A Co-culture System of C2C12 and MN1 for Neuromuscular Junction (NMJ)

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Abstract

With an increasingly aging population in the world, there are more people suffering from neurodegenerative diseases. This kind of situation creates an urgent need to research more on the factors causing neurodegenerative diseases and methods to treat and prevent neurodegenerative diseases. My project is aimed to establish a muscle/neuron, C2C12 and MN1, co-culture system mimicking neuromuscular junction (NMJ) where molecular, biochemical and immunological studies like qPCR, enzymatic detection and immunofluorescence can be performed in order to provide an in vitro platform to address various biological questions related to the disease progression or treatment. One of the immediate questions of my interest is how cellular retinoic acid-binding protein 1 (Crabp1) and Ca2+/calmodulin-dependent protein kinase II (CaMKII) may play roles in NMJ formation and plasticity at the cellular and molecular level. Understanding this question will provide insight into potential new therapeutic strategies for managing NMJ-related neurodegenerative diseases.

Keywords:

Neurodegenerative diseases, Motor neuron, Skeletal muscle, In vitro models, Synapse, Neuromuscular junction (NMJ).

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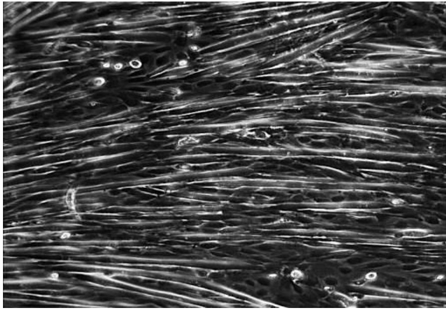

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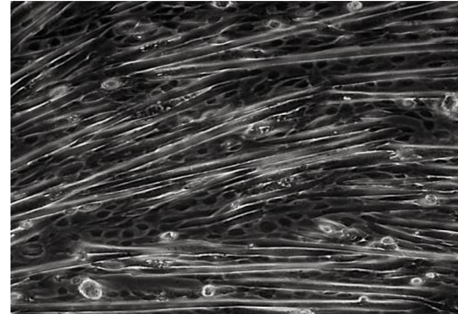

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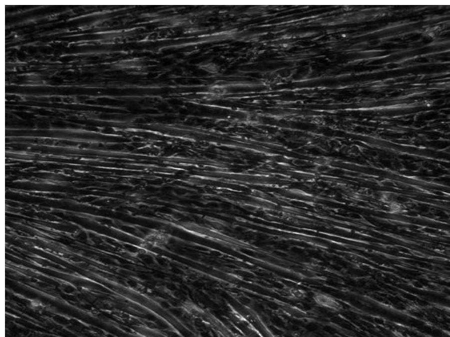

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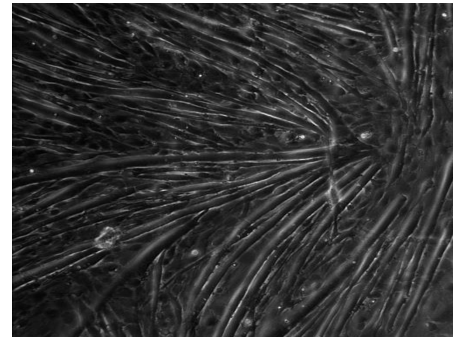

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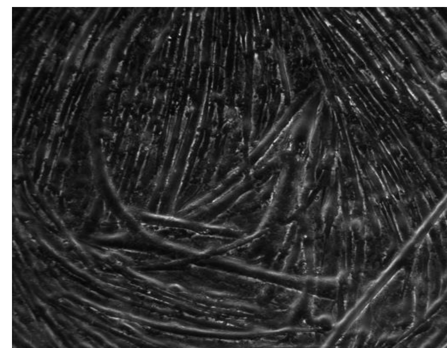

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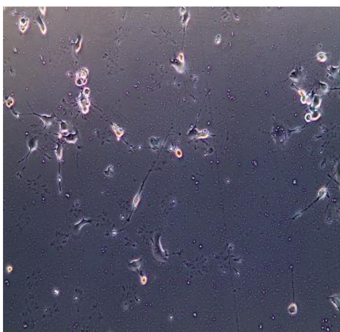

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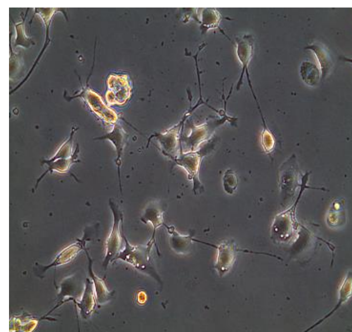

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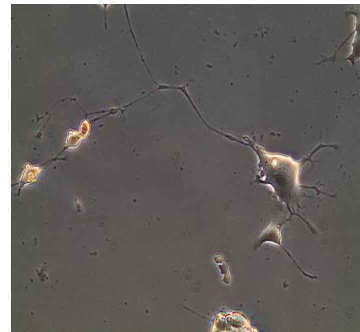

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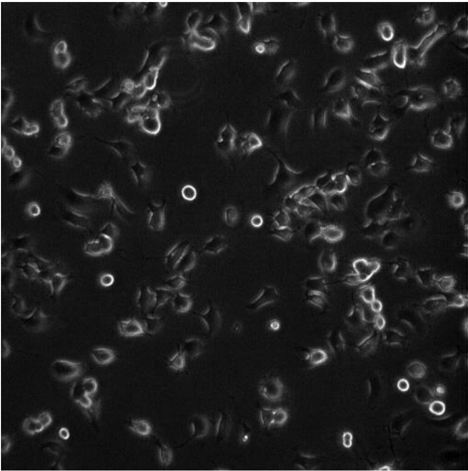

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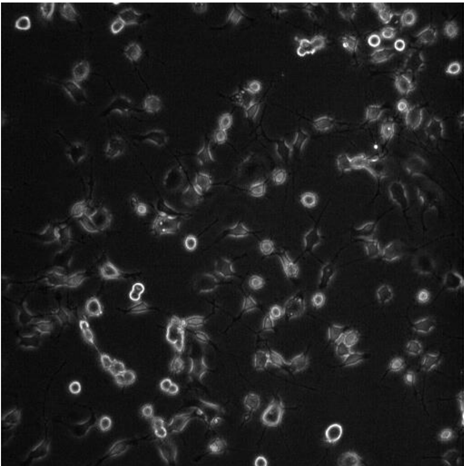


I. Introduction

i. Neurodegenerative diseases

Neurodegenerative diseases are chronic and progressive neurological disorders that could damage or destroy neurons' functions. There are many neurodegenerative diseases including Amyotrophic Lateral Sclerosis (ALS), Alzheimer's disease (AD), sarcopenia as well as Huntington's disease (HD). They have been big problems for public health and millions of people in the world are being affected by neurodegenerative diseases. Aging is a common factor that leads to many neurodegenerative diseases. If nerve cells that are located in any part of our body are injured or die, neurodegenerative diseases will occur. Following aging and neurodegenerative diseases, "old" cells can accumulate in the nervous system and people with this condition will show neurodegenerative-like symptoms. Current treatments for these diseases can only relieve some physical or mental problems but cannot slow or stop the disease progression. Old people are easier to be affected by neurodegenerative disease and this influence can be severe and dramatic. With the more aging population in the world, there are increasing people suffering from neurodegenerative diseases in the next few decades. This phenomenon reminds us of the importance and necessity to develop and apply new therapies and approaches for people with neurodegenerative diseases [1].

ii. Cellular retinoic acid-binding protein 1 (CRABP1)

Cellular retinoic acid-binding protein 1 (CRABP1) is a kind of highly conserved cytosolic protein that can bind to retinoic acid (RA). Crabp1 exerts its function by binding to retinoic acid (RA) which is a kind of metabolite of vitamin A and important in the central nervous system (CNS) development. After binding with retinoic acid, Crabp1 functions by recruiting signaling components such as those in the ERK pathway (for cancers) and the CaMKII pathway (for muscle and neuron). A recent paper shows Crabp1 is highly expressed in spinal motor neurons and the

Crabp1 level in patients with neurodegenerative diseases will decrease. This raises a question what kind of role Crabp1 plays in neurodegenerative diseases and how we can find the target and thus develop new therapies for them [52].

iii. Motor neuron

Motor neuron degeneration and dysfunction are common factors of many neurodegenerative diseases including amyotrophic lateral sclerosis and spinal cord injury as discussed above. And motor neuron recently becomes an important research target due to its involvement in many diseases. A motor neuron (or motoneuron) is a kind of neuron that will integrate directly or indirectly with effector organs (muscle and glands). The structure of motor neuron consists of a cell body, axon, and dendrite. The main locations of the cell body are brainstem, motor cortex, and spinal cord. Axon and dendrite are firstly derived from the cell body and then project to cells inside the spinal cord or outside the spinal cord. Upper motor neurons and lower motor neurons are two kinds of motor neurons that are classified by their location and function. Upper motor neuron axons integrate directly or indirectly (through interneurons) with lower motor neuron and the lower motor neuron is a kind of efferent neuron which can transit signal from the spinal cord to effectors (mainly muscles and glands)[2][3]. All motor neurons of vertebrate animals are cholinergic neurons and use acetylcholine as a neurotransmitter and can carry signals from pre-synapse to post-synapse.

Neurogenesis is a process in which neural stem cells divide into differentiated neurons. It occurs in the embryo stage and does not stop developing until childhood. Fully differentiated neurons are no longer capable of mitosis and degeneration. After the beginning of motor neuron formation, the further specification will classify motor neurons to five kinds of columns. These five motor columns will be located in different regions in the spinal cord and thus target different muscles and glands [4][5][6]. By far, nearly all of the researches for neurodegenerative diseases are focused on clarifying what causes neurons to die and neurodegeneration and their processes.

But neurodegenerative diseases can be regarded as “chronic”, which means neurons will go through some abnormal processes before dying. Finally, neurons will die following a lot of abnormal biological processes since their vulnerability to many pathological conditions and they also possess plasticity. This phenomenon suggests it is better to clarify these abnormal changes at the onset of neurodegeneration rather than until the cells die. Thus, a MNs cell culture model which is amenable and easy to monitor is needed [7]. Some researchers use established cell lines to study neuron related diseases. For example, motoneuron-like cell line NSC-34 is a cell line produced by the fusion of neuroblastoma with mouse motoneuron-enriched primary spinal cord cells. It has been used as a motor neuron cell model, but its use is limited since its low sensitivity to excitotoxic stimulation [9]. MN1 is a cholinergic motor neuron cell line derived from a fusion of N18TG2 (mouse neuroblastoma cell line) with embryonic mouse spinal cord motor neurons. Mouse neuroblastoma cells make MN1 possess the ability to unlimitedly proliferate, which is important in vitro experiments.

iv. Skeletal muscle

Skeletal muscles are tissues that are constituted with many muscle fibers with the same orientation and high density. The muscle fibers are composed of multinucleated cells differentiated from myoblasts[10]. 40% of lean body mass is skeletal muscle. Skeletal muscles are important for nearly all the motor movements and decreased skeletal muscle could lead to morbidity and mortality in many pathogenic conditions [11]. With people growing older, skeletal muscle mass will decrease by 8% per decade, leading to lower mobility. And accelerated muscle degeneration more than 8% per decade compared with normal people will show up in patients with congenital or non-congenital skeletal myopathy[10][12]. Motor neuron also participates in the developmental process of skeletal muscle. The numbers of myotubes needed for normal function and the expression of many muscle-related genes are influenced and partially controlled by the connection with the motor neurons. In other words,

changes in presynaptic nerve terminals affect the interaction and thus the postsynaptic terminals [13].

1. Skeletal muscle development and differentiation

Skeletal muscle development is an important process that is complex but coordinated. Briefly, the process of development includes muscle precursor cells formation and differentiation, myoblast trans-location and differentiation, and cell fusion to myotube. During the process, satellite cells (a kind of muscle stem cells) firstly differentiate to myogenic precursor cells which are located in the paraxial mesoderm. Then myogenic precursor cells trans-locate to the place for function and paraxial mesoderm is separated into different somites that stay beside the neural tube. Subsequently, myoblasts are differentiated to myocytes and the fusion of myocytes finally constitutes myotubes with more than one nucleus. Supplemented with enough fibroblasts or other growth factors will induce the proliferation of myoblast while culturing in vitro. But if there are not enough growth factors, the myoblast will stop proliferation and start to differentiate until the myotubes are formed[13][14]. In vivo, there are many different kinds of extracellular matrix (ECM) surrounding the skeletal muscle cells. Collagen and laminin are two major components of the extracellular matrix (ECM) and they are important for many biological processes of muscle such as the cell morphology, cell-cell interaction, cell proliferation, and differentiation, and normal muscle structure and functionality. Skeletal muscle fibers are embedded in a complex extracellular matrix (ECM) composed of primarily collagen and laminin [10][12] [15][16]. In vitro, to better mimic the in vivo surroundings of skeletal muscle cells, researchers use different biomaterials such as collagens, alginate hydrogels, gelatin, and Matrigel to replace the ECM in both 2D and 3D models[10][17].

2. Skeletal muscle models

Currently, animal models are normally used as a model system to research on the normal structure and function skeletal muscle as well as muscle-related diseases or therapeutic development[19]. Animal models seem to be a good choice to test the

responses in the whole organism level, but they need higher expenses and the throughput is low. In addition, it is nearly impossible to get cellular and molecular information and cause-effect results using the animal model systems because confounding factors exist in the animal model system and they may interrupt the experiment results [12][18]. Thus establishing amenable, stable, and easy-to-control in vitro skeletal muscle models will provide new hope for treating and preventing many skeletal muscle-related human diseases as mentioned above. It is essential that we can use the in vitro skeletal muscle models to clarify and elucidate some basic knowledge of the skeletal muscle development, degeneration, disease, and identifies new therapeutic targets as well as therapeutics tests with the benefits mentioned before [10][18]. Skeletal myoblasts are cultured on the extracellular matrix (ECM)-coated plastic dish and are surrounded by medium supplemented with specific nutrients for proliferation or differentiation to construct the skeletal muscle models in vitro [21]. In the in vitro experiments, the extracellular matrix (ECM) is used to increase cell adhesion and myogenesis development. In the beginning, using a high-serum proliferation medium until about 90% confluence and change the medium from high-serum proliferation medium to low-serum differentiation medium. In the next several days, myoblasts differentiate to myocytes and form myotubes which have high expression of muscle-related markers. But the culturing skeletal muscle is easy to delaminate from synthetic culture dishes because of the severe contractile property of myotubes. This places a big challenge for mature myotube formation which needs long term maintenance [18][20]. Thus, two-dimensional (2D) in vitro skeletal muscle models are not good enough to maintain the contractile characteristic due to the delamination. This severely limits the use and application of skeletal muscle and neuromuscular junction (NMJ) in vitro culture models[18][20]. To engineer a model for a longer-term and mature myotubes maintenance to carry on researches on skeletal muscles and neuromuscular junction (NMJ), there are papers establishing 3D in vitro skeletal muscle models[10][22][23][24]. This kind of model enables skeletal

myoblasts to differentiate to skeletal myotubes on micropatterned gelatin hydrogels or other material that could provide 3D surrounding for myoblast differentiation[12]. There are many currently used scaffolds for 3D models such as electrospun polylactic acid (PLA) nanofibrous, bio-printed silk fibroin cantilevers and micropatterned gelatin hydrogels. They all can provide an environment that is highly similar to the in vivo surroundings with which the cells can interact with each other and attach to the scaffolds. The 3D models can not only mimic the property of extracellular matrix (ECM) but also help muscle development and longer maintenance of cell culture[10][22][23][24][25]. In the 3D surroundings of the exogenous materials mimicking extracellular matrix (ECM), skeletal myotubes can be maintained for multiple weeks compared to the normal synthetic plastic dish with higher muscle protein expression, accelerating sarcomere formation, more power of contractile property as well as up-regulation of muscle-specific or non-specific markers (controlling skeletal muscle development) and produces a structurally and functionally more matured myotube [23][26][27].

The major sources of in vitro skeletal muscle cells are primary myoblasts or proliferative cell lines such as the C2C12 cell line. C2C12 cell line is an established cell line of satellite cells from skeletal muscle of C3H mouse[15][16]. It is a widely used skeletal muscle cell source for muscle[28][29] and NMJ[30][31] models because C212 is easy to culture, and it can successfully differentiate to myotube in just several days. Currently, important findings of muscle function have been made via the C2C12 myoblast cell line. Sarcopenia is a kind of symptom that people will lose muscle while growing older and it is a severe medical problem for public health. Furthermore, the neuromuscular junction will go through functional or structural changes following aging, implying that the signaling pathways involved in neuromuscular junction maintenance might be interrupted along with muscle degeneration [13]. Thus, an in vitro skeletal muscle model is needed for the researches of related diseases.

v. Synapse and Neuromuscular junction (NMJ)

Synapse is the structure at which a nerve action potential transmits from one neuron to another. Synapses mainly include three parts: The presynaptic terminal which will release neurotransmitter; The synaptic cleft between the two cells; The postsynaptic membrane that contains receptors. Synapse is a basic structural and functional constitution of regulation of the nervous system. By releasing a kind of ingredient, the synapse can exert its signal transmission function to downstream cells. This ingredient is called neurotransmitter and it is normally stored in presynaptic vesicles [7]. This process would make successful communication between presynaptic and postsynaptic terminals and finally finish tasks our body needs to do. The postsynaptic terminal can be neuron cells to conduct neuron-neuron communication or muscle cells to instruct neuron-muscle-induced contraction at the neuromuscular junction (NMJ). Neuron system possesses high plasticity through a complex process and this process can modulate inside signaling pathways to keep the whole system in "normal" status even under abnormal pressure[32]. However, the normal structural and functional balance will be broken and cannot regenerate or repair which finally leads to neurodegenerative-like symptoms once the whole system is damaged of any component in the process of plasticity and predispose dysfunction or disruption of the neuronal pathway [7][32].

In the spinal cord, most of the motor actions including voluntary and involuntary actions depend on the sophisticated neuronal regulatory system. Different kinds of neurons participate in this complex and coordinated network and this phenomenon is called center pattern generators. There are many different kinds of cell types in the neuronal regulatory system such as interneurons, motor neuron and non-neuronal cells such as astrocyte and microglia cells. Astrocyte cells will promote the development of the regulatory network and microglial cells can act as a kind of immune cells to protect neurons against attack. Each kind of neurons finally integrates with motor neurons to activate or inhibit the action potential and

downstream motor neurons will integrate with muscles to regulate motor or other functions. Thus, it is important to clarify the NMJ mechanism which is mainly composed of motor neurons and muscle myotubes and would allow us to better understand its formation and development[33].

The neuromuscular junction (NMJ) is a specialized synapse between the motor neuron and skeletal muscle. It functions to fast and stably transmit the electrical signal from motor neurons to skeletal muscles and is one of the most studied synaptic structure because of its essential role in nearly all motor functions, such as locomotion, respiration and speech[13][25].

1. NMJ formation and development

The major parts of motor neurons are soma, dendrites and axons. The soma is located in the spinal cord and sending dendrites or axons to the surrounded environment and form neuromuscular junctions the muscles [13]. There are three basic components in the NMJ structure: (1) the presynaptic motor neuron terminal that contains synapse vesicles releasing the neurotransmitter; (2) the narrow synaptic cleft; (3) the deeply folded postsynaptic muscle terminal that harvesting the acetylcholine receptors. There are voltage-gated calcium and sodium channels in the presynaptic and postsynaptic terminals which can help the transmission of the action potential. There are also two other components participating the action potential transmission: Schwann cells (SCs, a kind of specialized glial cell, which is derived from neural crest cells) surrounding the presynaptic motor neuron terminal and wrap the axons of motor neurons to increase the impulse transmission speed and thus promise high efficiency of neural communication. Because SCs' importance in the regulation of the networks, it has been widely researched on NMJ-related structure, function and diseases, which provides us a more comprehensive overview of NMJ. And the basal lamina containing different kinds of proteins providing molecules for structural and functional development [13][35] [36]. In vertebrate voluntary muscles, this system uses acetylcholine (ACh) to be neurotransmitter and acetylcholine (ACh) binds with

nicotinic ACh receptor (AChR) located in postsynaptic membrane to exert its function. The acetylcholine (ACh) can be broken down later by ACh esterase (AChE) to timely stop the transmission. AChRs are composed of five protein subunits and it is called ligand-gated ion channels[34] [35]. Before the presynaptic motor neuron terminal integration, the AChRs appear to be thin and lower-grade clusters located in some areas of muscle fibers (muscle pre-patterning). When the muscle fibers contact with the presynaptic terminals, the AChRs will increase even it is useless and mature to a broader, more complex and higher-grade cluster. During the formation of NMJ, there is a big change of the structure called synapse elimination to eliminate the remaining useless synapses. And the morphology of AChRs also changes from oval plaques shape to induced, pretzel-like shape. During an embryonic stage, one muscle fibers may be integrated with more than one motor axons to make sure there is not muscle without its control neuron and it can be controlled by the right neuron. Two weeks after birth, synapse elimination happens according to the activity of neurons and muscles. The useless neurons are lost and one muscle fiber is integrated by only one axons. This process is important for the development of NMJ structure and function. After the synapse reduction, the remaining axons integrations are stronger and more functional which could provide stable and strengthened control of the nervous system to muscle contraction. With NMJs aging, AChRs are gradually fragmented and denervated that also appears in some disease models[36][37]. Receiving action potentials transited by upstream neurons, voltage-gated calcium channel will open and promotes the immigration of synaptic vesicles to the presynaptic membrane and Ach will be released to the middle cleft. Then Ach diffuses and binds with AChRs in the post-synaptic membrane to cause muscle contraction by calcium stimulation[13][36].

2. Neuromuscular junction (NMJ)-related diseases

The NMJ is necessary for normal motor functions and thus important for our life [35]. Neuromuscular diseases are a series of disorders where nerves or muscles get injured

and thus lead to abnormal function [38]. Neuromuscular junction (NMJ) has been studied in a lot of neurodegenerative diseases, which also called neuromuscular diseases [34]. There is no treatment or prevention therapeutics currently for NMJ regeneration.

Several characteristics of the NMJ are important and essential for the normal signal transduction: 1) motor neuron terminal size which is also related to active zones. 2) voltage-gated calcium channel numbers which is linked with synaptic vesicle migration. 3) Ach production for signal emission. 4) AChRs production for signal acceptance. 5) acetylcholine esterase (AChE) production for signal termination. Neuromuscular disease symptoms such as muscle weakness, decreased motor skills will show up if impairment of any component or structure happens and influence the transmission of the action potential[13]. The dysfunction can be originated from structural and functional deficits in the NMJ in part or as a whole. For example, Lambert–Eaton myasthenic syndrome is due to NMJ deficits[35], amyotrophic lateral sclerosis is due to soma deficits, muscular dystrophy is due to muscle deficits, brachial plexopathies is due to axons deficits[38][39].

3. Neuromuscular junction (NMJ) models

Building and maintaining NMJ is an urgent and important system because of the significant role it plays in neural communication and skeletal muscle contraction. In the past two decades, there is rapid progress in understanding the molecular mechanism of NMJ [13]. But it is difficult to clarify and manipulate the molecular mechanism between the motor neuron and skeletal muscle in vivo, which requires a more precise system for the cellular and molecular investigation[25][32]. On the contrary, in vitro models are easy and simple to control variables and clarify the pathways and analyze or interpret the experimental results[40]. Currently, there are many animal preclinical studies to evaluate potential therapeutics for neuromuscular diseases [41][42][43]. But most of the time, the evaluations are not satisfactory and the following clinical trials are failed. Because compared with in vivo animal models,

the in vitro NMJ coculture models will have better evaluation results and comparatively successful clinical trials, it will be advantageous to establish the in vitro NMJ coculture model. Using this kind of in vitro models constructed between motor neurons and skeletal muscles, we can study NMJ development at the cellular and molecular level for proliferation, differentiation and maturation. And it could be a robust and powerful platform to do all the tests under neurodegenerative disease conditions such as evaluate pharmaceuticals and get some information on drug parameters. In detail, using this kind of system, researchers found cholesterol-lowering statins that are often used may lead to the death of skeletal muscles and aggravate the peripheral neuropathy process and extent. And Ach, AChR, AChE synthesis has been explored for modulating NMJ function. In the future, there will be more related researches that will finally be used on new therapeutic development. In summary, methods that can be used to establish functional NMJ cultures are an urgent need [22][23][25].

The in vitro neuron and muscle co-culture was first tried at least a century ago. It is gradually applied to the motor neuron and skeletal muscle co-culture to carry on in-depth investigation [23]. To date, to establish this co-culture system, researchers have tried cells from different kinds of species in both 2D and 3D modes including mouse [44], rat [45], Xenopus[46] and chick[47]. Co-culture systems from different species of motor neurons and skeletal muscles are also developed, such as rat–human[48], mouse–chick[50], mouse–human[49] and human embryonic stem cell (hESC)-derived MNs-C2C12 myotubes[25].

Traditionally, 2D co-culture models have been established and tested [25][40][51]. But in recent years, following 3D skeletal muscle model development, researchers are trying to establish 3D coculture models [10][22][23][24]. 2D models have been tested and validated that it cannot precisely mimic the in vivo surroundings. Transiting 2D system to a 3D system is a significant breakthrough since cells grown on 3D

coculture systems are more mature regarding both the structural and functional aspects[22].

II. Aims and Hypothesis

1. Aim:

This project is aimed to establish a fresh coculture model for neuromuscular junction (NMJ) and thus provide a platform for exploring how cellular retinoic acid-binding protein 1 (Crabp1) and Ca2+/calmodulin-dependent protein kinase II (CaMKII) may play roles in NMJ formation and plasticity at the cellular and molecular level.

2. Hypothesis:

(1) C2C12 and MN1 can be good cell models to study neuromuscular junction (NMJ).

(2) The formed NMJ can be used in neurodegenerative disease-related research.

III. Experimental design and Methods

i. C2C12 cell culture and differentiation

C2C12 skeletal myoblasts were maintained in a proliferation medium containing Dulbecco's Modified Eagle Medium (DMEM) with high glucose, 10% fetal bovine serum and 1% penicillin-streptomycin were supplemented in the C2C12 skeletal myoblasts proliferation medium. C2C12 skeletal myoblasts were seeded on 6-well plastic plates coated with 0.1% gelatin solution (STEMCELL™ Technologies). The density of C2C12 for seeding is 80000 cells per cm2 and it was cultured under the condition of 5% CO2 atmosphere at 37 °C. To differentiate C2C12 myoblasts, firstly seeding and culturing the cells in the proliferation medium for 48 hours until cells became confluence. Then the culture medium was changed to two kinds of mediums for following experiments: (1) DMEM with high glucose supplemented with 1% horse serum and 1% penicillin-streptomycin (C2C12 differentiation medium); (2) DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) with GlutaMAX™ supplement and supplemented with 1% fetal bovine serum, 1% non-Essential Amino Acids (NEAA) and 0.5% penicillin-streptomycin (MN1

differentiation medium) with or without Y27632. After incubation for 4 and 8 days under the above medium, the samples were visualized using a microscope equipped with the Infinity Analyze imaging software and then proceed to RNA extraction.

ii. MN1 cell culture and differentiation

MN1 cells were maintained in a proliferation medium containing Dulbecco's Modified Eagle Medium (DMEM) with high glucose, 10% fetal bovine serum and 1% penicillin-streptomycin. MN1 cells were seeded on 6-well plastic plates coated with P-D-L (Cultrex®, R&D Systems), Geltrex (Gibco by Life Technologies™) or Matrigel. The density of C2C12 for seeding is 3000 cells per cm2 and it was cultured under the condition of 5% CO_2 atmosphere at 37 °C. To differentiate MN1 cells, firstly seeding and culturing the cells in the proliferation medium for about 12 hours until cells attached to the plastic surface. Then changing the culture medium to the following two kinds of mediums: DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) with GlutaMAX™ supplement and supplemented with 1% fetal bovine serum, 1% non-Essential Amino Acids (NEAA) and 0.5% penicillin-streptomycin with or without Y27632. After incubation for 4 days under the above medium, the samples were visualized using a Leica microscope equipped with the Infinity Analyze imaging software and then proceed to RNA extraction.

iii. C2C12 skeletal C2C12 and MN1 cells coculture

C2C12 muscle cells were proliferated and differentiated as described above. The only difference is that C2C12 were seeded on a plastic coverslip in the bottom of 6-well plates for the convenience of immunostaining. On the fourth day of C2C12 differentiation as described before, seeding MN1 cells on top of myotubes. Then changing medium to a motor neuron differentiation medium of DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) with GlutaMAX™ supplement and supplemented with 1% fetal bovine serum, 1% non-Essential Amino Acids (NEAA) and 0.5% penicillin-streptomycin (+Y27632) for the coculture set-up for 4 days co-culture.

iv. RNA extraction

1. RNA preparation

1.1 Wash C2C12 cell samples from 4 and 8 days after differentiation and MN1 cell samples from 4 days after differentiation three times using Phosphate-buffered saline (PBS).

1.2 Adding 0.5 ml of TRIZOL Reagent to each well of 6-well plates to lyse cells and pipette the cell lysate.

2. Phase separation:

Place the samples at room temperature for about 5 minutes. Then add 0.1 ml chloroform. Vortex the tubes and place them at room temperature for about 2 minutes. Centrifuge the samples at 12,000 g, 15 minutes, 4°C.

3. RNA precipitation

Pipette the upper aqueous phase out and then add 250ul 100% isopropanol to the aqueous phase. Place it for 10 minutes at room temperature. Centrifuge the samples at 12000 g, 10 minutes, 4°C.

4. RNA wash

Pipette the supernatant out and discard. Wash the pellet using 500ul 75% ethanol and briefly vortex. Centrifuge the tube at 7500 g, 5 minutes, 4°C. Vacuum dry the pellet.

5. RNA resuspension

Add non-RNase water to dissolve the RNA pellet. Place it for 15 minutes at 60°C. Store under -20°C if not immediately use.

v. PCR

The whole PCR process includes two processes: a reverse transcription reaction (RT-PCR) to produce cDNA from mRNA and qPCR amplification. The template for RT-PCR is mRNA using reverse transcriptase. After the production of single-stranded cDNA, it will be the template for qPCR. Special primers we need for the specific mRNA regions will optimize qPCR to get the product of our interest. This technique possesses high sensitivity and it just needs a small amount of sample.

(1) Reverse-Transcription PCR (RT-PCR):

Measuring the concentration of RNA using Nano Drop (Thermo Scientific™) and take 2ug RNA for each sample to PCR tube and add non-RNase water to the total volume of 10ul. Then add the mixture of the following Reverse-Transcription reagents per tube:

10 X PCR buffer	2ul
dNTP Mix, 100mM	0.8ul
10 X RT random primers	2ul
Multi-scribe reverse transcriptase	1ul
RNase inhibitor	1ul
RNase-free water	3.2ul

The mixture was incubated for 10min at 25°C, and two cycles for 60min at 37°C, then for 5min at 85°C. Dilute the solution 10 times before the next qPCR step.

(2) qPCR (quantitative PCR or Real-Time PCR)

Take 5ul sample from last step and the following 10ul mixture together to a PCR tube:

2 X SYBR buffer	7.5ul
Primer	0.5ul
ddH2O	2ul

(The 2 X SYBR buffer contains everything needed for amplification)

Denaturation at 95 °C for 10min, 40 cycles at 95 °C for the 30s and 60°C for 60s, then 95 °C for 60s, 55 °C for 30s and 95 °C for 30s, followed by a melt curve analysis. Target gene expression was assessed using the comparative method and all values were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

vi. Immunostaining

1. Retrieve 6-well plates with coverslips and place on ice to avoid severe contraction while fixation to maintain the morphology and location of the co-

culture system.

2. Rinse cells with non-sterile Phosphate Buffered Saline (PBS) for 5 minutes, three times.
3. Add 1 mL of 100% ice-cold methanol to each well on ice for 5 minutes for fixation.
4. Aspirate the fixing solution and rinse cells with PBS for 5 minutes, three times. Block specimen in 2% Bovine serum albumin (BSA, blocking buffer) for 60 minutes.
5. Prepare primary antibody by diluting in 2% BSA as following:
   mouse anti-sarcomeric α -actinin (Sigma, 1:200) primary antibody
   synaptophysin (DSHB; 1:200)
   Tuj-1 (abcam, 1:1000)
6. Pipette 200 μL primary antibody solution on the plastic coverslips under 4°C overnight.
7. Rinse the coverslips with PBS 5 minutes, three times.
8. Prepare secondary antibody by diluting in PBS as following:
   - a-Bungarotoxin-488 (1:500)
   - Donkey-anti-rabbit-594 (1:1000)
   - Donket-anti-mouse-Cy5 (1:400)
9. Pipette 200μL secondary antibody solution on the plastic coverslips at room temperature for 60 minutes.
10. Aspirate the secondary antibody and coverslip with DAPI (1:1000) for 10 minutes.
11. Rinse the coverslips with PBS for 5 minutes, three times.
12. For every coverslip, pipette a drop of the antifade mounting medium (1-1-1000) on a microscope slide and seal the coverslip.
13. Cure the slides 15 minutes at room temperature. Using the clear mail polish to seal the edges until it is dry. Store the slides in the dark.

14. Using a confocal microscope to capture images.

IV. Result

1. C2C12 myotubes can be maintained for at least 8 days.

Figure 1. C2C112-diff day 4-17

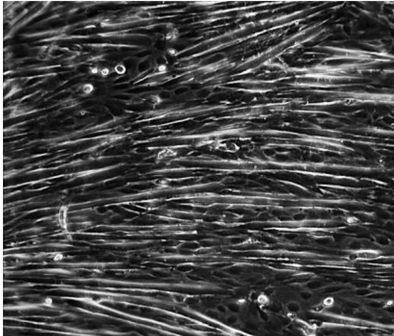
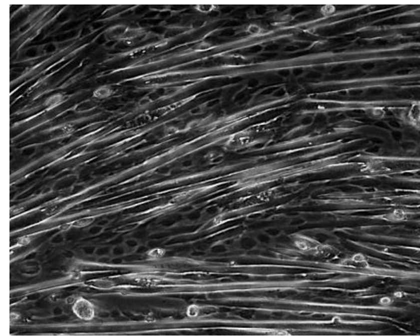


Figure 2. C2C12-diff day 8-17



2.1. MN1 differentiation medium is not supportive of myoblast differentiation compared to the C2C12 differentiation medium because the myotubes are thinner and broken using the MN1 differentiation medium.

2.2. Beside Skeletal muscle, there are two other kinds of muscles including cardiac muscle and smooth muscle. Sarcomeres in skeletal muscles are parallel bundles with each other, but sarcomeres in cardiac muscles are not parallel and they can branch with each other with irregular angles[14].

Y27632, as a selective Rho-associated protein kinase (ROCK), seems to push C2C12 cells to differentiate to a state in which the myotube morphology is similar to cardiac muscle.

Figure 3. MN-diff day 4-18

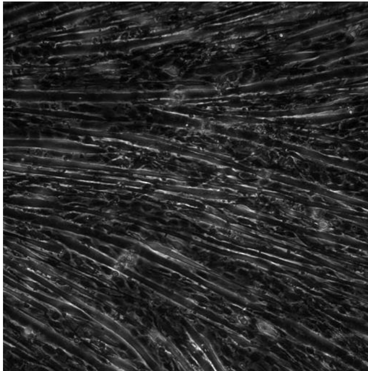


Figure 4. MN-diff + Y27 day4-18

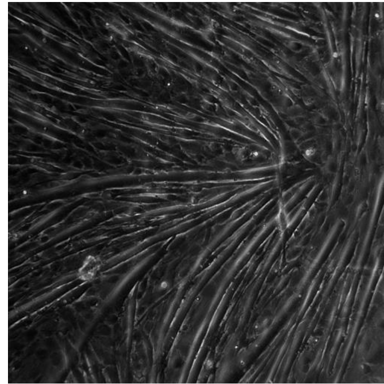
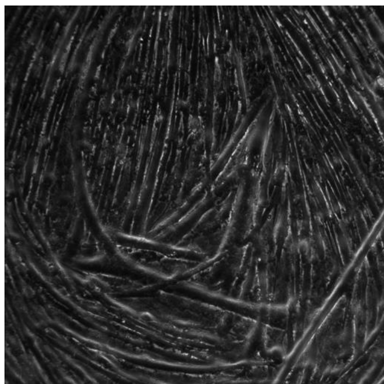


Figure 5. MN-diff day 8-18

Figure 6. MN-diff day 8 + Y27-18



3.1. MN1 cells can successfully project axons and dendrites after 4 days of differentiation.

Figure 7. MN1 differentiation medium day 4 (+Y27):

Figure 7-1-18

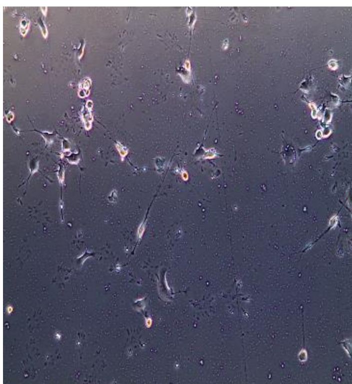


Figure 7-2-18

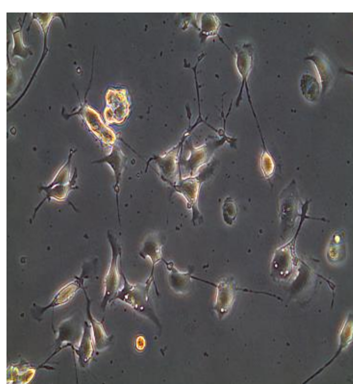
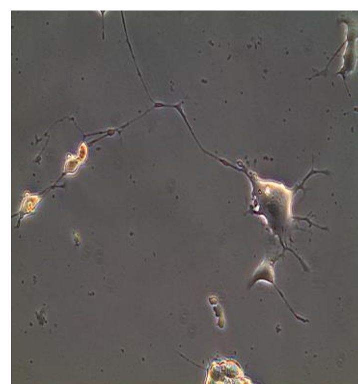


Figure 7-3-18



3.2. Y27632 promotes axons projection and elongation.

Figure 8-1. MN1 differentiation medium Day 4-19

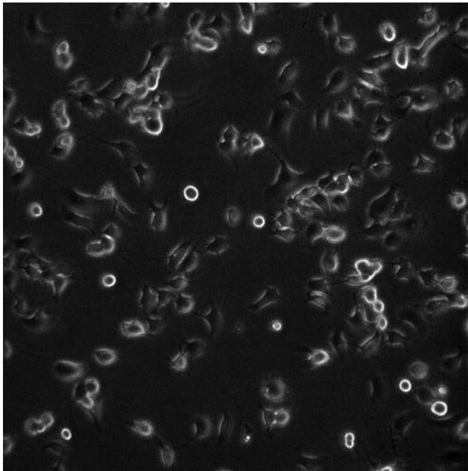
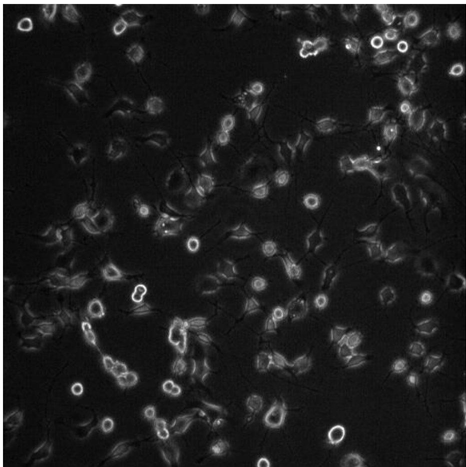


Figure 8-2. MN1 differentiation medium day 4 + Y27-19



V. Discussion

With the increasing aged population, neurodegenerative diseases that are associated with motor neuron and skeletal muscle dysfunction will manifest as a big medical problem for our society. Thus, a robust and controllable in vitro co-culture model of motor neuron and skeletal muscle is urgently needed for disease investigation and therapeutic development. Establishing new co-culture models will definitely provide new in vitro cell models and thus fresh resources and new opinion for neuromuscular disease research and its cure. But unfortunately, many models have their deficits to

be improved, which poses a great challenge for us but also provides us with a broader research space.

i. Cell sources

1. Skeletal muscle cell sources:

C2C12 cell line is an established cell line of satellite cells from skeletal muscle of the mouse and has been widely used in both muscle and NMJ models (as mentioned above). It is easy to culture and differentiates into myotubes in several days. But established immortal cell lines are less closely to myogenesis property than do primary myoblast, which will hinder the in-depth research of skeletal myotube. Compared with established cell lines, the primary culture of satellite cells beside myofiber will have more advantages and mimic in vivo situations better. So the primary culture of skeletal muscle is another popular in vitro skeletal muscle cell source. But the disadvantage is it is easy to be contaminated with other cells and the isolation techniques are not mature enough [10].

2. Spinal motor neuron cell sources:

MN1 is a cholinergic motor neuron cell line derived from a fusion of N18TG2 (mouse neuroblastoma cell line) with embryonic mouse spinal cord motor neurons. Mouse neuroblastoma cells make MN1 possess the ability to unlimitedly proliferate. But the same as the C2C12 cell line, established immortal cell lines are less closely to motor neuron cell property than do primary spinal motor neurons.

There are two other motor neuron cell sources: stem cell derived MNs and embryonic mouse primary spinal MNs.

Stem cell (mouse ES cells and human iPS cells)-derived MNs can unlimitedly expand and store, which makes it a good tool for drug screening, proteomics, and biochemistry. However, when utilizing embryonic neurons as a cell model to understand some molecular signaling pathways or diseases, we need to consider one point that embryonic neurons' property is different from adult neurons since they are just in their "infant" state. iPS cell derived MNs enable us to proliferate and research

MNs from adult patients, which provides a powerful tool to explore the diseases. But iPS cell-derived MNs' properties are more similar to embryonic spinal MNs than to their original adult patients and this hinders the in-depth research.

Primary spinal MNs are fully differentiated and possess a more genuine spinal motor neuron's characteristic. But primary MNs are easy to be contaminated by other kinds of cell types during dissection and the dissection techniques now are not mature enough. The MN yields are very low and have not been widely replicated. In addition, organotypic spinal cord slice is also a source of primary spinal motor neurons, which provides spinal motor neuron a similar embedded surrounding compared with naked neuron culture [8].

ii. Challenges and Improvements of Skeletal Muscle and Motor Neuron Co-culture

1. In vivo, spinal MNs are supported and embedded by glial cells such as astrocytes and microglia for structural or functional development. But in vitro studies, there is no such "embedded" surrounding and cells are "naked" alone, which is not enough for precisely mimicking the environment in vivo. Currently, there are researches incorporating glial cells with motor neurons, but the results were not as we expected and failed [8].
2. Most of the coculture systems use a single medium for both skeletal muscles and spinal motor neurons and this is not a good choice for the development of mature and functional neuromuscular junction since different kinds of cells need different nutrients to grow and differentiate. By manipulation motor neurons and muscle cells in separate compartments, the maturation of myofiber can be better and this enables more functional neuromuscular junction. There have been modified Campenot and microfluidic chambers which can separate motor neuron and skeletal muscle cells to better model the system. However. They have not been widely applied due to its high cost and low yield [23].
3. Nearly all of the in vitro co-culture systems use mediums with serum. Because

there is a much unknown variable that is not amenable for many applications such as testing the drug's effect and repeating the same experiments in serum, a serum-free system is needed. Currently, several serum-free systems of in vitro motor neuron and skeletal muscle co-culture have been studied and developed, which provides a system that is easy to manipulate and highly reproducible [40].

4. Translating in vitro animal coculture system research data or result to clinical application has been a big problem and fewer drugs have been approved because there is a species gap between animals and humans. To solve the problem, many researches have established human in vitro models which will make great breakthroughs to the clinical application of the co-culture models [40].

In summary, although many in vitro skeletal muscle and motor neuron co-culture systems have been established and their functionality has been validated at some extent, there are still many improvements breakthrough needed to be done, including finding the best cell sources, optimizing separate culture environment for both skeletal muscle and motor neuron, improving the application to clinical use and incorporating glial cells with motor neuron to promote maturation.

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