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Neuromuscular Junction Formation between Human Stem cell-derived Motoneurons and Human Skeletal Muscle in a Defined System

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Abstract

Functional in vitro models composed of human cells will constitute an important platform in the next generation of system biology and drug discovery. This study reports a novel human-based in vitro Neuromuscular Junction (NMJ) system developed in a defined serum-free medium and on a patternable non-biological surface. The motoneurons and skeletal muscles were derived from fetal spinal stem cells and skeletal muscle stem cells. The motoneurons and skeletal myotubes were completely differentiated in the co-culture based on morphological analysis and electrophysiology. NMJ formation was demonstrated by phase contrast microscopy, immunocytochemistry and the observation of motoneuron-induced muscle contractions utilizing time lapse recordings and their subsequent quenching by D-Tubocurarine. Generally, functional human based systems would eliminate the issue of species variability during the drug development process and its derivation from stem cells bypasses the restrictions inherent with utilization of primary human tissue. This defined human-based NMJ system is one of the first steps in creating functional in vitro systems and will play an important role in understanding NMJ development, in developing high information content drug screens and as test beds in preclinical studies for spinal or muscular diseases/injuries such as muscular dystrophy, Amyotrophic lateral sclerosis and spinal cord repair.

Keywords

stem cell; co-culture; in vitro; nerve tissue engineering; skeletal muscle; in vitro test

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1. Introduction

For centuries, animals and animal-derived tissues have been the major tools for understanding biological systems, human diseases, the development of therapeutic strategies and for screening drugs. However, translating the animal data to clinical applications has been problematic and has led to fewer drugs being approved and an increasing cost in the drug discovery process [1]. Human-based functional *in vitro* systems in defined, serum-free medium are the logical next step in bridging the gap between discovery research and clinical application as well as for the next generation of systems biology tools [2].

While some functional *in vitro* systems composed of human cells have been reported for liver [3], skin [4, 5], cardiomyocytes [6, 7] and for motoneurons [8, 9], no functional systems derived from human stem cells has been reported for the neuromuscular junction. Systems based on functional Neuromuscular Junctions (NMJ) are of particular interest due to the fact that NMJs represents a synapse-based model that would be clinically applicable to spinal cord injury, muscle and motoneuron-related diseases such as Amyotrophic lateral sclerosis (ALS) [10], spinal muscular atrophy [11] and muscular dystrophy [12]. An *in vitro* co-culture system composed of human motoneurons (MNs) and skeletal muscle (SKM) would be useful for studies ranging from understanding NMJ synaptogenesis, investigating pathogenesis for NMJ related diseases, screening therapeutic candidates and conducting drug efficacy/toxicology evaluation. The obvious advantages of human-based *in vitro* systems compared to *in vivo* systems reside in that they are much simpler and therefore it is straightforward to manipulate the system variables, to dissect the mechanisms or pathways and to analyze the results.

To date, multiple motoneuron-muscle co-cultures have been described in *Xenopus* [13, 14], chick [15-17], mouse [18, 19] and rat [20, 21], as well as cross-species experiments with mouse MN-chick muscle [19, 22] and human embryonic stem cell (hESC) -derived MNs-C2C12 myotubes [23]. However, all of these *in vitro* motoneuron-muscle co-culture systems use serum containing media and a biological substrate [15-17, 20, 21]. Serum brings in unknown variable that is not amenable for reproducible assays. Moreover, serum contains many factors which can confound the elucidation of a drug's effect on single cell analysis or with functional constructs. In addition, a recent report suggested inhibition of full functional *in vitro* development of myelination by serum [24]. Thus, some serum-free systems have been developed in an attempt to eliminate the inherent variability with serum [25] and NMJ formation in serum-free media has been demonstrated in rat [26] and cross species between human MN and rat muscle [27]. In general, *in vitro* systems composed of animal-derived components have provided the scientific community with readily available models for understanding NMJ synaptogenesis and NMJ-related diseases. However, due to species-specific differences, there is the problem of extrapolating the findings from animal systems to human systems especially for drug discovery and toxicology leading to clinical applications.

The major hurdles in building *in vitro* biological systems consisting of human components are the limitations due to tissue source. The emergence of stem cell biology in recent years however provides an avenue to not only have an unlimited supply of human cells for tissues, but also to provide genetic diversity in the systems especially give the great potential of induced pluripotent stem cells (iPSC). Human MNs have been successfully differentiated *in vitro* from embryonic stem cells (ESC) [23, 28], neural progenitors [29] and even induced pluripotent stem cells [9]. In addition, human ESC-derived MNs have been investigated for their capability of innervating C2C12 cells in a serum-containing system [30-34], and MNs derived from human fetal spinal cord stem cells were demonstrated to be able to form functional NMJs with rat myotubes derived from embryonic skeletal muscles in a defined

serum-free system [27]. Separately, cloned human skeletal muscle satellite cells have been used extensively for the study of in vitro NMJ formation or related diseases in combination with rat spinal explants or dissociated MNs in serum-containing systems [30-34].

In this study, we endeavored to develop an entirely human-based in vitro neuromuscular junction system, in which both MNs and SKMs were derived from stem cells, in a defined, serum-free system. This system would greatly facilitate not only research related to human NMJ-related diseases, but lead the way to a host of functional in vitro systems derived entirely from human stem cells.

2. Materials and Methods

2.1. DETA Surface Modification

Glass coverslips (6661F52, 22×22 mm No. 1; Thomas Scientific, Swedesboro, NJ, USA) were cleaned using HCl/methanol (1:1) for at least 2 hours, rinsed with water, soaked in concentrated H₂SO₄ for at least 2 hours and rinsed with water. Coverslips were boiled in nanopure water and then oven dried. The trimethoxysilylpropyl-diethylenetri-amine (DETA, T2910KG; United Chemical Technologies Inc., Bristol, PA, USA) film was formed by the reaction of cleaned surfaces with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (T2904; Fisher, Suwanee, GA, USA). The DETA coated coverslips were heated to ~80°C, then cooled to room temperature (RT), rinsed with toluene, reheated to approximately the same temperature, and then cured for at least 2 hours at 110°C. Surfaces were characterized by contact angle and X-ray photoelectron spectroscopy as described previously [35-37].

2.2. Co-culture of human motoneurons and human skeletal muscle

The co-cultures were established according to the procedures depicted in Fig 1. The human spinal cord stem cell line (hSCSC) was isolated and established as described in [38-40]. MNs were differentiated from this cell line as described in [29]. Briefly, $\sim 1 \times 10^6$ hSCSCs were plated in one 60 mm paraflex cell culture dish (Nunc, Cat #174888) and differentiated 4 days in the priming media followed by 6 days in differentiation media. The composition of the priming media and differentiation media were previously described [29].

Human skeletal muscle stem cells (hSKM SCs)/progenitors were isolated, proliferated and differentiated as described in [41]. Briefly, primary human skeletal muscle cells were isolated by needle biopsy [42] and were expanded in myoblast growth medium (MGM; SkGM (Cambrex Bio Science, Walkersville, MD) plus 15% (v/v) fetal bovine serum). Biopsies were performed on adult volunteers according to procedures approved by the Institutional Clinical Review Board of the Miriam Hospital. Cell preparations on average were 70% myogenic based on desmin-positive staining [43]. Myoblast fusion into postmitotic myofibers was induced by incubation in differentiation medium (high-glucose DMEM (Invitrogen, Carlsbad, CA) supplemented with insulin (10 µg/ml), bovine serum albumin (50 µg/ml), epidermal growth factor (10 ng/ml) and gentamicin (50 µg/ml)).

For each culture, hSKM SCs/progenitors were plated on DETA coverslips at a density of 20 cells/mm² in hSKM Growth Medium (Lonza, Cat# CC-3160) and fed every 2 days by changing the whole medium. On day 7, myoblast fusion was induced by switching to the differentiation medium. The cells were fed every 2 days by changing half of the medium. On day 7 after differentiation, the MNs differentiated from hSCSCs were harvested and plated on the induced myotubes at a density of 200 cells/mm² and the medium was changed to co-culture medium (Table 1). Two days later, the medium was fed by co-culture medium (without G5) by changing half of the medium. Thereafter, the cultures were fed every two days using NBActive4 (Brain Bits) by changing half of the medium.

2.3. Immunocytochemistry and Microscopy

Cells on DETA coverslips were fixed in freshly prepared 4% paraformaldehyde in PBS for 15 min. For the co-staining with BTX-488, cultures were incubated with BTX-488 (Invitrogen, Cat# B13422) at 1×10^{-8} M for 1 hr in a 37°C incubator before fixation. Cells were then washed twice in Phosphate Buffered Saline (PBS) (pH 7.2, w/o Mg^{2+} , Ca^{2+}) for 10 min at room temperature and then permeabilized with 0.1% triton X-100/PBS for 15 min. Non-specific binding sites were blocked using Blocking Buffer (5% Donkey serum plus 0.5% BSA in PBS) for 45 min at room temperature. Cells were incubated with primary antibodies overnight at 4°C. After being washed with PBS 3×10 min, the cells were incubated with secondary antibodies for 2.5 hours at room temperature. The cells were then washed with PBS 3×10 min and mounted utilizing Vectashield with 4'-6-Diamidino-2-Phenylindole (dapi) (Vector laboratories, Inc.). Primary antibodies used in this study include: Rabbit-anti- β III Tubulin (Sigma, 1:1500), Mouse-anti-synaptophysin (Antibodies Inc., 1:100). The monoclonal antibody against muscle heavy chain (MHC, F1.625, 1:10) was obtained from the Developmental Studies Hybridoma Bank which is under the auspices of the NICHD and maintained by the University of Iowa. Secondary antibodies include: Donkey-anti-Mouse-488 (Invitrogen, 1:250) and Donkey-anti-Rabbit-594 (Invitrogen, 1:250). All antibodies were diluted in Blocking Buffer.

2.4. Electrophysiological Recording

Electrophysiological properties of spinal cord stem cell-derived motoneurons and human myotubes were investigated after ~10 days using whole-cell patch-clamp recording techniques [36]. The recordings were performed in a recording chamber located on the stage of a Zeiss Axioscope 2FS Plus upright microscope [44].

Motoneurons were identified visually under an infrared DIC-videomicroscope. The largest multipolar or round cells (15-25 μ m diam) with bright illuminance in the culture were tentatively identified as motoneurons [45, 46]. Patch pipettes with a resistance of 6-10 M Ω were made from borosilicate glass (BF 150-86-10; Sutter, Novato, CA) with a Sutter P97 pipette puller (Sutter Instrument Company). Current-clamp and voltage-clamp recordings were made utilizing a Multiclamp 700A amplifier (Axon, Union City, CA). The pipette (intracellular) solution contained (in mM) K-gluconate 140, $MgCl_2$ 2, Na_2ATP 2, Phosphocreatine 5, Phosphocreatine kinase 2.4 mg and Hepes 10; pH 7.2. After the formation of a gigaohm seal and membrane puncture, the cell capacitance was compensated. The series resistance was typically < 23 M Ω , and it was compensated > 60% using the amplifier circuitry. Signals were filtered at 3 kHz and sampled at 20 kHz using a Digidata 1322A interface (Axon instrument). Data recording and analysis were performed with pClamp8 software (Axon instrument). Membrane potentials were corrected by subtraction of a 15 mV tip potential, which was calculated using Axon's pClamp8 program. Membrane resistance and capacitance were calculated using 50 ms voltage steps from -85 to -95 mV without any whole-cell or series resistance compensation. The resting membrane potential and depolarization-evoked action potentials were recorded in current-clamp mode. Depolarization-evoked inward and outward currents were examined in voltage-clamp mode.

2.5. Monitoring the contraction of human skeletal muscles in the co-culture and the determination of the effect of (+)-tubocurarine chloride pentahydrate (d-tubocurarine or curare) on the NMJs by video recording

Functional NMJ formation was investigated in the co-culture system 1~2 weeks after MN plating utilizing video recordings. In each experiment, the cells were maintained in NBActiv4 media at 37°C, in 5.0% CO_2 and 95% (or higher) humidity in a 6-well plate set in an insulated temperature controlled chamber (Okalabs) and imaged on the stage of a Zeiss Axiovert microscope 200. The videos were recorded by a Hamamatsu digital camera (Model

C8484-05G) at a frame rate of 8 frames/sec using Windows Movie Maker software. For the synapse blocking experiments, 100 μ l of the Nicotinic cholinergic antagonist, (+)-tubocurarine chloride pentahydrate (also known as curare, cat. no. 93750, Sigma) (stock 250 μ M, final 8 μ M), was applied to the bath solution to block the acetylcholine receptors present in the NMJs. This concentration was chosen based on a previous study [37].

3. Results

3.1. Morphological Evaluation

The procedure of co-culturing motoneurons and skeletal muscles (SKMs) is described in detail in the Material and Methods. Briefly, human SKM stem cells/progenitors were grown to confluence before differentiation induction (Fig 2A). After switching to differentiation media, the fusion of myocytes was initiated and multi-nuclei myotubes formed gradually and were prevalent from day 4 in the culture (Fig 2B). Differentiated human motoneurons (hMNs) were cultured as in Guo et al. [29] and were plated on top of the differentiated myotubes and simultaneously the medium was switched to a co-culture medium. Both hSKMs and hMNs survived well in the co-culture media and the differentiation of both hMNs and hSKMs was evident after one week (Fig 2C). The hMNs were morphologically identifiable in the co-culture and sent out axons either along or ending at the myotubes (Fig 2D, F). In addition, a large number of myotubes exhibited striated band patterns (Fig 2E, F). This characteristic A & I band patterning is due to differential light diffraction from organized myofibrillar proteins that form sarcomeres within the myotubes, and is observed with mature in vivo muscle fibers [47, 48]. The striated patterns indicated the formation of the basic contractile apparatus for skeletal muscle, implying that these myofibers were structurally and functionally mature. Another observation was that adjacent myotubes tended to align with the same basic orientation, thus forming “patches” of myofiber clusters in the culture. This alignment could originate from early myocyte alignment which is a pre-requisite for myotube fusion, which then remained as the myotubes continued to mature (Fig 2 C, E, F). This phenomenon is a reminiscent of native muscle structure which is composed of many aligned myofibers that are bundle to form muscles during development.

Multiple plating conditions were examined to determine the optimal culture procedures. Plating differentiated motoneurons on the hSKMs before extensive myotube formation caused the myocyte fusion to proceed sub-optimally when switched to the co-culture medium, with the formation of only a minimal number of multi-nuclei myotubes. The viability and morphological differentiation of the replated motoneurons was also poor, overall indicating that the co-culture media was not favorable for the fusion of human myocytes, and the successful establishment of the co-culture system required the pre-differentiation of the SKMs. This was reasonable considering that muscle cells release the neurotrophins BDNF, GDNF and NT-3/4 to support MN survival and attract neurite outgrowth of motoneurons during development [49-51]. Another observation was that when the co-culture was fed for four days with co-culture medium containing G5, undesired proliferation from undifferentiated spinal stem cells was observed. When G5 was removed from the co-culture medium completely, however, the replated motoneurons survived poorly. To mediate this complication G5 was kept in the original plating medium for the co-culture and then was gradually removed after two days.

3.2. Electrophysiology of hMNs and hSKMs in the co-culture

Both MNs and SKMs are electrically active cells and their electrophysiological maturity is an important monitor for their functional integrity. Motoneurons process and transmit action potentials by regulating the conduction through ion channels embedded in the cell membrane, which can be monitored by recording changes in membrane conductances or the membrane

potential. Similarly, the excitation of skeletal muscles relies on the proper function of membrane ion channels which when activated generate action potentials, which stimulates Ca^{++} release to induce muscle contraction. The electrophysiological properties of the MNs and myotubes in the co-culture were evaluated using voltage and current clamp recordings for each cellular component. Representative voltage-clamp and current-clamp recordings for the MNs and myotubes are shown in Fig 3. The electrical properties of MNs in the co-culture system, such as membrane resistance, resting membrane potential, Na^+/K^+ current amplitude, the ability to repetitively fire and the amplitude of action potential (AP), were comparable to results described previously [29, 36], indicating they are functionally mature. The electrical properties for the myotubes, such as the dynamics and amplitude of action potentials and inward/outward currents, were also comparable to previously published results [52]. The ability to fire action potentials upon excitation, taken together with the formation of a striated myotube structure (Fig 2E, F) as well as the appearance over time of robust contractions (Supplementary videos) indicate that these myotubes were also functionally mature.

3.3. Neuromuscular Junction (NMJ) formation demonstration by morphological and immunocytochemical analysis

After one week of co-culture, the morphology of the hMNs and hSKM myofibers were well defined and easily distinguishable, and it was observed that the hMNs axons terminated and even branched at contact points on the myofibers. Immunocytochemical analysis with β -III Tubulin for the neurons and myosin heavy chain (MHC) for the myofibers confirmed these observations. In the co-culture system the nerve endings branched in the vicinity of the myotubes and the terminals wrapped around the myotubes as shown in Fig 4A. This image reproduces previous findings during NMJ formation which indicated that synaptogenesis is a dynamic process directly correlated to the active branching and remodeling of axon terminal arbors [53, 54].

The presence of NMJs in the culture were confirmed by the co-immunostaining of BTX-488 (α -bungarotoxin, Alexa Fluor® 488 conjugate) which binds to AchRs on the myotubes, and synaptophysin, a synaptic vesicle protein. As shown in Fig 4 B & C, synaptophysin-positive terminals co-localized with AchR clusters, a strong indication for NMJ formation.

3.4. NMJ formation demonstration by videotape analysis

Numerous muscle contractions in the absence of any stimulus could be observed in the co-culture approximately one week after the introduction of the hMNs. This was based on observations from more than 15 coverslips out of 5 independent platings. All video graph experiments were conducted in a system in which the cultures were kept in the chamber with 5% CO_2 at 37°C. Multiple contractions with steady rhythms were observed in the culture as seen in Video 1. Occasionally, as recorded in Video 2, pulse contractions were also observed suggesting bursting stimulation from the MNss. The pulse contractions demonstrated in the video lasted ~ 30 sec and recurred every 1~2 min (Only a ~30 sec video is included due to file size limitations). These recurrent contractions continued for 6 min and then ceased.

All the previous studies describing human myoblasts in the co-cultures with fetal rat spinal cord explants consistently indicated that hSKMs don't exhibit spontaneous contraction, indicating that contractions in the co-culture were from innervation [31-34, 55, 56]. To determine that this was also true for this system, the hMN-hSKM co-culture was challenged with tubocurarine to confirm the source of initiation. As shown in Video 3, one of the two contractions completely ceased after the application of 100 μl of tubocurarine (250 μM) to the culture (final 8 μM), while the other contraction was weakened, probably due to the incomplete blocking of the AchRs. Similarly, pulse contractions in the co-culture were also

silenced with the challenge of tubocurarine as demonstrated in Video 4. The silencing of myotube contractions by tubocurarine confirmed the neuronal initiation of these contractions and the formation of NMJs. The experiment with curare was repeated 4 times in different cultures with the same result.

4. Discussion

This study reports a novel *in vitro* human-based functional NMJ system which supported the differentiation of both MNs and SKMs derived from human stem cells. Functional NMJ formation in this system was demonstrated by both immunostaining and a curare functional assay. This system was established in a chemically defined, serum-free medium on the biomimetic, non-biological synthetic DETA substrate.

Developing human-based functional system has attracted interests of multi-disciplinary scientific endeavors, from studying human physiology, investigating disease etiology and developing therapeutic design, to generating high throughput systems for drug screening [57], and integrating these systems with bio-hybrid prosthetic devices. It is of particular interest to translational medicine. To date, studies concerning human diseases have been heavily relying on animal-based models. However, animal models do not recreate the full process of human diseases and the therapies designed or drugs developed based on animal models often have problems when translated to human systems [58]. Therefore, functional *in vitro* systems composed of human cells in a defined, serum-free system, especially those reproducing fundamental neurological processes, will be a key component in transforming current methods of the understanding of human physiology, disease etiology, drug discovery and toxicology.

In response to this demand, various *in vitro* NMJ co-culture systems with human cells have been reported, but all were combined with animal cellular components, and most contained serum in the medium. Nevertheless, these systems have been utilized in multiple applications. For instance, they have been employed to study the functional integrity of MNs differentiated from human stem cells by testing their capability of innervating muscle [23, 27], to investigate the functional maturation process of SKMs derived from human SKM stem cells/progenitors in the co-culture with rat spinal explants [59-61], the mechanisms of NMJ formation on human SKMs [30-34, 55, 56, 59-63], and the pathogenesis of some spinal muscular diseases [64]. The system developed in this study, by the co-culture of human stem cell-derived MNs and SKMs, provides a model closer to the human condition that is capable of addressing not only all the above issues, but also those associated with neurological and/or muscular disease modeling, drug discovery and regenerative medicine for neuromuscular diseases or injuries. Particularly, this serum-free system provides a defined list of the factors supporting the functional human-based NMJ formation *in vitro*. These factors will provide important guidance for regenerative medicine when designing therapeutics for neuromuscular diseases/injuries. Moreover, this human-based functional NMJ system can act as a model to understand the synaptogenesis/regeneration/repair or maintenance of the human NMJ. It can also be modified to represent different disease models, for instance an ALS disease model by introducing adverse factors, such as activated glia cells or stress factors, which can then be applied for etiological studies, therapeutic target design, and drug screening.

The human-based NMJ system in this study was developed by utilizing human stem cells. The characteristics of stem cells, self-renewability and multi-potency, make them an ideal biomaterial either for replacement or regeneration therapy via *in vivo* transplantation, or for generating *in vitro* models for disease study and drug screening [1, 65]. Utilizing stem cells would circumvent the problem of tissue source limitation, and hence ensure a cell supply for

repetitive or large scale experiments. The utilization of stem cells also provides the opportunity for genetic diversity in developing in vitro model systems. In particular, stem cells can be genetically modified into specific disease models [66]. Specifically, the development of iPS (induced Pluripotent Stem) technology has achieved the reprogramming of human skin fibroblasts to a pluripotent state [67]. It was further reported that iPS (iPS cells) generated from the fibroblasts of an ALS patient can be differentiated into MNs [9]. In addition, skeletal muscle stem/progenitor cells have been successfully generated from murine iPS [68, 69]. Hence, generating patient-specific disease models is becoming feasible and these models are expected to be extremely useful for disease study and therapeutic design. Consequently, the NMJ system developed in this study would not only be an important functional model for the applications as listed before, it also establishes a platform for generating more relative disease-specific models by the utilization of the continuing progress in the field of stem cell biology.

The NMJ system reported here was established in serum-free media, on a biomimetic, synthetic DETA surface. Serum brings in undesirable and unaccountable variability because it contains various undefined factors such as growth factors, hormones, antibodies etc., which vary from source to source. The defined feature makes this system more reproducible and easy to analyze, which is essential for their application in either mechanism dissection or drug screening. The DETA surface is a self-assembled monolayer that can be formed on any hydroxylated surface [70], it is amenable to photolithographic patterning [71] and it is non-degradable by cells due to its non-biological origins and covalent attachment to the surface [72, 73]. Especially, this surface is compatible with high throughput BioMEM (Microelectromechanical) devices such as multi-electrode arrays (MEA) and cantilever devices [74]. Thus, this self-assembled surface enables the integration of this system with the next generation of high-content and ultimately high-throughput screening technologies.

5. Conclusion

This study reports a novel human based NMJ in vitro culture system. This human cell-based system would bridge the gap between findings from animals and their clinical applications. The stem cell origin for both motoneurons and skeletal muscles would enable the formation of these cultures in large quantities which would be important for high throughput drug screening. The serum-free medium allows this system highly re-producible and easy to manipulate. The patternable surface gives the power to the system to engineer the interface to be compatible with BioMEMs devices as well. All of these attributes indicate that this system would facilitate not only the studies concerning human NMJ development and regulation, both in vitro and in vivo, but also the research fields targeting NMJ-related diseases and treatment, such as by developing high information content drug screen systems and test beds in pre-clinical studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Janne J, Johan H, Petter B. Human embryonic stem cell technologies and drug discovery. *J Cell Physiol.* 2009; 219:513–519. [PubMed: 19277978]
2. Griffith LG, Swartz MA. Capturing complex 3D tissue physiology in vitro. *Nat Rev Mol Cell Bio.* 2006; 7:211–224. [PubMed: 16496023]
3. Cai J, Zhao Y, Liu Y, Ye F, Song Z, Qin H, et al. Directed differentiation of human embryonic stem cells into functional hepatic cells. *Hepatology.* 2007; 45:1229–1239. [PubMed: 17464996]
4. Guenou H, Nissan X, Larcher F, Feteira J, Lemaitre G, Saidani M, et al. Human embryonic stem-cell derivatives for full reconstruction of the pluristratified epidermis: a preclinical study. *The Lancet.* 2009; 374:1745–1753.
5. Schlüter H, Kaur P. Bioengineered human skin from embryonic stem cells. *The Lancet.* 2009; 374:1725–1726.
6. Vidarsson H, Hyllner J, Sartipy P. Differentiation of human embryonic stem cells to cardiomyocytes for in vitro and in vivo applications. *Stem Cell Rev.* 2010; 6:108–120. [PubMed: 20091143]
7. White SM, Claycomb WC. Embryonic stem cells form an organized, functional cardiac conduction system in vitro. *Am J Physiol Heart Circ Physiol.* 2005; 288:H670–679. [PubMed: 15471973]
8. Agholme L, Lindström T, Kågedal K, Marcusson J, Hallbeck M. An in vitro model for neuroscience: Differentiation of SH-SY5Y cells into cells with morphological and biochemical characteristics of mature neurons. *J Alzheimers Dis.* 2010; 20:1069–1082. [PubMed: 20413890]
9. Dimos JT, Rodolfa KT, Niakan KK, Weisenthal LM, Mitsumoto H, Chung W, et al. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science.* 2008; 321:1218–1221. [PubMed: 18669821]
10. Boillée S, Velde CV, Cleveland DW. ALS: A disease of motor neurons and their nonneuronal neighbors. *Neuron.* 2006; 52:39–59. [PubMed: 17015226]
11. Stavarachi M, Apostol P, Toma M, Cimponeriu D, Gavrilla L. Spinal muscular atrophy disease: a literature review for therapeutic strategies. *J Med Life.* 2010; 3:3–9. [PubMed: 20302191]
12. Emery AEH. The muscular dystrophies. *The Lancet.* 2002; 359:687–695.
13. Lu B, Czernik AJ, Popov S, Wang T, Poo MM, Greengard P. Expression of synapsin I correlates with maturation of the NMJ synapse. *Neuroscience.* 1996; 74:1087–1097. [PubMed: 8895877]
14. Peng HB, Yang JF, Dai Z, Lee CW, Hung HW, Feng ZH, et al. Differential effects of neurotrophins and Schwann cell-derived signals on neuronal survival/growth and synaptogenesis. *J Neurosci.* 2003; 23:5050–5060. [PubMed: 12832528]
15. Fischbach GD. Synapse formation between dissociated nerve and muscle cells in low density cell cultures. *Dev Biol.* 1972; 28:407–429. [PubMed: 4338026]
16. Fischbach GD, Cohen SA. The distribution of acetylcholine sensitivity over uninervated and innervated muscle fibers grown in cell culture. *Dev Biol.* 1973; 31:147–162. [PubMed: 4787184]
17. Frank E, Fischbach GD. Early events in neuromuscular junction formation in vitro. *J Cell Biol.* 1979; 83:143–158. [PubMed: 511937]
18. Harper JM, Krishnan C, Darman JS, Deshpande DM, Peck S, Shats I, et al. Axonal growth of embryonic stem cell-derived motoneurons in vitro and in motoneuron-injured adult rats. *PNAS.* 2004; 101:7123–7128. [PubMed: 15118094]
19. Miles GB, Yohn DC, Wichterle H, Jessell TM, Rafuse VF, Brownstone RM. Functional properties of motoneurons derived from mouse embryonic stem cells. *J Neurosci.* 2004; 24:7848–7858. [PubMed: 15356197]
20. Daniels MP, Lowe BT, Shah S, Ma JX, Samuelson SJ, Lugo B, et al. Rodent nerve-muscle cell culture system for studies of neuromuscular junction development: Refinements and applications. *Microsc Res Tech.* 2000; 49:26–37. [PubMed: 10757876]
21. 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; 15:7401–7416. [PubMed: 7472493]
22. Soundararajan P, Lindsey BW, Leopold C, Rafuse VF. Easy and rapid differentiation of embryonic stem cells into functional motoneurons using sonic hedgehog-producing cells. *Stem Cells.* 2007; 25:1697–1706. [PubMed: 17395777]

23. Li XJ, Du ZW, Zarnowska ED, Pankratz M, Hansen LO, Pearce RA, et al. Specification of motoneurons from human embryonic stem cells. *Nat Biotechnol.* 2005; 23:215–221. [PubMed: 15685164]
24. Rumsey JW, Das M, Stancescu M, Bott M, Fernandez-Valle C, Hickman JJ. Node of Ranvier formation on motoneurons in vitro. *Biomaterials.* 2009; 30:3567–3572. [PubMed: 19361859]
25. Walsh K, Megyesi J, Hammond R. Human central nervous system tissue culture: A historical review and examination of recent advances. *Neurobiol Dis.* 2005; 18:2–18. [PubMed: 15649692]
26. Das M, Rumsey JW, Bhargava N, Stancescu M, Hickman JJ. A defined long-term in vitro tissue engineered model of neuromuscular junctions. *Biomaterials.* 2010; 31:4880–4888. [PubMed: 20346499]
27. Guo XF, Das M, Rumsey JW, Gonzalez M, Stancescu M, Hickman JJ. NMJ formation between human stem cell derived motoneurons and rat skeletal muscle in a defined system. *Tissue Eng Part C.* 2010; 16:1347–1355.
28. Li XJ, Hu BY, Jones SA, Zhang YS, LaVaute T, Du ZW, et al. Directed differentiation of ventral spinal progenitors and motor neurons from human embryonic stem cells by small molecules. *Stem Cells.* 2008; 26:886–893. [PubMed: 18238853]
29. Guo XF, Johe K, Molnar P, Davis H, Hickman JJ. Characterization of a human fetal spinal cord stem cell line NSI-566RSC and its induction to functional motoneurons. *J Tissue Eng Reg Med.* 2010; 4:181–193.
30. Gajsek N, Jevsek M, Mars T, Mis K, Pirkmajer S, Brecelj J, et al. Synaptogenetic mechanisms controlling postsynaptic differentiation of the neuromuscular junction are nerve-dependent in human and nerve-independent in mouse C2C12 muscle cultures. *Chem Biol Interact.* 2008; 175:50–57. [PubMed: 18691702]
31. Guettier-Sigrist S, Coupin G, Warter JM, Poindron P. Cell types required to efficiently innervate human muscle cells in vitro. *Exp Cell Res.* 2000; 259:204–212. [PubMed: 10942592]
32. Kobayashi T, Askanas V, Engel WK. Human muscle cultured in monolayer and cocultured with fetal rat spinal cord: importance of dorsal root ganglia for achieving successful functional innervation. *J Neurosci.* 1987; 7:3131–3141. [PubMed: 3668620]
33. Mars T, King MP, Miranda AF, Walker WF, Mis K, Grubic Z. Functional innervation of cultured human skeletal muscle proceeds by two modes with regard to agrin effects. *Neuroscience.* 2003; 118:87–97. [PubMed: 12676140]
34. Mars T, Yu KJ, Tang XM, Miranda AF, Grubic Z, Cambi F, et al. Differentiation of glial cells and motor neurons during the formation of neuromuscular junctions in cocultures of rat spinal cord explant and human muscle. *J Comp Neurol.* 2001; 438:239–251. [PubMed: 11536191]
35. Das M, Bhargava N, Gregory C, Riedel L, Molnar P, Hickman JJ. Adult rat spinal cord culture on an organosilane surface in a novel serum-free medium. *Vitro Cell Dev Biol Anim.* 2005; 41:343–348.
36. Das M, Molnar P, Devaraj H, Poeta M, Hickman J. Electrophysiological and morphological characterization of rat embryonic motoneurons in a defined system. *Biotechnol Prog.* 2003; 19:1756–1761. [PubMed: 14656152]
37. Das M, Rumsey JW, Gregory CA, Bhargava N, Kang JF, Molnar P, et al. Embryonic motor neuron-skeletal muscle co-culture in a defined system. *Neuroscience.* 2007; 146:481–488. [PubMed: 17383103]
38. Cizkova D, Kakinohana O, Kucharova K, Marsala S, Johe K, Hazel T, et al. Functional recovery in rats with ischemic paraplegia after spinal grafting of human spinal stem cells. *Neuroscience.* 2007; 147:546–560. [PubMed: 17524565]
39. Xu L, Yan J, Chen D, Welsh AM, Hazel T, Johe K, et al. Human neural stem cell grafts ameliorate motor neuron disease in SOD-1 transgenic rats. *Transplantation.* 2006; 82:865–875. [PubMed: 17038899]
40. Yan J, Xu L, Welsh AM, Hatfield G, Hazel T, Johe K, et al. Extensive neuronal differentiation of human neural stem cell grafts in adult rat spinal cord. *PLoS Medicine.* 2007; 4:318–332.
41. Thorrez L, Shansky J, Wang L, Fast L, VandenDriessche T, Chuah M, et al. Growth, differentiation, transplantation and survival of human skeletal myofibers on biodegradable scaffolds. *Biomaterials.* 2008; 29:75–84. [PubMed: 17928049]

42. Hennessey JV, Chromiak JA, Dellaventura S, Guertin J, Maclean DB. Increase in percutaneous muscle biopsy yield with a suction-enhancement technique. *J Appl Physiol.* 1997; 82:1739–1742. [PubMed: 9173935]
43. Powell C, Shansky J, DelTatto M, Forman DE, Hennessey J, Sullivan K, et al. Tissue engineered human bioartificial muscles expressing a foreign recombinant protein for gene therapy. *Hum Gen Ther.* 1999; 10:565–577.
44. Gao BX, Ziskind-Conhaim L. Development of ionic currents underlying changes in action potential waveforms in rat spinal motoneurons. *J Neurophysiol.* 1998; 80:3047–3061. [PubMed: 9862905]
45. Gao BX, Ziskind-Conhaim L. Development of Glycine- and GABA-gated currents in rat spinal motoneurons. *J Neurophysiol.* 1995; 74:113–121. [PubMed: 7472315]
46. Takahashi T. Intracellular recording from visually identified motoneurons in rat spinal cord slices. *Proc R Soc Lond B Biol Sci.* 1978; 202:417–421. [PubMed: 29296]
47. Flucher BE, Phillips JL, Powell JA, Andrews SB, Daniels MP. Coordinated development of myofibrils, sarcoplasmic reticulum and transverse tubules in normal and dysgenic mouse skeletal muscle, in vivo and in vitro. *Dev Biol.* 1992; 150:266–280. [PubMed: 1551475]
48. Kontogianni-Konstantopoulos A, Ackermann MA, Bowman AL, Yap SV, Bloch RJ. Muscle giants: Molecular scaffolds in sarcomerogenesis. *Physiol Rev.* 2009; 89:1217–1267. [PubMed: 19789381]
49. Funakoshi H, Belluardo N, Arenas E, Yamamoto Y, Casabona A, Persson H, et al. Muscle-derived neurotrophin-4 as an activity-dependent trophic signal for adult motor neurons. *Science.* 1995; 268:1495–1499. [PubMed: 7770776]
50. Henderson CE, Phillips HS, Pollock RA, Davies AM, Lemeulle C, Armanini M, et al. GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. *Science.* 1994; 266:1062–1064. [PubMed: 7973664]
51. Zurn AD, Winkel L, Menoud A, Djabali K, Aebischer P. Combined effects of GDNF, BDNF, and CNTF on motoneuron differentiation in vitro. *J Neurosci Res.* 1996; 44:133–141. [PubMed: 8723221]
52. Das M, Gregory CA, Molnar P, Riedel LM, Hickman JJ. A defined system to allow skeletal muscle differentiation and subsequent integration with silicon microstructures. *Biomaterials.* 2006; 27:4374–4380. [PubMed: 16647113]
53. Alsina B, Vu T, Cohen-Cory S. Visualizing synapse formation in arborizing optic axons in vivo: dynamics and modulation by BDNF. *Nat Neurosci.* 2001; 4:1093–1101. [PubMed: 11593233]
54. Cohen-Cory S. The developing synapse: Construction and modulation of synaptic structures and circuits. *Science.* 2002; 298:770–776. [PubMed: 12399577]
55. Askanas V, Kwan H, Alvarez RB, Engel WK, Kobayashi T, Martinuzzi A, et al. De novo neuromuscular junction formation on human muscle fibres cultured in monolayer and innervated by foetal rat spinal cord: Ultrastructural and ultrastructural-cytochemical studies. *J Neurocytol.* 1987; 16:523–537. [PubMed: 3681352]
56. Grubic Z, Komel R, Walker WF, Miranda AF. Myoblast fusion and innervation with rat motor nerve alter distribution of acetylcholinesterase and its mRNA in cultures of human muscle. *Neuron.* 1995; 14:317–327. [PubMed: 7857641]
57. Stett A, Egert U, Guenther E, Hofmann F, Meyer T, Nisch W, et al. Biological application of microelectrode arrays in drug discovery and basic research. *Anal Bioanal Chem.* 2003; 377:486–495. [PubMed: 12923608]
58. Schnabel J. Neuroscience: Standard model. *Nature.* 2008; 454:682–685. [PubMed: 18685674]
59. Kobayashi T, Askanas V. Acetylcholine receptors and acetylcholinesterase accumulate at the nerve-muscle contacts of de novo grown human monolayer muscle cocultured with fetal rat spinal cord. *Exp Neurol.* 1985; 88:327–335. [PubMed: 3987861]
60. Bandi E, Jevsek M, Mars T, Jurdana M, Formaggio E, Sciancalepore M, et al. Neural agrin controls maturation of the excitation-contraction coupling mechanism in human myotubes developing in vitro. *Am J Physiol Cell Physiol.* 2008; 294:C66–73. [PubMed: 18003748]

61. Jurdana M, Fumagalli G, Grubic Z, Lorenzon P, Mars T, Sciancalepore M. Neural agrin changes the electrical properties of developing human skeletal muscle cells. *Cell Mol Neurobiol*. 2009; 29:123–31. [PubMed: 18807173]
62. Gajsek N, Jevsek M, Grubic Z. Expression of MuSK in in vitro-innervated human muscle. *J Mol Neurosci*. 2006; 30:27–8. [PubMed: 17192614]
63. Jevsek M, Mars T, Mis K, Grubic Z. Origin of acetylcholinesterase in the neuromuscular junction formed in the in vitro innervated human muscle. *Eur J Neurosci*. 2004; 20:2865–2871. [PubMed: 15579140]
64. Séverine GS, Gilliane C, Serge B, Jean-Marie W, Philippe P. Muscle could be the therapeutic target in SMA treatment. *J Neurosci Res*. 1998; 53:663–669. [PubMed: 9753194]
65. Rubin LL. Stem cells and drug discovery: The beginning of a new era. *Cell*. 2008; 132:549–552. [PubMed: 18295572]
66. Karumbayaram S, Kelly TK, Paucar AA, Roe AJ, Umbach JA, Charles A, et al. Human embryonic stem cell-derived motor neurons expressing SOD1 mutants exhibit typical signs of motor neuron degeneration linked to ALS. *Dis Model Mech*. 2009; 2:189–95. [PubMed: 19259395]
67. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007; 131:861–72. [PubMed: 18035408]
68. Mizuno Y, Chang H, Umeda K, Niwa A, Iwasa T, Awaya T, et al. Generation of skeletal muscle stem/progenitor cells from murine induced pluripotent stem cells. *FASEB J*. 2010; 24:2245–2253. [PubMed: 20181939]
69. Darabi R, Pan W, Bosnakovski D, Baik J, Kyba M, Perlingeiro R. Functional Myogenic Engraftment from Mouse iPS Cells. *Stem Cell Reviews and Reports*. 2011 April. online.
70. Hickman JJ, Bhatia SK, Quong JN, Shoen P, Stenger DA, Pike CJ, et al. Rational pattern design for in-vitro cellular networks using surface photochemistry. *J Vac Sci & Tech A-Vac Surf Films*. 1994; 12:607–616.
71. Ravenscroft MS, Bateman KE, Shaffer KM, Schessler HM, Jung DR, Schneider TW, et al. Developmental neurobiology implications from fabrication and analysis of hippocampal neuronal networks on patterned silane- modified surfaces. *J Am Chem Soc*. 1998; 120:12169–12177.
72. Schaffner AE, Barker JL, Stenger DA, Hickman JJ. Investigation of the factors necessary for growth of hippocampal neurons in a defined system. *J Neurosci Methods*. 1995; 62:111–9. [PubMed: 8750092]
73. Stenger DA, Pike CJ, Hickman JJ, Cotman CW. Surface determinants of neuronal survival and growth on self-assembled monolayers in culture. *Brain Res*. 1993; 630:136–47. [PubMed: 8118680]
74. Wilson K, Das M, Wahl KJ, Colton RJ, Hickman JJ. Measurement of contractile stress generated by cultured muscle on silicon cantilevers. *PLoS One*. 2010; 5:e11042. [PubMed: 20548775]

Figure 1.
Schematic diagram of the culture protocol and timeline.

Figure 2.

Phase contrast micrographs A. Myocytes in the expansion phase before differentiation was induced. Scale bar: 100 μm. B. Multi-nuclei myotubes induced after differentiation. Scale bar: 25 μm. C. Myotubes and neurons exhibited healthy morphology after plating in the co-culture system. Scale bar: 50 μm. D. Physical connections were established between neurons and myotubes in the co-culture (indicated by arrows) Scale bar: 40 μm. E. Striated myotubes were present in the co-culture system as indicated by the yellow arrow. Scale bar: 25 μm. F. A micrograph of a neuron with MN morphology that extended an axon (red arrow) towards a striated myotube (yellow arrow). Scale bar: 25 μm.

Figure 3.
A&B. Representative voltage clamp (A) and current clamp (B) trace recording from the myotubes in the co-culture. C&D. Representative voltage clamp (C) and current clamp (D) trace recording from the motoneurons in the co-culture. Insert pictures indicate the cells from which the recordings were obtained.

Figure 4. Synapse formation as indicated by immunocytochemical analysis. A. Immunocytochemical identification of each component of the co-culture. Co-immunostaining of MHC (myosin heavy chain) and β III Tubulin in a 19 day co-culture demonstrated that axonal terminals branched in the vicinity of the myotube and wrapped heavily around the myotube. Scale bar: 50 μ m. B&C. Potential synaptic sites (yellow arrows in B and arrowheads in C) demonstrated by co-localization of nerve terminals (indicated by synaptophysin) and AchR (indicated by BTX-488) at day 15 in coculture. Scale bar: 100 μ m in B and 50 μ m in C.

Table 1
Composition of Enriched Co-culture Media

Component	Full Name	Concentration	Company	Catalog Number
Neurobasa /Neurobasal A			Invitrogen	10888/21103
B27 (50X)		1X	Invitrogen	17504-044
Glutamax (100X)		1X	Invitrogen	35050
GDNF	Glial-derived Neurotrophic Factor	10 ng/ml	Cell Sciences	CRG400B
BDNF	Brain-derived Neurotrophic Factor	20 ng/ml	Cell Sciences	CRB600B
Shh	Sonic Hedgehog, N-terminal peptide	50 ng/ml	R&D	1845-SH-025
RA	Retinoic Acid	0.1 uM	Sigma	R2625
IGF-1	Insulin-like Growth Factor -I	10 ng/ml	PeptoTech	100-11
cAMP	Adenosine 3',5'-cyclic Monophosphate	1 uM	Sigma	A9501
CNTF	Ciliary Neurotrophic Factor	5 ng/ml	Cell Sciences	CRC400A
NT-3	Neurotrophin-3	20 ng/ml	Cell Sciences	CRN500B
NT-4	Neurotrophin-4	20 ng/ml	Cell Sciences	CRN501B
Vitronectin		100 ng/ml	Sigma	V8379
Laminin	Mouse Laminin	4 µg/ml	Invitrogen	23017-015
G5 (100X)		1X	Invitrogen	17503-012
Agrin		100 ng/ml	R&D	550-AG-100