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Skeletal Muscle Tissue Engineering: A Maturation Model Promoting Long Term Survival of Myotubes, Structural Development of the Excitation - Contraction Coupling Apparatus and Neonatal Myosin Heavy Chain Expression

Mainak Das^{*}, John W Rumsey^{*}, Neelima Bhargava, Maria Stancescu, and James J Hickman^{**}

Hybrid Systems Lab, NanoScience Technology Center, Suite 402, Research Pavilion, 12424, Research Parkway, University of Central Florida, Orlando, FL 32826, USA

Abstract

The use of defined *in vitro* systems to study the developmental and physiological characteristics of a variety of cell types is increasing, due in large part to their ease of integration with tissue engineering, regenerative medicine, and high-throughput screening applications. In this study, myotubes derived from fetal rat hind limbs were induced to develop several aspects of mature muscle including: sarcomere assembly, development of the excitation-contraction coupling apparatus and myosin heavy chain (MHC) class switching. Utilizing immunocytochemical analysis, anisotropic and isotropic band formation (striations) within the myotubes was established, indicative of sarcomere formation. In addition, clusters of ryanodine receptors were colocalized with dihydropyridine complex proteins which signaled development of the excitation-contraction coupling apparatus and transverse tubule biogenesis. The myotubes also exhibited MHC class switching from embryonic to neonatal MHC. Lastly, the myotubes survived significantly longer in culture (70–90 days) than myotubes from our previously developed system (20–25 days). These results were achieved by modifying the culture timeline as well as the development of a new medium formulation. This defined model system for skeletal muscle maturation supports the goal of developing physiologically relevant muscle constructs for use in tissue engineering and regenerative medicine as well as for high-throughput screening applications.

Keywords

Cell culture; Silane; Muscle; In Vitro Test; Surface Analysis; Surface Modification

Introduction

Skeletal muscle differentiation and maturation is a complex process involving the synergy of different growth factors and hormones interacting over a broad time period [1–4]. The differentiation process is further complicated by neuronal innervation, where neuron to muscle cell signaling can regulate myosin heavy chain (MHC) gene expression and acetylcholine receptor clustering [5–7]. Consequently, understanding of the role of growth factors, hormones and cellular interactions in skeletal muscle differentiation would be a key step in generating physiologically relevant tissue engineered constructs, developing advanced strategies for

^{**}Corresponding author jhickman@mail.ucf.edu.

^{*}These authors have equal contribution

regenerative medicine and integrating functional skeletal muscle with bio-hybrid MEMS devices for non-invasive interrogation in high-throughput screening technologies.

In order for skeletal muscle myotubes developed *in vitro* to be useful in tissue engineering applications, they must exhibit as many of the functional characteristics of *in vivo* skeletal muscle fibers as possible. During muscle fiber development *in vivo*, several critical structural changes occur that indicate functional maturation of the extrafusal myotubes. These changes include sarcomere organization, clustering and colocalization of ryanodine (RyR) and dihydropyridine (DHPR) receptors and MHC class switching [8,9]. Each of these structural changes reflects the physiological maturation of the skeletal muscle and is critical for consistent muscular contraction. For example, organization of the contractile proteins myosin and actin into sarcomeric units gives skeletal muscle myotubes organized and structured contraction, a property lacking in smooth muscle. The organization of sarcomeres in skeletal muscle gives rise to anisotropic and isotropic bands of proteins (A and I bands) and gives skeletal muscle a striated appearance. The clustering and colocalization of RyR and DHPR is indicative of transverse tubule (T-tubule) biogenesis and excitation contraction coupling. This developmental step structurally links electrical excitation to the internal contractile system by providing close apposition of DHPR located in the T-tubule and RyR located in the sarcoplasmic reticulum. Finally, a properly functioning skeletal muscle must express the appropriate MHC proteins required for the task it must perform. For example, different muscle fibers express different MHC proteins depending on the rate of contraction and force generation required by the work to be done. Without these modifications, an *in vitro* model of skeletal muscle maturation cannot achieve full physiological relevance.

One approach for identifying the role of specific growth factors and hormones in muscle differentiation is to develop an *in vitro* model system consisting of a serum-free medium supplemented with the factors of interest [10]. Such a model provides the opportunity to evaluate the role of each factor individually or in combination with others known or believed to be important in skeletal muscle development. It also has the benefit of removing factors that may be present in serum that would inhibit maturation. 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, non-hydrophilic surface for cell growth and may partially mimic the three dimensional features of an extracellular matrix, which may be responsible for the robust growth found for different cell types on this synthetic substrate [11–15]. Previously, studies have demonstrated the usefulness of the DETA silane substrate for *in vitro* culture systems [10,16,17]. 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 [17] and it promotes long-term cell survival because it is non-digestible by matrix metalloproteinases secreted by the cells [15,18].

A defined system that promoted differentiation of different skeletal muscle phenotypes was developed earlier and resulted in the formation of contractile myotubes, but with only short-term survival [12,15]. In a technological advance we have also developed a novel bio-hybrid system to integrate functional myotubes with cantilever based bio-MEMS devices for the study of muscle physiology, neuromuscular junction formation, bio-robotics applications and for use in a model of the stretch reflex arc [19]. More recently, using our defined model system, we have achieved a significant breakthrough by creating mechanosensitive intrafusal myotubes *in vitro* [20]. Intrafusal fibers are the myotubes present in the muscle spindle which functions as the sensory receptor of the stretch reflex circuit [7] and combined with extrafusal fibers represent the primary component necessary to reproduce functional muscle activity *in vitro*.

This system has been utilized as a model for different developmental and functional applications, however, further improvements were necessary to enhance the physiological relevance of the skeletal muscle myotubes [19,20]. Specifically, in order to create a working model of the stretch reflex arc, myotubes are needed that more accurately represent extrafusal fibers *in vivo*. In this study, we have now demonstrated sarcomere assembly, the development of the excitation-contraction coupling apparatus and myosin heavy chain (MHC) class switching. These results suggest we have discovered a group of biomolecules that act as a molecular switch promoting the transition from embryonic to neonatal MHC expression as well as other structural adaptations resulting in the maturation of skeletal muscle *in vitro*. The discovery of these biomolecular switches will be a powerful tool in regenerative medicine and tissue engineering applications such as skeletal muscle tissue grafts. It should also be useful in the creation of higher content high-throughput screening technologies.

Materials and Methods

Surface modification and characterization

Glass coverslips (Thomas Scientific 6661F52, 22 × 22mm 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 [12]. 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 [12].

Skeletal muscle culture and serum free medium

Skeletal muscle was dissected from the thighs of the hind limbs of fetal rat (17–18 days old). 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 300g 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 [12]. 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. Table 1 and Table 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 full medium was replaced after four days with NBactiv4 medium according to the protocol in Figure 1 [21]. Thereafter three-fourths of the medium was changed every three days with NBactiv4.

Immunocytochemistry of skeletal muscle myotubes

Coverslips were prepared for immunocytochemical analysis as previously described. Briefly, 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) for 10 minutes, and blocked for 2h with 10% goat serum and 1% BSA. Cells were incubated overnight with primary antibodies against embryonic myosin heavy chain (F1.652) (dilution $>1:5$), neonatal myosin heavy chain (N3.36) (1:5) (Developmental Studies Hybridoma Bank), ryanodine receptor (AB9078, Millipore) (1:500) and dihydropyridine binding complex ($\alpha 1$ -Subunit) (MAB 4270, Millipore) (1:500) diluted in the blocking solution. Cells were washed with PBS and incubated with the appropriate secondary antibodies for two hours in PBS. After two hours the coverslips were rinsed with PBS and mounted on glass slides and evaluated using confocal microscopy [14].

AChR labeling of myotubes

AChRs were labeled as described previously by incubating cultures with 5×10^{-8} M of α -bungarotoxin, Alexa Fluor® 488 conjugate (B-13422; Invitrogen) for 1.5 h at 37°C [5,14]. Following incubation in α -bungarotoxin, the cultures were fixed as above for subsequent staining with embryonic myosin heavy chain (F1.652) antibodies.

Patch clamp electrophysiology of the myotubes

Whole-cell patch clamp recordings were performed in a recording chamber located on the stage of a Zeiss Axioscope 2FS Plus upright microscope as described previously [15,20]. The chamber was continuously perfused (2 ml/min) with the extracellular solution (Leibovitz medium, 35°C). Patch pipettes were prepared from borosilicate glass (BF150-86-10; Sutter, Novato, CA) with a Sutter P97 pipette puller and filled with intracellular solution (K-gluconate 140 mM, EGTA 1 mM, MgCl_2 2 mM, Na_2ATP 2 mM, phosphocreatine 5mM, phosphocreatine kinase 2.4 mM, Hepes 10 mM; pH=7.2). The resistance of the electrodes was 6–8M Ω . Voltage clamp and current clamp experiments were performed with a Multiclamp 700A amplifier (Axon Laboratories, Union City, CA). Signals were filtered at 2 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface. Data recording and analysis were done with pClamp 8 software (Axon Laboratories). Membrane potentials were corrected by subtraction of a 15 mV tip potential, which was calculated using Axon's pClamp 8 program. Sodium and potassium currents were measured in voltage clamp mode using voltage steps from a -85 mV holding potential. Action potentials were evoked with 1 second depolarizing current injections from a -85 mV holding potential [12,15].

Results

DETA surface modification and characterization

Static contact angle and XPS analysis was used for the validation of the surface modifications and for monitoring the quality of the surfaces. Stable contact angles ($40.64^{\circ} \pm 2.9$ /mean $\pm$ SD) throughout the study indicated high reproducibility and quality of the DETA surfaces and these characteristics were similar to previously published results [14,15]. Based on the ratio of the N (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 [22].

Development of the serum free medium formulation and culture timeline for long-term survival and maturation of myotubes

The serum free medium composition was developed semi-empirically. The final medium is derived from two different medium compositions described in Table 1 and Table 2. Table 1 constitutes the same medium composition used previously for a motoneuron-muscle co-culture

and adult spinal cord neurons culture [11,14]. Table 2 is composed of twelve additional factors that had been shown to promote skeletal muscle maturation and neuromuscular junction formation individually. The final medium was prepared by mixing these two mediums in a 1:1 v/v ratio. After the first 4 days of culture the whole medium was replaced with NBactiv4 medium [21]. Thereafter, every three days three-fourths of the medium was changed with NBactiv4. The culture technique has been illustrated in the flowchart in Figure 1.

Using this new medium formulation and timeline, myotubes were successfully cultured for more than 90 days. Figure 2 indicates 50 days old myotubes in culture. As the myotubes aged and grew they began to form the characteristic anisotropic (A band) and isotropic (I band) banding pattern observed with mature *in vivo* muscle fibers [9,23]. This banding pattern is caused by differential light diffraction due to the organization of myofibril proteins forming sarcomeres within the myotubes [9,23]. The arrowheads in the images (Figure 2 A–D) indicate myotubes where sarcomeric organization has occurred and is visualized by the appearance of the A and I bands.

Myotube expression of fetal myosin heavy chain

The myotubes formed were evaluated for the expression of fetal MHC to establish a baseline as comparison to our previous results [12]. In Figure 3, the different myotube phenotypes formed at approximately day 50 *in vitro* are shown. The myotubes ranged from having clustered nuclei (Figure 3 A–D) to having diffuse nuclear organization (Figure 3 E–H). The arrowheads in the images indicate the characteristic striations.

Differential expression of neonatal MHC protein in the myotubes

In order to determine if the myotubes were maturing in a physiologically relevant way as they aged *in vitro*, the expression of neonatal MHC protein was evaluated. After approximately 50 days *in vitro*, 25% of the myotubes expressed neonatal MHC (Figure 4 A–M). Additionally, the myotubes were stained to detect the clustering of acetylcholine receptors (AChR) using alpha bungarotoxin (Figure 5 B, F). This clustering of the AChR receptors, which are induced by the motoneuron protein agrin *in vivo*, are locations on the myotube where neuromuscular junction formation occurs and another indication of functional myotube formation.

Formation of the excitation – contraction coupling apparatus

The presence of the ryanodine (RyR) receptor and dihydropyridine (DHPR) receptor clusters, as well as their colocalization *in vivo*, represents the development of excitation-contraction coupling apparatus in skeletal muscle myotubes [8,9,24]. The clustering of both RyR and DHPR receptors was observed on the myotubes after 30 days in culture (Figure 5 A–D). The clustering and colocalization of the RyR + DHPR clusters was observed with a number of different myotube morphologies (Figure 5 E–L). This functional adaptation illustrated that the medium formulation facilitated not only the structural maturation but also the functional maturation of myotubes in this *in vitro* system. The clustering of the RyR + DHPR receptors was also observed in 90 day old myotubes, indicating that the older myotubes maintained their functional integrity (Figure 6 A–F).

Myotube electrophysiology

The myotubes contracted spontaneously in the culture and the contractions began generally by day four and continued throughout the life of the culture. Most of the myotubes expressed functional voltage gated sodium, potassium and calcium ion channels as reported previously [12]. The voltage clamp electrophysiology of the myotubes indicated the inward and outward currents that demonstrates functional sodium and potassium channels (Figure 7 A). Figure 7B shows the single action potential fired by the myotubes in current clamp mode. The electrical

properties of the myotubes were also consistent with the formation of mature, functional myotubes.

Discussion

In this report we have documented the development of a system for long-term, functional, skeletal muscle culture. This system was in response for the need to develop more physiologically relevant skeletal muscle myotubes for functional *in vitro* systems. For our specific research, they are a component needed for the creation of a realistic model of the stretch reflex arc as well as their integration with bio-MEMS cantilevers for screening applications. The results indicate that we have achieved three significant structural modifications within the myotubes, causing both the developmental profile and functionality of the fibers to better mimic *in vivo* physiology. This skeletal muscle maturation resulted from modifications to the cell culture technique, a new medium formulation and the use of NBactiv4 as the maintenance medium.

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 [3,4,25]. Table 1 and Table 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 used for motoneuron-muscle co-culture and adult spinal cord neuron culture [11,14]. Table 2 lists the twelve additional factors we have identified in muscle development and neuromuscular junction formation. The use of NBactiv4 for the maintenance of the cells significantly improved the survival of the skeletal muscle derived myotubes despite the original development of NBactiv4 for the long-term maintenance and synaptic connectivity of fetal hippocampal neurons *in vitro* [21].

We observed a ratio of 25% neonatal to 75% embryonic MHC expression of the myotubes after 50 days, which contrasts with the previous study in which MHC expression was strictly embryonic. We believe that the myotubes matured in this culture system because the long-term survival provided adequate time for the myotubes to respond to the additional growth factors, which activated the necessary signaling pathways to achieve MHC class switching [26]. This suggests that a different growth factor profile could be utilized to activate alternative signaling pathways and drive myotube differentiation down other pathways. For example, the effects of adding steroid hormones like testosterone to the system could be critically examined.

The colocalization of RyR and DHPR clusters in the myotubes indicated the formation of the excitation-contraction coupling apparatus and was another indication of functional maturation in the fibers. Excitation-contraction coupling is the signaling process in muscle by which membrane depolarization causes a rapid elevation of the cytosolic Ca^{2+} generating contractile force [27]. The close proximity of the DHPR and RyR complexes occurs at specialized junctions established between the transverse tubule and sarcoplasmic reticulum (SR) membranes in skeletal muscle myotubes [28]. At these junctions, T-tubule depolarization is coupled to Ca^{2+} release from the SR resulting in muscle contraction [29,30]. This structural adaptation represents a significant functional change due to the fact that excitation-contraction coupling is required for successful extrafusal muscle fiber development as well as neuromuscular junction formation [8,9,23]. This improved model provides the potential to study excitation-contraction coupling in a defined system as well as for myotonic and myasthenic diseases.

Conclusions

The development of sarcomeric structures, the excitation-contraction coupling apparatus and MHC class switching in the skeletal muscle myotubes is a result of the improvements to the model system documented in this research. This improved system supports the goal of creating physiologically relevant tissue engineered muscle constructs and puts within reach the goal of developing functional skeletal muscle grafts. This defined model can also be used to map the developmental pathway regulating the formation of the excitation-contraction coupling apparatus as well as MHC class switching. Furthermore, we believe this serum-free culture system will be a powerful tool in developing advanced strategies for regenerative medicine in muscular dystrophies, stretch reflex arc development and integrating 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.
Schematic diagram of the culture protocol and timeline.
Culture method and medium change protocol for skeletal muscle culture

Figure 2.
A, B, C and D: Phase pictures of 50 day old myotubes in culture. Red arrows indicate characteristic striations in most of the myotubes. Scale bar: 75 microns.

Figure 3. Myotubes stained with antibodies against the embryonic myosin heavy chain (F 1.652) proteins at day 50. Scale bar: 75 microns

A. Panel showing phase + fluorescent picture of the myotubes.

B. Panel A observed only in fluorescent light (white arrows indicate the striations).

C. Panel showing phase + fluorescent image of the myotubes.

D. Panel C observed only in fluorescent light.

E. Panel showing phase + fluorescent micrograph of the myotubes (white arrow indicates the striations).

F. Panel E observed only in fluorescent light (white arrow indicates the striations).

G. Panel showing phase + fluorescent picture of the myotubes (white arrow indicates the striations).

H. Panel G observed only in fluorescent light (white arrow indicates the striations).

Figure 4. Myotubes immunostained with neonatal myosin heavy chain (N3.36) and alpha-bungarotoxin at day 50. Scale bar: 75 microns

- A. Phase picture of 2 myotubes indicated by white arrows.
- B. Both the myotubes shown in phase (panel A) have acetylcholine receptor clustering indicated by alpha-bungarotoxin staining.
- C. Only one myotube out of the two seen in panel A stained for N3.36.
- D. Double stained image of panel A with alpha-bungarotoxin and N3.36.
- E. Phase image of 6 myotubes indicated by white arrows.
- F. All the myotubes shown in phase (panel E) have acetylcholine receptor clustering shown by alpha-bungarotoxin staining.
- G. None of the myotubes in panel E stained for N3.36.
- H, I, J. Differential staining of the myotubes with N3.36.
- K, L, M. Differential staining of the myotubes with N3.36.

Figure 5. Ryanodine receptor and DHPR receptor clustering in 30 day old skeletal muscle culture. Scale bar 75 microns

- A. A phase and fluorescent-labeled micrograph of the myotubes.
- B. Merged fluorescent picture of the ryanodine receptor (green) and DHPR receptor (red) clustering on the myotubes shown in panel A.
- C. Ryanodine receptors (green) on the myotubes shown in panel A.
- D. DHPR receptors (red) on the myotubes shown in panel A.
- E. Phase and fluorescent-labeled micrograph of the myotubes.
- F. Merged fluorescent picture of the ryanodine receptor (green) and DHPR receptor (red) clustering on the myotubes (panel E).
- G. Ryanodine receptor (green) on the myotubes (panel E).
- H. DHPR receptors (red) on the myotubes (panel E).
- I. Phase and fluorescent-labeled micrograph of the myotubes.
- J, K and L. Merged fluorescent micrograph of the ryanodine receptor (green) and DHPR receptor (red) clustering on the myotubes (panel I) at three different planes (white arrows indicate the striations and the receptor clustering).

Figure 6. Ryanodine receptor and DHPR receptor clustering in a 90 day old skeletal muscle culture.
Scale bar: 75 microns

- A. Phase and fluorescent-labeled micrograph of the myotubes.
- B. Merged fluorescent micrograph of the ryanodine receptor (green) and DHPR receptor (Red) clustering on the myotubes (panel A).
- C. Ryanodine receptor (green) on the myotubes (panel A).
- D. DHPR receptors on the myotubes (panel A).

E and F. Same panels at a different plane indicating the merged fluorescent picture of the Ryanodine receptor (green) and DHPR receptor (red) clustering on the myotubes.

Figure 7. Patch clamp electrophysiology of the myotubes

A. Representative voltage clamp trace obtained after patching a 48 days old myotube in culture.
B. Representative current clamp trace of the same myotube for which voltage clamp trace had been obtained. Inset showing the picture of the patched myotubes.

Table 1

Composition of medium 1

S. No	Component	Amount	Catalogue #	Source	References
1.	Neurobasal	500 ml	10888	Gibco/ Invitrogen	[28]
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	[7,28]
5.	G5 Supplement (100X)	5 ml	17503-012	Gibco/ Invitrogen	[29–38]
6.	VEGF 165 r Human	10 µg	P2654	Gibco/ Invitrogen	[39–42]
7.	Acidic FGF	12.5 µg	13241-013	Gibco/ Invitrogen	[29,36,38,43–48]
8.	Heparin Sulphate	50 µg	D9809	Sigma	[29,36,38,43–48]
9.	LIF	10 µg	L5158	Sigma	[49–57]
10.	Vitronectin (Rat Plasma)	50 µg	V0132	Sigma	[58,59]
11.	CNTF	20 µg	CRC 401B	Cell Sciences	[60–64]
12.	NT 3	10 µg	CRN 500B	Cell Sciences	[65]
13.	NT 4	10 µg	CRN 501B	Cell Sciences	[66, 67]
14.	GDNF	10 µg	CRG 400B	Cell Sciences	[68–72]
15.	BDNF	10 µg	CRB 600B	Cell Sciences	[67, 73, 74]
16.	CT-1	10 µg	CRC 700B	Cell Sciences	[75–83]

Table 2

Composition of medium 2

No	Component(s)	Amount	Catalogue	Source	References
1.	NBactiv4	500 ml	10888	Invitrogen/Gibco	[31]
2.	Antibiotic-antimycotic	5 ml	15240-062	Invitrogen/Gibco	
3.	Cholesterol (250X)	5ml	12531	Invitrogen/Gibco	[50]
4.	TNF- α , human	10 μ g	T6674	Sigma-Aldrich	[51]
5.	PDGF BB	50 μ g	P4056	Sigma-Aldrich	[38,52]
6.	Vasoactive intestinal peptide (VIP)	250 μ g	V6130	Sigma-Aldrich	[53]
7.	Insulin-like growth factor 1	25 μ g	I2656	Sigma-Aldrich	[54]
8.	NAP	1mg	61170	AnaSpec, Inc.	[55]
9.	r-Apolipoprotein E2	50 μ g	P2002	Panvera, Madison, WI	[56]
10.	Laminin, mouse purified	2 mg	08-125	Millipore	[57,58]
11.	Beta amyloid (1-40)	1mg	AG966	Millipore	[59]
12.	Human Tenascin-C protein	100 μ g	CC065	Millipore	[60]
13.	rr-Sonic hedgehog, Shh N-terminal	50 μ g	I314-SH	R&D Systems	[61,62]
14.	rr-Agrin (C terminal)	50 μ g	550-AG-100	R&D Systems	[63]