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A Defined Long-Term In Vitro Tissue Engineered Model of Neuromuscular Junctions

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Abstract

Neuromuscular junction (NMJ) formation, occurring between motoneurons and skeletal muscle, is a complex multistep process involving a variety of signaling molecules and pathways. *In vitro* motoneuron-muscle co-cultures are powerful tools to study the role of different growth factors, hormones and cellular structures involved in NMJ formation. In this study, a serum-free culture system utilizing defined temporal growth factor application and a non-biological substrate resulted in the formation of robust NMJs. The system resulted in long-term survival of the co-culture and selective expression of neonatal myosin heavy chain, a marker of myotube maturation. NMJ formation was verified by colocalization of dense clusters of acetylcholine receptors visualized using alpha-bungarotoxin and synaptophysin containing vesicles present in motoneuron axonal terminals. This model will find applications in basic NMJ research and tissue engineering applications such as bio-hybrid device development for limb prosthesis and regenerative medicine as well as for high-throughput drug and toxin screening applications.

Keywords

In Vitro Test; Muscle; Nerve Tissue Engineering; Neural Cell; Surface Modification; Self Assembly

Introduction

The neuromuscular junction (NMJ), formed between motoneurons and skeletal muscle fibers, is one of the most studied synaptic structures [1]. In a mammalian vertebrate, whenever an action potential is fired by a motoneuron, pre-synaptic vesicles loaded with the neurotransmitter acetylcholine (ACh) are released in the synaptic cleft [2]. The released ACh diffuses across the synaptic cleft and binds to the post-synaptic terminals in the muscle enriched with receptors for acetylcholine (AChRs). This leads to muscle contraction. In this transmission process the electrical impulses (action potentials) generated by the motoneuron are converted to chemical signals, then the chemical signals are converted into a mechanical signal in the form of muscle contraction. Therefore, not only do NMJs represent an

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important system for studying synapse formation and maturation, but also for studying how cells interconvert messages between electrical, chemical and mechanical modalities.

In vivo, NMJ formation is a multistep process, requiring the spatial and temporal interaction of growth factors, hormones and cellular structures that results in a pre-synaptic axonal terminal interfaced with a region of the skeletal muscle membrane (post-synaptic) prepatterned with AChRs [3,4]. *In vitro* culture models represent a powerful cell biology tool to study the role of these different growth factors, hormones and cellular structures involved in NMJ formation in a defined, controlled system. Consequently, the development of an *in vitro* system resulting in NMJ formation would facilitate investigations into the roles of specific factors involved in and required for the process to occur efficiently. However, limited success has been achieved in developing a long-term *in vitro* system for NMJ formation in the absence of serum containing media and biological substrates. These issues limit the reproducibility of *in vitro* studies and their translation to tissue engineering applications and high-throughput assay development. For example, the concentration and/or temporal application of medium components could be investigated to determine their influence on NMJ formation, maturation and maintenance. Such a system also benefits from the absence of factors that may be present in serum that would inhibit these processes. Employing a non-biological growth substrate such as trimethoxy-silylpropyl-diethylenetriamine (DETA) provides an additional measure of control. DETA is a silane molecule that forms a covalently bonded monolayer on glass coverslips, resulting in a uniform, hydrophilic surface for cell growth. The use of DETA surfaces is advantageous from a tissue engineering perspective because it can be covalently linked to virtually any hydroxylated surface, it is amenable to patterning using standard photolithography [5] and it promotes long-term cell survival because it is non-digestible by matrix metalloproteinases secreted by the cells [6,7]. It is also possible that its structural relationship to the growth factor spermidine, which has recently been shown to prolong cell life [8], contribute to its unique ability to enable long-term healthy cell cultures.

Previously, we developed a defined *in vitro* model facilitating the short-term co-culture of motoneurons and skeletal muscle that resulted in NMJ formation [9]. This model also utilized a biocompatible silane substrate and a serum-free medium formulation. However, further improvements were necessary to enhance the physiological relevance of the NMJ development system. Limitations of the previous model were that it did not support long-term tissue engineering studies and therefore, could not mimic several of the muscle maturation processes observed *in vivo* by obtaining myotubes that more accurately represent mature extrafusal fibers.

In this study, we have demonstrated a co-culture system that enable sarcomere assembly in the skeletal muscle myotubes as evidenced by A band I band formation, increased NMJ density and selective myosin heavy chain (MHC) class switching. These results suggest we have discovered a group of biomolecules that act as molecular switches promoting NMJ formation and maturation as well as skeletal muscle fiber maturation to the extrafusal phenotype. This model system will be a powerful tool in basic NMJ research, tissue engineered NMJ systems, bio-hybrid device development for limb prosthesis and in regenerative medicine. It could also be useful in new screening modalities for drug development and toxicology investigations.

Materials and Methods

Surface modification and characterization

Glass coverslips (Thomas Scientific 6661F52, 22 × 22 mm No.1) were cleaned using an O₂ plasma cleaner (Harrick PDC-32G) for 20 minutes at 100 mTorr. The DETA (United

Chemical Technologies Inc. T2910KG) films were formed by the reaction of the cleaned glass surface with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904). The DETA coated coverslips were then heated to approximately 100°C, rinsed with toluene, reheated to approximately 100°C, and then oven dried [10]. Surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, Cam 200) and by X-ray photoelectron spectroscopy (XPS) (Kratos Axis 165). XPS survey scans, as well as high-resolution N1s and C1s scans utilizing monochromatic Al K α excitation were obtained [10].

Skeletal muscle culture in serum free medium

Skeletal muscle was dissected from the thighs of the hind limbs of fetal rat (17–18 days old). Briefly, rats were anaesthetized and killed by inhalation of an excess of CO₂. This procedure was in agreement with the Animal Research Council of University of Central Florida, which adheres to IACUC policies. The tissue was collected in a sterile 15 mL centrifuge tube containing 1 mL of phosphate-buffered saline (calcium- and magnesium-free) (Gibco 14200075). The tissue was enzymatically dissociated using 2 mL of 0.05% of trypsin-EDTA (Gibco 25300054) solution for 30 minutes in a 37°C water bath at 50 rpm. After 30 minutes the trypsin solution was removed and 4 mL of Hibernate E + 10% fetal bovine serum (Gibco 16000044) was added to terminate the trypsin reaction. The tissue was then mechanically triturated with the supernatant being transferred to a 15 mL centrifuge tube. The same process was repeated two times by adding 2 mL of L15 + 10% FBS each time. The 6 mL cell suspension obtained after mechanical trituration was suspended on a 2 mL, 4% BSA (Sigma A3059) (prepared in L15 medium) cushion and centrifuged at 300 \times g for 10 minutes at 4°C. The pellet obtained was washed 5 times with L15 medium then resuspended in 10 mL of L15 and plated in 100 mm uncoated dishes for 30 minutes. The non-attached cells were removed and then centrifuged on a 4% BSA cushion [10]. The pellet was resuspended in serum-free medium according to the protocol illustrated in Figure 1 and plated on the coverslips at a density of 700–1000 cells/mm². The serum-free medium containing different growth factors and hormones was added to the culture dish after one hour. The final medium was prepared by mixing medium one (Table 1) and medium two (Table 2) in a 1:1 v/v ratio. Figure 1 indicates the flowchart of the culture protocol. Tables 1 and 2 list the growth factor and hormone supplement compositions of medium one and medium two. The cells were maintained in a 5% CO₂ incubator (relative humidity 85%). The entire medium was replaced after four days with NBactiv4 medium according to the protocol in Figure 1 [11]. Thereafter three-fourths of the medium was changed every three days with NbActiv4.

Rat embryonic motoneuron isolation and co-culture

Rat spinal motoneurons were purified from ventral cords of embryonic day 14 (E14) embryos. Briefly, rats were anaesthetized and killed by inhalation of an excess of CO₂. This procedure was in agreement with the Animal Research Council of University of Central Florida, which adheres to IACUC policies. Ventral spinal cord cells from the embryo were collected in cold Hibernate E + GlutaMAXTM + antibiotic-anti-mycotic + B27. The cells were dissociated with 0.05% trypsin-EDTA (Invitrogen) treatment for 15 minutes. The dissociated cells were layered over a 4 mL step gradient (Optiprep diluted 0.505: 0.495 (v/v) with Hibernate E + GlutaMAXTM + antibiotic anti-mycotic + B27 and then made to 15%, 20%, 25% and 35% (v/v) in Hibernate E + GlutaMAXTM + antibiotic-anti-mycotic + B27 followed by centrifugation for 15 min, using 200 \times g at 4°C. After centrifugation, four bands of cells were obtained. The motoneurons with large somas constituted the uppermost band. These cells present in the uppermost band were collected in fresh Hibernate E + GlutaMAXTM + antibiotic-anti-mycotic + B27 and centrifuged for 5 minutes at 200 \times g and 4°C. The pelleted motoneurons were resuspended in plating medium then plated on top of

muscle cells at a density of 100 cells/mm². Motoneuron plating was performed 30 minutes after plating of the muscle cells.

Immunocytochemistry

Neonatal myosin heavy chain (neonatal MHC)—Coverslips were rinsed with PBS, fixed in 20°C methanol for 5–7 min, washed in PBS, incubated in PBS supplemented with 1% BSA and 0.05% saponin (permeabilization solution), and blocked for 30 min in a permeabilization solution + 10% goat serum (blocking solution). Cells were incubated overnight with primary antibody against neonatal MHC (N3.36, IgG, Developmental Studies Hybridoma Bank) diluted (1:5) in the blocking solution. Cells were washed with PBS and incubated with AlexaFluor secondary antibody (Invitrogen) (diluted in PBS) for 2 h. The secondary antibody solution was removed and the cells were rinsed using PBS. The coverslips were dried and mounted on glass slides using VectaShield+DAPI mounting medium (Vector Laboratories H-1200) and viewed on a confocal microscope (UltraVIEW™ LCI, PerkinElmer).

Double staining with Neurofilament 150 and Neonatal myosin heavy chain—Co-cultures were processed for immunocytochemistry as described above. Next, cells were incubated overnight at 4°C with rabbit anti-neurofilament M polyclonal antibody, 150 kD, (Chemicon, AB1981, diluted 1:2000) and neonatal MHC (N3.36, IgG, Developmental Studies Hybridoma Bank diluted 1:5). After overnight incubation, the coverslips were rinsed three times with PBS and then incubated with the AlexaFluor secondary antibodies (Invitrogen) for 2h. After rinsing three times in PBS, the coverslips were mounted with Vectashield+DAPI mounting medium onto glass slides. The coverslips were visualized and images collected using a confocal microscope (UltraVIEW™ LCI, PerkinElmer). Controls without primary antibody were negative.

AChR + synaptophysin co-staining—AChRs were labeled as described previously by incubating cultures with 5×10^{-8} M of α -bungarotoxin, Alexa Fluor® 488 conjugate (Molecular Probes, B-13422) for 1.5 hr at 37°C before observation [9]. Labeled cultures were fixed with glacial acetic acid and ethanol, washed with PBS, dried, mounted and examined by confocal microscopy. The coverslips which were used for double staining with AChR + synaptophysin for locating the NMJ were processed further. After 1.5 hr of α -bungarotoxin labeling of the AChR receptors, the coverslips were fixed, blocked, permeabilized and incubated overnight with synaptophysin antibody (MAB368, diluted 1:1000; Millipore/Chemicon), the pre-synaptic marker present in motoneuron axonal terminals.

Data Analysis

The statistics shown in Table 3 were calculated using the following procedure. One coverslip was randomly selected from each experiment (typically, there are six coverslips per experiment). 25 non-overlapping fields of view were used to characterize each coverslip. At the magnification used, 25 fields covers over 40% of the surface area of the coverslip. The values of n shown in the table indicate the number of experiments used to obtain each result.

Results

DETA surface modification and characterization

Static contact angle and XPS analysis were used for the validation of the surface modifications and for monitoring the quality of the surfaces. Stable contact angles ($40.64^\circ \pm 2.9/\text{mean} \pm \text{SD}$) throughout the study indicated high reproducibility and quality of the

DETA surfaces and these characteristics were similar to previously published results [6,7,9,10,12]. Based on the ratio of the N 1s (401 and 399 eV) and the Si 2p_{3/2} peaks, XPS measurements indicated that a reaction-site limited monolayer of DETA was formed on the coverslips [13].

Temporal growth factor application

The formation of the maximal number of neuromuscular junctions was observed using the temporal growth factor application technique (Table 3) described in Figure 1. Upon plating of the motoneurons and skeletal muscle, the cells were treated with medium containing factors that promoted both growth and survival as well as enhancement of NMJ formation (Table 1, Table 2 and Table 3). After 3 days in culture, the entire medium was removed and switched to a minimal formulation, NBactiv4, which facilitated both long-term survival and further development of the NMJs (Figure 1). Further 3/4th of the NB4Activ medium per well was removed and replaced with an equal volume of fresh NB4Activ medium. When compared to the continuous application of growth factors, the timed application resulted in cultures that lasted for up to 7 weeks as opposed to 10–12 days.

Culture morphology of motoneuron and skeletal muscle myotube interactions

Phase contrast microscopy was used to visualize motoneuron axons appearing to interact with skeletal muscle myotubes between days 12–15 (Table 3, Figure 2, A–D). Some of the axonal processes appear to branch and terminate on the myotubes. Furthermore, many of the myotubes exhibited characteristic striation patterns (Table 3) observed after sarcomere formation when the fibers reached approximately 25–30 days in culture (Figure 3, A–D). Quantification of the appearance of striations after this time indicated that the co-cultures contained about twice the number of myotubes showing striations.

Immunocytochemical characterization of motoneuron and skeletal muscle co-culture

The characteristic protein expression patterns of the motoneurons and myotubes in co-culture were evaluated at day 25. Immunocytochemistry was used to visualize the neurofilament protein expression in the motoneurons and neonatal myosin heavy chain (MHC) expression for the myotubes (Figure 4, A–B). Motoneuron processes were clearly indicated interacting with the skeletal muscle myotubes. A band/I band formation was more visible in the myotubes after staining with the neonatal myosin heavy chain antibody (Table 3). The immunocytochemical analysis supported the morphological analysis, which had indicated the presence of striations in double the number of myotubes as observed with the muscle only controls.

Neuromuscular junction formation

In order to determine neuromuscular junction formation using this novel medium formulation, the clustering of AChRs using alpha bungarotoxin and their colocalization with synaptophysin vesicles was analyzed immunocytochemically. The colocalization of these two synaptic markers indicated the proximity of pre-synaptic and post-synaptic structures and was a positive indication of NMJ formation. This technique was used to identify the colocalization of synaptophysin vesicles with the AChR clusters (Figure 5, A–D). The axon + myotube interactions that did not result in the colocalization of pre-synaptic and post-synaptic structures were also identified (Figure 6, A–B). The observation of the negative result defines the difference between colocalization and non-colocalization and emphasizes the positive result observed in this system. Figure 7 illustrates NMJ formation between a myotube in culture that did not stain for neonatal myosin heavy chain and a motoneuron (Table 3).

Discussion

This work documents the substantial improvement of an *in vitro* model system for NMJ formation. Specifically, we observed enhanced survivability of the culture resulting in our ability to conduct long-term studies on the motoneuron-skeletal muscle co-cultures. This increased survivability resulted in maturation of the skeletal muscle myotubes and a significant improvement in the number of NMJs formed in culture.

Previously, we developed the first defined culture model to co-culture embryonic motoneuron and fetal skeletal muscle, however this model was not suitable for long-term tissue engineering studies and the myotubes in the culture only expressed an early muscle marker, i.e. fetal myosin heavy chain and none of the myotubes exhibited characteristic striations. In this study, significant improvement over our previous motoneuron-skeletal muscle co-culture model system was documented. This new culture model supported long-term co-culture of both motoneuron and muscle, resulted in a more adult-like morphology of the muscle and a higher density of neuromuscular junctions (NMJ). Our findings were supported by morphological and immunocytochemical data and results were summarized in Table 3.

We developed this serum-free medium, supplemented with growth factors that supported the survival, proliferation and fusion of fetal rat myoblasts into contractile myotubes, in a semi-empirical fashion. The rationale for selecting the growth factors was based on the distribution of their cognate receptors in the developing myotubes in rat fetus [14–16]. Tables 1 and 2 reference the literature where these individual growth factors, hormones and neurotransmitters were observed to support muscle and neuromuscular junction development. The composition in Table 1 is the formulation used for a previously published medium utilized for motoneuron-muscle co-culture and adult spinal cord neuron culture [9,17–19]. Table 2 lists the twelve additional factors identified in muscle development and neuromuscular junction formation that enabled the increased survivability of the system. Further addition of the factors in Table 2 promoted formation of characteristic striation in the muscle in the culture. The use of NBactiv4 for the maintenance of the cells significantly improved the survival of the skeletal muscle derived myotubes despite the fact that the original purpose of the development of NBactiv4 was for the long-term maintenance and synaptic connectivity of fetal hippocampal neurons *in vitro* [11].

In our previous co-culture model, we did not observe the expression of neonatal MHC proteins in the myotubes. Interestingly, when this same medium and protocol was used to culture pure skeletal muscle we observed certain striking differences. The pure muscle culture survived longer, exhibited characteristic striations, but only a very small percentage of myotubes expressed N3.36 [20]. To the best of our understanding, the N3.36 expression in skeletal muscle in culture is influenced by the motoneurons either physically or by certain trophic factors secreted in the presence of this modified medium and NBactiv4. This observation needs further studies in order to dissect the molecular pathways regulating N3.36 expression in pure skeletal muscle culture and in skeletal muscle-motoneuron co-culture. Also, the potential regulation of MHC class switching independent of neuronal innervation/denervation represents an interesting topic for further study and would have application in therapies for muscle-nerve diseases such as ALS, spinal muscular atrophy, spinal cord injury and myasthenia gravis.

Conclusions

The development of robust NMJ formation, long-term survival of motoneuron+skeletal muscle co-cultures and selective MHC class switching is documented in this research. This

improved system supports the goal of creating a physiologically relevant tissue engineered motoneuron+skeletal muscle construct and puts within reach the goal of developing functional bio-hybrid devices to analyze NMJ activity. This defined model can also be used to map the developmental pathway regulating NMJ formation and MHC class switching. Furthermore, we believe this serum-free culture system will be a powerful tool in developing advanced strategies for regenerative medicine in ALS, stretch reflex arc development and integrating motoneuron+skeletal muscle with bio-hybrid prosthetic devices. Due to the use of a serum-free defined culture system this also has applications for new high-throughput screening systems for use in drug discovery research and toxicology investigations.

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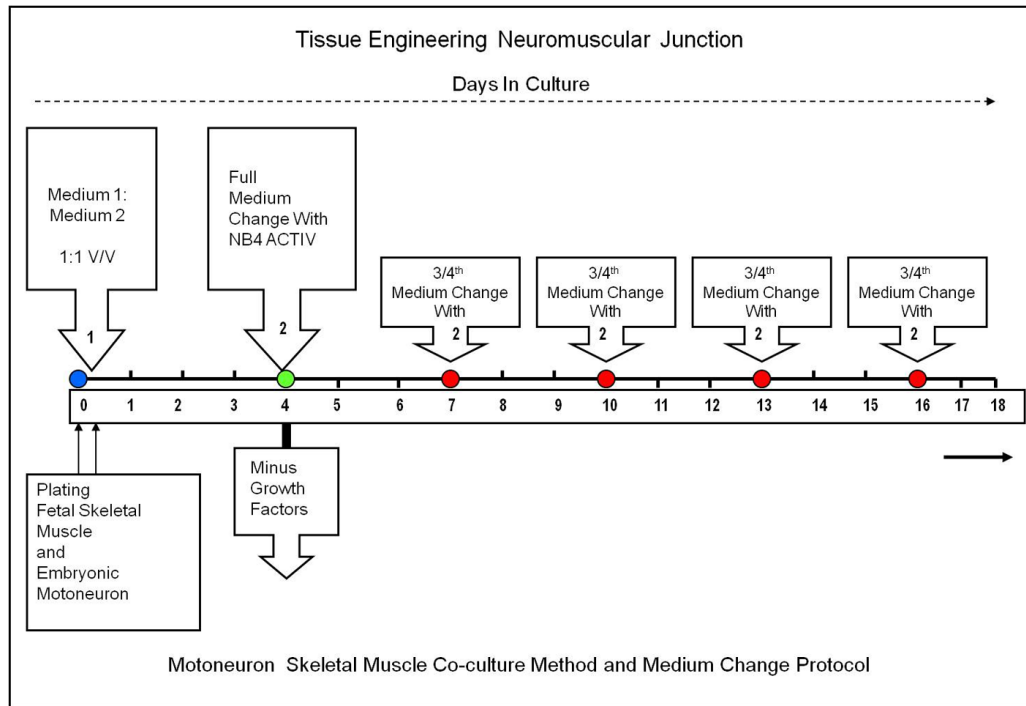


Figure 1. Protocol for long-term NMJ formation in a motoneuron and skeletal muscle co-culture.

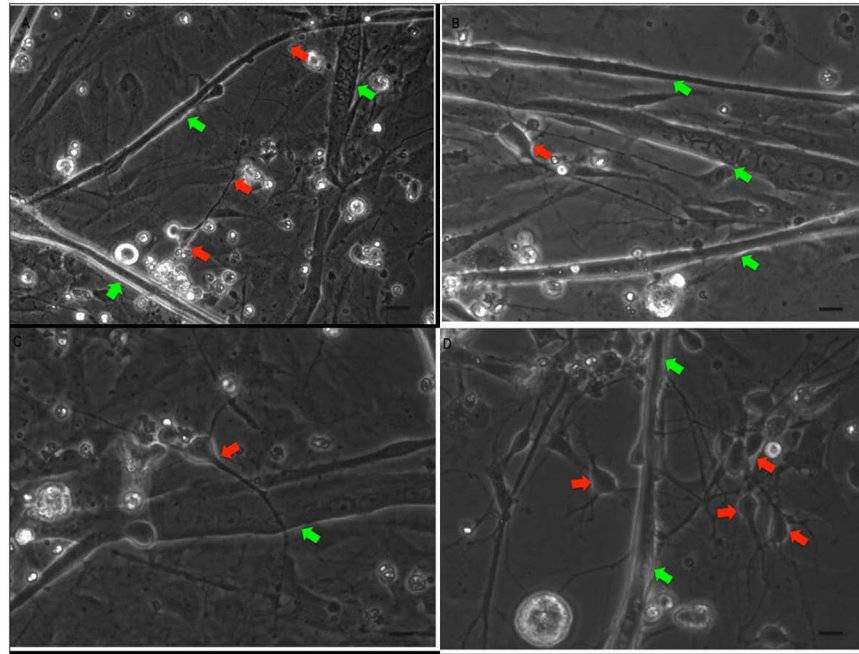


Figure 2. Phase contrast micrographs of the motoneuron and skeletal muscle co-culture between days 12–15
(A–D) Red arrows indicated the distinct morphology of the motoneuron and its processes. Green arrows indicate the myotubes. Scale bar = 25 μm .

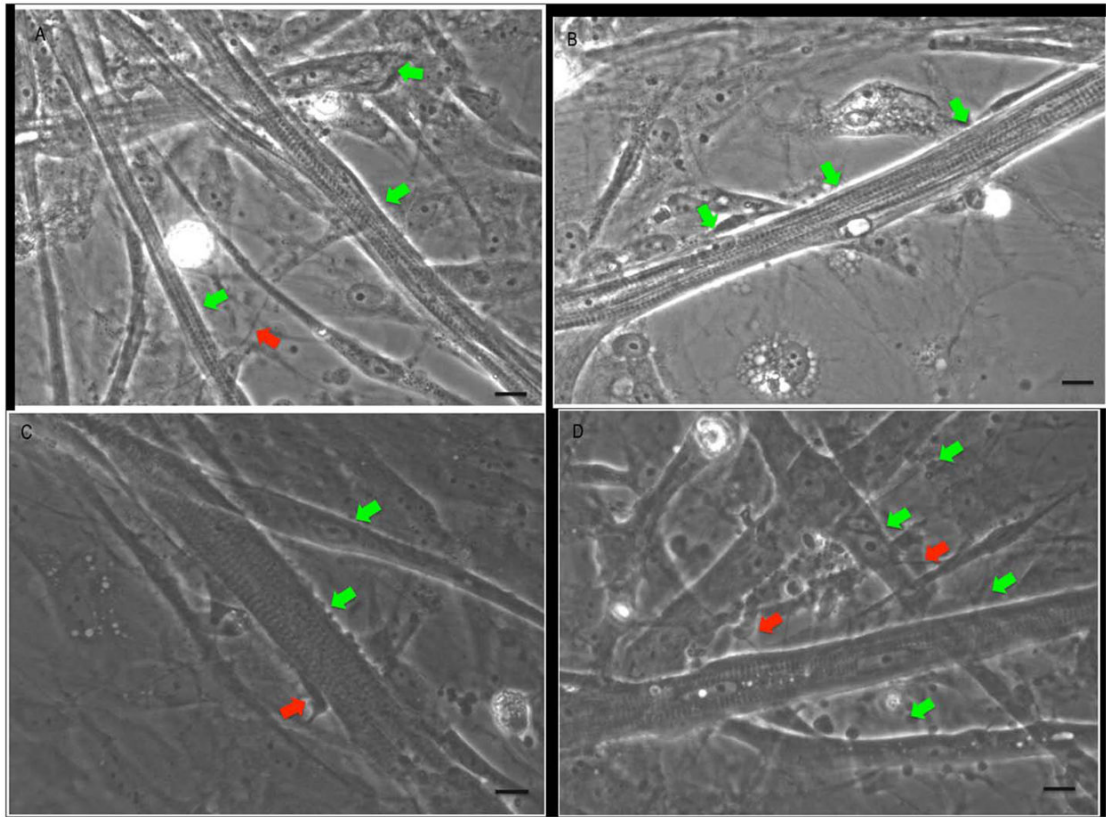


Figure 3. Phase contrast pictures of the co-cultures between days 25–30
(A, B) The myotubes exhibited characteristic striations. (C, D) Myotubes with striations and myotubes without striations. Red arrows indicate the motoneuron cell body and the processes. The green arrows indicate the myotubes. The scale bar for A, B = 40 μm . The scale bar for C, D = 25 μm .

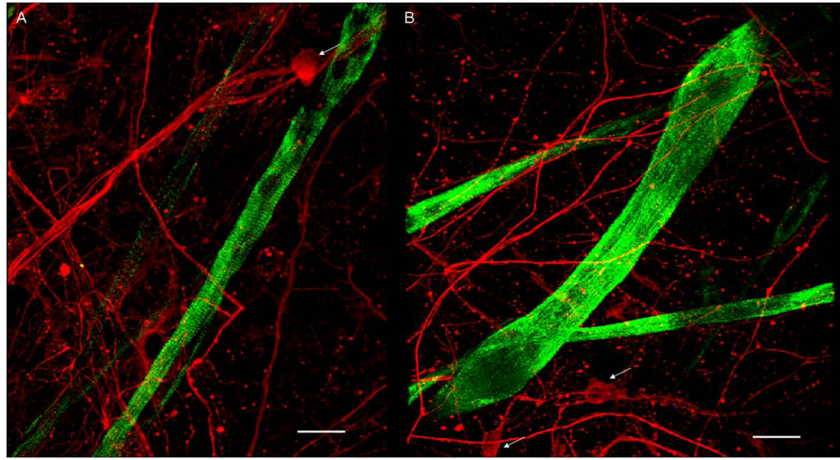


Figure 4. Immunocytochemistry of co-cultures at day 25
(A–B) NF-150 (red) indicates the large motoneurons and their processes (white arrows). The striated myotubes (green) stained for nMHC (N3.36). Scale bar = 50 μm .

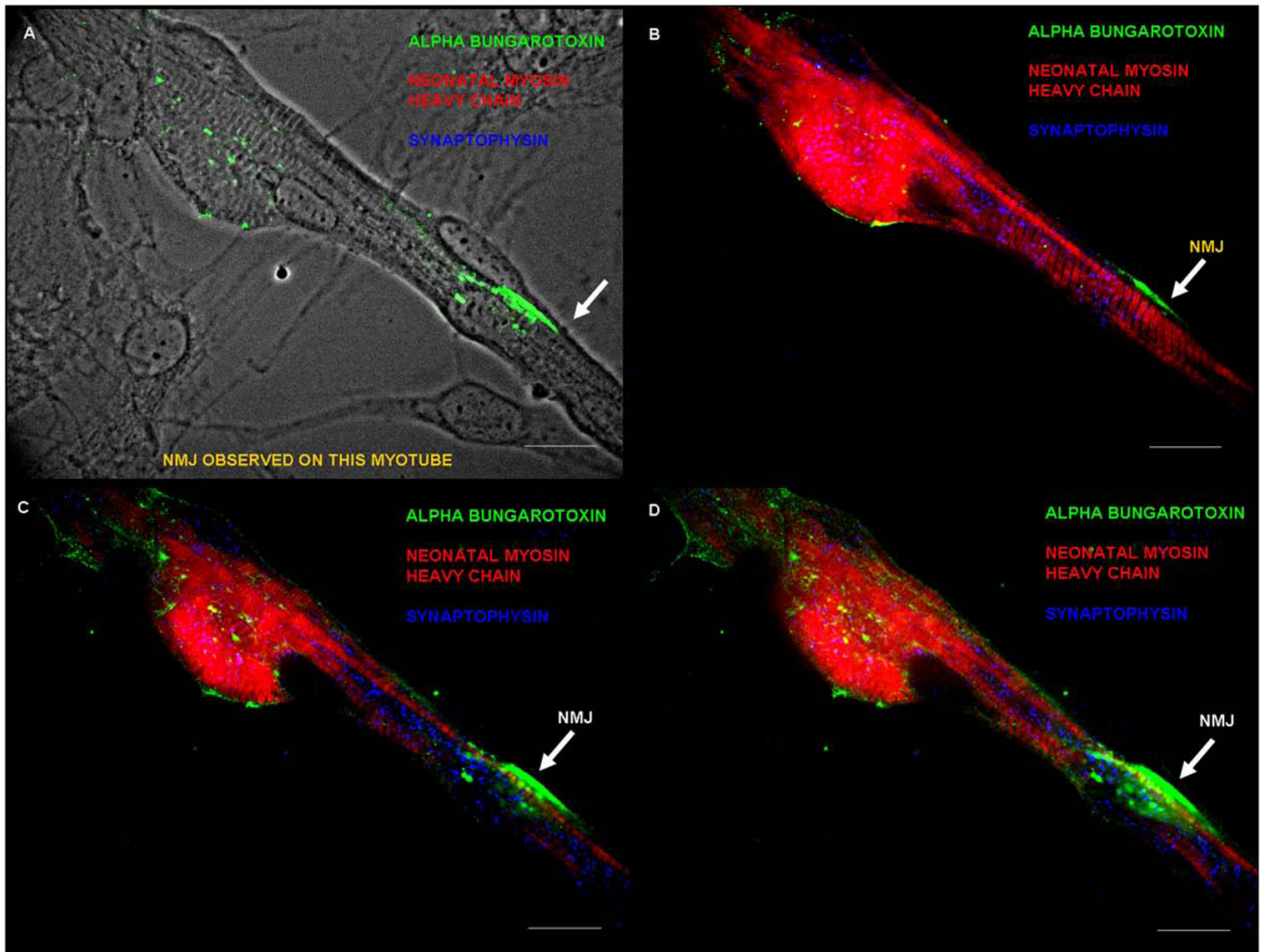


Figure 5. Neuromuscular junction (NMJ) formation between day 30–40

(A) Phase picture of the myotube indicating the alpha-bungarotoxin staining in green, (B) Triple stain, showing the close proximity of alpha-bungarotoxin (green) and synaptophysin (blue) indicating synapse formation at a specific confocal plane and myotube striations are indicated in red (nMHC), (C–D) NMJ observed at two different planes using confocal microscopy. A much more dense clustering of synaptophysin and alpha-bungarotoxin was observed in these planes.

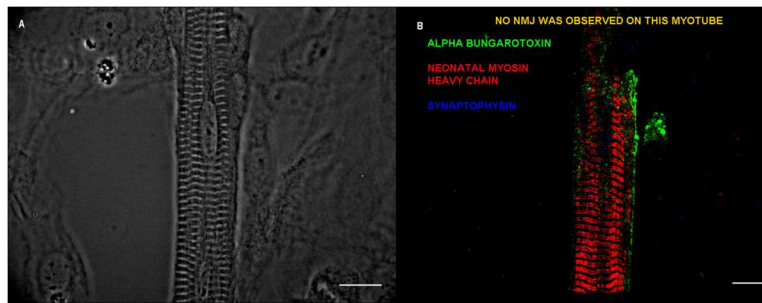


Figure 6. Striated myotube development in the absence of NMJ formation

(A, B) No NMJs were observed on this striated myotube. (A) A phase picture of the myotube, (B) Immunostained picture of the same myotube with alpha-bungarotoxin, N3.36 and synaptophysin. Scale bar = 50 μ m.

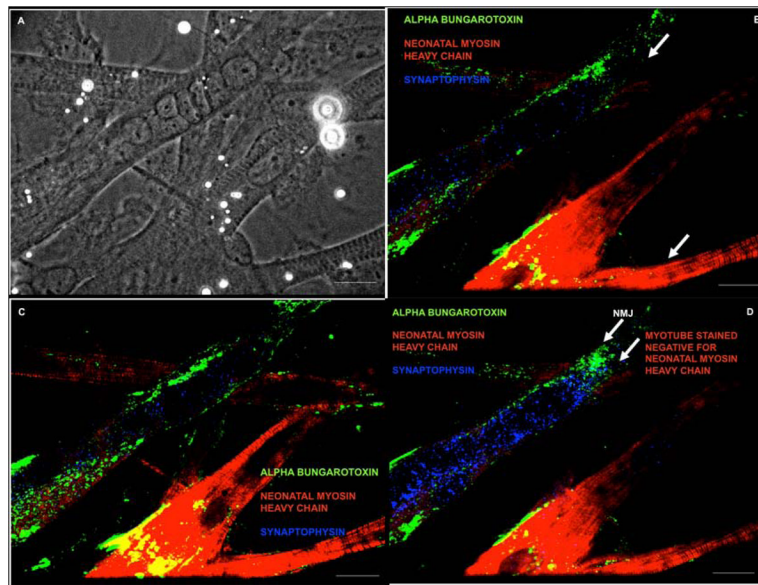


Figure 7. NMJ formation on a N3.36 (-) myotube

(A) Phase picture showing the different morphologies of myotubes in the co-culture, (B–D) NMJ formation was observed on a myotube that was negative for N3.36. Culture stained with alpha-bungarotoxin, N3.36 and synaptophysin. Possibly the myotube on which NMJ was formed was immature and had not yet expressed the neonatal myosin heavy chain marker (N3.36).

Table 1

Composition of medium 1.

S. No	Component	Amount	Catalogue #	Source	References
1.	Neurobasal	500 ml	10888	Gibco/Invitrogen	[21]
2.	Antibiotic-Antimycotic	5 ml	15240-062	Gibco/Invitrogen	
3.	Glutamax	5 ml	35050-061	Gibco/Invitrogen	
4.	B27 Supplement	10 ml	17504-044	Gibco/Invitrogen	[6,21]
5.	G5 Supplement (100X)	5 ml	17503-012	Gibco/Invitrogen	[22]
6.	VEGF ₁₆₅ Human	10 µg	P2654	Gibco/Invitrogen	[23]
7.	Acidic FGF	12.5 µg	13241-013	Gibco/Invitrogen	[24,25]
8.	Heparin Sulphate	50 µg	D9809	Sigma	[24,25]
9.	LIF	10 µg	L5158	Sigma	[26-28]
10.	Vitronectin (Rat Plasma)	50 µg	V0132	Sigma	[29,30]
11.	CNTF	20 µg	CRC 401B	Cell Sciences	[31,32]
12.	NT 3	10 µg	CRN 500B	Cell Sciences	[33]
13.	NT 4	10 µg	CRN 501B	Cell Sciences	[34]
14.	GDNF	10 µg	CRG 400B	Cell Sciences	[35,36]
15.	BDNF	10 µg	CRB 600B	Cell Sciences	[37]
16.	CT-1	10 µg	CRC 700B	Cell Sciences	[38,39]

Table 2

Composition of medium 2.

S. No	Component(s)	Amount	Catalogue	Source	References
1.	NBActiv4	500 ml	NBActiv4-500	BrainBits	
2.	Glutamax	5 ml	35050-061	Invitrogen/Gibco	
3.	Antibiotic-antimycotic	5 ml	15240-062	Invitrogen/Gibco	
4.	B27 supplement	10 ml	17504-044	Invitrogen/Gibco	[6,21]
5.	Cholesterol (250X)	5 ml	12531	Invitrogen/Gibco	
6.	TNF-alpha, human	10 µg	T6674	Sigma-Aldrich	[40,41]
7.	PDGF BB	50 µg	P4056	Sigma-Aldrich	[26,42]
8.	Vasoactive intestinal peptide (VIP)	250 µg	V6130	Sigma-Aldrich	[43]
9.	Insulin-like growth factor 1	25 µg	I2656	Sigma-Aldrich	[27,40]
10.	NAP	1 mg	61170	AnaSpec, Inc.	[44]
11.	Recombinant Apolipoprotein E2	50 µg	P2002	Panvera, Madison, WI	[45]
12.	Laminin, mouse purified	2 mg	08-125	Millipore	[46]
13.	Beta amyloid (1-40)	1 mg	AG966	Millipore	[47,48]
14.	Human Tenascin-C protein	100 µg	CC065	Millipore	[49]
15.	rr- Sonic hedgehog, Shh N-terminal	50 µg	1314-SH	R&D Systems	[15,50,51]
16.	rr (Agrin C terminal)	50 µg	550-AG-100	R&D Systems	[52]

Table 3
Comparison of the culture following temporal application of the growth factor and continuous application of the growth factors

	Temporal application of growth factors	Continuous application of growth factors
Quality of the coculture based on visual observations	Visually individual myotubes and motoneurons could be distinctly seen in the culture by day 10. Myotube formation is initiated on day 4 and continued through day 12. Large, multipolar motoneurons with long processes were clearly seen by day 10. The maturation and appearance of striated myotubes were seen after day 15, and by day 25 a large population of myotubes exhibited striation patterns.	When growth factors were applied continuously, the cells proliferated uncontrollably. The uncontrolled growth of different cell types in the culture prevented the myotube formation process. This resulted in detachment of the cells from the substrate by day 9 and the culture to die out by day 12. The formation of very few myotubes was observed in the culture during this 10–12 day period.
Survival of the culture	Cultures survived for more than 5 weeks with a maximum up to 7 weeks (n>25); where n was the total number of trials.	Cultures survived for 10–12 days (n>7); where n was the total number of trials.
Fraction of myotubes indicating evidence of neuromuscular junction (NMJ) formation between day 12–15 in the co-culture	% Myotubes with NMJ: 20.36 ± 3.75 (mean ± standard error, n=11)	Since the cultures died by day 10–12 and there were hardly any myotube formation, no NMJ quantification was done on these cultures.
Fraction of myotubes exhibiting characteristic striation patterns in in pure muscle culture as well as in co-culture between day 20–25	In pure muscle culture: 40.95 ± 3.06 (mean ± standard error, n=8) In coculture: 70.33 ± 2.17 (mean ± standard error, n=8)	Since there was little evidence of myotube formation, and the cultures did not survive beyond day 10, no estimation was possible.
Fraction of myotubes exhibiting N3.36 protein expression in pure muscle culture as well as in co-culture between day 20–25	In pure muscle culture: 29.95 ± 2.96 (mean ± standard error, n=12) In coculture: 60.33 ± 3.79 (mean ± standard error, n=12)	No N3.36 expression was observed in any of the surviving myotubes.