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INVITED REVIEW

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Creating stem cell-derived neuromuscular junctions in vitro

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Abstract

Recent development of novel therapies has improved mobility and quality of life for people suffering from inheritable neuromuscular disorders. Despite this progress, the majority of neuromuscular disorders are still incurable, in part due to a lack of predictive models of neuromuscular junction (NMJ) breakdown. Improvement of predictive models of a human NMJ would be transformative in terms of expanding our understanding of the mechanisms that underpin development, maintenance, and disease, and as a testbed with which to evaluate novel therapeutics. Induced pluripotent stem cells (iPSCs) are emerging as a clinically relevant and non-invasive cell source to create human NMJs to study synaptic development and maturation, as well as disease modeling and drug discovery. This review will highlight the recent advances and remaining challenges to generating an NMJ capable of eliciting contraction of stem cell-derived skeletal muscle in vitro. We explore the advantages and shortcomings of traditional NMJ culturing platforms, as well as the pioneering technologies and novel, biomimetic culturing systems currently in use to guide development and maturation of the neuromuscular synapse and extracellular microenvironment. Then, we will explore how this NMJ-in-a-dish can be used to study normal assembly and function of the efferent portion of the neuromuscular arc, and how neuromuscular disease-causing mutations disrupt structure, signaling, and function.

KEYWORDS
disease modeling, drug discovery, hiPSC, neuromuscular junction, NMJ, stem cells

1 | INTRODUCTION

The motor neuron-muscle synapse is the primary site of neuromuscular interactions and has been studied widely due to its large size and relative accessibility compared to synapses in the central nervous system. The vertebrate neuromuscular junction (NMJ) is composed of the presynaptic lower motor neuron originating from the ventral horn of the spinal cord (Figure 1A), the postsynaptic muscle myofiber (Figure 1B), and terminal Schwann cells associated with the synapse (Figure 1C).[1-4] Muscle contraction occurs when acetylcholine (ACh) is released from synaptic vesicles in the presynaptic nerve terminal and

Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; ALS, amyotrophic lateral sclerosis; BioMEMS, biological microelectromechanical systems; BMP4, bone morphogenetic protein 4; CREB, cAMP-response element binding protein; Dok7, Downstream-of-tyrosine-kinase 7; Dvl, Dishevelled; ECM, extracellular matrix; ESC, embryonic stem cell; FUS, fused in sarcoma; GDNF, glial cell-derived neurotrophic factor; GGT, geranylgeranyl transferase 1; hiPSC, human induced pluripotent stem cell; iPSC, induced pluripotent stem cell; LRP4, lipoprotein receptor-related protein 4; MuSK, muscle-specific kinase; nAChR, nicotinic acetylcholine receptor; NMJ, neuromuscular junction; PAK1, p21 kinase; PBMC, peripheral blood mononuclear cell; PDMS, polydimethylsiloxane; SA-β-Gal, senescence-associated beta-galactosidase; SMA, spinal muscular atrophy; TGFβ, transforming growth factor β; TSC, terminal Schwann cell.

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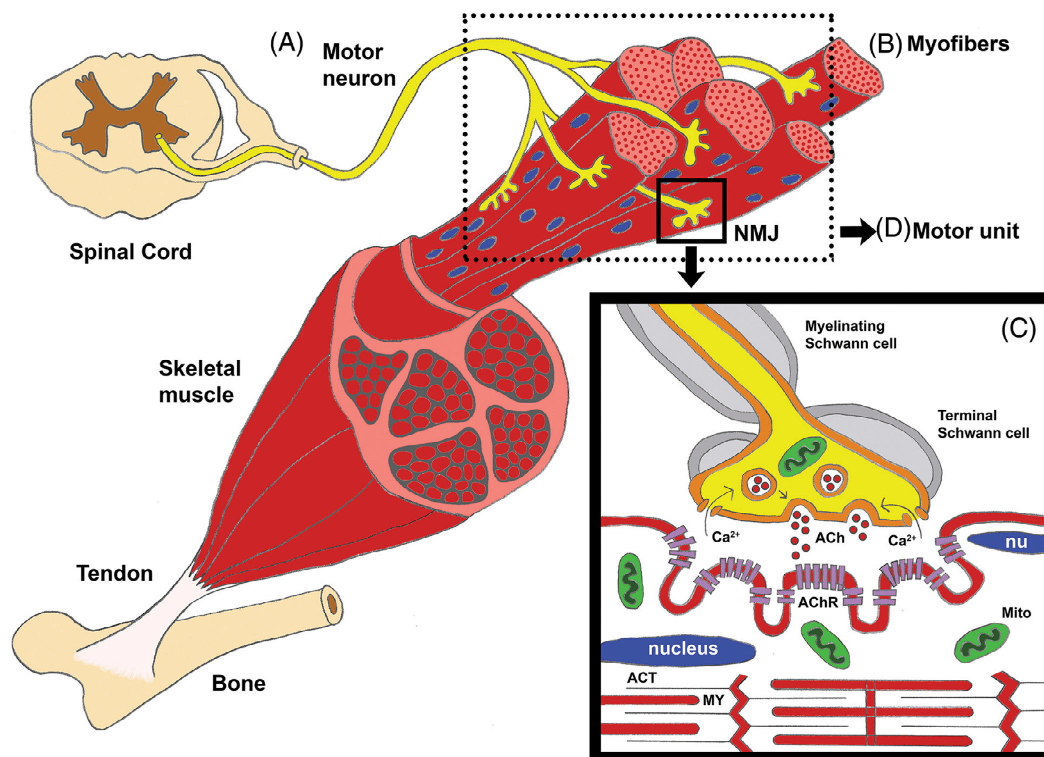

FIGURE 1 The vertebrate neuromuscular junction. A, Axons from motor neurons extend from the ventral horn of the spinal cord and innervate myofibers in skeletal muscle. B, Skeletal muscle is composed of bundles of myofibers. Each myofiber contains a multitude of actin (ACT) and myosin (MY) contractile filaments called myofibrils. C, The neuromuscular junction is the synapse between a motor end plate and a myofiber. Muscle contraction occurs when motor neurons release ACh into the synapse, which binds to AChR in the myofiber. D), A motor unit is comprised of a single motor neuron and all of the myofibers it innervates. Mito = mitochondria (green), nu = nucleus (blue), $Ca2^{+}$ = calcium ion

bind to nicotinic acetylcholine receptors (nAChR) in the postsynaptic motor end plate of the muscle fiber (Figure 1C). Interactions at the NMJ are required for normal development and synapse function, and are necessary for NMJ maintenance and survival.[5-7] Disease pathology in neuromuscular disorders is often coupled with maturation defects in the NMJ, which typically precede the onset of progressive disease symptoms.[8-15] Abnormalities associated with the NMJ may initiate complete denervation of muscles, escalating subsequent muscle degeneration.[16] The neuromuscular synapse is therefore an important site to assess early onset of disease pathology.

Several obstacles, however, prevent systematic experimental analysis of NMJs in patients with neuromuscular disorders. The inherent variability in the pathophysiology of many neuromuscular syndromes makes accurate modeling of them difficult and invasive biopsies are required to thoroughly examine disease pathology.[17] Furthermore, these samples yield NMJs from a specific point in time and do not reveal temporal pathological changes associated with disease progression. Animal models of NMJ disorders have provided valuable insight into disease etiology. For example, Murray et al. used a spinal muscular atrophy (SMA) mouse model to show that denervation of NMJs was present before the onset of detectable disease symptoms.[13] Similarly, Frey et al. used SOD1^{G93A} mice to show significant denervation in limb muscles occurred about 40 days before progressive muscle weakness was displayed.[10] A wide range of animal models, from dogs and zebrafish to invertebrate models like *Drosophila* and *C. elegans* have been used to elucidate neuromuscular disease pathophysiology.[18-21] While these models have revealed a wealth of information, they have critical limitations. There is increasing evidence that animal models do not fully recapitulate human disease phenotypes. Numerous clinical trials have failed due to preliminary studies in animal models inadequately predicting human responses to compound exposure.[22-29] Human NMJs are distinct from murine neuromuscular synapses, both morphologically and molecularly. Human NMJs are smaller and more fragmented with thinner pre-terminal axons.[30,31] Human NMJs have extensive postsynaptic folds, compared to other vertebrate models, which are thought to amplify transmitter action.[31] Healthy, adult human NMJs are relatively stable and do not undergo extensive degeneration and remodeling over time as has been shown in murine models.[32,33] Comparison of the proteome between human and murine NMJs has revealed significant differences in gene expression levels in molecular pathways known to impact NMJ development and function, including synaptic signaling, axonal guidance, and cAMP-response element binding protein (CREB) signaling in neurons.[30] These differences highlight the importance of using human NMJs to study neuromuscular development and maintenance, as well as pathological mechanisms of human neuromuscular disorders.

Human induced pluripotent stem cells (hiPSCs) are emerging as a promising model with which to study human NMJ dynamics. Patient-derived NMJs would help identify pathological mechanisms in diseases with multifaceted genetics. Furthermore, CRISPR-created variants of patient-derived cell lines could help detect downstream effects of single gene mutations. Ultimately, iPSC-based models would enable analysis of patient-specific responses to therapeutic strategies in vitro, thus validating personalized treatment for people suffering from peripheral neuropathies and ensuring greater confidence that observed responses will translate to human tissues in vivo.[34,35] Given the value of iPSCs for disease modeling and therapeutic efficacy evaluations, as well as the importance of accurately modeling NMJ development, this review will discuss recent efforts to create stem cell-derived neuromuscular junctions using classical culturing techniques, as well as novel biomimetic platforms. We will highlight strengths and limitations of each approach, emphasizing gaps in our knowledge and specific areas that require further refinement to fully recapitulate and analyze NMJ formation and function in vitro.

2 | MOLECULAR MECHANISMS OF NMJ DEVELOPMENT

Recapitulating the molecular mechanisms of synaptogenesis and NMJ maturation may be key to exploiting stem cell-derived in vitro NMJ models. While this process remains incompletely understood, numerous studies have revealed key pathways modulating synaptic development and communication at the human NMJ.[2,3,36,37] Neuromuscular synapse formation and maturation is tightly regulated through a series of complex interactions between motor neurons, muscle fibers, and Schwann cells. Initially, myofibers accumulate AChRs in their centers in a process called prepatterning.[37-39] The primitive clustering of receptors may serve as guidance cues for migrating axons, but their exact role has yet to be fully elucidated. Receptor density increases in clusters where axons make contact with muscle, while non-innervated AChRs disperse over time. The postsynaptic membrane invaginates forming junctional folds at the primitive NMJ inducing a morphological shift from a plaque-like shape to a mature branching pattern resembling a pretzel.[1,2] Signaling cues from the pre- and post-synaptic terminals, as well as from Schwann cells, regulate NMJ development, maturation, and maintenance. One of the most well characterized pathways regulating NMJ development is the agrin/LRP4/MuSK pathway. The sulfate proteoglycan protein, Agrin, is synthesized by motor neurons and is essential for stabilizing synaptic AChR clusters.[40,41] Agrin binds the muscle-bound transmembrane lipoprotein receptor-related protein 4 (LRP4), which triggers dimerization and phosphorylation of muscle-specific tyrosine kinase receptor (MuSK, Figure 2).[43,44] MuSK appears to be a key postsynaptic organizer and regulator of NMJ development and maturation. MuSK deficient mice do not have prepatterned muscle fibers and they do not form AChR clusters or NMJs in response to innervation.[38,39,45,46] MuSK interacts with a variety of molecules to regulate

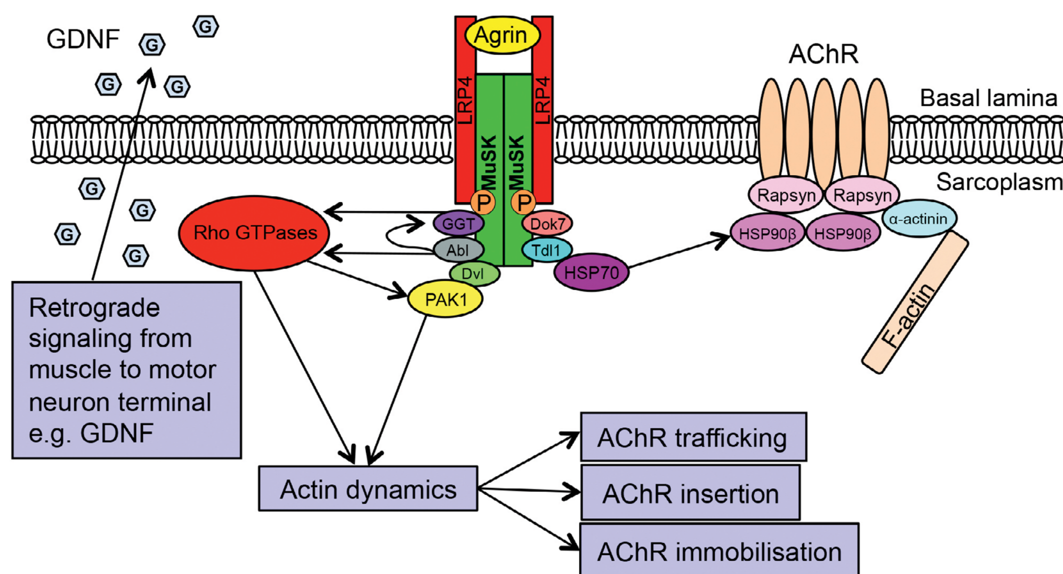

FIGURE 2 The Agrin/LRP4/MuSK organizing complex. Wnt signaling molecules bind to MuSK, activating Dvl in the cytoplasm. The Dvl protein bridges MuSK with PAK1 to bring about structural changes in the cytoskeleton. Upon innervation, neural agrin binds to LRP4 on the muscle membrane. LRP4 interacts with MuSK causing dimerization and phosphorylation (P). Downstream-of-tyrosine- kinase 7 (Dok7) also binds to MuSK and is required to complete the MuSK multi-protein complex stabilizing AChR clusters at the NMJ. The Agrin/LRP4/MuSK complex mediates interactions with an array of proteins including the tyrosine kinase Abl and the metalloenzyme GGT, as well as Rac and Rho GTPases. The Agrin/LRP4/MuSK complex recruits the adaptor protein Rapsyn to developing AChRs. Rapsyn interacts with both AChRs and the actin crosslinker α-actinin to anchor the AChR cluster to the cytoskeleton. The MuSK complex recruits heat shock proteins to prevent rapid degradation of Rapsyn and promote long term stabilization of AChRs at the NMJ. Retrograde transport of GDNF from muscle to the motor neuron works to stabilize and mature the pre-synaptic terminal. Figure adapted from Refs. 3,42

neuromuscular development. Prior to innervation, Wnt signaling molecules bind to MuSK, which is thought to activate Dishevelled (Dvl) in the cytoplasm.[47] The Dvl protein bridges MuSK with the p21 kinase (PAK1) to bring about structural changes in the cytoskeleton, which allows MuSK to induce AChR clustering by regulating actin dynamics (Figure 2).[47,48] Downstream-of-tyrosine-kinase 7 (Dok7) also binds to MuSK and is required to complete the

MuSK multi-protein complex stabilizing AChR clusters at the NMJ through a series of complex mechanisms (Figure 2).[49,50] The Agrin/LRP4/MuSK complex mediates interactions with an array of proteins including the tyrosine kinase Abl and the metalloenzyme geranylgeranyl transferase 1 (GGT), as well as Rac and Rho GTPases (Figure 2).[51-53] GGT facilitates Rho GTPase activation, which controls cytoskeletal changes in actin involved in AChR trafficking, membrane insertion and immobilization.[52,53] The Agrin/LRP4/MuSK complex leads to the recruitment of the adaptor protein Rapsyn to developing AChRs and promotes stabilization of the receptors at the synapse.[54,55] Rapsyn interacts with both AChRs and the actin crosslinker α-actinin to anchor the AChR cluster to the cytoskeleton (Figure 2).[56] Rapsyn has an incredibly short half-life, therefore it is thought that the MuSK complex recruits heat shock proteins to prevent rapid degradation of Rapsyn and promote long term stabilization of AChRs at the NMJ (Figure 2).[57-59] Terminal Schwann cells aid in eliminating redundant nerve terminals by releasing transforming growth factor β (TGFβ), thereby aiding in NMJ maturation.[60,61] Glial cell-derived neurotrophic factor (GDNF) from the myofiber also regulates maturation of the presynaptic terminal through retrograde signaling and promotes superfluous synapse elimination (Figure 2).[62] Together, these molecular events work to induce, stabilize, and mature neuromuscular synapses.

Exogenous application of small molecules, like neural agrin and growth factors, may promote robust NMJ development and maturation in stem cell-derived NMJ cultures. Agrin has been shown to enhance AChR development in vitro in primary muscle fibers, but has yet to be tested in stem cell derived skeletal muscle co-cultures.[40,63] Treatment with growth factors can augment the density of axonal branches and induce skeletal muscle hypertrophy.[64-66] Overexpression of GDNF in vivo triggers hyperinnervation of skeletal muscle in mice.[62] Determining the optimal concentration of GDNF in stem cell co-cultures remains unexplored and may be key to promote normal physiological levels of muscle innervation in vitro. A wide variety of growth factors are commonly used in differentiating both iPSC-derived motor neurons and skeletal muscle, but their precise modulation in developing NMJ cultures has yet to be studied. In fact, neuronal co-culture with skeletal muscle alone may affect intrinsic growth factor production in cultured cells, further hampering our ability to control concentrations to promote synaptogenesis. Systematic experimental analysis of applying small molecules and growth factors will be necessary to create stem cell-derived NMJ models that recapitulate in vivo development. The pathways discussed here are a small fraction of the known molecular cascade of events controlling vertebrate NMJ formation and maturation, however, the complete molecular network is beyond the scope of this review. For detailed and comprehensive reviews on the molecular mechanisms of NMJ development, see Wu et al.[3] and Ferraro et al.[67]

3 | STEM CELL DERIVED MOTOR NEURONS, MYOTUBES, AND TERMINAL SCHWANN CELLS

Induced pluripotent stem cells are readily becoming an invaluable tool to decipher neuromuscular development and disease mechanisms. Stem cell-derived tissues created from the same individual ensures a complete patient-specific neuromuscular disease profile, thereby eliminating possible cellular mismatch through mechanisms that are not fully understood. A major strength of this approach is the ability to pair normal neurons with diseased muscle and vice versa to figure out where the root of the phenotype lies. Incomplete cellular differentiation and maturation, however, has been a critical roadblock to using iPSCs for studying adult-onset diseases and drug discovery studies. Immature, fetal-like cells may not display advanced or late-onset disease phenotypes and underdeveloped cells may respond differently to pharmacological assays compared to fully mature, neuromuscular synapses in adult tissue.[68-74] Innovative cell culturing technologies inspired by a greater understanding of native embryogenesis are beginning to produce more mature cellular phenotypes. These recent advancements will enable the de novo creation of functional neuromuscular junctions in vitro that far surpass previous attempts and will undoubtedly change the future of personalized neuromuscular disease therapies.

In the past decade, iPSCs have been created by a variety of reprogramming methods and starting cell types, including skin fibroblasts,[75] peripheral blood mononuclear cells (PBMCs),[76-78] adipose tissue,[79] keratinocytes,[80,81] and urine cells.[82,83] Urine-derived iPSCs are gaining in popularity as a new stem cell source due to the safe and non-invasive nature of somatic cell collection. A recent study claims exfoliated renal system epithelial cells are the cell type that is reprogrammed in voided urine, however, these cells have yet to be well characterized.[84] Regardless, the resulting iPSCs possess self-renewal and multilineage differentiation potential.[83-88] Yi et al. successfully differentiated urine-derived iPSCs into motor neurons. Furthermore, they compared motor neurons differentiated from urine and blood-based iPSCs and found there was no significant difference in differentiation potential.[89] Numerous groups have made iPSC-derived motor neurons, including terminally differentiated cells from patients with neuromuscular diseases.[90-94] Late-onset disease phenotypes, however, may not be accurately displayed in iPSC-derived motor neurons. Differentiated cells that have been reprogrammed back into a pluripotent state may not retain the epigenetic and phenotypic features of aged cells.[95-97] Furthermore, iPSC-derived motor neurons are functionally immature and must undergo lengthy culture times to achieve mature cell traits.[98]

Direct reprogramming of patient fibroblasts is another approach to generate motor neurons that may be better suited to study late-onset neuromuscular diseases, such as amyotrophic lateral sclerosis (ALS). Transdifferentiated motor neurons retain characteristics of aged cells, including loss of heterochromatin and nuclear organization, extensive DNA damage, and increased senescence-associated beta-galactosidase (SA-β-Gal) activity.[99] Direct reprogramming bypasses

the pluripotent state by using transcription factors and small molecules to direct differentiated cells to neuronal fates.[100-102] While this strategy yields functional motor neurons with high efficiencies, cells are typically reprogrammed using viral vectors to deliver transcription factors. One potential problem with this approach is the possibility of random integration of viral DNA into the host cell genome. Viral DNA insertional mutagenesis could possibly mask or change patient-specific disease phenotypes. Both direct reprogramming and iPSC-based models have advantages and shortcomings, but overall, both have produced comprehensive and efficient protocols to create high quality motor neurons, a key component of the NMJ. For a review of reprogramming methods, see Morris and Daley.[103]

Another vital component of the NMJ is skeletal muscle. Methods to generate mature, functional iPSC-derived myotubes have proved to be more challenging, but new protocols are emerging with promising results. Since the advent of pluripotent stem cells, there have been two basic strategies to derive myogenic precursor cells. Most studies have used direct reprogramming via forced expression of myogenic transcription factors, such as *MYOD*, *PAX3*, or *PAX7*, using a viral gene delivery approach.[104-108] A drawback of direct reprogramming, in addition to the possible integration of viral DNA into the host cell genome, is that expressing one master transcription factor will only turn on a subset of muscle genes. Without the full complement of transcripts critical for myogenesis, the skeletal muscle produced may not fully recapitulate native developmental processes. An alternative method to generate skeletal muscle progenitors from iPSCs avoids these shortcomings by using stepwise induction protocols with small molecules and growth factors that recapitulate embryonic myogenesis. Chal et al. used reporter iPSC lines and gene expression analysis to show that activating the Wnt signaling pathway by using a GSK-3β inhibitor (CHIR-99021) directs cells to paraxial mesoderm fates.[109] Furthermore, they used a small molecule inhibitor of bone morphogenetic protein 4 (BMP4) (LDN-193189) to prevent cells from becoming lateral plate mesoderm. This method, in combination with growth factors to promote myogenesis, produced striated, spontaneously contracting, skeletal muscle myotubes from iPSCs after about 4 weeks of differentiation in 2D cultures, without using cell sorting or purification techniques. Other groups have used similar strategies, some combined with cell sorting and purification, to obtain myogenic progenitors from human iPSCs.[110-113] For a comprehensive review of skeletal muscle cell induction from pluripotent stem cells see Kodaka et al. and Miyagoe-Suzuki and Takeda.[114,115]

Terminal Schwann cells (TSCs) are the third cell type found in normal NMJs. This cell is distinct from myelinating Schwann cells and forms a cap around the nerve-muscle contact (Figure 1C).[1-3] TSCs have been shown to play critical roles in the development and maintenance of the synapse.[116-121] While these cells have successfully been differentiated from iPSCs,[122-124] they have yet to be included into NMJ culturing platforms. Recent studies, however, have shown that TSCs can form spontaneously in vitro from both myogenic cells in 2D cultures and axial stem cells in 3D organoids.[125,126] Terminal Schwann cells were localized to NMJs, capping neuronal terminals, establishing that all three cell types can be cultured simultaneously. Adding TSCs into NMJ cultures in a controlled and deliberate process will be an exciting avenue for future studies and may help to generate complete neuromuscular synapses, recapitulating in vivo NMJ formation.

4 | 2D IN VITRO NMJ MODELS

Stem cell-derived models of single, mostly homogeneous cell types exist and have been used successfully to reveal a wide range of developmental processes and complex disease mechanisms.[83,109,127-129] Co-culturing different cell types together to study tissue interactions during development, however, is considerably more challenging to establish and maintain over time. Numerous biological factors control and strictly regulate cell migration and cell–cell interactions. Extracellular guidance cues, including substrate topography and morphogen gradients established by soluble factors can direct cell identity, polarity and movement.[130-133] Ligands must also be presented in the right spatial and temporal orientation to engage cell-surface receptors during a specific window of development.[134-136] Different cell types also have widely divergent metabolic needs, requiring specialized culture media. Further confounding these in vitro models, metabolic needs for each cell type change as the cells proliferate, differentiate and mature over time. All of these factors combined make co-culturing challenging, even for two different cell types. Many of these obstacles, however, are currently being surmounted through the application of innovative technologies that incorporate endogenous molecular and architectural cues to mimic the native microenvironment. These novel, biomimetic platforms are transforming stem cell culturing methods and opening new avenues to study developmental processes, including muscle development and synaptogenesis, synaptic maintenance and repair, and pathological mechanisms associated with neuromuscular disorders. Below, we highlight some of the issues associated with maintaining neuromuscular co-cultures for NMJ studies and provide examples of how innovative culture techniques have been used to address these problems.

Traditional two-dimensional (2D) culture platforms have been used widely for NMJ models and are advantageous for their simplicity (Figure 3A). These systems are typically arranged as two layers, with differentiated myotubes on the bottom and motor neurons uniformly plated on top. Early co-culture models investigated the development, function, and maintenance of NMJs.[137-141] More recent studies have shown that co-culture actually facilitates myotube development and subsequent NMJ formation.[142] Myogenic differentiation can be improved further by temporally adjusting delivery of growth factors.[143]

Stem cell-derived motor neurons were first incorporated into 2D nerve-muscle co-cultures in 2010,[144] showing that derived cell types could form synapses in vitro. Moreover, stem cell-derived motor neurons can recapitulate disease phenotypes and, notably, can be used to measure the efficacy of pharmacological drugs on disease pathology.[145] Since then, a variety of studies have used stem cell-derived motor neurons, myotubes, or both to uncover intricate molecular mechanisms of synaptogenesis, synapse functionality, and NMJ

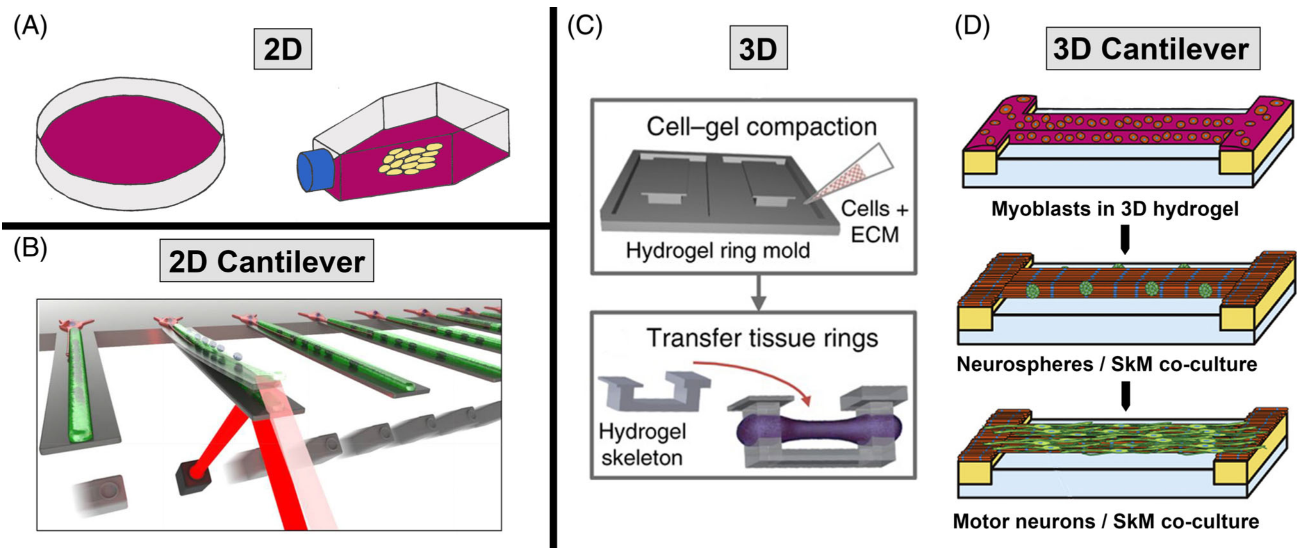

FIGURE 3 NMJ culturing platforms in 2D and 3D. A, Petri dish and T-flask commonly used for 2D cultures. B, The 2D cantilever with skeletal muscle strips (green) and neurospheres in an automated laser detection system to measure cantilever deflection and muscle contractile force. Figure reprinted from 189, World Scientific Open, DOI: 10.1142/S233954781350001. C, 3D hydrogel NMJ tissue ring molds anchored onto a hydrogel skeleton. The skeleton provides tension in the hydrogel and muscle cells align in the direction of strain. Figure reprinted from 183, Creative Commons (CC BY) DOI: 10.1038/micronano.2017.15. D, 3D hydrogel seeded with skeletal muscle myoblasts on PDMS cantilevers. Myoblasts fuse to form skeletal muscle (SkM) strips. Neurospheres (green) are seeded onto SkM strips (red) and axons migrate forming NMJs. Figure from 190. Reprinted from biomaterials, Vol 34(37), Morimoto Y, Kato-Negishi M, Onoe H, Takeuchi S. The 3D neuron-muscle constructs with neuromuscular junctions, pg 9413–9419 (2013) with permission from Elsevier, license #5075180760794

formation using the relatively simple 2D platform.[146-148] Notably, it was shown that motor neurons and myotubes could be differentiated from the same donor line and subsequently co-cultured in a common medium for several weeks.[146-148] Diseased iPSC-derived motor neurons and myotubes from patients with fused in sarcoma (FUS)-ALS have since been used to reveal endplate maturation defects in the NMJ due to toxic localization of FUS in both motor neurons and myotubes affecting NMJ maintenance and stability.[149] The 2D platform is a straightforward culturing method, capable of exposing disease pathology using relatively simple molecular assays.

One drawback of high density, 2D co-cultures is the difficulty in isolating individual motor neuron-myotube cell pairs for functional analysis. Umbach et al. found a way around this obstacle by plating motor neurons and myotubes together in low density co-cultures.[150] Dual patch clamp recordings confirmed both spontaneous and multi-quantal evoked synaptic communication. Functional NMJs were only observed for about a week, however, limiting the efficacy of this model for large scale drug discovery assays using iPS-derived cell types.

Another obstacle in using hiPSC-derived cells in 2D cultures is the heterogenous mix of cell types typically seen during differentiation. Puttonen et al. seeded monolayer cultures from hiPSC-derived neurospheres and observed spontaneously contracting, multinucleated myotubes along with motor neurons.[148] While the authors did not specifically set out to differentiate myogenic cells from hiPSCs, their study demonstrates that myotubes can form spontaneously in heterogenous stem cell cultures and likely communicate with surrounding cells through signaling molecules, paracrine factors, and direct cell–cell junctions. In fact, obtaining highly pure motor neuron or skeletal muscle cell populations from small molecule directed differentiation of hiPSCs without using cell purification strategies is a common obstacle.[151,152] On the other hand, heterogenous cell cultures may be an asset for NMJ models. Multiple cell types, including glial cells, are involved with in vivo NMJ formation, maintenance, and repair.[1-3] Furthermore, variable cell–cell interactions are often coupled with disease pathology in neuromuscular disorders. Heterogeneous cell populations may provide a more realistic environment for modeling NMJ disorders.[34,153]

The 2D culture platforms are relatively inexpensive and have been instrumental in developing protocols for motor neuron-muscle co-cultures. They have demonstrated that motor neurons and myotubes can be derived from the same individual and cultured together to form functional NMJs. This is a critical first step to establish parameters and minimum factors necessary to model genetic, neuromuscular diseases in vitro. The primary downside with 2D platforms is the difficulty in tracking and interrogating individual cell pairs with high confidence in densely populated cultures. Moreover, standard cell culture dishes typically have flat, rigid substrates, which restricts cellular migration and alters cell morphology and possibly gene expression. The extreme disparity between 2D culture conditions and the in vivo microenvironment likely contributes to underdeveloped myotube formation and weak synaptogenesis. Many 2D studies report randomly oriented myotubes, fetal-like hiPSC-derived myofibers, and asynchronous myotube contractions.[143,144,150] Altering the topography of the 2D substrate is one strategy researchers are using to more accurately replicate in vivo extracellular architecture to control myotube alignment and maturation.

5 | 2D MECHANICALLY PATTERNED NMJ MODELS

Myotubes align with remarkable precision in living systems, creating structurally ordered, anisotropic muscular tissue. This highly organized morphology is absolutely critical to achieve coordinated, force generating muscle contraction. The native extracellular microenvironment plays a critical role in this process, providing directional cues to dictate cell alignment.[154,155] The extracellular matrix (ECM) is composed of various protein fibers that have nanoscale features that connect to and interact with muscle cells. The quantity and composition of individual fibers within the ECM specifies the rigidity and topography of the microenvironment. Numerous studies have shown that nanoscale topographical cues augment myogenic cell alignment when compared to unpatterned surfaces.[156-161] Moreover, surface patterning enhances myotube development by producing wider muscle fibers with increased numbers of nuclei per myotube.[160] ACHRs are also larger, suggesting greater receptor clustering.[160,162,163] This is supported at the transcriptional level through upregulation of LRP4 and MuSK, thereby promoting improved levels of myotube prepatterning.[43,57] A main drawback of this platform, like other 2D systems, is that myotubes tend to detach from the substrate after about a week of differentiation. This has been observed in other studies using patterned and unpatterned substrates and is likely due to a combination of forceful myotube contraction, which increases with greater maturation, and increasing passive tension as cells develop in vitro.[164]

6 | 2D ELECTRICALLY STIMULATED NMJ MODELS

Current NMJ models strive to replicate the native neuromuscular microenvironment by incorporating endogenous cues that are known to provide developmental instructions during synaptogenesis, and synapse maturation. Motor neurons and myotubes are both electrically excitable and artificial electrical stimulation has been shown to promote maturation in individual cultures of both cell types.[165-167] NMJ development in co-cultures is also enhanced, giving rise to more mature phenotypes and increased neuronal and myogenic gene expression.[168] The exact mechanisms that facilitate this process are largely unknown, but studies have shown that electrical stimulation fosters NMJ stability[169,170] and accelerates NMJ development through activation of ADAM19/neuregulin/ErbB signaling pathways.[171] Biomemetic techniques that recapitulate in vivo processes are essential to produce normal and disease tissues that progress through all developmental stages, particularly late stages, when certain disease phenotypes are established. Careful experimental analysis is necessary, however, to optimize electrical stimulation parameters that induce maximum cell maturation without damaging sensitive myotube and neuronal developmental mechanisms.

7 | 2D OPTOGENETIC NMJ MODELS

Optogenetic technology allows for light-mediated activation of genetically engineered cells, including neurons and myotubes.[172-175] Ion channels in these cells induce action potentials when stimulated with specific wavelengths of light, which allows for precise stimulation of cells in heterogenous cell cultures. Steinbeck and colleagues plated purified, Channelrhodopsin2 engineered human embryonic stem cell (ESC) -derived motor neurons under control of the human synapsin promoter onto human fetal or adult primary myoblast-derived skeletal muscle and observed muscle contraction in response to light after 6–8 weeks of co-culture.[176] This method of neuronal stimulation ensures that muscle contraction is being modulated through bona fide NMJ signaling as there is no artificial electrical stimulation in the system. Another advantage of this approach is that chronic stimulation regimens can be used, simulating exercise programs to induce greater synapse, as well as myotube, maturation. Another benefit might be introducing other cell types, such as terminal Schwann cells, to more

TABLE 1 Summary of in vitro NMJ maturation enhancers

Application	Advantage	Disadvantage	References
Surface patterning	• Increased cell alignment/anisotropy • Increased cell fusion • Larger myofiber bundles • Directed axon pathfinding	• Increased time and cost to manufacture • Size and width of ridges and grooves in pattern must be optimized for each cell type • Orientation of pattern relative to cell alignment and axonal pathfinding trajectories must be optimized	160
Electrical conditioning	• Simulates exercise or work leading to larger myotubes and increased hypertrophy • Increased Sarcomerogenesis and contractile force • Increased Ca^{2+} handling	• Over stimulation can damage or kill cells	168,171
Optogenetics	• Precise, controlled stimulation over duration and amplitude • Fine temporal resolution of cellular activation and inhibition • Highly reproducible and stable responses • Increased myotube hypertrophy	• Cell transfection and transgenic cell lines might alter disease phenotypes or behavior • May require implantation of an optical fiber in 3D or thick constructs • Increased cost	176

accurately mimic the in vivo NMJ. Table 1 compares the advantages and disadvantages of NMJ developmental and maturation enhancers.

Recent 2D models have provided an enormous amount of insight into the mechanisms that promote NMJ formation in vitro. Their relative simplicity makes for an attractive platform to quickly assess the viability of novel NMJ formation and maturation technologies. 2D systems, however, do not fully recapitulate in vivo architecture, likely impeding long-term culture. This impacts the ability of cells to achieve mature phenotypes and limits the ability to study NMJ maintenance and survival over time. New models are beginning to address this problem by creating matrices that support NMJ culture in three dimensions.

8 | 3D NMJ MODELS

The native NMJ microenvironment provides critical chemical and mechanical cues to cells that facilitate cell migration, differentiation and maturation. Surrounding cells with ECM permits unobstructed movement, which facilitates the natural mechanics and shape of muscle cells.[177] Furthermore, axons are able to migrate freely in response to chemical cues from myogenic cells and the ECM. The 2D systems limit cell migration and overall development by prohibiting movement and contact with neighboring cells in all directions.

Models in 3D can be relatively simple, using ECM only to separate motor neurons and myotubes, essentially creating a sandwich. This method has been shown to promote mature pretzel-like clustering of AChRs at the NMJ.[178] Time lapse photography is possible in this system and can be used to visualize cell migration in real time. Layers of Matrigel, a proprietary blend of proteins found in ECM that support myogenic differentiation,[179,180] above and below contracting muscle fibers may serve to stabilize them, helping to prevent delamination from the substrate.

More complex 3D systems can incorporate mechanical cues to mimic in vivo processes. Stretching cells through mechanical loading has been shown to significantly increase muscle specific proteins that facilitate development of contractile machinery.[181,182] Stretching regimens in both 2D and 3D systems emulate exercise induced muscle development, which stimulates cell alignment cues. Stretching muscle in 2D, however, may not be informative for the purposes of NMJ modeling as fibers may detach from the substrate with increased tension. Mechanical stretch in 3D systems can be achieved by applying tension to the matrix surrounding the cells and muscle fibers align with the direction of strain (Figure 3C).[183]

Culturing neurons and myotubes together in 3D enables cells to receive extracellular cues from all membrane surfaces, more faithfully reproducing the in vivo environment. Matrigel and fibronectin are popular 3D matrices; however, collagen has also been shown to promote neuromuscular interactions and facilitate NMJ formation.[184] This is not surprising considering the high concentration of collagen in the ECM of native skeletal muscle.[185] This highlights the importance of experimenting with different matrices to optimize 3D scaffold parameters for specific cell types. Compliant ECM allows cells to freely migrate, while providing structural support to establish cell–cell signaling, fusion, and anisotropic alignment. This enhances cell differentiation and maturation, leading to larger myotubes and enhanced NMJ development. Long culture times are also possible with 3D systems, enabling access to diverse cell types. Moreover, cells can be evaluated at different stages of development, elucidating temporal changes in disease phenotypes. On the other hand, thick ECM and high cell density in 3D cultures can lead to visualization and imaging difficulties, as well as unequal oxygen and nutrient distribution, causing cell death. Furthermore, patch clamp and electrophysiological evaluation is more challenging and often impossible in 3D systems.

9 | CANTILEVER NMJ MODELS

Microscale cantilevers have conventionally been used to investigate muscle contractile function.[186-188] Generally, cantilevers are composed of a rigid post or beam anchored at one end to a supporting structure. Bending of the post can then be used to derive the amount of force acting on the cantilever. A novel study in 2013 reported an automated system to evaluate NMJ functionality using silicon cantilevers (Figure 3B).[189] A custom-designed scanning laser and photodetector system analyzed 32 individual cantilevers simultaneously in real time under automated control. Cantilever deflection in response to muscle contraction was used to measure the effect of controlled motor neuron stimulation providing functional force assessments in an automated, multiplexing, high-throughput platform. There are several advantages to this system over other NMJ models. Automated scanning of 32 isolated cantilevers provides significant statistical power. Moreover, muscle contractile force changes, especially in diseased NMJ systems, indicate the level of functional rescue in response to drug treatment. Additionally, this system facilitates analysis of individual NMJs, as opposed to multiple NMJs acting in concert.

Cantilevers are useful in a variety of different configurations and can be used in single or multi-post arrays. They can be used in 3D systems and combined with anchoring points to induce strain on skeletal muscle (Figure 3D).[190] Anchoring points have been shown to promote anisotropy because cells align in the direction of strain.[74,170] Cantilevers can be used to apply tension to cells in 3D hydrogels, inducing mechanical stress and enhancing cell alignment and myotube maturation.[191] A primary benefit of using cantilevers is that it provides a method to quantitatively measure the contractile force of myotubes in culture. This readout delivers a diagnostic tool to gauge the effect of pharmacological drugs on synaptic transmission, maintenance and repair in healthy and diseased NMJs. Furthermore, these systems are capable of multiplexing and automation, which is advantageous for high throughput or semi-high throughout pharmacological assays.

10 | BIOMEMS NMJ MODELS

Biological microelectromechanical systems (BioMEMS), sometimes referred to as “lab on a chip” are miniature, compartmentalized systems, normally used to study neuronal based co-cultures. Two

chambers are separated with a physical barrier and contain numerous microchannels for axonal growth. They are usually composed of polydimethylsiloxane (PDMS) and are typically the size of a microscope slide to facilitate optical examination. These systems can be used with broad field electrical stimulation to excite motor neurons for functional assays, including drug efficacy and toxicity trials in diseased NMJ constructs (Figure 4A).[192,193] Furthermore, BioMEMS are ideal systems to examine disease pathology in the pre- and post-synaptic junction. Compartmentalization allows for temporal and spatial interrogation of each cell type and is useful when applying therapeutics targeting only one component of the NMJ. The use of PDMS could potentially be problematic in these platforms, however, as PDMS has been associated with leaching of uncured oligomers and absorption of small, hydrophobic molecules.[194]

11 | MICROFLUIDIC NMJ MODELS

Microfluidic technology has become a widely popular platform to create stem cell NMJ models due to the ability to construct a microenvironment that resembles in vivo design. These devices are oxygen permeable, enabling long-term culture of cells. They are compartmentalized systems, maintaining physical and fluidic isolation of different cell populations, enabling different tissue specific media applications. They can be manufactured quickly and arranged in various geometries, including multi-chambered arrays. Individual compartments and channels are amendable to surface modification, such as microgrooves, to augment cell differentiation and axonal migration (Figure 4B).[195-197] Microfluidic platforms can help distinguish between proximal and distal effects of applied factors,

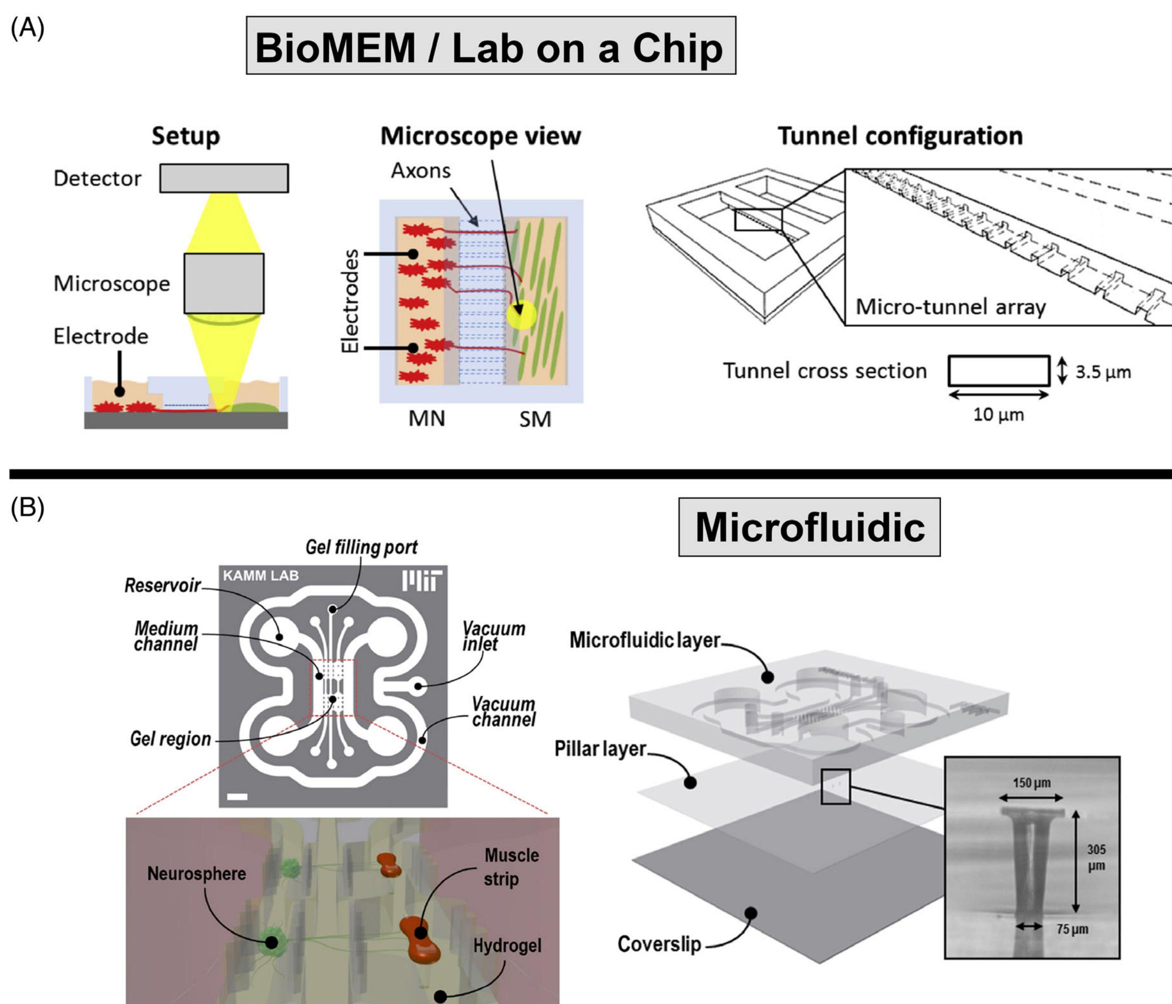

FIGURE 4 BioMEM and microfluidic NMJ culturing platforms. A, BioMEMS showing the setup and microscope view with motor neurons (MN) and skeletal muscle (SM) in separate chambers. Axons migrate through an array of micro-tunnels to innervate SM. Electrodes in the motor neuron chamber induce action potentials that stimulate muscle contractions. Figure from 192. Reprinted from biomaterials, Vol 166, Santhanam N, Kumanchik L, Guo X, Sommerhage F, Cai Y, Jackson M, et al. Stem cell derived phenotypic human neuromuscular junction model for dose response evaluation of therapeutics, pg 64–78 (2018) with permission from Elsevier, license #5075181320930. B, Microfluidic device with separate chambers for neurospheres (green) and muscle strips (red) embedded in a 3D hydrogel. Micro-tunnels for axonal migration connect the two chambers. Figure from 201. Reprinted through the creative commons attribution license 4.0 (CC BY). DOI: 10.1126/sciadv.1501429

revealing key mechanisms of NMJ function, cell signaling, and transport processes.[198-200]

A key advantage of microfluidics is the ability to incorporate multiple technologies and cell types into one system. Optogenetically engineered motor neurons enable controlled neuronal stimulation, while culturing 3D skeletal muscle tissues on compliant pillars provides a way to quantitatively measure contractile force (Figure 4B).[201,202] These technologies, when used together, provide a system to functionally and systematically compare healthy and diseased cell types to create a platform for disease modeling, as well as, neuromuscular drug screening trials.

Microfluidic technology is similar to BioMEMS and both offer many of the same advantages and disadvantages over other platforms. They both use extremely small cell and fluid volumes, minimizing reagent cost and reducing waste. They are both compartmentalized, allowing separation of cell types and subsequent individual interrogation of pre- and post-synaptic mechanisms. Both systems can be integrated with electric and mechanical stimulation and miniaturized biosensors for automation, multiplexing, and real-time data analysis. Both systems are high throughput capable, making them appealing models for hiPSC-derived NMJ disease modeling and drug discovery. Small cell volumes, however, can be difficult to seed. Furthermore, microscale fluid volumes are affected by capillary forces and surface topography, limiting the experimental reproducibility between different devices. Novel, complex chip designs will also likely require skilled production personnel and significant protocol validation to improve repeatability among different labs and individual operators. Table 2 compares the advantages and disadvantages of current NMJ culture platforms.

TABLE 2 Summary of serum-free in vitro NMJ platforms

Platform	Advantage	Disadvantage	References
2D	• Simplicity • Inexpensive • Well established protocols • Easily reproducible • Easy to observe and image • Relatively homogenous cultures • Easy to scale up	• Flat, rigid substrates • Fixed device architecture • Does not mimic the native environment • Limited cell–cell, cell-ECM interactions • High reagent consumption • Cells anchored to plate, which alters morphology and possibly gene expression • Chemical gradients are difficult to achieve • Typically end point analysis only	142-145,147,148,150
3D	• ECM surrounds cells on all sides • More similar to native architecture • Complex cell–cell interactions • Increased cell migration • Cells retain natural morphology	• Can be difficult to image • Not compatible with patch clamp technology • Unequal distribution of nutrients and oxygen leading to cell death • May require additional equipment and handling expertise • Reduced reproducibility • Scaffolds may not be compatible with downstream applications	178,183,190
Cantilever	• Functional readouts possible • Can be automated • Capable of multiplexing • Real-time analysis	• Increased fabrication time and expense • Specialized equipment required	189,191
Microfluidics, BioMEMs/lab on a Chip	• Low fluid volumes reduce waste and reagent cost • Easily integrated with imaging platforms • Compartmentalized, allowing separation of cell types • Flexibility of device design • Chemical concentration gradients possible • Real-time and direct downstream analysis • Short diffusion distances result in faster response times and analysis • Integrated with electric and mechanical stimulation and miniaturized biosensors • Automation • High throughput capable • Multiplexing	• Small cell volumes can be difficult to seed • Leaching of surface material (e.g. PDMS) • Complex chip design • Increased fabrication time and labor intensive • Specialized manufacturing equipment and skilled production personnel required • Novel devices lack significant protocol validation • Microliter scale fluid volumes are affected by surface topography and capillary forces reducing experimental reproducibility • Increased manufacturing cost	192,193,195-198,201

12 | CONCLUSION AND FUTURE PERSPECTIVES

Very few in vitro NMJ models have been composed entirely of human cell types and even fewer have used hiPSC-derived motor neurons and myotubes. This is likely due to the challenges of differentiating mature, hiPSC-derived skeletal muscle. Moreover, limited cell viability post differentiation in stem cell co-cultures restricts the amount of time NMJs have to fully develop. Methods that incorporate environmental cues native to the physiological NMJ niche, like electrical conditioning, topographical patterning, mechanical stretching regimens, compliant substrates, 3D technology, and anchoring points, are significantly improving hiPSC developmental phenotypes. Given that many of these technologies are being incorporated into NMJ systems, often in combinatorial approaches, it is likely that all hiPSC-based co-cultures will be used more widely in the near future.

New technologies will also enable improved assessment of NMJ function, which has been a major shortcoming of many in vitro models. The majority of NMJ studies have relied on visual inspection, using immunofluorescence and brightfield imaging, and electrophysiological recordings, using dual patch clamp technology, to assess NMJ formation. Few of these methods produce functional data in terms of muscle contractile force in response to motor neuron stimulation. Functional readouts are ideal to compare potential rescue of pharmacological drugs on pre- and post-synaptic mechanisms in neuromuscular diseases. Several studies have been successful in quantitatively measuring NMJ-induced muscle contraction and preliminary NMJ drug response testing. The platforms reviewed here, as well as new and improved strategies, will be instrumental in creating models to generate all human based stem cell-derived neuromuscular junctions for in vitro disease modeling and drug discovery. Moreover, this would enable a method to bin heterogenous diseases involving NMJ dysfunction into subclasses for clinical trial inclusion/exclusion criteria to better predict drug efficacy.

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ETHICAL STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

CONFLICTS OF INTEREST

The authors declare no financial holdings or conflicts of interest for all work presented in this Review.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

REFERENCES

1. Sanes JR, Lichtman JW. Development of the vertebrate neuromuscular junction. *Annu Rev Neurosci*. 1999;22(1):389-442.
2. Sanes JR, Lichtman JW. Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat Rev Neurosci*. 2001;2:791-805.
3. Wu H, Xiong WC, Mei L. To build a synapse: signaling pathways in neuromuscular junction assembly. *Development*. 2010;137:1017-1033.
4. Kuo IY, Ehrlich BE. Signaling in muscle contraction. *Cold Spring Harb Perspect Biol*. 2015;7(2):a006023.
5. Nishimune H, Stanford JA, Mori Y. ROLE of exercise in maintaining the integrity of the neuromuscular junction. *Muscle Nerve*. 2014;49(3):315-324.
6. Nelson PG, Fields RD, Yu C, Neale EA. Mechanisms involved in activity-dependent synapse formation in mammalian central nervous system cell cultures. *J Neurobiol*. 1990;21(1):138-156.
7. Valdez G. Effects of disease-afflicted and aging neurons on the musculoskeletal system. *Bone*. 2019;122:31-37.
8. Sleigh JN, Grice SJ, Burgess RW, Talbot K, Cader MZ. Neuromuscular junction maturation defects precede impaired lower motor neuron connectivity in Charcot Marie- tooth type 2D mice. *Hum Mol Genet*. 2014;23(10):2639-2650.
9. Moloney EB, de Winter F, Verhaagen J. ALS as a distal axonopathy: molecular mechanisms affecting neuromuscular junction stability in the presymptomatic stages of the disease. *Front Neurosci*. 2014;8:252.
10. Frey D, Schneider C, Xu L, Borg J, Spooren W, Caroni P. Early and selective loss of neuromuscular synapse subtypes with low sprouting competence in motoneuron diseases. *J Neurosci*. 2000;20(7):2534-2542.
11. Fischer LR, Culver DG, Tennant P, et al. Amyotrophic lateral sclerosis is a distal axonopathy: evidence in mice and man. *Exp Neurol*. 2004;185(2):232-240.
12. Gould TW, Buss RR, Vinsant S, et al. Complete dissociation of motor neuron death from motor dysfunction by Bax deletion in a mouse model of ALS. *J Neurosci*. 2006;26(34):8774-8786.
13. Murray LM, Comley LH, Thomson D, Parkinson N, Talbot K, Gillingwater TH. Selective vulnerability of motor neurons and dissociation of pre- and post- synapticpathology at the neuromuscular junction in mouse models of spinal muscular atrophy. *Hum Mol Genet*. 2008;17(7):949-962.
14. Kong L, Wang X, Choe DW, et al. Impaired synaptic vesicle release and immaturity of neuromuscular junctions in spinal muscular atrophy mice. *J Neurosci*. 2009;29:842-851.
15. Vaughan SK, Kemp Z, Hatzipetros T, Vieira F, Valdez G. Degeneration of proprioceptive sensory nerve endings in mice harboring amyotrophic lateral sclerosis-causing mutations. *J Comp Neurol*. 2015;523(17):2477-2494.
16. Sleigh JN, Burgess RW, Gillingwater TH, Cader MZ. Morphological analysis of neuromuscular junction development and degeneration in rodent lumbrical muscles. *J Neurosci Methods*. 2014;227:159-165.
17. Tazir M, Hamadouche T, Nouioua S, Mathis S, Vallat JM. Hereditary motor and sensory neuropathies or Charcot-Marie-tooth diseases: an update. *J Neurol Sci*. 2014;347(1):14-22.
18. Rich MM, Wang X, Cope TC, Pinter MJ. Reduced neuromuscular quantal content with normal synaptic release time course and depression in canine motor neuron disease. *J Neurophysiol*. 2002;88:3305-3314.
19. Feiguin F, Godena VK, Romano G, D'Ambrogio A, Klima R, Baralle FE. Depletion of TDP-43 affects *Drosophila* motorneurons terminal synapsis and locomotive behaviour. *FEBS Lett*. 2009;583:1586-1592.
20. Wang J, Farr GW, Hall DH, et al. An ALS-linked mutant SOD1 produces a locomotor defect associated with aggregation and synaptic

dysfunction when expressed in neurons of *Caenorhabditis elegans*. *PLoS Genet*. 2009;5:1-14.
21. Ramesh T, Lyon AN, Pineda RH, et al. A genetic model of amyotrophic lateral sclerosis in zebrafish displays phenotypic hallmarks of motor neuron disease. *Dis Model Mech*. 2010;3:652-662.
22. Kola I, Landis J. Can the pharmaceutical industry reduce attrition rates? *Nat Rev Drug Discov*. 2004;3(8):711-715.
23. Becker RE. Lessons from Darwin: 21st century designs for clinial trials. *Curr Alzheimer Res*. 2007;4:458-467.
24. Sena E, van der Worp HB, Howells D, Macleod M. How can we improve the pre-clinical development of drugs for stroke? *Trends Neurosci*. 2007;30:433-439.
25. Akhtar AZ, Pippin JJ, Sandusky CB. Animal models in spinal cord injury: a review. *Rev Neurosci*. 2008;19(1):47-60.
26. Geerts H. Of mice and men: bridging the translational disconnect in CNS drug discovery. *CND Drugs*. 2009;23:915-926.
27. van der Worp HB, Howells DW, Sena ES, et al. Can animal models of disease reliably inform human studies? *PLoS Med*. 2010;7(3):e1000245.
28. Greek R, Menache A. Systematic reviews of animal models: methodology versus epistemology. *Int J Med Sci*. 2013;10:206-221.
29. Mehta D, Jackson R, Paul G, Shi J, Sabbagh M. Why do trials for Alzheimer's disease drugs keep failing? A discontinued drug perspective for 2010-2015. *Expert Opin Investig Drugs*. 2017;26:735-739.
30. Jones RA, Harrison C, Eaton SL, et al. Cellular and molecular anatomy of the human neuromuscular junction. *Cell Rep*. 2017;21(9):2348-2356.
31. Slater CR. The structure of human neuromuscular junctions: some unanswered molecular questions. *Int J Mol Sci*. 2017;18(10):2183.
32. Valdez G, Tapia JC, Kang H, et al. Attenuation of age-related changes in mouse neuromuscular synapses by caloric restriction and exercise. *Proc Natl Acad Sci U S A*. 2010;107:14863-14868.
33. Willadt S, Nash M, Slater CR. Age-related fragmentation of the motor endplate is not associated with impaired neuromuscular transmission in the mouse diaphragm. *Sci Rep*. 2016;6:24849.
34. Thomson SR, Wishart TM, Patani R, Chandran S, Gillingwater TH. Using induced pluripotent stem cells (iPSC) to model human neuromuscular connectivity: promise or reality? *J Anat*. 2012;230:122-130.
35. Romano G, Morales F, Marino IR, Giordano A. A commentary on iPS cells: potential applications in autologous transplantation, study of illnesses and drug screening. *J Cell Physiol*. 2014;229:148-152.
36. Fox MA, Umemori H. Seeking long-term relationship: axon and target communicate to organize synaptic differentiation. *J Neurochem*. 2006;97:1215-1231.
37. Kummer TT, Misgeld T, Sanes JR. Assembly of the postsynaptic membrane at the neuromuscular junction: paradigm lost. *Curr Opin Neurobiol*. 2006;16:74-82.
38. Yang X, Arber S, William C, et al. Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. *Neuron*. 2001;30:399-410.
39. Lin W, Burgess RW, Dominguez B, Pfaff SL, Sanes JR, Lee KF. Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. *Nature*. 2001;410:1057-1064.
40. Campanelli JT, Hoch W, Rupp F, Kreiner T, Scheller RH. Agrin mediates cell contact-induced acetylcholine receptor clustering. *Cell*. 1991;67:909-916.
41. Gautam M, Noakes PG, Moscoso L, et al. Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell*. 1996;85:525-535.
42. Smith AST. Development and Characterisation of a Novel Myotube-Motoneuron 3D Co-Culture System. Doctoral thesis, UCL (University College London) 2012.
43. Kim N, Stiegler AL, Cameron TO, et al. Lrp4 is a receptor for Agrin and forms a complex with MuSK. *Cell*. 2008;135(2):334-342.
44. Zhang B, Luo S, Wang Q, Suzuki T, Xiong WC, Mei L. LRP4 serves as a coreceptor of agrin. *Neuron*. 2008;60(2):285-297.
45. DeChiara TM, Bowen DC, Valenzuela DM, et al. The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. *Cell*. 1996;85:501-512.
46. Ghazanfari N, Fernandez KJ, Murata Y, et al. Muscle specific kinase: Organiser of synaptic membrane domains. *Int J Biochem Cell Biol*. 2011;43:295-298.
47. Jing L, Lefebvre JL, Gordon LR, Granato M. Wnt signals organize synaptic prepattern and axon guidance through the zebrafish unplugged/MuSK receptor. *Neuron*. 2009;61:721-733.
48. Henríquez JP, Salinas PC. Dual roles for Wnt signalling during the formation of the vertebrate neuromuscular junction. *Acta Physiol (Oxf)*. 2012;204(1):128-136.
49. Bergamin E, Hallock PT, Burden SJ, Hubbard SR. The cytoplasmic adaptor protein Dok7 activates the receptor tyrosine kinase MuSK via dimerization. *Mol Cell*. 2010;39:100-109.
50. Okada K, Inoue A, Okada M, et al. The muscle protein Dok-7 is essential for neuromuscular synaptogenesis. *Science*. 2006;312:1802-1805.
51. Finn AJ, Feng G, Pendergast AM. Postsynaptic requirement for Abl kinases in assembly of the neuromuscular junction. *Nat Neurosci*. 2003;6:717-723.
52. Luo ZG, Je H-S, Wang Q, et al. Implication of geranylgeranyltransferase I in synapse formation. *Neuron*. 2003b;40:703-717.
53. Weston C, Gordon C, Teressa G, Hod E, Ren X-D, Prives J. Cooperative regulation by Rac and Rho of agrin-induced acetylcholine receptor clustering in muscle cells. *J Biol Chem*. 2003;278:6450-6455.
54. Mittaud P, Camilleri AA, Willmann R, Erb-Vogtli S, Burden SJ, Fuhrer C. A single pulse of agrin triggers a pathway that acts to cluster acetylcholine receptors. *Mol Cell Biol*. 2004;24:7841-7854.
55. Borges LS, Yechikhov S, Lee YI, et al. Identification of a motif in the acetylcholine receptor β subunit whose phosphorylation regulates Rapsyn association and postsynaptic receptor localization. *J Neurosci*. 2008;28:11468-11476.
56. Dobbins GC, Luo S, Yang Z, Xiong WC, Mei L. Alpha-Actinin interacts with rapsyn in agrin- stimulated AChR clustering. *Mol Brain*. 2008;1:18.
57. Luo S, Zhang B, Dong X-P, et al. HSP90 beta regulates rapsyn turnover and subsequent AChR cluster formation and maintenance. *Neuron*. 2008;60:97-110.
58. Apel ED, Glass DJ, Moscoso LM, Yancopoulos GD, Sanes JR. Rapsyn is required for MuSK signaling and recruits synaptic components to a MuSK-containing scaffold. *Neuron*. 1997;18:623-635.
59. Gautam M, Noakes PG, Mudd J, et al. Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn deficient mice. *Nature*. 1995;377:232-236.
60. Feng Z, Ko CP. Schwann cells promote synaptogenesis at the neuromuscular junction via transforming growth factor-beta1. *J Neurosci*. 2008;28:9599-9609.
61. Fuentes-Medel Y, Ashley J, Barria R, Maloney R, Freeman M, Budnik V. Integration of a retrograde signal during synapse formation by glia-secreted TGF-beta ligand. *Curr Biol*. 2012;22:1831-1838.
62. Nguyen QT, Parsadanian A, Snider WD, Lichtman JW. Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. *Science*. 1998;279(5357):1725-1729.
63. Bakooshli MA, Lippmann ES, Mulcahy B, Iyer NR, Nguyen CT, Tung K. A three-dimensional culture model of innervated human skeletal muscle enables studies of the adult neuromuscular junction and disease modeling. *Elife*. 2019;8:E44530.
64. Caroni P, Grandes P. Nerve sprouting in innervated adult skeletal muscle induced by exposure to elevated levels of insulin-like growth factors. *J Cell Biol*. 1990;110(4):1307-1317.

65. Cohen-Cory S, Fraser SE. Effects of brain-derived neurotrophic factor on optic axon branching and remodeling *in vivo*. *Nature*. 1995; 378(6553):192-196.
66. Zanou N, Gailly P. Skeletal muscle hypertrophy and regeneration: interplay between the myogenic regulatory factors (MRFs) and insulin-like growth factors (IGFs) pathways. *Cell Mol Life Sci*. 2013;70 (21):4117-4130.
67. Ferraro E, Molinari F, Berghella L. Molecular control of neuromuscular junction development. *J Cachexia Sarcopenia Muscle*. 2012;3(1): 13-23.
68. Martin NR, Passey SL, Player DJ, et al. Factors affecting the structure and maturation of human tissue engineered skeletal muscle. *Biomaterials*. 2013;34(23):5759-5765.
69. Juhas M, Engelmayr GC, Fontanella AN, Palmer GM, Bursac N. Biomimetic engineered muscle with capacity for vascular integration and functional maturation in vivo. *Proc Natl Acad Sci U S A*. 2014; 111(15):5508-5513.
70. Nam KH, Smith AS, Lone S, Kwon S, Kim DH. Biomimetic 3D tissue models for advanced high-throughput drug screening. *J Lab Autom*. 2015;20(3):201-215.
71. Sposito T, Preza E, Mahoney CJ, et al. Developmental regulation of tau splicing is disrupted in stem cell-derived neurons from frontotemporal dementia patients with the 10 + 16 splice-site mutation in MAPT. *Hum Mol Genet*. 2015;24(18):5260-5269.
72. Vera E, Studer L. When rejuvenation is a problem: challenges of modeling late-onset neurodegenerative disease. *Development*. 2015; 142(18):3085-3089.
73. Hosoya M, Czysz K. Translational prospects and challenges in human induced pluripotent stem cell research in drug discovery. *Cell*. 2016; 5(4):E46.
74. Smith AST, Davis J, Lee G, Mack DL, Kim DH. Muscular dystrophy in a dish: engineered human skeletal muscle mimetics for disease modeling and drug discovery. *Drug Discov Today*. 2016;21(9):1387-1398.
75. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007;131:861-872.
76. Loh YH, Agarwal S, Park IH, et al. Generation of induced pluripotent stem cells from human blood. *Blood*. 2009;113(22):5476-5479.
77. Giorgetti A, Montserrat N, Aasen T, et al. Generation of induced pluripotent stem cells from human cord blood using OCT4 and SOX2. *Cell Stem Cell*. 2009;5(4):353-357.
78. Haase A, Olmer R, Schwanke K, et al. Generation of induced pluripotent stem cells from human cord blood. *Cell Stem Cell*. 2009;5(4): 434-441.
79. Heng BC, Heinimann K, Miny P, et al. mRNA transfection-based, feeder-free, induced pluripotent stem cells derived from adipose tissue of a 50-year-old patient. *Metab Eng*. 2013;18:9-24.
80. Aasen T, Raya A, Barrero MJ, et al. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nat Biotechnol*. 2008;26(11):1276-1284.
81. Illing A, Stockmann M, Swamy T, et al. Definitive endoderm formation from plucked human hair-derived induced pluripotent stem cells and SK channel regulation. *Stem Cell Int*. 2013;2013:360573.
82. Zhou T, Benda C, Duzinger S, et al. Generation of induced pluripotent stem cells from urine. *Nat Protoc*. 2012;7(12):2080-2089.
83. Guan X, Mack DL, Moreno CM, et al. Dystrophin- deficient cardiomyocytes derived from human urine: new biologic reagents for drug discovery. *Stem Cell Res*. 2014;12(2):467-480.
84. Cheng L, Lei Q, Yin C, Wang HY, Jin K, Xiang M. Generation of urine cell-derived non- integrative human iPSCs and iNSCs: a step-by-step optimized protocol. *Front Mol Neurosci*. 2017;10:348.
85. Guan J, Niu X, Gong F, et al. Biological characteristics of human urine derived stem cells: potential for cell-based therapy in neurology. *Tissue Eng Part A*. 2014;20:1794-1806.
86. Bharadwaj S, Liu G, Shi Y, et al. Multi-potential differentiation of human urine-derived stem cells: potential for therapeutic applications in urology. *Stem Cells*. 2013;31:1840-1856.
87. Zhou T, Benda C, Duzinger S, et al. Generation of induced pluripotent stem cells from urine. *J Am Soc Nephrol*. 2011;22:1221-1228.
88. Li D, Wang L, Hou J, et al. Optimized approaches for generation of integration-free iPSCs from human urine-derived cells with small molecules and autologous feeder. *Stem Cell Rep*. 2016;6(5):717-728.
89. Yi H, Xie B, Liu B, et al. Derivation and identification of motor neurons from human urine-derived induced pluripotent stem cells. *Stem Cells Int*. 2018;2018:3628578.
90. Dimos JT, Rodolfa KT, Niakan KK, et al. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Nature*. 2008;321(5893):1218-1221.
91. Stockmann M, Linta L, Föhr KJ, et al. Developmental and functional nature of human iPSC derived motoneurons. *Stem Cell Rev*. 2011;9: 475-492.
92. Sances S, Bruijn LI, Chandran S, et al. Modeling ALS with motor neurons derived from human induced pluripotent stem cells. *Nat Neurosci*. 2016;16(4):542-553.
93. Fuller HR, Mandefro B, Shirran SL, et al. Spinal muscular atrophy patient iPSC-derived motor neurons have reduced expression of proteins important in neuronal development. *Front Cell Neurosci*. 2015;9:506.
94. Toma JS, Shettar BC, Chipman PH, et al. Motoneurons derived from induced pluripotent stem cells develop mature phenotypes typical of endogenous spinal motoneurons. *J Neurosci*. 2015;35(3):1291-1306.
95. Patterson M, Chan DN, Ha I, et al. Defining the nature of human pluripotent stem cell progeny. *Cell Res*. 2012;22:178-193.
96. Seibler P, Graziotto J, Jeong H, Simunovic F, Klein C, Krainc D. Mitochondrial Parkin recruitment is impaired in neurons derived from mutant PINK1 induced pluripotent stem cells. *J Neurosci*. 2011;31: 5970-5976.
97. Lapasset L, Milhavet O, Prieur A, et al. Rejuvenating senescent and centenarian human cells by reprogramming through the pluripotent state. *Genes Dev*. 2011;25:2248-2253.
98. Sances S, Bruijn LI, Chandran S, et al. Modeling ALS with motor neurons derived from human induced pluripotent stem cells. *Nat Neurosci*. 2016;19:542-553.
99. Tang Y, Liu M-L, Zang T, Zhang C-L. Direct reprogramming rather than iPSC-based reprogramming maintains aging hallmarks in human motor neurons. *Front Mol Neurosci*. 2017;10:359.
100. Son EY, Ichida JK, Wainger BJ, et al. Conversion of mouse and human fibroblasts into functional spinal motor neurons. *Cell Stem Cell*. 2011;9:205-218.
101. Liu ML, Zang T, Zou Y, et al. Small molecules enable neurogenin 2 to efficiently convert human fibroblasts into cholinergic neurons. *Nat Commun*. 2013;4:2183.
102. Liu Y, Miao Q, Yuan J, et al. Ascl1 converts dorsal midbrain astrocytes into functional neurons in vivo. *J Neurosci*. 2015;35:9336-9355.
103. Morris S, Daley G. A blueprint for engineering cell fate: current technologies to reprogram cell identity. *Cell Res*. 2013;23:33-48.
104. Dekel I, Magal Y, Pearson-White S, Emerson CP, Shani M. Conditional conversion of ES cells to skeletal muscle by an exogenous MyoD1 gene. *New Biol*. 1992;4(3):217-224.
105. Abujarour R, Bennett M, Valamehr B, et al. Myogenic differentiation of muscular dystrophy-specific induced pluripotent stem cells for use in drug discovery. *Stem Cells Transl Med*. 2014;3(2):149-160.
106. Tanaka A, Woltjen K, Miyake K, et al. Efficient and reproducible myogenic differentiation from human iPS cells: prospects for modeling Miyoshi myopathy in vitro. *PLoS One*. 2013;8(4):e61540.
107. Shoji E, Sakurai H, Nishino T, et al. Early pathogenesis of Duchenne muscular dystrophy modelled in patient-derived human induced pluripotent stem cells. *Sci Rep*. 2015;5:12831.

108. Tedesco FS, Gerli MF, Perani L, et al. Transplantation of genetically corrected human iPSC-derived progenitors in mice with limb-girdle muscular dystrophy. *Sci Trans Med*. 2012;4(140):140ra89.
109. Chal J, Oginuma M, Al Tanoury Z, et al. Differentiation of pluripotent stem cells to muscle fiber to model Duchenne muscular dystrophy. *Nat Biotechnol*. 2015;33(9):962-969.
110. Choi IY, Lim HT, Estrellas K, et al. Concordant but varied phenotypes among Duchenne muscular dystrophy patient-specific myoblasts derived using a human iPSC-based model. *Cell Rep (Cambridge)*. 2016;15(10):2301-2312.
111. Borchin B, Chen J, Barberi T. Derivation and FACS-mediated purification of PAX3+/PAX7+ skeletal muscle precursors from human pluripotent stem cells. *Stem Cell Rep*. 2013;1(6):620-631.
112. Shelton M, Metz J, Liu J, et al. Derivation and expansion of PAX7-positive muscle progenitors from human and mouse embryonic stem cells. *Stem Cell Rep*. 2014;3(3):516-529.
113. Hosoyama T, McGivern JV, Van Dyke JM, Ebert AD, Suzuki M. Derivation of myogenic progenitors directly from human pluripotent stem cells using a sphere-based culture. *Stem Cells Trans Med*. 2014; 3(5):564-574.
114. Kodaka Y, Rabu G, Asakura A. Skeletal muscle cell induction from pluripotent stem cells. *Stem Cells Int*. 2017;2017:1376151.
115. Miyagoe-Suzuki Y, Takeda S. Skeletal muscle generated from induced pluripotent stem cells – induction and application. *World J Stem Cells*. 2017;9(6):89-97.
116. Reddy LV, Koirala S, Sugiura Y, Herrera AA, Ko CP. Glial cells maintain synaptic structure and function and promote development of the neuromuscular junction *in vivo*. *Neuron*. 2003;40(3):563-580.
117. Smith IW, Mikesh M, Lee Y, Thompson WJ. Terminal Schwann cells participate in the competition underlying neuromuscular synapse elimination. *J Neurosci*. 2013;33(45):17724-17736.
118. Feng Z, Ko CP. The role of glial cells in the formation and maintenance of the neuromuscular junction. *Ann N Y Acad Sci*. 2008;1132: 19-28.
119. Griffin JW, Thompson WJ. Biology and pathology of nonmyelinating Schwann cells. *Glia*. 2008;56(14):1518-1531.
120. Ko CP, Robitaille R. Perisynaptic Schwann cells at the neuromuscular synapse: adaptable, multitasking glial cells. *Cold Spring Harb Perspect Biol*. 2015;7(10):a020503.
121. Barik A, Li L, Sathyamurthy A, Xiong WC, Mei L. Schwann cells in neuromuscular junction formation and maintenance. *J Neurosci*. 2016;36(38):9770-9781.
122. Kreitzer FR, Salomonis N, Sheehan A, et al. A robust method to derive functional neural crest cells from human pluripotent stem cells. *Am J Stem Cells*. 2013;2(2):119-131.
123. Wang A, Tang Z, Park IH, et al. Induced pluripotent stem cells for neural tissue engineering. *Biomaterials*. 2011;32(22):5023-5032.
124. Liu Q, Spusta SC, Mi R, et al. Human neural crest stem cells derived from human ESCs and induced pluripotent stem cells: induction, maintenance, and differentiation into functional schwann cells. *Stem Cells Transl Med*. 2012;1(4):266-278.
125. Lin C-Y, Yoshida M, Li L-T, et al. iPSC-derived functional human neuromuscular junctions model the pathophysiology of neuromuscular diseases. *JCI Insight*. 2019;4(18):e124299.
126. Faustino Martins J-M, Fischer C, Urzi A, et al. Self- Organizing 3D Human Trunk Neuromuscular Organoids. *Cell Stem Cell*. 2020;26(2): 172-186.
127. Xi H, Fujiwara W, Gonzalez K, et al. In vivo somitogenesis guides somite development from HPSCs. *Cell Rep*. 2017;18(6):1573-1585.
128. Pioner JM, Guan X, Klaiman JM, et al. Absence of full- length dystrophin impairs normal maturation and contraction of cardiomyocytes derived from human induced pluripotent stem cells. *Cardiovasc Res*. 2020;116 (2):368–382.
129. Zaunbrecher RJ, Abel AN, Beussman K, et al. Cronos titin is expressed in human cardiomyocytes and necessary for normal sarcomere function. *Circulation*. 2019;140(20):1647-1660.
130. Laird DJ, von Andrian UH, Wagers AJ. Stem cell trafficking in tissue development, growth, and disease. *Cell*. 2008;132:612-630.
131. O'Donnell M, Chance RK, Bashaw GJ. Axon growth and guidance: receptor regulation and signal transduction. *Annu Rev Neurosci*. 2009;32:383-412.
132. Weijer CJ. Collective cell migration in development. *J Cell Sci*. 2009; 122(Pt. 18):3215-3223.
133. Aman A, Piotrowski T. Cell migration during morphogenesis. *Dev Biol*. 2010;341:20-33.
134. Hathaway HJ, Shur BD. Novel cell surface receptors during mammalian fertilization and development. *Bioessays*. 1988;9(5):153-158.
135. Ailhaud G. Cell surface receptors, nuclear receptors and ligands that regulate adipose tissue development. *Clin Chim Acta*. 1999;286 (1–2):181-190.
136. Uings IJ, Farrow SN. Cell receptors and cell signaling. *Mol Pathol*. 2000;53:295-299.
137. Giller ELJ, Schrier BK, Shainberg A, Fisk HR, Nelson PG. Choline acetyltransferase activity is increased in combined cultures of spinal cord and muscle cells from mice. *Science*. 1973;182(4112):588-589.
138. Nelson PG, Fields RD, Yu C, Liu Y. Synapse elimination from the mouse neuromuscular junction in vitro: a non-Hebbian activity-dependent process. *J Neurobiol*. 1993;24(11):1517-1530.
139. Dutton EK, Uhm CS, Samuelsson SJ, Schaffner AE, Fitzgerald SC, Daniels MP. Acetylcholine receptor aggregation at nerve-muscle contacts in mammalian cultures: induction by ventral spinal cord neurons is specific to axons. *J Neurosci*. 1995;15(11):7401-7416.
140. Daniels MP, Lowe BT, Shah S, et al. Rodent nerve–muscle cell culture system for studies of neuromuscular junction development: refinements and applications. *Microsc Res Tech*. 2000;49(1):26-37.
141. Li MX, Jia M, Jiang H, Dunlap V, Nelson PG. Opposing actions of protein kinase A and C mediate Hebbian synaptic plasticity. *Nat Neurosci*. 2001;4(9):871-872.
142. Das M, Rumsey JW, Gregory CA, et al. Embryonic motoneuron-skeletal muscle co-culture in a defined system. *Neurosci*. 2007;146: 481-488.
143. Das M, Rumsey JW, Bhargava N, Stancescu M, Hickman JJ. A defined long-term *in vitro* tissue engineered model of neuromuscular junctions. *Biomaterials*. 2010;31(18):4880-4888.
144. Guo X, Das M, Rumsey J, Gonzalez M, Stanescu M, Hickman J. Neuromuscular junction formation between human stem-cell-derived motoneurons and rat skeletal muscle in a defined system. *Tissue Eng Part C Methods*. 2011;16(6):1347-1355.
145. Yoshida M, Kitaoka S, Egawa N, et al. Modeling the early phenotype at the neuromuscular junction of spinal muscular atrophy using patient-derived iPSCs. *Stem Cell Rep*. 2015;4(4):561-568.
146. Chipman PH, Zhang Y, Rafuse VF. A stem-cell based bioassay to critically assess the pathology of dysfunctional neuromuscular junctions. *PLoS One*. 2014;9:e91643.
147. Demestre M, Orth M, Föhr KJ, et al. Formation and characterisation of neuromuscular junctions between hiPSC derived motoneurons and myotubes. *Stem Cell Res*. 2015;15(2):328-336.
148. Puttonen K, Ruponen M, Naumenko N, Hovatta OH, Tavi P, Koistinaho J. Generation of functional neuromuscular junctions from human pluripotent stem cell lines. *Front Cell Neurosci*. 2015;9:473.
149. Picchiarelli G, Demestre M, Zuko A, et al. FUS-mediated regulation of acetylcholine receptor transcription at neuromuscular junctions is compromised in amyotrophic lateral sclerosis. *Nat Neurosci*. 2019;22 (11):1793-1805.
150. Umbach JA, Adams KL, Gundersen CB, Novitch BG. Functional neuromuscular junctions formed by embryonic stem cell-derived motor neurons. *PLoS One*. 2012;7(5):e36049.

151. Chiu AY, Rao MS. Cell-based therapy for neural disorders - anticipating challenges. *Neurotherapeutics*. 2011;8(4):744-752.
152. Salani S, Donadoni C, Rizzo F, Bresolin N, Comi GP, Corti S. Generation of skeletal muscle cells from embryonic and induced pluripotent stem cells as an *in vitro* model and for therapy of muscular dystrophies. *J Cell Mol Med*. 2012;16(7):1353-1364.
153. Palma E, Inghilleri M, Conti L, et al. Physiological characterization of human muscle acetylcholine receptors from ALS patients. *Proc Natl Acad Sci U S A*. 2011;108(50):20184-22088.
154. Gillies AR, Lieber RL. Structure and function of the skeletal muscle extracellular matrix. *Muscle Nerve*. 2011;44(3):318-331.
155. Trotter JA, Purslow PP. Functional morphology of the endomysium in series fibered muscles. *J Morphol*. 1992;212(2):109-122.
156. Clark P, Coles D, Peckham M. Preferential adhesion to and survival on patterned laminin organizes myogenesis *in vitro*. *Exp Cell Res*. 1997;230(2):275-283.
157. Evans DJ, Britland S, Wigmore PM. Differential response of fetal and neonatal myoblasts to topographical guidance cues *in vitro*. *Dev Genes Evol*. 1999;209(7):438-442.
158. Huang NF, Patel S, Thakar RG, et al. Myotube assembly on nanofibrous and micropatterned polymers. *Nano Lett*. 2006;6(3):537-542.
159. Yang HS, Ieronimakis N, Tsui JH, et al. Nanopatterned muscle cell patches for enhanced myogenesis and dystrophin expression in a mouse model of muscular dystrophy. *Biomaterials*. 2014;35(5):1478-1486.
160. Happe CL, Tenerelli KP, Gromova AK, Kolb F, Engler AJ. Mechanically patterned neuromuscular junctions-in-a-dish have improved functional maturation. *Mol Biol Cell*. 2017;28(14):1950-1958.
161. Vincent LG, Choi YS, Alonso-Latorre B, del Alamo JC, Engler AJ. Mesenchymal stem cell durotaxis depends on substrate stiffness gradient strength. *Biotechnol J*. 2013;8(4):472-484.
162. Marques MJ, Conchello JA, Lichtman JW. From plaque to pretzel: fold formation and acetylcholine receptor loss at the developing neuromuscular junction. *J Neurosci*. 2000;20(10):3663-3675.
163. Huh KH, Fuhrer C. Clustering of nicotinic acetylcholine receptors: from the neuromuscular junction to interneuronal synapses. *Mol Neurobiol*. 2002;25(1):79-112.
164. Wang PY, Thissen H, Tsai WB. The roles of RGD and grooved topography in the adhesion, morphology, differentiation of C2C12 skeletal myoblasts. *Biotechnol Bioeng*. 2012;109(8):2104-2115.
165. Nikolic N, Bakke SS, Kase ET, et al. Electrical pulse stimulation of cultured human skeletal muscle cells as an *in vitro* model of exercise. *PLoS One*. 2012;7(3):e33203.
166. van der Schaft DW, van Spreeuwel AC, Boonen KJ, Langelaan ML, Bouten CV, Baaijens FP. Engineering skeletal muscle tissues from murine myoblast progenitor cells and application of electrical stimulation. *J Vis Exp*. 2013;19(73):e4267.
167. Wehrle U, Düsterhöft S, Pette D. Effects of chronic electrical stimulation on myosin heavy chain expression in satellite cell cultures derived from rat muscles of different fiber-type composition. *Differentiation*. 1994;58(1):37-46.
168. Charoensook SN, Williams DJ, Chakraborty S, Leong KW, Vunjak-Novakovic G. Bioreactor model of neuromuscular junction with electrical stimulation for pharmacological potency testing. *Integr Biol*. 2017;9:956-967.
169. Tomori K, Ohta Y, Nishizawa T, Tamaki H, Takekura H. Low-intensity electrical stimulation ameliorates disruption of transverse tubules and neuromuscular junctional architecture in denervated rat skeletal muscle fibers. *J Muscle Res Cell Motil*. 2010;31(3):195-205.
170. Johnson AM, Connor NP. Effects of electrical stimulation on neuromuscular junction morphology in the aging rat tongue. *Muscle Nerve*. 2011;43(2):203-211.
171. Fukazawa T, Matsumoto M, Imura T, Khalesi E, Kajiume T, Kawahara Y. Electrical stimulation accelerates neuromuscular junction formation through ADAM19/neuregulin/ErbB signaling *in vitro*. *Neurosci Lett*. 2013;545:29-34.
172. Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K. Millisecond-timescale, genetically targeted optical control of neural activity. *Nat Neurosci*. 2005;8(9):1263-1268.
173. Zhang F, Vierock J, Yizhar O, et al. The microbial opsin family of optogenetic tools. *Cell*. 2011;147(7):1446-1457.
174. Sakar MS, Neal D, Boudou T, et al. Formation and optogenetic control of engineered 3D skeletal muscle bioactuators. *Lab Chip*. 2012;12(23):4976-4985.
175. Raman R, Cvetkovic C, Uzel SG, et al. Optogenetic skeletal muscle-powered adaptive biological machines. *Proc Natl Acad Sci U S A*. 2016;113(13):3497-3502.
176. Steinbeck JA, Jaiswal MK, Calder EL, et al. Functional connectivity under optogenetic control allows modeling of human neuromuscular disease. *Cell Stem Cell*. 2016;18(1):134-143.
177. Hinds S, Bian W, Dennis RG, Bursac N. The role of extracellular matrix composition in structure and function of bioengineered skeletal muscle. *Biomaterials*. 2011;32(14):3575-3583.
178. Vilmont V, Cadot B, Ouanounou G, Gomes ER. A system for studying mechanisms of neuromuscular junction development and maintenance. *Development*. 2016;143(13):2464-2477.
179. Kleinman HK, Martin GR. Matrigel: basement membrane matrix with biological activity. *Semin Cancer Biol*. 2005;15(5):378-386.
180. Grefte S, Vullinghs S, Kuijpers-Jagtman AM, Torensma R, Von den Hoff JW. Matrigel, but not collagen I, maintains the differentiation capacity of muscle derived cells in vitro. *Biomed Mater*. 2012;7(5):055004.
181. Moon d G, Christ G, Stitzel JD, Atala A, Too JJ. Cyclic mechanical preconditioning improves engineered muscle contraction. *Tissue Eng*. 2008;14:473-482.
182. Candiani G, Riboldi SA, Sadr N, Lorenzoni S, Neuenschwander P, Montevecchi FM. Cyclic mechanical stimulation favors myosin heavy chain accumulation in engineered skeletal muscle constructs. *J Appl Biomater Biomech*. 2010;8(2):68-75.
183. Cvetkovic C, Rich MH, Raman R, Kong H, Bashir R. A 3D-printed platform for modular neuromuscular motor units. *Microsyst Nanoeng*. 2017;3:17015.
184. Smith AS, Passey SL, Martin NR, et al. Creating interaction between tissue-engineered skeletal muscle and the peripheral nervous system. *Cells Tissues Organs*. 2016;202(3–4):143-158.
185. Gillies AR, Bushong EA, Deerinck TJ, Ellisman MH, Lieber RL. Three-dimensional reconstruction of skeletal muscle extracellular matrix ultrastructure. *Microsc Microanal*. 2014;20(6):1835-1840.
186. Wilson K, Molnar P, Hickman JJ. Integration of functional myotubes with a Bio-MEMS device for non-invasive interrogation. *Lab Chip*. 2007;7(7):920-922.
187. Wilson K, Das M, Wahl KJ, Colton RJ, Hickman J. Measurement of contractile stress generated by cultured rat muscle on silicon cantilevers for toxin detection and muscle performance enhancement. *PLoS One*. 2010;5(6):e11042.
188. Smith AS, Long CJ, McAleer C, Bobbitt N, Srinivasan B, Hickman JJ. Utilization of microscale silicon cantilevers to assess cellular contractile function in vitro. *J Vis Exp*. 2014;3(92):e51866.
189. Smith AS, Long CJ, Pirozzi K, Hickman JJ. A functional system for high-content screening of neuromuscular junctions in vitro. *Dent Tech*. 2013;1(1):37-48.
190. Morimoto Y, Kato-Negishi M, Onoe H, Takeuchi S. Three-dimensional neuron-muscle constructs with neuromuscular junctions. *Biomaterials*. 2013;34(37):9413-9419.
191. Dixon TA, Cohen E, Cairns DM, Rodriguez M, Mathews J, Jose RR. Bioinspired three- dimensional human neuromuscular junction development in suspended hydrogel arrays. *Tissue Eng Part C Methods*. 2018;24(6):346-359.

192. Santhanam N, Kumanchik L, Guo X, et al. Stem cell derived phenotypic human neuromuscular junction model for dose response evaluation of therapeutics. *Biomaterials*. 2018;166:64-78.
193. Tong Z, Seira O, Casas C, et al. Engineering a functional neuro-muscular junction model on a chip. *RSC Adv*. 2014;4:54788-54797.
194. Regehr KJ, Domenech M, Koepsel JT, et al. Biological implications of polydimethylsiloxane-based microfluidic cell culture. *Lab Chip*. 2009;9(15):2132-2139.
195. Park HS, Liu S, McDonald J, Thakor N, Yang IH. Neuromuscular junction in a microfluidic device. *Conf Proc IEEE Eng Med Biol Soc*. 2013;2013:2833-2835.
196. Southam KA, King AE, Blizzard CA, McCormack GH, Dickson TC. Microfluidic primary culture model of the lower motor neuron–neuromuscular junction circuit. *J Neurosci Methods*. 2013;218(2):164-169.
197. Ionescu A, Zahavi EE, Gradus T, Ben-Yaakov K, Perlson E. Compartmental microfluidic system for studying muscle–neuron communication and neuromuscular junction maintenance. *Eur J Cell Biol*. 2016;95(2):69-88.
198. Zahavi EE, Ionescu A, Gluska S, Gradus T, Ben-Yaakov K, Perlson E. A compartmentalized microfluidic neuromuscular co-culture system reveals spatial aspects of GDNF functions. *J Cell Sci*. 2015;128(6):1241-1252.
199. Paratcha G, Ledda F. GDNF and GFRalpha: a versatile molecular complex for developing neurons. *Trends Neurosci*. 2008;31:384-391.
200. Kanning KC, Kaplan A, Henderson CE. Motor neuron diversity in development and disease. *Annu Rev Neurosci*. 2010;33:409-440.
201. Uzel SG, Platt RJ, Subramanian V, Pearl TM, Rowlands CJ, Chan V. Microfluidic device for the formation of optically excitable, three-dimensional, compartmentalized motor units. *Sci Adv*. 2016;2(8):e1501429.
202. Osaki T, Uzel SGM, Kamm RD. 3D model of amyotrophic lateral sclerosis (ALS) from human iPS-derived muscle cells and optogenetic motor neurons. *Sci Adv*. 2018;4(10):eaat5847.

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