showed a relatively normal distribution while the histogram of spontaneous contraction frequency was more dispersed. The results were even more noticeable when box and whisker plot was created. Induced contraction by glutamate is, therefore, preferable to spontaneous contraction as it is more controlled and should yield more reliable measurement for contraction frequency and in turn, lead to a more accurate evaluation for drug potency based on muscle contraction in later experiment.

8.3.3 The NMJ model responded to drugs affecting neurotransmission in a dose-dependent manner

To demonstrate utility of the NMJ model, the cocultures were used to study the potency of pharmacological agents involved in NMJ signal transmission and function. To this end, we examined all five interconnected mechanisms including ACh synthesis, ACh vesicular storage, ACh synaptic release, AChR activation, and ACh inactivation in the synaptic cleft (Figure 35A). Different concentrations of drugs were added to the coculture, with a specific exposure time set
Figure 34 Histogram and box and whisker plot of contraction frequencies of spontaneous and glutamate-induced contraction

for each drug, and contraction videos were recorded and analyzed. Dose–response curves exhibited concentration-dependent responses of NMJ to pharmacological agents. Specifically, acetyethylcholine mustard hydrochloride, a precursor for ethylcholine mustard aziridinium ion, blocked the synthesis of ACh and decreased the contraction frequency of the myotubes, with the half maximal inhibitory concentration (IC$_{50}$) of 1.29 µM (Figure 35B). Vesamicol, a vesicular acetylcholine transporter (AChT) inhibitor, prevented filling of synaptic vesicles with ACh and
inhibited neuromuscular transmission, with IC\textsubscript{50} of 113.60 nM (Figure 35C). BoT-A inhibited neurotransmission by preventing ACh release. BoT-A cleaved SNAP-25, a t-SNARE protein essential for neurotransmitter vesicle fusion with the plasma membrane of motoneuron terminals, with IC\textsubscript{50} of 69.07 pM (Figure 35D). Pancuronium interfered with NMJ function by reversibly and competitively binding to AChRs and thereby reducing the contraction frequency of skeletal muscle, with IC\textsubscript{50} of 10.71 µM (Figure 35E). Neostigmine enhanced neuromuscular activities by inhibiting acetylcholinesterase (AChE), a potent enzyme that catalyzed ACh, and terminated synaptic transmission, with the half maximal effective concentration (EC\textsubscript{50}) of 0.375 µM (Figure 35F). Similar experiments were performed to assess the potency of these drugs using motoneurons derived from mouse ESCs and skeletal muscle transdifferentiated from MEFs. Acetyethylcholine mustard hydrochloride had an IC\textsubscript{50} of 1.33 µM (Figure 36B), vesamicol had an IC\textsubscript{50} of 98.08 nM (Figure 36C), BoT-A had an IC\textsubscript{50} of 50.01 pM (Figure 36D), pancuronium had an IC\textsubscript{50} of 5.337 µM (Figure 36E) and neostigmine had an EC\textsubscript{50} of 0.461 µM (Figure 36F).
Figure 35 Pharmacokinetic studies of various drugs affecting NMJ formed from motoneurons and C2C12.

(A) A schematic shows the different cellular mechanisms involved in neuromuscular transmission. (B), (C), (D), (E), (F) Dose-dependent responses of coculture contraction frequency exposed to different drugs. (B) Acetylcholylcholine mustard hydrochloride blocked the synthesis of acetylcholine (ACh) by irreversibly inhibiting choline acetyltransferase (ChAT). A half maximal inhibitory concentration (IC$_{50}$) was 1.29 µM. (C) Vesamicol inhibited vesicular acetylcholine transporter (AChT) and prevented filling of synaptic vesicles with ACh. Its IC$_{50}$ was 113.6 nM. (D) Botulinum toxin A (BoT-A) prevented ACh release by cleaving SNAP-25, a t-SNARE protein essential for fusion of neurotransmitter vesicles with the plasma membrane of motoneuron terminals. Its IC$_{50}$ was 69.07 pM. (E) Pancuronium blocked neurotransmission by reversibly and competitively binding to acetylcholine receptors (AChRs). Its IC$_{50}$ was 10.71 µM. (F) Neostigmine increased neuromuscular activities by inhibiting acetylcholinesterase (AChE). Its half maximal effective concentration (EC$_{50}$) was 0.357 µM. Quantitative data presented as mean ± SD, n = 6 biological replicates.
Figure 36: Potency studies of various drugs affecting NMJ formed from motoneurons and transdifferentiated myocytes.

(A) A schematic shows the different cellular mechanisms involved in neuromuscular transmission. (B), (C), (D), (E), (F) Dose-dependent responses of coculture contraction frequency exposed to different drugs. (B) Acetyethylcholine mustard hydrochloride blocked the synthesis of acetylcholine (ACh) by irreversibly inhibiting choline acetyltransferase (ChAT). A half maximal inhibitory concentration (IC$_{50}$) was 1.22 µM. (C) Vesamicol inhibited vesicular acetylcholine transporter (AChT) and prevented filling of synaptic vesicles with ACh. Its IC$_{50}$ was 98.08 nM. (D) Botulinum toxin A (BoT-A) prevented ACh release by cleaving SNAP-25, a t-SNARE protein essential for fusion of neurotransmitter vesicles with the plasma membrane of motoneuron terminals. Its IC$_{50}$ was 50.01 pM. (E) Pancuronium blocked neurotransmission by reversibly and competitively binding to acetylcholine receptors (AChRs). Its IC$_{50}$ was 0.501 µM. (F) Neostigmine increased neuromuscular activities by inhibiting acetylcholinesterase (AChE). Its half maximal effective concentration (EC$_{50}$) was 0.461 µM. Quantitative data presented as mean ± SD, n = 6 biological replicates.
8.4 Discussion

<table>
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<th>Pharmacological agents</th>
<th>Pharmacological potency in C2C12/motoneurons NMJ model</th>
<th>Pharmacological potency in transdifferentiated myocytes/motoneurons NMJ model</th>
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<tr>
<td>AF-64A</td>
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<td>Vesamicol</td>
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<td>IC$_{50}$ = 98.08 nM</td>
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<tr>
<td>Neostigmine</td>
<td>EC$_{50}$ = 0.357 µM</td>
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</table>

Table 6 A summary table comparing drug potencies deriving from two *in vitro* NMJ models

This study is the first to create a functional *in vitro* NMJ model using ESC-derived motoneurons and myotubes derived from C2C12 and MEF-transdifferentiated myocytes to demonstrate the utility of the *in vitro* NMJ models in evaluating the pharmacological potency of drugs affecting neuromuscular synaptic communication. The coculture protocols are slightly different between the two models, for instance, sequences of media because we have to adjust the media regimen to facilitate the reprogramming of MEFs into myocytes for the skeletal muscle component of the NMJ model. Moreover, we believe that the unconverted fibroblasts did not substantially affect the development and the performance of the NMJ because we did not observe significant differences in the morphology, gene expressions and drug potencies between the two models. As shown previously, both cocultures could form functional NMJ models and effectively capture the functionality during synaptic transmission and the drug-induced response. Importantly, they exhibited a concentration-dependent response to the applied drugs and their potencies for each drug are comparable as shown in Table 6.
However, comparing the potency results from the NMJ models to the values found in literature was challenging because there is limited data of drug potency testing in *in vitro* NMJ models and the data obtained from other models still have sizeable ranges of potency values as shown in Table 7. The ranges are wide as these numbers were derived from different models using different cell sources, such as *in vitro* cell-based model models, *in vivo* models or human. In general, the results were within the cited ranges. Moreover, the model was very sensitive to drug concentrations as it could detect the concentration less than 1 nM in case of BoT-A. These encouraging results support the potential use of the established models in drug screening and cytotoxicity testing.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC$_{50}$ Values from Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylethylcholine mustard hydrochloride [136-138]</td>
<td>0.9 µM - 5.1 µM</td>
</tr>
<tr>
<td>Vesamicol [139-141]</td>
<td>14.7 nM - 0.17 µM</td>
</tr>
<tr>
<td>BoT-A [134, 142, 143]</td>
<td>25 pM - 2 nM</td>
</tr>
<tr>
<td>Pancuronium [144-147]</td>
<td>5.5 nM - 5.62 µM</td>
</tr>
<tr>
<td>Neostigmine [148-152]</td>
<td>0.23 nM - 10 µM</td>
</tr>
</tbody>
</table>

Table 7 IC$_{50}$ values of the drugs used in the study from literature search

The excitation–contraction coupling mechanism in skeletal muscle begins with the propagation of an AP along the plasma membrane and the T-tubule system. Once dihydropyridine receptors (DHPRs) detect changes of membrane potential, they will interact with sarcoplasmic reticulum ryanodine receptors, causing release of Ca$^{2+}$. Intracellular Ca$^{2+}$ then binds to troponin, allowing actin-myosin interaction and muscle contraction. A spontaneous contraction is a property of the contractile system at low actin-myosin affinity. Even low Ca$^{2+}$ or
changes in ionic strength can have effects on actin-myosin interactions [153]. Intrinsic contractions take place independent of synaptic activities. Its frequency and intensity are more diverse and are not under our manipulation; the distribution of contraction frequency is more dispersed when compared to induced contraction via glutamate stimulation. This is our underlying rationale for choosing the frequency of induced contraction instead of spontaneous contraction. According to the histograms and box and whisker plot showing distribution of contraction frequency in Figure 34, the deviation of induced contraction was less, leading to smaller variation of the measurement. A potential mechanism of intrinsic contraction involves the spontaneous activation of a T-type calcium current in the sub-threshold range of the membrane potential, which raises the membrane potential to the AP threshold and the release of Ca^{2+} stored in the sarcoplasmic reticulum [102]. It was shown that most spontaneous contractions of rat myotubes in culture were blocked by 100 nM TTX. This suggests that Na^+ play an important role in the DHPR-mediated detection of changes in membrane potential at the level of the T-tubules [102]. Optogenetics can be utilized to study calcium transients during contraction and further investigate the mechanism of action [154]. On the other hand, electrically induced contraction occurs when electrical stimulation directly depolarizes the membrane of muscle cells and motoneurons, where increased voltages cause an activation of voltage-gated ion channels, leading to an influx of extracellular Na^+/Ca^{2+} and allowing the membrane potential to reach the threshold easier. These activities can be blocked pharmacologically using Na^+ or Ca^{2+} channel blockers. We did not directly measure the intensity or the force of contraction, but we hypothesize that the force induced by electrical stimulation should be higher as more muscle fibers could be depolarized by electrical stimulation, leading to increased activation of voltage-gated ion channels, APs and contractions. For the dissertation, the emphasis is on the NMJ-
driven contraction, which relies mostly on the synaptic activities and communication between motoneurons and muscle. Depolarization and APs in motoneurons causes the release of ACh neurotransmitter from axon terminals to the synaptic cleft, which later binds to AChRs on the skeletal plasma membrane, resulting in membrane depolarization and muscle contraction. The synaptic activities can be selectively isolated in the coculture experiment by various pharmacological interventions, including, inhibiting ACh synthesis, blocking ACh storage into neurotransmitter vesicles, preventing the release of ACh from the neurotransmitter vesicles into the synaptic cleft and inhibiting the binding of ACh to the AChRs on the postsynaptic muscle membrane. These interventions should not affect intrinsic activity of skeletal muscle and that is why we selected pharmacological agents that affect these pathway to evaluate the functionality of the NMJ in vitro models.

In summary, we established an in vitro NMJ model that incorporated electrical stimulation and performed systematic analysis to show the formation and function of the NMJ. The model provides a simple and robust method for analyzing pharmacological potency using drug-induced contraction frequency to quantify cell response to different concentration of applied drugs. The proposed NMJ model also offers application in therapeutic agent screening, cytotoxicity testing and disease modeling to study the relative roles of motoneuron and muscle in neurodegenerative diseases.
IV Final Perspectives
9 Perspectives

9.1 Overall Conclusions

NMJ is widely studied as a model to investigate synaptogenesis and pathophysiological development of neurodegenerative diseases [55-58]. To date, coculture methods established from various species have been described, including mouse [155-157], rat [158, 159] and heterologous cocultures derived from motoneurons and muscle cells of different species, such as rat-human [160], mouse-human [161] and mouse-chick [162]. These cocultures, however, resulted in the formation of immature myotubes and sarcomeric structures. Moreover, these models did not incorporate important factors intrinsic to the native physiological niche of NMJ, and the utility of the in vitro models of NMJ for investigating the pharmacological potency of drugs affecting synaptic communication has not been demonstrated.

The goal of this thesis research is to create a stable and functional in vitro model of NMJ for physiologic and pharmacologic studies. To this end, we established a coculture protocol for NMJ formation from motoneurons and myocytes with the application of developmentally relevant electrical signals. We first determined the optimal culture medium that can support proper growth and development of both the muscle and motoneurons. Serum and supplemented growth factors, based on important cognate receptors during NMJ formation, were essential for development and long-term maintenance of NMJ. In addition, we evaluated the optimal electrical stimulation to further enhance the biomimicry and physiological relevance of the model while
ensured that the stimulation did not elicit detrimental effects on cell survival. Many studies have shown that electrical stimulation can promote muscle cell maturation, orientation, and differentiation leading to greater contraction of myotubes [92], and to benefit neural development by guiding axonal growth [127], improving synaptic connectivity [128], and enhancing neural regeneration and reinnervation [129]. An optimal electrical stimulation should attain a desirable physiological response with minimal damage to the stimulated tissue. The stimulating voltage of 3V was chosen because based on our study, 3V did not cause significant cell death compared to the control, unlike 5V and 7V, which led to substantial death of myocytes. In addition, a frequency of 1 Hz and a pulse duration of 2 milliseconds were utilized because they were adequately long to depolarize cells and also sufficient for the double layers on stimulation carbon electrodes to dissipate between subsequent pulses [94]. Monophasic square-wave pulses were chosen due to the simplicity and compatibility with carbon rod electrodes [95]. Electrical stimulation was introduced to the muscle/motoneuron culture to mimic the native NMJ niche, where electrical signals are vital for NMJ development and function. We confirmed that electrical stimulation led to upregulation of motoneuron- and muscle-specific genes and improved cellular structures. A possible explanation for the beneficial effects of electrical stimulation is that the sub-threshold stimulation provided electrical activities that were absent in an in vitro culture environment, but needed in the activity-dependent development of motoneurons and myotubes. We hypothesized that positive effects were attributed to increased calcium transients and contractions. Even though the stimulation regimen used in the study could not directly induce contraction, an increase in cell depolarization due to electrical stimulation could lead to more contractions. When TTX was added to the coculture, induced calcium transients as a result of electrical stimulation and contraction activities were significantly
suppressed. The reduction of spontaneous contraction activities and calcium transients by TTX can potentially explain why the benefits of electrical stimulation were abolished, further substantiating our hypothesis regarding activity-dependent benefits of electrical stimulation.

This study is the first to create a functional *in vitro* NMJ model using ESC-derived motoneurons and MEF-transdifferentiated myocytes to demonstrate potential application of the NMJ model for testing potency of pharmacological agents that affect mechanisms involving in neuromuscular transmission. The coculture effectively captured the drug-induced response and functionality during signal transduction, and it exhibited a dose-dependent response to the applied drugs, allowing the evaluation of therapeutic potency, including $IC_{50}$ and $EC_{50}$. The model was also very sensitive to drug concentrations as it could detect the concentration less than 1 nM in case of BoT-A. The experiment is reproducible and could be used as a cytotoxicity platform in clinical applications. In conclusion, the optimized coculture medium implemented in conjunction with electrical stimulation resulted in a biomimetic model that effectively captured the critical characteristics and functions of the NMJ.

### 9.2 Applications, Limitations and Future Work

*In vitro* models will always have a place in drug screening and cytotoxicity testing because of the high throughput capability and simplicity. This is particularly true for 2D model even though 3D culture might be more biomimetic. For example, the established *in vitro* NMJ models offer an alternative method to test the median lethal dose (LD$_{50}$) of BoT-A. Currently, the potency testing of some BoT-A products for therapeutic or cosmetic applications is based on an *in vivo* LD$_{50}$ assay, which evaluates the amount of BoT-A needed to kill 50% of a group of mice that have been injected intraperitoneally [163]. For each batch of BoT-A, at least 100 mice are used to assess the LD$_{50}$ level and the toxin causes diffuse muscular paralysis, impaired vision
and paralysis of the diaphragm, followed by suffocation and death of mice [164]. Manufacturers use the LD\(_{50}\) to grade the strength of their products and the in vivo method has frequently brought up controversies regarding ethical issues involving animal experiments. The criticism is even sterner as BoT-A is widely used for cosmetic procedures, instead of therapeutic applications. Currently, many countries ban the testing of cosmetic products and ingredients on animals, but since BoT-A is also used in therapeutic treatment, it is classified as medicine and can be tested on animals. In addition to the ethical concerns, there are other downfalls associated with the mouse LD\(_{50}\) assay as the bioassay is inherently variable and has a large intra/inter-laboratory variability [165]. The mouse LD\(_{50}\) assay has also been found inadequate for evaluating differences in the potency of different manufacturer’s products, suggesting that the assay is inefficient at detecting some relevant factor [166]. Lastly, the mouse assay takes 1-4 days to provide results, delaying production, diagnosis and detection applications that rely on this assay. Consequently, an alternative method to replace the in vivo mouse LD\(_{50}\) assay is in dire need for both the ethical concerns over the animal use and the scientific concerns over the inaccurate animal bioassay, especially now where there is growing demand and production of BoT-A products. A number of alternative in vivo, ex vivo, and in vitro methods for BoT-A potency testing have been in the development and some are validated as shown in the following table. We believe that our cell-based NMJ models can offer a simple and reliable platform to assess the LD\(_{50}\) level because the results showed reproducible dose-dependent responses of the models to BoT-A. Our models are also very sensitive as it can detect BoT-A at the concentration less than 1 nM. In addition, the use of stem cells allows large-scale experiments and minimizes genetic variation among assays, creating a unified standard for the LD\(_{50}\) testing. Therefore, we believe that the established in vitro models of NMJ can provide utility in cytotoxicity testing,
leading to a more systematic, accurate and humane assay for the potency and quality control tests used to produce BoT-A products.

<table>
<thead>
<tr>
<th>Name of Test</th>
<th>System</th>
<th>Endpoint</th>
<th>Duration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse hind-limb assay</td>
<td>in vivo</td>
<td>local paralysis</td>
<td>2 days</td>
<td>Pearce, et al., 1994; 1995a; 1995b; Roger Aoki K., 2002; Aoki, 2001</td>
</tr>
<tr>
<td>Mouse abdominal pteosis assay</td>
<td>in vivo</td>
<td>local paralysis</td>
<td>&lt; 1 day</td>
<td>Takahashi et al., 1990a; 1990b; Sesardic, et al., 1996</td>
</tr>
<tr>
<td>Mouse phrenic nerve-hemidiaphragm assay</td>
<td>ex vivo</td>
<td>muscle contraction</td>
<td>&lt; 1 day</td>
<td>Dressler, et al., 2005; Bigalke, et al., 2001; Bigalke, 2001; Kalandakanand and Coffield (2001; 2001);</td>
</tr>
<tr>
<td>SNAP-25/Endopeptidase assay</td>
<td>in vitro</td>
<td>molecular disruption of nerve transmission</td>
<td>&lt; 1 day</td>
<td>Ekon et al., 1997; Hallis (1996); Schmidt, et al. (2001); Wietome et al., 1999; Ferraciti, et al., 2005;</td>
</tr>
<tr>
<td>Endopep-MS</td>
<td>in vitro</td>
<td>molecular disruption of nerve transmission</td>
<td>&lt; 1 day</td>
<td>Boyer et al., 2005; Barr, et al., 2005; Kalb, et al., 2005</td>
</tr>
<tr>
<td>Human foot muscles extensor digitorum brevis</td>
<td>in vivo</td>
<td>muscle contraction</td>
<td>1-7 days; up to 90 days</td>
<td>Bigalke, 2001; Bigalke, et al., 2001; Eleoacta, et al., 2002; Jost, et al., 2005</td>
</tr>
</tbody>
</table>

Table 8 Alternative methods to the in vivo mouse LD50 assay for BoT-A
Adapted from Stephens, Martin L. Test Method Nomination to the ICCVAM, 2005.

*In vitro* models can also play an important role in disease models, especially during an early stage of investigation because cell-based models allow the use of patient-derived cells, and the system is high throughput, well defined and tunable to answer different applications [167]. For example, a study conducted by Yoshida, et al. used SMA patient-derived iPSCs to create an *in vitro* model of NMJ to evaluate the efficacy of therapeutic candidate, valproic acid and antisense oligonucleotide, and investigate pathophysiological mechanisms of SMA [58]. The models could provide researchers with a fundamental understanding of disease development and
enable collection of potentially predictive data of experimental treatments before advancing to animal studies and clinical trials [52-54]. With more awareness of neuromuscular diseases, increase in resources dedicated to NMJ-related research and continuing progress in cell biology, genetics and regenerative medicine, ongoing effort to find effective treatments is heading in a promising direction.

There are limitations found in the NMJ models created in the study. First, even though the current models could demonstrate dose-dependent responses to the applied drugs and allowed drug potency calculation, there are some flaws with the design of the experiments for pharmacological testing that could be practically addressed to improve the outcome of the study. For example, the induced contractions of the coculture were measured 5 minutes after adding glutamate and the limitation of this approach is that the characteristic activities due to the effect of the drugs might not be effectively captured as the activities might already become plateaued. To better assess neuromuscular activities, contraction should be measured right after glutamic stimulation. Better yet, additional data of calcium activities or electrophysiology would further provide comprehensive insight into the effects of the drugs. In addition, a more defined system can be created by applying optogenetic techniques [168-170]; motoneurons can be genetically engineered to express channelrhodopsin proteins, light-activated cation channels, which enable light to regulate depolarization, calcium transients and other cellular processes. To derive motoneurons with channelrhodopsin proteins, channelrhodopsin gene can be introduced into ESCs by gene delivery techniques, such as transduction and transfection. The ESCs will then be differentiated to motoneurons following the process described earlier in the study. An advantage of optogenetics is the ability to selectively activate motoneurons, resulting in cleaner results as glutamate, though not significant, can also affect muscular activities. The system can provide a
contactless and more defined way of investigating pharmacological effects on NMJ transmission.

To better mimic the intrinsic NMJ milieu, other beneficial factors in addition to electrical stimulation can be incorporated, for example, mechanical stimulation, topological manipulation and substrate stiffness. Even though 2D culture offers simple and high-throughput platform for drug testing, 3D in vitro models can encompass these additional features to further replicate the in vivo mechanical and chemical microenvironment capable of facilitating cell maturation and neuromuscular function [155, 171, 172]. For instance, a controllable in vitro model of the NMJ can be created from encapsulation of both muscle and motoneurons into micropatterned hydrogels. We can apply bioengineering techniques to precisely control the factors of interest in the cellular environment, including the composition and biomechanics of the hydrogel, geometry of patterning and the application patterns of electrical and mechanical stimuli. We hypothesize that the provision of an in vivo-like environment directing the organized growth of motoneurons and myocytes will enhance functional coupling of the cells and enable quantitative studies of regulatory factors, underlying mechanisms and a more physiologically relevant systems for drug screening.

Another aspect of the current models that can be further improved in future studies is an integration of microfabricated coculture platform for motoneurons and skeletal muscle to better mimic the native environment of NMJ. In the body, motoneurons are morphologically separated from, but functionally connected with skeletal muscle. Therefore, we can design the biomimetic coculture platform such that axon terminals of motoneurons can interact with myotubes and form NMJ, but motoneurons and myotubes are cultured in separate compartments. The potential design of the coculture platform is composed of two culture compartments connected to each
other through 8 microconduits. The conduits allow only axons of motoneurons to go through since the size of the microchannel is smaller than the size of motoneuron cell body (10-20 µm). The compartments and microconduits are situated on microelectrode arrays (MEA) substrate with multiple electrodes (small blue circles), which can stimulate and record the electrical activity of the coculture system. The expected dimension is 2 cm X 2 cm. Having separate compartments for motoneurons and myotubes not only mimics the native state, but also allows easy monitoring. We can easily locate motoneurons and muscle, and selectively stimulate certain type of cells without significantly interfering another. An advantage of MEA substrate and multiple electrodes is that non-invasive and multisite stimulation and recording system with novel interface circuit modules are possible. This allows not only flexible selection of stimulation sites, but also rapid switching of the selected sites between stimulation and recording. As a result, almost continuous monitoring of extracellular signals at all the substrate-

Figure 37 Potential design for coculture platform
embedded electrodes, including those used for stimulation, is possible. Moreover, the platform will be compatible with high-throughput BioMEM (microelectromechanical) devices, enabling the integration of this system with the next generation of high-throughput screening technologies.

In our study, cells are derived from mouse, including mESC-derived motoneurons, C2C12 myoblast cell line and MEF-derived myocytes. The main reasons for using murine cells are the convenience of cell sources, their ability to capture the key functionality of skeletal muscle, motoneurons and NMJ, and ease of manipulation, for example, MEFs are more amenable to cell reprogramming, compared to human fibroblasts [173]. However, mouse cells cannot fully recapitulate the clinical, physiological and biochemical manifestations of human cells [174, 175]. The results from mouse models, therefore, might not be accurately predictive of responses found in human. Potential factors contributing to possible discrepancy include interspecies variations in gene expression, anatomical and physiological differences. Nonetheless, mouse models should be able to recapitulate the general characteristics of NMJ and provide valuable insights regarding synaptogenesis and neuromuscular transmission.

To improve the physiological relevance of the model and bridge the gap between discovery research and clinical applications, human-based functional model is the logical next step and there are multiple potential cell sources. Motoneurons can be derived from stem cells or iPS cells of healthy individuals or patients with NMJ-related diseases [41, 176-180] and skeletal muscle can be derived from primary cells, satellite cells, stem cells or iPS cells of healthy individuals or patients with NMJ-related diseases [40, 173, 181-183]. Multitude of established techniques for human cell derivation broaden the versatility of the in vitro models such that they can be used in a variety of real-world applications, including drug screening or disease models. In the context of SMA, the model of neuromuscular interactions will serve as a basis for several
key mechanistic and translational approaches in the future, including extension of the model to
diseased cells by combining SMA ESC/iPSC-derived motoneurons [41] and primary or
immortalized muscle cells from SMA biopsies; validation of the relative role of muscles and
motoneurons in the pathogenesis of SMA, which can be important information for targeting of
therapeutic agents; testing of drugs, CRISPR/Cas9, microRNA and viral cDNAs/shRNAs for
their ability to correct the SMA phenotype; and patient-specific disease models and model
modification to represent different neuromuscular disease models, which will help defining new
potential therapeutic targets linked to neuromuscular dysfunction.

In summary, the proposed additional studies to overcome the shortfalls of the current
cocultures should materially improve and further equip the models with the state-of-the-art
features of an in vitro NMJ system, such as 3D model [155, 171] that offer better biomimetic
characteristics, an integration of optogenetic techniques [184], and the use of autologous cells for
homologous coculture systems, which hold promise for personalized disease therapies [58, 184].
All in all, NMJ model is essential for gaining acumen in the development of NMJ, the
pathogenesis of NMJ-related diseases and the screening of therapeutic candidates. This research
combined with the continuing progress in the field of genetics and regenerative medicine can
potentially constitute an important platform in the next generation of cytotoxicity screening,
system biology and drug discovery.
V Bibliography and Appendices
Bibliography


96. Maidhof, R., et al., *Biomimetic perfusion and electrical stimulation applied in concert improved the assembly of engineered cardiac tissue*. Journal of tissue engineering and regenerative medicine, 2012. 6(10).


Appendices

Journal publication as a culmination of the thesis work can be accessed at

Charoensook S, Williams D, Chakraborty S, Leong K and Vunjak-Novakovic G. Bioreactor
Model of Neuromuscular Junction with Electrical Stimulation for Pharmacological Potency

Online supplementary videos available at
https://www.dropbox.com/sh/ey910l0c2yayqbw/AAAVUSho1dKo3U2V4cnBkB-ka?dl=0.