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**Development of an *In Vitro* Model of Neuronal Differentiation
and Nerve-Target Interaction**

by

Charmaine Ferguson

Thesis submitted to the department of Cellular and Molecular Medicine in
partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

My objective was to develop a nerve source for use in a 3-dimensional *in vitro* model to study neurite extension and target innervation. A hybrid neuroblastoma-DRG cell line (ND-C) with properties of neuronal precursors was examined for its ability to effectively differentiate into neurons, produce extensive neurites, and interact with target cells. Neuronal differentiation of ND-Cs was optimized through a combinatorial approach that involved culturing the cells in the presence of various types of culture media, ECM, and soluble factors in a dose dependent fashion. The proportion of neurons and the length of neurites generated following culture in the differentiation promoting conditions were assessed. Immunostaining and RT-PCR analysis of differentiated ND-Cs revealed the expression of markers specific for terminally differentiated neurons. ND-Cs were able to extend neurites within a 3D collagen matrix. In co-culture, neurites from ND-Cs formed contacts with muscle and epithelial targets, indicating the plasticity of the cell line.

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LIST OF ABBREVIATIONS

- Ach: Acetylcholine
- AchR: Acetylcholine receptor
- ANOVA: Analysis of variance
- ARA-C: Cytosine arabinoside or Arabinosylcytosine
- ATCC: American Type Culture Collection
- BMP: Bone morphogenetic protein
- CREB: cAMP response element binding protein
- cAMP: Cyclic adenosine monophosphate
- COX: Cyclooxygenase
- DAPI: 4',6-Diamidino-2-phenylindole
- db cAMP: Dibutyl cyclic adenosine monophosphate
- DMEM: Dulbecco's Modified Eagles Medium
- DMSO: Dimethylsulfoxide
- DRG: Dorsal root ganglia
- EC: Embryonic carcinoma
- ECM: Extracellular matrix
- EDTA: Ethylenediaminetetra acetic acid
- EGF: Epidermal growth factor
- EGFR: Epidermal growth factor receptor
- FBS: Fetal bovine serum
- GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
- GEF: Guanine nucleotide exchange factor
- GFAP: Glial fibrillary acidic protein

HCEC: Human corneal epithelial cell line

KSFM: Keratinocyte Serum-Free Medium

MEK: Mitogen-activated or extracellular signal-regulated protein kinase

MMLV: Moloney Murine Leukemia Virus

nAChR: Nicotinic acetylcholine receptors

ND-C: Mouse neuroblastoma x neonatal rat dorsal root ganglia hybrid cell line

NF-200: Neurofilament-200

NF-M: Neurofilament-M

NGF: Nerve growth factor

PBS: Phosphate buffer saline

PG: Prostaglandin

PIP3: Phosphatidyl inositol-triphosphate

PI-3K: Phosphoinositide 3-kinase

PKA: Protein kinase A

PKC: Protein kinase C

PLC γ : Phospholipase C

RA: Retinoic Acid

RAc: Retinal Acetate

RT-PCR: Reverse transcriptase polymerase chain reaction

SHEM: Supplemented hormonal epithelial medium

TAE: Tris-Acetate-EDTA

TBS: Tris-Buffered Saline: TBS

TGF β : Transforming growth factor β

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1. INTRODUCTION

1.1 Neurite Extension

During embryonic development, neurons must extend their neurites over great distances to locate and form synapses with appropriate targets. The growth cone is the motile structure at the end of the extending neurite (Gourdon-Weeks, 2000; Suter and Forscher, 2000). Growth cones sense and respond to a variety of repulsive or attractive cues in the local microenvironment, causing neurites to either retract or advance and ultimately extend along the correct pathway despite a maze of possible routes and a countless number of potential yet inappropriate synaptic targets. Understanding how the growth cone interprets guidance cues to mediate directed neurite extension is a fundamental goal in developmental biology

1.1.1 Growth cone motility

Motility of the growth cone is driven by cytoskeletal reorganization. Actin is the primary component of the growth cone cytoskeleton. At the leading edge of the growth cone are membrane extensions called lamellipodia, which are generated by the polymerization of free actin monomers (G-actin) into filamentous actin (F-actin) networks. Actin monomers are continuously translocated toward the tip of the lamellipodia, forcing the cell membrane to extend outward at a speed of approximately 4 μ m/min. (Lin et al.1996). As the growth cone extends, older parts of the actin network are depolymerized allowing actin monomers to be recycled to the leading edge of the network. Four key components have been shown to regulate actin polymerization: actin related protein (*ARP*) 2/3 is required to nucleate new actin formation; Profilin promotes

actin assembly; *CapZ* restricts the length of growing filaments; and actin-depolymerizing factor (*ADF*)/cofilin acts to depolymerize the older parts of the network (Badour, 2003, Schuler, 1997, Copper, 2000, Bamberg, 1999). *Rho* GTPases, which are molecular switches that regulate cellular processes by binding and hydrolyzing GTP, are likely involved in the regulation of the actin polymerization machinery (Meyer, 2002). In addition to cytoskeleton rearrangement, growth cones must be able to grip the surrounding substrate in order to extend. The necessary traction is provided by adhesion molecules that include: cell adhesion molecules (CAMs); integrins; cadherins; and immunoglobulin-CAMs (Takuro, 2004).

1.1.2 Axonal guidance

Growth cones express an array of different guidance receptors on their surfaces. Whether a given guidance cue is interpreted as attractive or repulsive depends on the identity of the receptors and the temporal and spatial exposure to the specific cue. A variety of axonal guidance systems have been identified. There are long-range guidance cues that are secreted by intermediate or final target cells to create a chemotactic gradient along the pathway of the extending growth cone. For example, during development of the mammalian neural tube, the *DCC* (*deleted in colon cancer*) axonal guidance receptor interacts with a gradient of netrin (a molecule structurally related to laminin) to guide the axons of commissural neurons toward the ventral midline but repel motor neurons away from the midline (Keino-Masu 1996). There are also short range guidance cues that are secreted by cells in the surrounding tissue or bound to their membranes to influence the direction of the growth cone at specific choice points. *Ephrins* (*Eph*) are membrane

bound ligands that bind to *Eph* receptors on growth cones and drive axonal pathfinding to establish a topographical map for retinotectal development (Holder and Klein, 1999;Wilkinson, 2001). Upon detection of guidance cues, intracellular signaling cascades promote cycles of extension and retraction of the growth cone via actin polymerization/depolymerization. As mentioned the signal transduction network is thought to include the Rho family of GTPases.

1.2. Neuron-Target Interaction

1.2.1 Neuron-target trophic interactions

It is well established that nerves play an important role in the homeostasis of the tissue they innervate. Denervation often results in trophic changes in the respective target cells. For example, denervated skin is prone to ulcerations (Head, 1905); loss of sensory nerves in the cornea leads to impaired epithelial wound healing (Lim, 1976); and loss of nerves in the intestine leads to adverse changes in smooth muscle cell proliferation (Blennerhassett, 2000).

Neurons in turn depend on target cells for trophic support. Nerve growth factor (NGF) is the most studied target-derived neurotrophic factor. NGF is produced by many targets, including muscle cells, fibroblasts and intestinal epithelial cells, and is essential for the survival and maintenance of sympathetic and sensory ganglion cells (reviewed by Huang and Reichardt, 2001). Investigation into the biological role of NGF has provided the foundation of our understanding regarding the process by which target tissue influences the survival of the neurons that innervate them. In addition to NGF, many other target-derived trophic factors have been identified including brain derived

neurotrophic factor (*BDNF*), neurotrophin 3 (*NT-3*), neurotrophin 4 (*NT-4*) and glial derived neurotrophic factor (*GDNF*).

The neurotrophic hypothesis stipulates that in order to ensure an optimal amount of target innervation, neurons are initially overproduced during development. However, once they innervate their targets they compete for limited amounts of target-derived neurotrophic factors. Neurons that do not get sufficient amounts of neurotrophin die by apoptosis (Davies, 1996). The neurotrophic hypothesis is based on three assumptions: first, neurons require a minimum amount of trophic factor for survival; secondly, target tissues synthesize and provide the appropriate trophic factor to neurons; finally, targets produce trophic factors in limited amounts, therefore the survival of neurons depends on neuronal competition.

1.2.2 Neuron plasticity and target cell induced differentiation

Studies have revealed that, in addition to promoting neuronal survival, target-derived factors mediate the acquisition of cellular identity (Francis and Landis 1999, McMahon et al. 1989, Devay et al. 1999, Raucher and Dryer, 1995). Immature neuronal properties are already established at cell cycle exit. However, it is thought that signals encountered en route to the target, or from target cells themselves, control the later steps of neuronal differentiation, such as the expression of neurotransmitter and ions channels. Since neuronal traits can be regulated by targets, phenotype is not fully determined at cell cycle arrest and can be manipulated. Therefore, post-mitotic neurons can demonstrate “plasticity”, which is the ability to recognize and adapt to local environmental changes.

Several studies have established that neurotransmitter and neuropeptide phenotype is plastic and influenced by interactions between neurons and targets. For example, sympathetic neurons *in vivo* undergo a switch from noradrenergic to cholinergic after they innervate the sweat glands in the footpads of rats (Francis and Landis, 1999). Another example is the change in neuropeptide expression that occurs in adult sensory neurons when a muscle and a cutaneous nerve are cross-anastomosed (McMahon et al. 1989).

The influence of target tissue on the expression of ion channels has also been observed. For example, there is a significant difference in the level of nicotinic acetylcholine receptor (nAChR) transcripts in sympathetic neurons cultured with cardiomyocytes vs. kidney cells (Devay et al. 1999). Another study showed that the expression of potassium channels can be induced in sympathetic neurons by a factor that is produced by cardiac myocytes (Raucher and Dryer, 1995).

1.3 Regeneration and Tissue Engineering

1.3.1 Regeneration of peripheral nerves

Peripheral nerves have the natural capacity to regenerate. When a nerve is transected the myelinated axons distal to the site of injury completely deteriorate. The neurilemma, however, remains intact and forms a tube directing axonal growth. Axons from the proximal nerve stump extend through the channels of the neurilemma and eventually reach the target tissue. Chemical and physical cues at the site of injury are responsible for stimulating and guiding neurite extension. However, the natural regeneration process is extremely slow (<1mm per day) (Sunderland et al. 1947).

Therefore, in cases of extensive nerve damage where the nerve gap is very large, the target tissue can atrophy or become altered before reinnervation is achieved (Jakubiec-Puka 1977, Beuerman et al 1980)

1.3.2 Nerve repair through tissue engineering

A tissue engineering approach to peripheral nerve repair aims to upregulate the natural regeneration response in order to attain rapid neuron outgrowth and timely reinnervation of the target tissue. This can be achieved by incorporating positive mediators of neurite extension into a semi-synthetic scaffold that, upon implantation, will actively support regeneration.

Our limited understanding of the molecular mechanisms of peripheral nerve regeneration makes the engineering of growth promoting scaffolds a technical challenge. Recent research has focused on identifying factors that mediate the regeneration process. Neurotrophic factors, such as nerve growth factor, and extracellular matrix components, including laminin and fibronectin, have been shown to play a role in peripheral nerve regeneration and can promote axonal outgrowth under experimental conditions (Markus, 2002). Molecules such as netrins, semaphorins and ephrins are known to guide neuron growth cones *in vivo* during development and regeneration (Drescher, 2004).

Piezoelectric materials (materials that generate a surface charge) have also been shown to stimulate neurite extension *in vitro* (Schmidt, 1997).

1.4 Neural progenitor cells

Neural precursors or progenitor cells are immature proliferating cells that give rise to a variety of specialized cells in the nervous system, including neurons and glia. Neural progenitors have proven to be very useful in studying neural differentiation and identify signals directing specialization. These cells can be isolated from embryonic nervous tissue and from the hippocampus and subventricular zone in adult tissue.

Neuroblastoma is a common pediatric tumour of the peripheral nervous system. The tumour develops when neural progenitors in the embryo fail to differentiate and mature into normal neural cells and continue proliferating. Neuroblastoma cells are known to display some properties of neural progenitors. They have been shown to differentiate in the presence of differentiation-inducing factors such as retinoic acid (RA), and cytosine arabinoside (ARA-C), cAMP and NGF (Ponzonin, 1991, Prasad, 1971, Waris, 1973). Therefore, neuroblastoma cells are frequently used as a model of neuronal precursor cells to study neural differentiation. In addition, neuroblastoma cell lines provide a homogeneous and renewable source of neurons.

The N-18TG2 neuroblastoma cell line is commonly used as a model for neuronal precursor cells. Unfortunately, N-18TG2's are inactive with regard to neurotransmitter phenotype (Amano et al 1972). It has been hypothesized that N18TG2 cells have a dysfunction in neurotransmitter production and are therefore unable to progress in their neuronal developmental program. This dysfunction can be overcome by hybridizing N-18TG2s with a cell type that actively synthesizes neurotransmitter. For example, N-18TG2 – glioma hybrid cells can produce acetylcholine (Hamprecht, 1977).

1.4.1 Neuroblastoma-based lines

ND-C is a cell line derived from the fusion of mouse neuroblastoma with neonatal rat dorsal root ganglia (Wood et al. 1990). This cell line is an attractive candidate for a neural progenitor. Due to their hybrid nature, ND-Cs are immortalized and renewable. Several derivative subclones have been established from parental lines that have more restricted differentiation potentials and some properties of specific post-mitotic neurons. For example, ND-Cs have been shown to extend short neurites and produce substance P when differentiated by the addition of cAMP to the culture media (Wood et al. 1990).

1.5 Factors Controlling Differentiation of Neuronal Precursors

Neuronal differentiation is a complex developmental program that involves the coordinated phosphorylation of cellular substrates and regulation of gene expression. With the advent of cell lines that are able to differentiate *in vitro*, researchers are now beginning to identify the different factors and signaling pathways that mediate neuronal differentiation. Neurotrophins, hormones, ECM components, signaling molecules and chemicals have all been shown to induced differentiation of neuronal precursor cells.

1.5.1 Ascorbic acid

Ascorbic acid (vitamin C) has been shown to enhance the differentiation of embryonic stem cells and cortical precursors cells into neurons (Lee, 2000, Lee, 2003). Although ascorbic acid acts as a free radical scavenger in the brain, differentiation does not depend on its antioxidant properties (Yan, 2001). Instead, it is thought that ascorbic

acid-induced differentiation is mediated through the bone morphogenetic protein (BMP) signaling pathway, with BMP-2 and BMP-7 as key mediators (Lee, 2003). Interaction of BMP-7 with Noggin is known to play a role in the induction of neural tissue during embryonic development. Genes upregulated due to ascorbic acid induced differentiation include synaptically localized adhesion molecules and factors for calcium signaling. Changes in calcium concentration can stimulate differentiation through a mechanism that involves phosphatidylinositol 3-kinase (PI3K) activation by calmodulin.

1.5.2 Cyclic Adenosine Monophosphate

Many studies have shown that hydrolyzed cyclic adenosine monophosphate (cAMP) derivatives can stimulate neuronal differentiation in neuronal precursors (Mattson, 1988, Ryddel and Greene, 1988, Nakao, 1998, Grewal, 2000, Ghil, 2000). It is well established that cAMP's effects are partially attributed to the direct binding of cAMP to protein kinase A (PKA). PKA regulates many different cellular functions, including differentiation. It is necessary for the initiation of neurite elongation and is important in stimulating synaptogenesis (Sanchez, 2004, Tojima, 2003). It is thought that PKA directly phosphorylates substrates involved in neurite outgrowth and elongation (i.e., microtubule associated proteins) and/or regulates transcription of neuronal genes by activation of the transcription factor CREB. PKA also acts through the mitogen-activated protein kinase (MEK) pathway.

In addition, cAMP induced differentiation is linked to the activation of the PI3K-Akt pathway. PI3K is essential for cAMP induced-neurite elongation (Sanchez, 2004). The inhibition of PI3K causes growth cone collapse and neurite retraction.

cAMP-regulated guanine nucleotide exchange factors (camp-GEFs) are recently identified members of the novel family of cAMP binding proteins that bind cAMP and selectively activate Ras superfamily guanine nucleotide binding protein (Rap1A) (Kawasaki, 1998). Rap1A in turn stimulates B-Raf (a member of the Raf family of serine/threonine kinases) and the mitogen activated protein kinase (MAPK) pathway, which include, extracellular-signal related kinase ERK, c-Jun amino terminal kinase (JNK) and p38.

cAMP has also been shown to transactivate the nerve growth factor receptor (Trk A) and the epidermal growth factor receptor (EGFR) (Piiper, 2002), both of which have been implicated in neuronal differentiation. However, cAMP transactivated receptor tyrosine kinases only elicit partial biological response compared to stimulation with their ligand (Piiper, 2002).

1.5.3 Nerve Growth Factor

Nerve growth factor (NGF) is a member of the neurotrophin family. It is largely known as a target-derived factor responsible for the survival and maintenance of sympathetic and sensory neurons during development and maturation. Many neuronal cell lines, notably PC12 cells, differentiate when exposed to NGF (Klein et al. 1991, Lee, 1982, Chen, 1990, Liesi, 1983).

NGF is synthesized and secreted by a variety of cell sources including immune cells (Levi Montalcini et al. 1996), cardiomyocytes (Long et al. 1990), fibroblasts (Olgart et al. 2001), vascular smooth muscle cells (Ueyama et al. 1991), and renal cells (Hammerman,

1995). The biological effects of NGF are mediated mainly through ligation with its high affinity receptor, TrkA. TrkA expression has been identified in several neuron population in the CNS and PNS, including neurons of the peripheral ganglia (dorsal root and sympathetic). In addition, many non-neural cells express TrkA, indicating a role for NGF in regulating non-neural tissues (Vega, 1994). Upon ligation, Trk A becomes phosphorylated and tyrosine kinase domains are activated. This initiates a phosphorylation of many cellular proteins including phospholipase-C γ 1, PI-3-kinase, the adapter protein Shc and the Src-associated neurotropic factor-induced tyrosine phosphorylated target (SNT) (Greene, 1995). These proteins couple the TrkA receptor to the MAPK signal cascade. Active ERK1 and ERK2 translocate to the nucleus where they phosphorylate transcription factors and initiate transcription of neuronal genes.

The PI3K-Akt pathway is also implicated in NGF-induced differentiation. As mentioned, PI3K becomes phosphorylated upon activation of the Trk A receptor. PI3K phosphorylates membrane phosphoinositides at the d-3 position. These 3' phosphorylated phospholipids act as second messengers, one of the major targets is Akt (protein kinase B). Akt is recruited to the cell membrane and is activated by further phosphorylation by phosphoinositide-dependent kinases I and II. Activated Akt has been shown to induce differentiation of PC12 cells (Kim, 2004) and induce neurite outgrowth in embryonic sensory neurons (Markus, 2002).

Ligand engagement of TrkA has also been shown to activate acidic sphingomyelinase, resulting in generation of ceramide (Dobrowsky et al. 1995). Ceramide has been shown to activate several signaling pathways, including the ERK and

Jun kinase cascades and NF κ B. Differentiation of many cell types has been achieved by the addition of exogenous ceramides (Kim, 1990).

1.5.4 Dimethyl sulfoxide

Dimethyl sulfoxide (DMSO) is a synthetic compound originally intended for use as an industrial solvent but has more recently been used for biology applications and as a therapeutic agent. It is an extremely powerful solvent that interacts strongly with hydrophilic compounds, altering their conformational structure. DMSO is widely used to differentiate P19 mouse embryonic carcinoma cell into cardiomyocyte and bipolar skeletal muscle cells (Skerjanc, 1999). However, treatment of neuroblastoma cells with DMSO provides morphologically stable, differentiated cultures of neurons (Clejan, 1996). The mechanism underlying DMSO-induced differentiation is largely unknown. DMSO stimulates the hydrolysis of sphingomyelin by sphingomyelinase to generate ceramide (Clejan, 1996). It has been hypothesized that ceramide is a transducer of DMSO mediated cell injury and initiates a program of cell cycle suppression which involves differentiation.

1.5.5 Transforming growth factor β

TGF β superfamily consists of numerous growth factors that regulate various cellular processes. TGF- β subtypes have been shown to play a role in the differentiation of many tissues including neocortical precursors and neuroblastoma cells (Li, 1998, Hogan, 1996, Mehler, 1997, Gomez Santos 2002). The TGF β family members bind to cell surface

receptors with serine-threonine kinase activity. These receptors phosphorylate and activate the Smad signal transduction pathway. A complex of Smads enters the nucleus and regulates transcriptional responses.

1.5.6 Dexamethasone

Dexamethasone is a synthetic glucocorticoid known to induce differentiation of various cell types. It is well recognized for its role in promoting osteogenic and neuronal differentiation in vitro and in vivo models (Kim, 2005, Hyeon, 2003, Ross, 2002, Nuttleman, 2006). It is hypothesized that dexamethasone mediates neuronal differentiation through the induction of cyclooxygenase-1 (Cox-1), which is a regulator of prostanoid synthesis (Hyeon, 2003). Prostaglandin E2 (PGE2) is a major enzymatic product of the COX mediated pathway. PGE2 is known to be involved in differentiation, and exogenous PGE2 triggers neurite outgrowth in neuroblastoma cells (Hyeon, 2003). Dexamethasone has been shown to regulate COX-1 at both the mRNA and protein levels (Schneider, 2001).

1.5.7 Induction of neurite outgrowth by extracellular matrix components

Laminin is a large glycoprotein found in basement membranes, such as those lining epithelia, surrounding blood vessels and nerves (Luckencill-Esds, 1997). The molecule has multiple binding domains, which interact with other extracellular matrix components and cell surface receptors. Several types of neuron cell surface receptors bind laminin, with $\alpha3\beta1$ and $\alpha6\beta1$ integrins, being among the best characterized.

Experimental results suggest that laminin interacts with integrins located on the tips of extending neurites. This interaction provides a signal to increase the velocity of axonal outgrowth (Luckencill-Esds, 1997). The exact mechanism by which laminin signaling becomes translated into changes in neuron behaviour is not clear. However, it likely involves the phosphorylation and /or dephosphorylation of laminin binding proteins, which then communicate with the cytoskeleton machinery of the cell.

Many *in vitro* assays have been performed to assess the influence of laminin on axonal outgrowth (Sephel et al. 1989, Sanes, 1989). Neurons cultured on laminin substrates have shown an increase in neurite extension compared to control surfaces, as seen in spinal cord neurons and PC12 cells grown in monolayer on laminin coated culture dishes or in 3-dimensional laminin-treated gels (Hantaz-Ambroise et al. 1987, Bellamkonda et al. 1995). Application of anti- β 1 integrin subunit blocking antibody, which is thought to block binding of laminin with β 1 integrin, was shown to significantly decrease neurite extension on laminin substrates (Smith et al. 1996). There are also a number of examples in which laminin has been used for experimental nerve repair applications *in vivo*. In a recent study, chitosan tubes covalently linked to laminin were used to bridge 10 mm nerve gaps in rat sciatic nerves (Suzuki et al. 2003). In comparison with untreated control tubes, the laminin treated tubes exhibited faster regeneration of the damaged nerve and better motor function recovery.

Fibronectin is also a large, multidomained glycoprotein. This molecule is ubiquitously distributed in extracellular matrices and it interacts with a wide variety of macromolecules and cell surface receptors. Specific binding domains on the fibronectin molecule have been shown to mediate neural adhesion and promote axonal growth

(Haugen et al. 1992). As with the case for laminin, the events down stream of the fibronectin-neuron interaction have not been elucidated.

Neurite extension is enhanced when fibronectin is used as a substrate for culturing neurons in 2- and 3-dimensional cultures (Baldwin et al. 1996). Furthermore, mutation of the fibronectin neural adhesion domain results in the failure of neurons to extend neurites onto these fibronectin substrates (Drake et al.1993). *In vivo* studies investigating the effect of exogenous fibronectin on regenerating nerves have also been performed. Oriented strands of fibronectin have been used to bridge 10 mm nerve defects in rats with successful results (Whitworth et al.1995).

1.6 Current models to study neurite extension and peripheral target innervation

1.6.1 Limitations of current models

The models currently used to study neurite extension and peripheral target innervation are flawed in their design. Many studies are conducted *in vivo* using rodent models or simple monolayer cell culture assays. Results from *in vivo* investigations in rodents are difficult to interpret due to confounding factors that cannot be monitored and controlled sufficiently (inflammatory response, humoral response) (Suzuki et al. 2003, Whitworth et al.1995). The use of primary neuronal cultures in monolayer *in vitro* experiments is problematic, as these are too simplistic and neuronal-target interactions cannot be assessed with sufficient mechanistic accuracy.

A 3-dimensional *in vitro* model that allows nerve-target interaction has been developed in our lab using dorsal root ganglia (DRG) isolated from chick embryos

(Suronen, 2003). While nerve-target interactions were documented, problems remained. For example, despite careful dissection and attempts to remove contaminating fibroblasts, these cells still persist. Extending filopodia from the fibroblasts could be mistaken for axons, resulting in inaccurate measurements of neurite extension.

Clearly, there is need for a new model to study neurite extension and peripheral target innervation. The new model should be *in vitro*, to control for confounding factors. It should utilize a neuronal source that provides a high yield of neurons with homogenous phenotype. The model should account for nerve-target cell interactions. Lastly, the model should be versatile and allow for accurate measurement of neurite extension.

1.7 Research Objectives

My objective was to investigate the plasticity and differentiation potential of a possible neuronal precursor line for use in an *in vitro* model to study neurite extension and target innervation.

1.7.1 In vitro model

The *in vitro* model I designed consists of a co-culture of neurons and target cells within a three dimensional scaffold (refer to Fig. 1). Within this trilaminar model, the basal layer contained the nerve source. The second layer was a hydrated collagen-based matrix that separates the neuron cell bodies from the target cells located on the third layer. When cultured under optimized conditions, neurites should extend through the matrix to make contact with target cells. Such a trilaminar arrangement exists in nature,

e.g. skin or cornea where nerve axons will traverse the ECM en route to their target cells (epidermis).

Skeletal muscle and corneal epithelial target cells served as the motor and sensory target cells, respectively. Specifically, C2C12 mouse myoblast cells (Blau et al. 1983), and P19 mouse teratocarcinoma cells were differentiated into myotubes and used as the muscle target. Immortalized human corneal epithelial cells (HCEC; Araki-Sasaki et al. 2000), were used as the epithelial target.

1.7.2 Nerve source for proposed model

The desired nerve source should be homogenous and renewable in order to provide a large quantity of terminally differentiated and phenotype-specific neurons. Further, the nerve source must generate long neurites that can extend through a 3-D matrix and contact target cells. Based on the properties of different neuronal lines and precursors described above (Section 1.4), the ND-C cell line was selected for evaluation as a nerve source for the proposed *in vitro* model. In order to confirm the various factors identified to promote neuronal differentiation and optimal extension, Sh-sy5y cells, a well characterized neuronal line, and DRG were used as a second line for comparison.

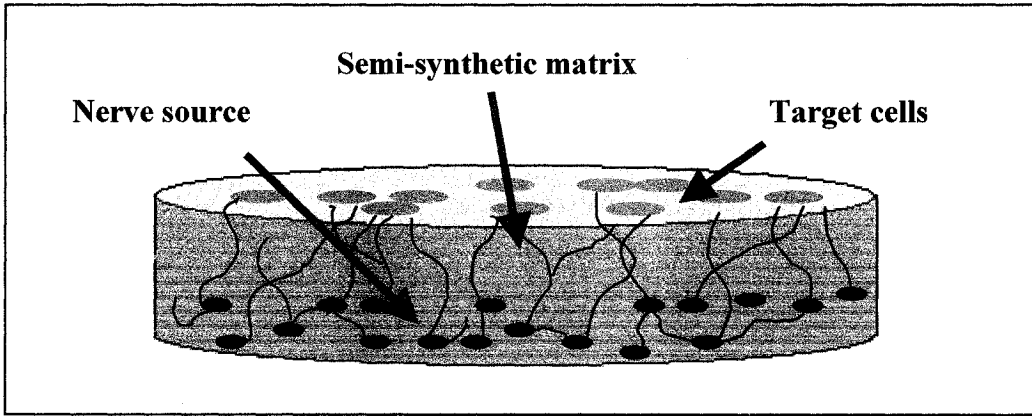


Figure 1. Diagram of proposed *in vitro* model

2. MATERIALS AND METHODS

2.1 Cell lines and growth conditions

ND-C cells were obtained from the European tissue culture collection. They were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Ontario, Canada) and 50 µg/ml gentamycin (Invitrogen, Ontario, Canada) in a humidified tissue culture incubator at 37 °C with atmosphere of 5% CO₂. Cells were seeded at a density of 100,000 cells per 100 x 20mm culture dish. The culture medium was replaced every other day. ND-Cs were passaged at 70% confluency. To harvest the cells, they were incubated for 3 minutes in 0.05% trypsin-ethylenediaminetetra acetic acid (trypsin/EDTA; Invitrogen, Ontario, Canada). The reaction was stopped by the addition of serum containing media. Dislodged cells were centrifuged for 5 min at 15000 rpm and then split 1:4.

An immortalized C2C12 myoblast cell line was used as the "muscle" target (Biau et al. 1983). The cells were provided by Dr. L. Megeney from the Ottawa Health Research Institute, Ottawa, Canada. C2C12s were seeded at a density of 100,000 cells per 100 x 20mm culture dish. Cultures were maintained in the same growth media formulation as the ND-Cs and passaged using the same procedure. To obtain differentiated myotubes, C2C12 cells were grown to (100%) confluency and the concentration of FBS in the media was reduced to 1.5%. Media was changed every other day and myotubes were used in assays after 9 days of culture in differentiation media.

A clonally derived P19 mouse teratocarcinoma α -actin promoter-driven puromycin-resistant cell line was also used as a muscle target (a gift from Dr. Ilona

Skerjanc, University of Ottawa; original p19 cells were acquired from ATCC, Number CRL-1825). Differentiation of P19 cells into muscle was initiated by allowing the cells to aggregate in suspension culture in Petri dishes in the presence of 0.8% dimethylsulfoxide (DMSO; EM Science, NJ, USA), while growing in α MEM media with 10% FBS (Invitrogen, Ontario, Canada) (McBurney et al., 1982). On day 4 of culture, the aggregates were re-plated into tissue culture dishes and they differentiated into skeletal myocytes by day 9 (Skerjanc, 1999). After 7 days of culture, 2 μ g/ml of puromycin (Invitrogen, Ontario, Canada) was added to the culture media to select for actin-expressing muscle cells.

An immortalized human corneal epithelial cell line (HCEC; Araki-Sasaki et al. 2000) that was previously used in our lab to tissue engineer innervated corneas was used as the “sensory” target (Suuronen et al. 2004). Cells were maintained in Keratinocyte Serum-Free Medium (KSFM; Invitrogen, Ontario, Canada) in a humidified tissue culture incubator. Media was changed every other day and cells were passaged using the same procedure as described for ND-Cs. However, HCECs were split 6:1.

Sh-sy5y cells (Odelstad L, 1981) were provided by Dr J. Dimitroulakos from the Ottawa Health Research Institute, Ottawa, Canada. Cells were maintained in DMEM/F12 media (Invitrogen, Ontario, Canada) and supplemented with 10% FBS (Invitrogen, Ontario, Canada). Media was changed every other day and cells were passaged using the same procedure as described for ND-Cs. To induce neuronal differentiation of Sh-sy5ys, 1 μ M retinoic acid (RA; Sigma Aldrich, Ontario, Canada) was added to the culture media when cells reached 50% confluency. Cells were then cultured in the RA supplemented differentiation media for 7 days.

2.2 Assessing the effect of culture media and substrates on neuronal differentiation and extension

A combinatorial assay was set up to evaluate the ability of different culture media and extracellular matrix (ECM) substrates to induce the neuronal differentiation of ND-Cs (as diagrammed in Fig. 2). ND-Cs were plated at a density of 100 000 cells/ well in a 24-well plate and cultured in 2 ml of media. The growth media used in the assay are as follows: Keratinocyte-serum free medium (KSFM; Invitrogen), which is supplemented with bovine pituitary extract and epidermal growth factor (EGF); Neurobasal serum-free medium (Invitrogen, Ontario, Canada) containing N2 and B27 supplements; StemPro-34 serum-free medium (Invitrogen, Ontario, Canada); DMEM medium (Invitrogen, Ontario, Canada) supplemented with FBS (Invitrogen, Ontario, Canada); and supplemented hormonal epithelial medium (SHEM), consisting of DMEM/F12 (Invitrogen, Ontario, Canada) supplemented with 15% FBS (Invitrogen, Ontario, Canada), 10 ng/ml epidermal growth factor (EGF; Sigma Aldrich, Ontario, Canada), 5 µg/ml insulin (Sigma, Aldrich, Ontario, Canada), 0.1 µg/ml cholera toxin A subunit (Sigma, Aldrich, Ontario, Canada), 0.5% Dimethylsulfoxide (DMSO; EM Science, NJ, USA.), 5 mM L-glutamine and 20 µg/ml gentamycin (Invitrogen, Ontario, Canada) (detailed base formulations for KSFM and StemPro-34 are given in the Appendix).

The ECM substrates assessed in the combinatorial assay included; rat tail type I collagen (BD biosciences, Ontario, Canada) at 100 µg/ cm², natural human fibronectin (BD Biosciences, Ontario, Canada) at 5 µg/cm² and mouse laminin (BD Biosciences, Ontario, Canada) at 20 µg/cm². To coat the culture dishes, approximately 50 µL of ECM

solution, just enough to cover the surface of the dish, was applied. The dish was then incubated at 37 °C for 1 hr. Residual liquid was aspirated off and media and cells were immediately added to the dish.

ND-Cs were cultured in the combinatorial assay for six days. The effect of the culture conditions on neuronal differentiation was determined by assessing the proportion of cells with a neuron-like morphology that were generated. The proportion of cells with neurites and the length of the neurites were considered to determine the effect of the culture condition on neurite extension.

Four trials were run for the combinatorial assay (n=4). A trial consisted of three independent plates, each assessing all of the culture conditions.





















	Uncoated	Collagen I	Fibronectin	Laminin
DMEM (FBS)				
KSFM (pituitary extract + EGF)				
SHEM (EGF)				
Neurobasal (N2 + B27 Supplements)				
Stem Pro-34				

Figure 2. Set up for multi-well dish used in combinatorial assay.

Phase contrast images of the cells were taken at 10X magnification using Zeiss Axiocam mounted on an Axioskop 2 inverted microscope (Zeiss, Oberkochen, Germany). The number of neurons per image were counted and then divided by the total number of cells in the image to give the percentage of neurons generated. Four to six pictures were taken per dish and then averaged to give a representative percentage value for that culture condition.

The final value for the percentage of neurons generated by a particular culture condition was calculated by taking the representative percentage value and then averaging the results from multiple trials. For a cell to be considered a neuron, it must have extended neurites that were at least twice the length of the diameter of the cell body.

2.3 Assessing the effect of supplements on neuronal differentiation and extension

Various supplements were evaluated for their ability to promote neuronal differentiation and enhance neurite extension of ND-Cs. ND-Cs were plated at a density of 100 000 cells/well in a 24-well plate. 500 µl of media was added to each well. Cells were grown on uncoated tissue culture dishes for six days in KSFM base media (Invitrogen, Ontario, Canada) supplemented with 10^{-7} M Dexamethasone (Sigma Aldrich, Ontario, Canada) 50 µg/ml asorbic acid (Fluka Biochemika, Switzerland), 20 µM dibutryl cyclic adenosine monophosphate (db cAMP; Sigma), 2 µM DMSO (EM Science, NJ, USA), or 1 µg/ml nerve growth factor (NGF; Sigma Aldrich, Ontario, Canada). Media was changed every other day. Immunocytochemistry was performed and the extent of neuronal differentiation and neurite extension was quantified (described below). The experiment was repeated four times (n=4). Each time, three dishes containing a particular supplement were assessed.

After identifying supplements that increased differentiation and extension, a concentration curve experiment was performed to determine the optimal concentration of the supplements of interest. ND-Cs were cultured for 6 days in KSFM base media (Invitrogen, Ontario, Canada) supplemented with different concentrations of dexamethasone, db cAMP, DMSO and NGF. Immunocytochemistry was performed to

determine which concentration induced the greatest neuronal differentiation and neurite extension. The experiment was repeated four times (n=4). Each time, three dishes containing a particular concentration of a supplement were assessed.

2.4 Immunocytochemistry to quantify neuronal differentiation and extension

ND-Cs in monolayer on tissue culture dishes were washed with phosphate buffer saline (PBS; 1.0 M, pH 7, Invitrogen, Ontario, Canada), and fixed in PBS containing 4% paraformaldehyde (PFA; Sigma Aldrich, Ontario, Canada) for 30 minutes at room temperature. Cells were then washed twice for 5 minutes with PBS. Double-labelled fluorescent immunostaining for neurofilament-200 (NF-200) and glial fibrillary acidic protein (GFAP) was then performed (see Table 1 for antibody details). Mouse anti-neurofilament-200 and rabbit anti-GFAP primary antibodies were diluted 1:200 in 0.3% Triton X-100 detergent in Tris-Buffered Saline (TBS) buffer containing Tris (Boehringer Mannheim, Ingelheim Germany) and NaCl (Sigma Aldrich, Ontario, Canada) and applied to the dish for 3 hours at room temperature. After washing with TBS three times for five minutes, fluor-Link Cy2 labelled goat anti-mouse (Amersham Biosciences, Buckinghamshire, United Kingdom) and alexa-fluor cy3 goat anti-rabbit (Sigma Aldrich, Ontario, Canada) secondary antibodies, diluted 1:400 in 0.3% Triton-X in TBS, were applied for 2 hours at room temperature. The dish was then washed again three times for five minutes with TBS. 4',6-Diamidino-2-phenylindole (DAPI; Sigma Aldrich, Ontario, Canada) was diluted 1:1000 in TBS and added to the dishes for 3 minutes. The dishes were again washed twice for 5 minutes in TBS. A drop of FluorSave reagent

(Calbiochem, Mississauga, Ontario, Canada) was added to each dish and a coverslip was applied over top of the cells.

Table 1: Summary of primary antibodies use in immunocytochemistry

ANTIBODY	ANTIGEN	MANUFACTURER
Mouse monoclonal anti-neurofilament 200 (clone NE14)	neurofilament-200	Sigma Aldrich, Ontario, Canada
Rabbit anti-gial fibrillary acidic protein (IgG fraction of antiserum)	glial fibrillary acidic protein	Sigma Aldrich, Ontario, Canada
Mouse monoclonal anti-myosin (skeletal)	myosin heavy chain	Sigma Aldrich, Ontario, Canada
Rabbit polyclonal to pan cytokeratin	Cytokeratin	Novus Biological, Littleton, CO, USA
Mouse anti-acetylcholine monoclonal antibody	acetylcholine	Novus Biological, Littleton, CO, USA

Fluorescent images of the cells were taken at the same 10X magnification using Zeiss Axiocam mounted on an Axioskop 2 inverted microscope (Zeiss, Oberkochen, Germany). The number of neurons or glial cells per image were counted and then divided by the total number cells in the image to give the percentage of neurons or glial cells generated. Four to six pictures were taken per dish and then averaged to give a representative percentage value for that culture condition.

The final value for the percentage of neurons/glial cells generated by a particular culture condition was calculated by taking the representative percentage value and then averaging the results from multiple trials. For a cell to be considered a neuron, it must have stained positive for NF-200 and have extended neurites that were at least twice the length of the diameter of the cell body. A glial cell was identified as being GFAP positive but NF-200 negative.

The length and number of processes extending from ND-Cs were measured using Northern Eclipse imaging software (Empix, Mississauga, ON, Canada). The average neurite length for each dish was calculated and then this value was averaged to give the mean neurite length for a particular culture condition. In addition, the measured lengths were categorized so that the frequency and distribution of neurite lengths for different culture conditions could be compared. Only one trial was performed to assess neurite extension from ND-C in 3D matrices with varying concentrations of collagen. Therefore, statistics could not be performed and results should be only considered preliminary.

Differences between mean values for the different culture conditions were compared using 1-way analysis of variance (ANOVA), followed by a Dunnett's test. For the combinatorial assay where ECM and media were assessed simultaneously, a 2-way ANOVA was used. A P-value of $P < 0.05$ was used for all significance comparisons. All analyses were done using SPSS software (SPSS Inc, Chicago, IL, USA).

2.5 Immunocytochemistry to show neuron-target cell interaction

Protocol for fixation, immunocytochemical staining and mounting is the same as the protocol used to quantify neuronal differentiation and extension (2.4). However, the primary antibodies employed were different. To demonstrate contact between ND-Cs and target cells, anti-myosin heavy chain and anti-neurofilament-200 primary antibodies were used to perform double-labelled fluorescent immunostaining on C2C12 or P19 and ND-C co-cultures, respectively. Anti-keratin and anti-NF-200 antibodies were used for the HCEC and NDC co-cultures (see Table 1 for antibody details).

Fluorescein conjugated α -bungarotoxin (Molecular Probes, Ontario, Canada) was used to visualize clustering of acetylcholine receptors at the site of contact between neurons and C2C12s. 10 nM α -bungarotoxin conjugate was applied to C2C12-neuron co-culture, after fixation and immunocytochemical staining with NF-200 as outlined in section 2.4, and incubated for 1 hour room temperature. Cells were washed 3 times for 5 minutes with TBS. Images of the cells were taken using the Zeiss Axiocam mounted on a Axioskop 2 inverted microscope (Zeiss, Oberkochen, Germany). Cells were visualized using a 63X oil immersion lens objective.

2.6 RT PCR analysis to show expression of neuron specific markers

ND-Cs were harvested after culturing for 6 days. Cells were lysed by adding TRIZOL reagent (Invitrogen, Ontario, Canada) directly to the culture dish and pipetting repeatedly. Total RNA was isolated according to the manufacturer's instructions.

Two μ g of total RNA was used for each reverse transcription reaction. Primer annealing was achieved by combining 1 μ g of reverse primer with RNA in sterile ddH₂O and incubating for 15 minutes at 65°C. First strand cDNA was synthesized by incubating RNA with 5X First Strand Buffer (Gibco BRL, Gaithersburg, MD, USA), 0.1 M DTT (Gibco BRL Gaithersburg, MD, USA), 10mM dNTP mix (10mM each dATP, dGTP, dCTP, and dTTP (at pH 7; Amersham Pharmacia Biotech, Germany)), 40 U of RNase Inhibitor (Invitrogen) and 200 U of Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RTase; Invitrogen, Ontario Canada) for 1 hour at 37 °C.

The polymerase chain reaction was performed in 25 μ L of PCR solution containing 10X PCR buffer (200 mM Tris-Hydrochloride (pH 8.4), 500 mM KCl; Gibco

BRL, Gaithersburg, MD, USA), 50 mM MgCl₂ (Gibco BRL, Gaithersburg, MD, USA), 10 mM dNTP mix, 10 μM of sense and antisense primer (Sigma-Genosys, Oakville, Ontario, Canada) and 5 U of *Taq* DNA Polymerase (Gibco BRL, Gaithersburg, MD, USA) in sterile ddH₂O. The following rat primers for glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and neurofilament-M protein (*nf-m*) were used: *gapdh* sense primer 5' ACC ACA GTC CAT GCC ATC AC -3', *gapdh* anti-sense primer 5' TCC ACC ACC CTG TTG CTG TA -3'; *nf-m* sense primer 5' GCC GAG CAG AAC AAG GAG GCC ATT-3', *nf-m* anti-sense primer 5' CTG GAT GGT GTC CTG GTA GCT GCT-3'. PCR amplification was performed in an Eppendorf Gradient Mastercycler under the following conditions: initial template denaturation at 94 °C for 15 minutes; 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 64 °C for 30 seconds and primer extension at 72°C for 30 seconds; heating at 72°C for 10 minutes carried out final extension of PCR products; and final products were stored at 4°C.

Separation of PCR products was performed on an ethidium bromide stained 2% agarose gel in 1X Tris-Acetate-EDTA (TAE) buffer. Gel was visualized by UV trans-illumination and images were taken using a digital camera connected to a gel documentation system (Gel Doc 2000, Bio-Rad Laboratories, California, USA).

2.7 Measurement of neurite extension in collagen-based hydrogels

Collagen-based hydrated matrix or hydrogels were prepared by combining type I rat tail collagen (0.3% w/v); Becton Dickinson, Oakville, Canada) with chondroitin-6-sulfate (CS; Sigma Aldrich, Oakville, Canada) (1:5 w/w ratio collagen: CS) on ice. The matrix was then cross-linked with glutaraldehyde (0.02% v/v), and residual aldehyde

groups were inactivated by reaction with 0.8% aqueous glycine (w/v) solution. This collagen-CS mixture is liquid at 4°C but forms a hydrated gel at 37°C. ND-Cs expressing green fluorescent protein were embedded within the matrix before being thermogelled at 37°C.

ND-Cs were cultured within the matrix for 6 days in optimized medium (KSFM base media supplemented with 10^{-7} M Dexamethasone (Sigma Aldrich, Ontario, Canada), 20 μ M dibutyl cyclic adenosine monophosphate (dB cAMP; Sigma Aldrich, Ontario, Canada), 2 μ M DMSO (EM Science, NJ, USA), and 1 μ g/ml nerve growth factor (Sigma Aldrich, Ontario, Canada). The protocol for fixation and mounting is the same as the protocol used to quantify neuronal differentiation and extension (2.4). Images of the cells were taken using Zeiss AxioCam mounted on a Axioskop 2 inverted microscope (Zeiss, Oberkochen, Germany).

2.8 Evaluating the Effect of the Optimized Media on Neurite Extension from Dorsal Root Ganglia Neurons

Dorsal root ganglia (DRG) were isolated from chick embryos on day 8 of incubation by dissection. They were placed on culture dishes that had been thinly coated with collagen matrix (see protocol for making collagen matrix section 2.7). The coating was applied to ensure adherence of the DRG to the culture dish. The DRG were cultured for six days in either unsupplemented KSFM base media, optimized media, standard DRG media (consisting of KSFM media (Invitrogen, Ontario, Canada) supplemented with 1% N2 and 2% B27 supplements (Life Technologies Ltd, Renfrew, Strathclyde, UK) and 1 nmol/ml retinal acetate (Sigma Aldrich, Ontario, Canada) or standard DRG media

supplemented with optimized supplements. DRG were fixed, mounted and immunostained for neurofilament-200 according to the protocol outlined in section 2.4. Images were taken of the DRG using the Zeiss Axiocam mounted on an Axioskop 2 inverted microscope (Zeiss, Oberkochen, Germany). Cells were visualized using a 10 X lens objective.

Neurite extension from the DRG was assessed by counting the number of neurites that were present at increasing radial distances from the center of the DRG. To accomplish this, Adobe Photoshop 7.0 was used to superimpose a concentric grid over the images to track and count the number of neurites present at intervals of 125 μm from the DRG center. The percentage of neurites reaching each interval was calculated by dividing the number of neurites counted at a given interval by the total number of neurites extending from the DRG. Several DRG were evaluated for each different type of media per trial and the results were averaged. The frequency of neurites reaching a given length interval for different culture conditions was compared.

Differences between mean values for the different culture conditions were demonstrated using a student t-test. A P-value of $P < 0.05$ was used for all significance comparisons. Analysis was done using SPSS software (SPSS Inc, Chicago, IL, USA)..

2.9 Evaluating the Effect of the Optimized Media on Differentiation of Sh-sy5y cells

Sh-sy5y cells were cultured for 6 days on uncoated tissue culture dishes, in either unsupplemented KSFM base media, optimized media, standard differentiation media (see section 1.1 for components of standard differentiation media) or standard differentiation media supplemented with optimized supplements. Fixation, mounting and

immunostaining for NF-200 were performed according to the protocol outlined in section 2.4. Images were taken using the Zeiss Axiocam mounted on an Axioskop 2 inverted microscope (Zeiss, Oberkochen, Germany). Cells were visualized using a 10 X lens objective. The extent of neuronal differentiation in the different culture conditions was assessed based on cell morphology and intensity of NF-200 staining.

3. RESULTS

3.1 Effect of culture media and ECM matrix molecules on differentiation

Figure 3 summarizes the effects of various culture media and ECM macromolecule culture substrates on the neuronal differentiation of ND-C cells. The choice of culture media had a significant effect on the proportion of ND-Cs that differentiated into neurons. KSFM media was shown to be the most effective in inducing neuronal differentiation. ND-Cs cultured in KSFM generated by far the greatest proportion of neurons, yielding on average 19 % neurons on uncoated dishes. Though not as outstanding as KSFM, Neurobasal was also significantly more effective in promoting neuronal differentiation compared to the SHEM, DMEM and StemPro media. The average yield of neurons produced on uncoated dishes with Neurobasal was 0.7%. SHEM media, DMEM and StemPro produced average neuronal yields of 2%, 0.6% and 0.4% respectively on uncoated dishes.

ECM macromolecules as culture substrates also had a significant effect on neuronal differentiation. ND-Cs cultured on laminin coated dishes generated significantly more neurons than ND-Cs grown on uncoated or collagen coated dishes. This is best exemplified in combination with the Neurobasal media supplements where the average neuronal yield on laminin coated dishes was 11%, while the collagen and uncoated dishes yielded 2% and 0.4% respectively. There is a trend suggesting that fibronectin coated culture dishes also increases the percent of neurons generated compared to uncoated and collagen coated dishes, however a statistical difference was not observed. In Neurobasal media, an average neuronal yield of 5% was observed on fibronectin coated dishes.

Results from a two-way ANOVA show that culture media and ECM macromolecule culture substrates have an interactive effect on neuronal differentiation ($P < 0.05$). This interaction is best observed between the substrates and Neurobasal media. Statistically, Neurobasal was shown to be more effective in promoting neuronal differentiation than SHEM, despite the fact that SHEM produced a greater neuronal yield on uncoated dishes. This is because when Neurobasal was combined with an ECM substrate, the resulting neuron yield was considerably higher than that achieved with SHEM media alone or in combination with a substrate. These results indicate Neurobasal has a substantial permissive effect on the neuronal differentiation of ND-C and can effectively support neuronal differentiation when used in combination with ECM substrates.

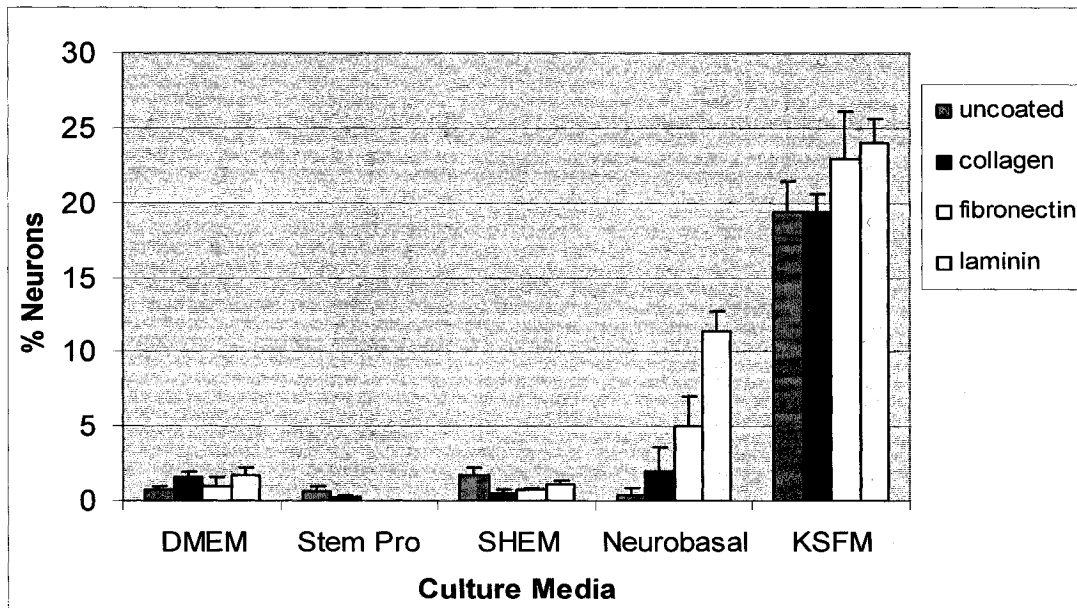


Figure 3. Effect of different culture media and ECM macromolecules culture substrates on neuronal differentiation of ND-Cs. KSFM and Neurobasal have a significant differentiating effect on ND-Cs. However, KSFM media generated by far the greatest proportion of neuron-like cells. Laminin also significantly increases the proportion of neurons generated. The effect of culture media and ECM substrates was proven to be interactive. Four trials of this experiment were performed ($n = 4$), with three repeats of each condition per trial.

After 6 days of culture in KSFM, ND-Cs extended long neurites, some of which reached lengths greater than 400 μm . These neurites were positively stained with a monoclonal antibody against NF-200 (Fig 4). In the other media types, ND-Cs extended short neurites (less than the diameter of the cell body) but did not stain positive for NF-200 (Fig 4.). Reverse transcriptase PCR (RT PCR) indicated the expression of neurofilament-M (*nf-m*) transcripts in ND-Cs cultured in KSFM (Fig 5).

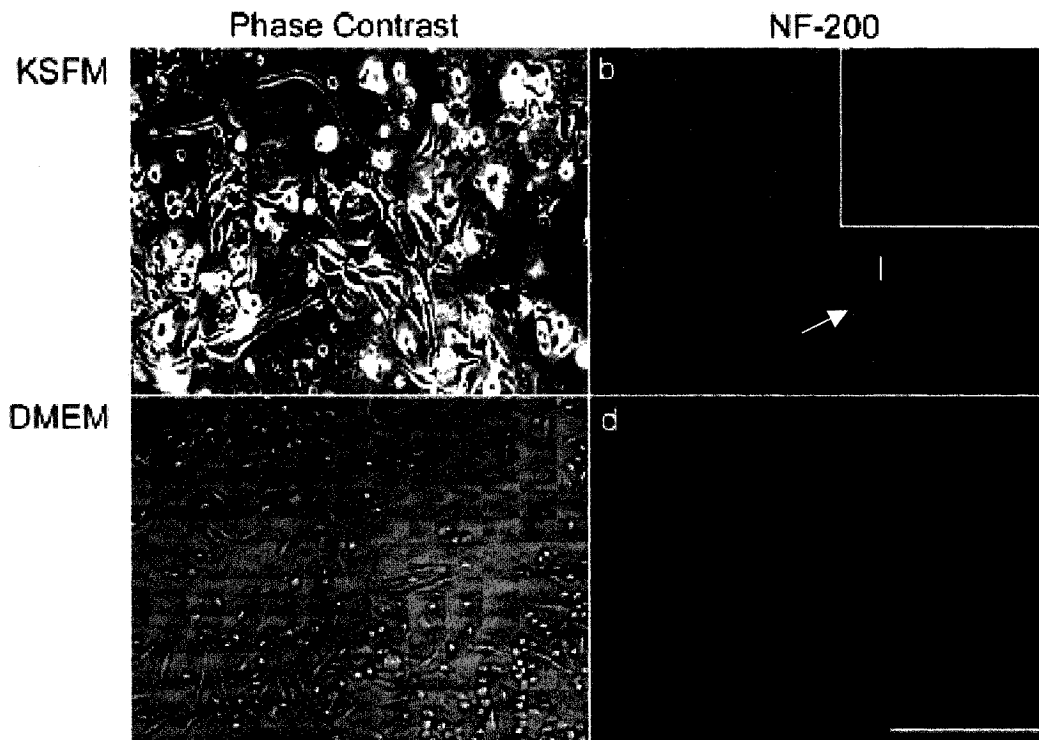


Figure 4. ND-Cs cultured in KSFM show extension of long neurites (arrow) (A) and express NF-200 antibody (C) compared to ND-Cs culture in DMEM (B and D). Immunocytochemical staining of ND-Cs with monoclonal NF-200 antibody (green) and DAPI staining (blue). Scale bar, 200 μm .

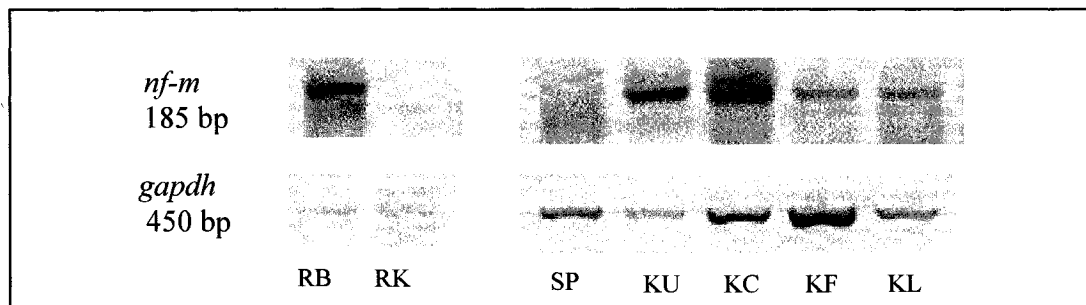


Figure 5. RT PCR analysis for expression of glyceraldehyde-3-phosphate dehydrogenase (*gapdh*; control) and *nf-m* transcripts. RB represent rat brain, RK represents rat kidney and SP represents ND-Cs cultured in Stem Pro media on uncoated dishes. KU, KC, KF, KL represent ND-Cs culture in KSFM on uncoated, collagen, fibronectin and laminin coated dishes respectively.

KSFM media is supplemented with bovine pituitary extract and epidermal growth factor (EGF). The components added to the KSFM base media alone or in combination did not generate more neurons than the KSFM base media on its own (Fig 6.), suggesting that it is the KSFM base media itself that was inducing neuronal differentiation. The proportion of glial cells generated was significantly greater in KSFM supplemented with EGF (34%), and EGF in combination with pituitary extract (28%) compared to KSFM with only pituitary extract (17%) and unsupplemented KSFM (11%). These results indicate that EGF was promoting the differentiation of ND-Cs into glial cells.

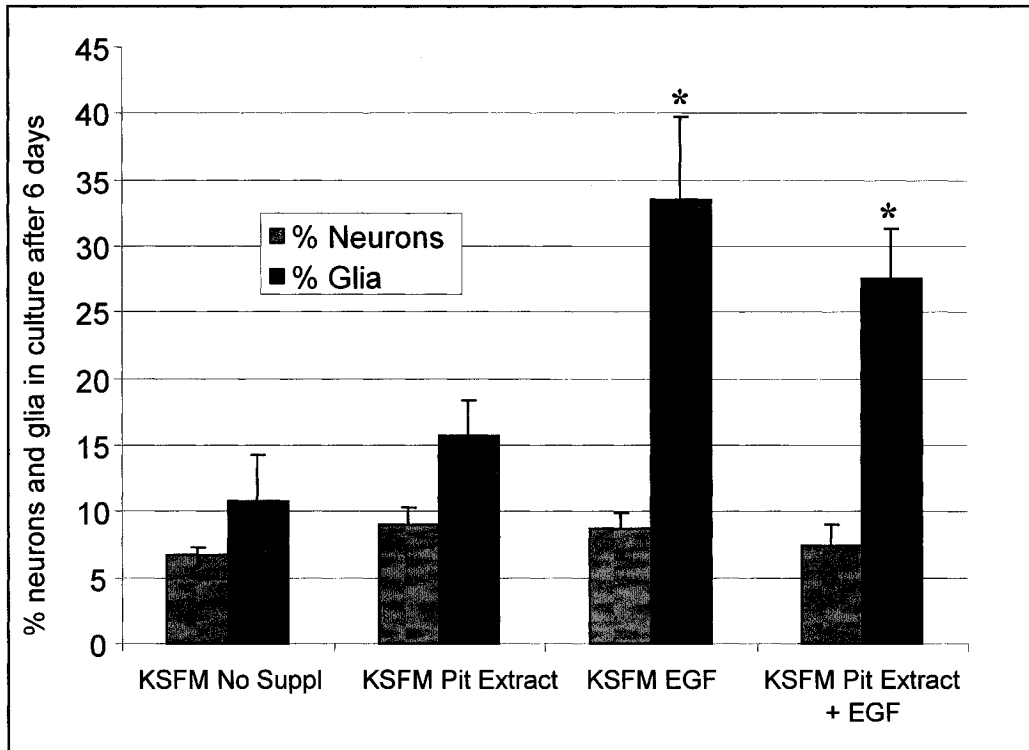


Figure 6. Neural differentiation induced by components of KSFM culture media. The components added to the KSFM base media alone or in combination did not generate more neurons than the KSFM base media on its own. EGF increased the proportion of glial cells that were generated. * indicates $p < 0.05$ by ANOVA, compared to KSFM with no supplement (negative control). Four trials of this experiment were performed ($n = 4$), with three repeats of each condition per trial.

3.2 Effect of ECM Substrates on Neurite Outgrowth

Using laminin and fibronectin as culture substrates increased the proportion of long neurites generated by ND-Cs (Fig. 7). Compared to ND-Cs culture on uncoated dishes, ND-Cs on fibronectin and laminin coated dishes showed a significant increase in the percentage of neurites with lengths within the 101-200 μm range. On uncoated dishes only 24% of neurites were within 101-200 μm , whereas on laminin and fibronectin coated dishes 32% and 34% of the neurites respectively were within this range. Using collagen as a substrate did not significantly affect the proportion of neurites in the 101-200 μm range. The ND-Cs cultured on laminin also extended significantly more neurites

that were greater than 200 μm in length. 14% of neurites were longer than 200 μm on the laminin coated dishes compared to 3% on uncoated dishes. Fibronectin and collagen generated 11% and 9% respectively in this range but these values were not significant different from those achieved on the uncoated dishes.

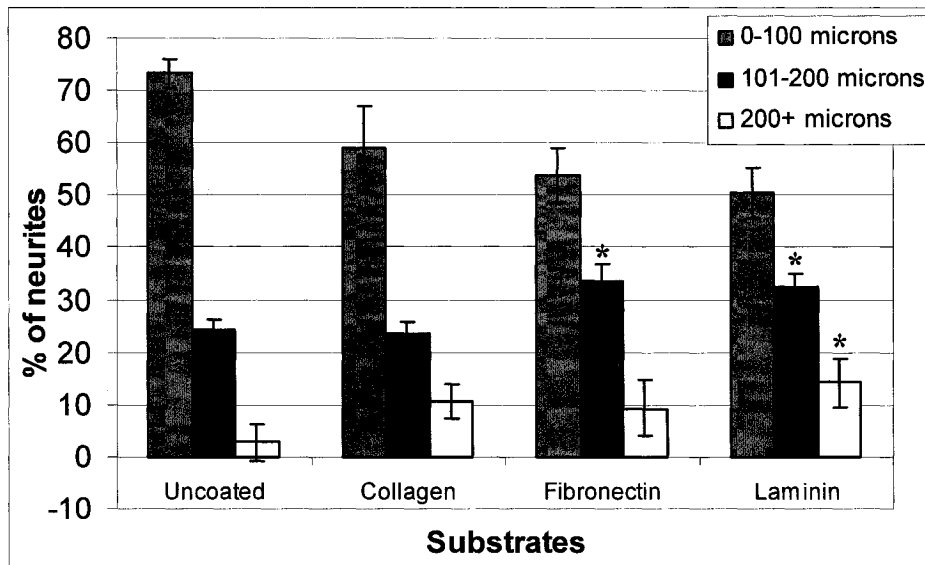


Figure 7. Extension of neurites from ND-Cs cultured in KSFM on differentially coated dishes. Compared to ND-Cs cultured on uncoated dishes, ND-Cs on fibronectin and laminin coated dishes generated a greater proportion of neurites in the 101-200 μm range. ND-Cs on laminin coated dishes also generated a greater proportion of neurons reaching over 200 μm in length. * indicates $p < 0.05$ by ANOVA, compared to uncoated dishes in the corresponding length category. Error bars represent standard error. Four trials of this experiment were performed ($n = 4$), with three repeats of each condition per trial.

3.3 Effect of supplements on neuronal differentiation and neurite outgrowth

The addition of dexamethasone, dimethyl sulfoxide (DMSO), dibutyryl cyclic adenosine monophosphate (db cAMP) or nerve growth factor (NGF) to the KSFM base media resulted in a significant increase in the proportion of neurons generated (Fig. 8). ND-Cs cultured in unsupplemented KSFM base media generated on average a 7% neuronal yield. The addition of dexamethasone, DMSO, db cAMP or NGF to the KSFM base media increased the neuronal yield to 16%, 14%, 11% and 15% respectively. When

all of the supplements were added to the KSFM base media simultaneously a 26% neuronal yield was observed, indicating that the differentiating effects of the supplements were not additive.

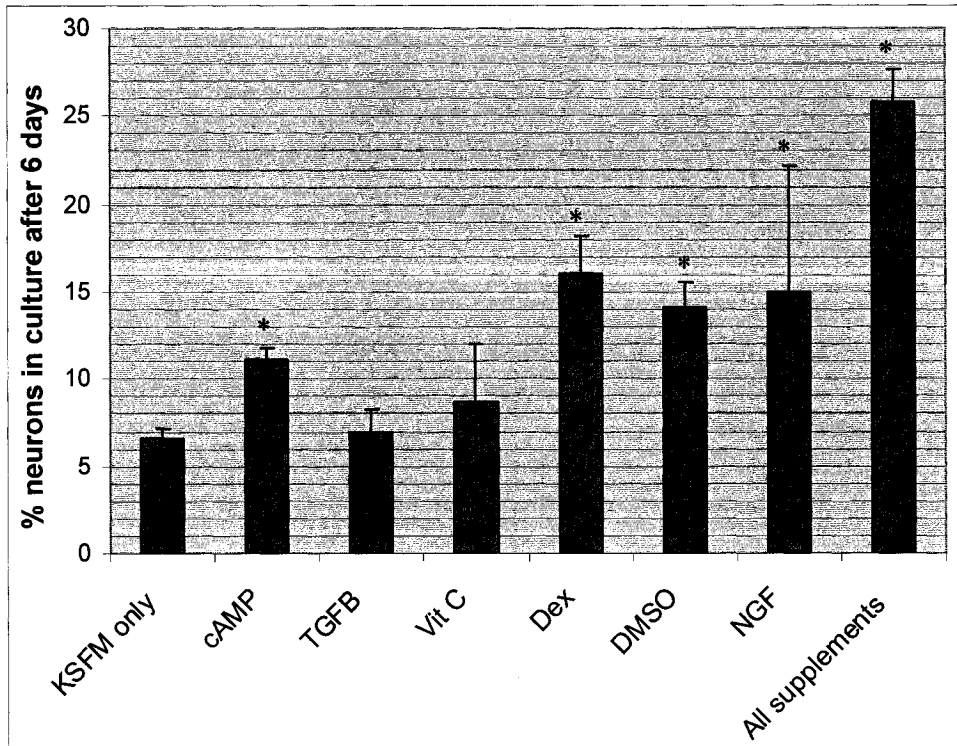


Figure 8. Effect of media supplements on neurite outgrowth. The addition of dexamethasone (dex), DMSO, cAMP or NGF to the KSFM base media resulted in a significant increase in the proportion of neurons generated. * indicates $p < 0.05$ by ANOVA, compared to unsupplemented KSFM. Error bars represent standard error. Four trials of this experiment were performed ($n = 4$), with three repeats of each condition per trial.

Figures 9a and 9b are different representations of the same data illustrating the distribution of neurite lengths in cultures with different supplements added to the media.

Figure 9a clearly shows that the presence of db cAMP in the culture media resulted in a greater proportion of long neurites. There were more neurites greater than $200 \mu\text{m}$ in the db cAMP supplemented media compared to the other media. However, this increase is

not statistically significant, due to the variability that is indicated by the large error bars in figure 9b.

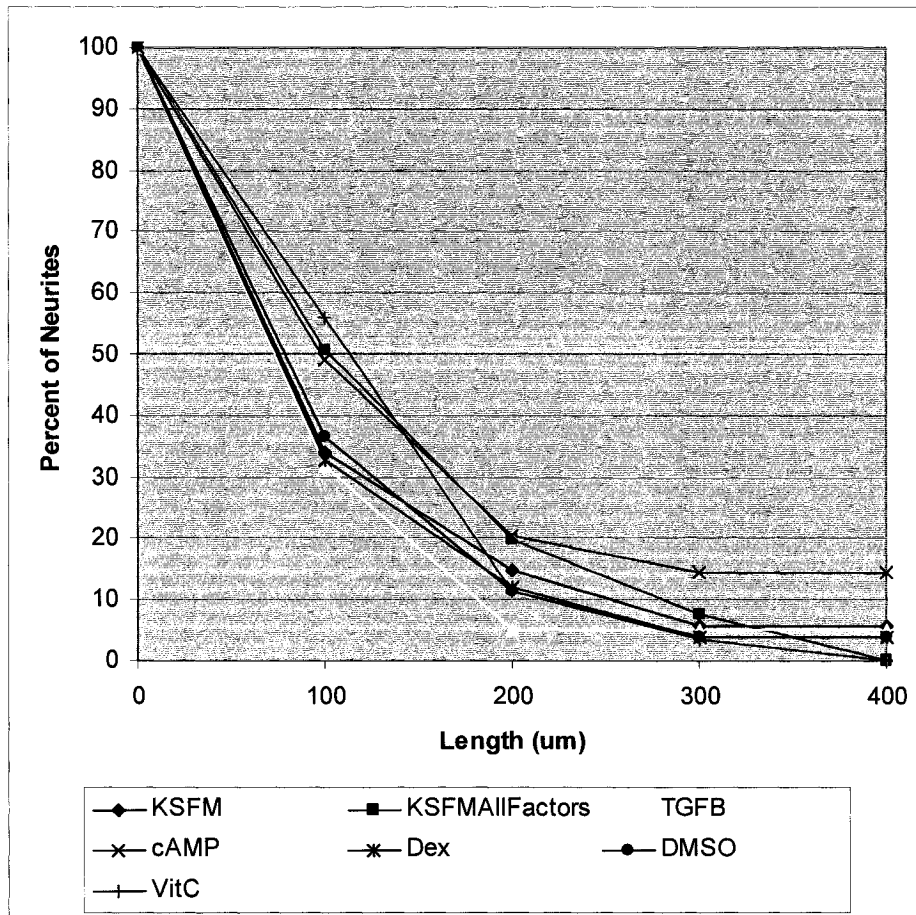


Figure 9a) Distribution of neurite lengths in cultures with various supplements added. ND-Cs cultured in media supplemented with cAMP extended a greater proportion of neurites longer than 200 μm.

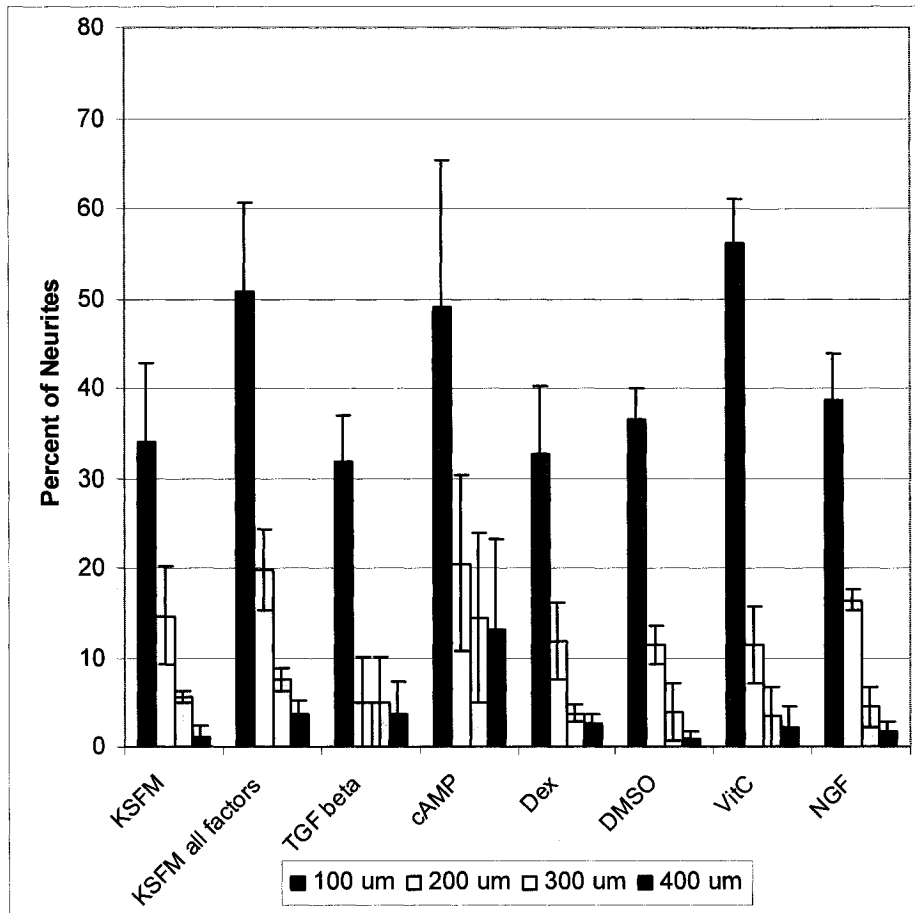


Figure 9b. Proportion of neurites reaching a given length in cultures with differentially supplemented media. The addition of db cAMP to the culture media increases the proportion of neurites reaching lengths of 300 μm or more. This increase is not statistically significant, due to the variability indicated by the large error bars. Four trials of this experiment were performed ($n = 4$), with three repeats of each condition per trial.

3.4. Optimal Conditions for Maximum Neuronal Differentiation and Neurite Extension

DMSO induced the greatest neuronal yield (36%) at a concentration of 1.5 μM (Fig 10). The DMSO concentration curve is bi-phasic. Media with a 1.0 μM or 2.0 μM concentration of DMSO generated neuronal yields of 16% and 10% respectively, significantly less neurons than the 1.5 μM media ($p < 0.05$). The db cAMP concentration curve shows that the greatest average yield of neurons was achieved at a concentration of 15 μM (Fig. 8). At a concentration of 15 μM , a 14% neuronal yield was generated.

Significantly smaller yields were obtained at a concentration of 10 μM (4%) and 25 μM (5%). No significant difference in neuronal differentiation was observed among the different concentrations evaluated for dexamethasone and NGF. (Fig. 10)

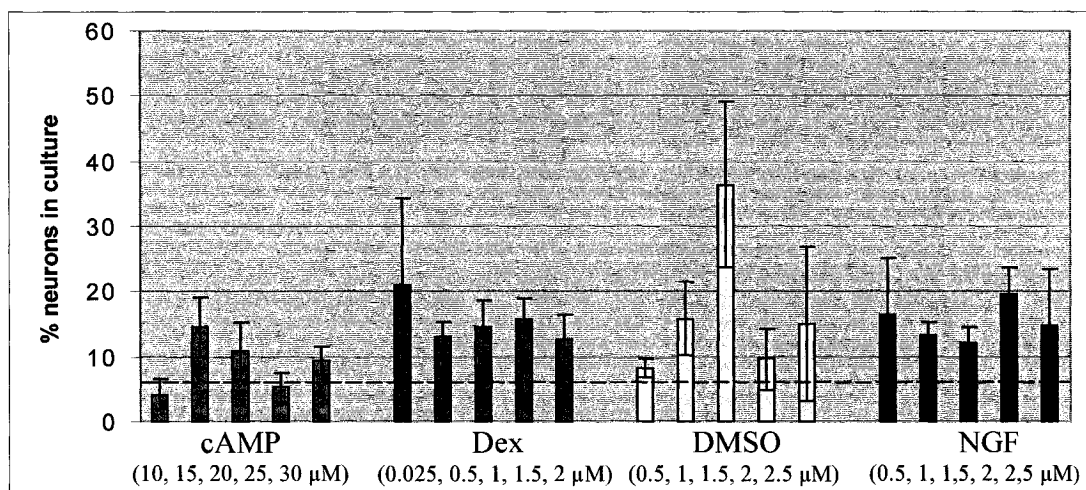


Figure 10. Concentration curve for media supplements to determine optimal concentration for inducing neuronal differentiation. The trend of the concentration curve for DMSO suggests that the greatest yield of neurons is achieved at a concentration of 1.5 μM . The cAMP concentration curve shows that the greatest neuronal yield at 15 μM . No significant difference in neuronal differentiation was observed among the different concentrations evaluated for dexamethasone and NGF. Six trials of this experiment were performed ($n = 6$), with three repeats of each condition per trial. Red dotted line indicates the percentage of neurons generated in KSFM base media without supplements.

Table 1 summarizes the selected concentrations of supplements that were used in the “optimized media”, which allows for maximum neuronal differentiation and neurite extension of ND-Cs.

Table 1. Selected concentrations of supplements in “optimized media”.

Supplement/Growth Factor	Selected Concentration
Dexamethasone	0.1 μM
DMSO	1.5 μM
NGF	1.5 μM
db cAMP	20 μM

Culturing ND-C in the optimized media resulted in an average yield of 26% neurons. When ND-Cs were cultured in the optimized media on a laminin substrate there was no significant difference in the percentage of neurons generated (Fig. 11).

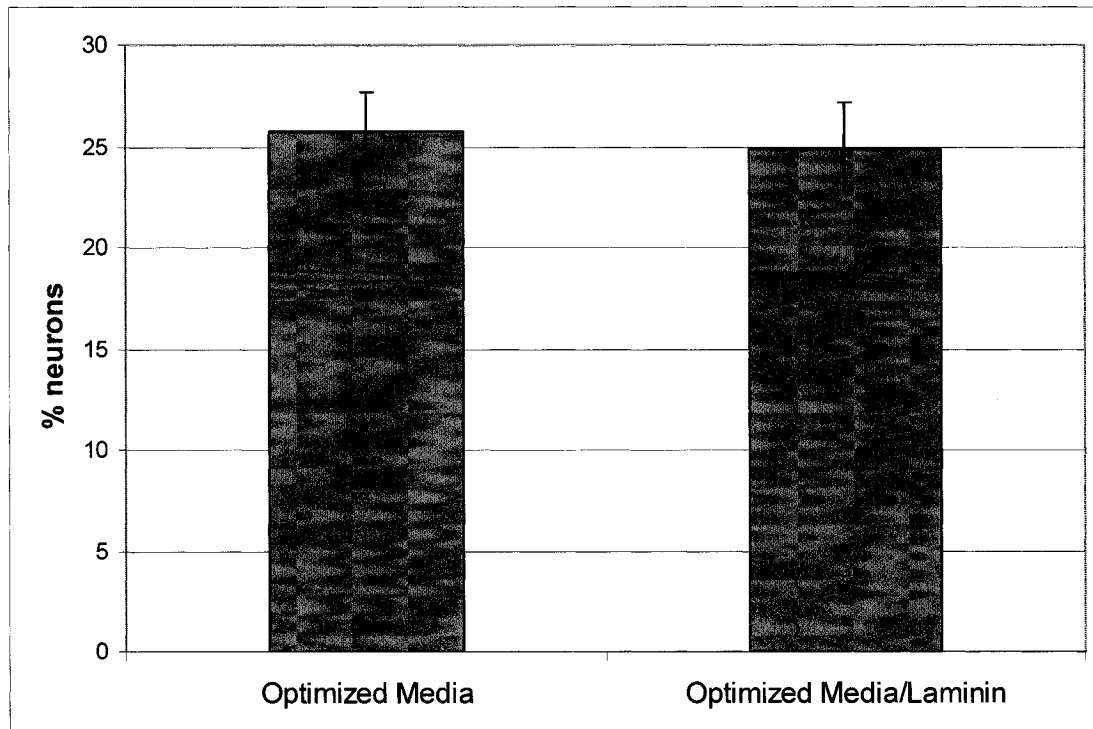


Figure 11. Comparison of the neurons generated from ND-C cultured in the optimized media culture on TC plastic and on a laminin substrate. No significant difference was observed when student t-test was performed. Four trials of this experiment were performed ($n = 4$), with three repeats of each condition per trial.

3.5 Extension of ND-Cs in 3-D Matrix

ND-Cs seeded within a semi-synthetic collagen based matrix extended long neurites after 6 days of culture in the optimized media (Fig. 12). 3D neurite extension was considerably greater in the optimized media than in KSFM base media. ND-Cs extended the greatest proportion of long neurites in gel with 0.06% collagen content (Fig. 13). In the 0.06% matrices with optimized media, 20% of the neurites were greater than 200 μm

in length. Neurites longer than 200 μm were not observed in gels with higher concentrations of collagen.



Figure 12. ND-C seeded within a semi-synthetic matrix with 0.06% collagen concentration. Stained with NF-200 antibody (green). DAPI nuclear staining in blue. Scale bar, 100 μm .

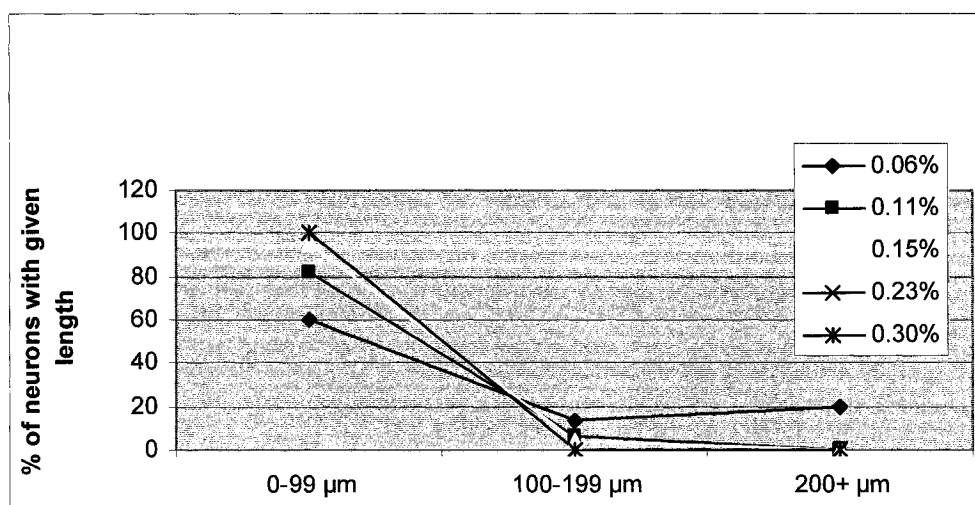
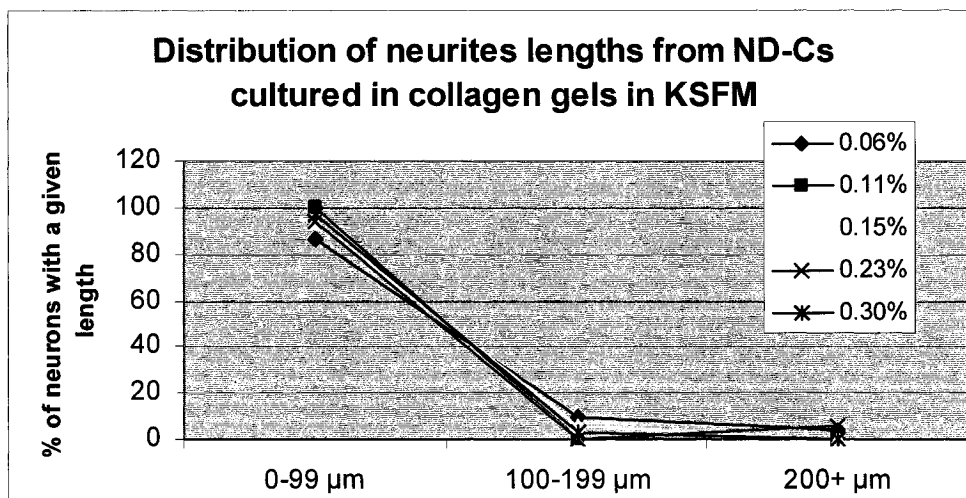


Figure 13. Distribution of neurite lengths from ND-C seeded into collagen gels of varying concentrations. 3D neurite extension was considerably greater in the optimized media (A) than in KSFM base media (B). In the 0.06% gel with optimized media ND-Cs extended the greatest proportion of neurites that were 200 μm or longer (B). One trial of this experiment were performed ($n = 1$), with three repeats of each condition per trial.

3.6 Achieving Contact Between Differentiated ND-Cs and Target Cells

Differentiated ND-Cs formed contacts with both epithelial cells and myotubes when grown in co-culture (Fig. 14). Acetylcholine was highly expressed in the neurites of ND-Cs forming contacts with C2C12 targets (Fig. 15). However, ND-Cs co-cultured

with epithelial targets did not express acetylcholine. These results therefore demonstrate the plasticity of ND-Cs, and also suggest that the target cell has an effect on the neurotransmitter phenotype of the differentiated ND-Cs.

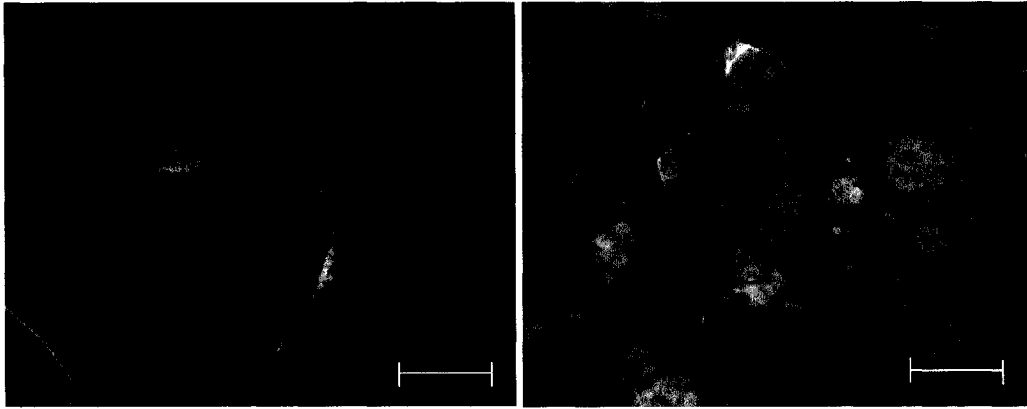


Figure 14. ND-Cs made contact with muscle and epithelial target cells. A) Neurites from ND-Cs stained with anti-NF-200 antibody (yellow) made contact with C2C12 myotubes stained with anti-myosin antibody (red). B) Neurites stained with anti NF-200 antibody (green) made contact with HCEC cells stained with anti-keratin antibody (red). DAPI nuclear staining in blue. Scale bar 20 μm (A) and 100 μm (B).

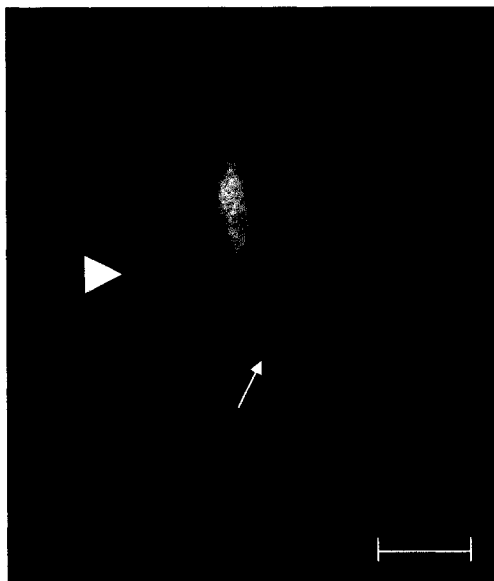


Figure 15. Acetylcholine positive ND-C (arrow) targeting C2C12 (arrow head). Immunocytochemical staining with anti- myosin antibody (red) and nicotinic acetylcholine (green). Scale bar, 20 μm .

Local accumulation of acetylcholine receptors was present at the site of contact between C2C12 myotubes and primary DRG neurons. Therefore, C2C12 myotubes, although lacking some of the properties of primary myotubes, are still capable of forming a neuromuscular junction with neurons (Fig. 16). Acetylcholine receptor clustering was observed at the site of contact between C2C12 myotubes and ND-Cs, indicating neuromuscular junction formation (Fig. 17).



Figure 16. C2C12s formed neuromuscular junctions with NF-200 immuno-stained dorsal root ganglia neurons (green), indicated by the clustering of acetylcholine receptors (red). DAPI nuclear staining in blue. Scale bar, 20 μ m.

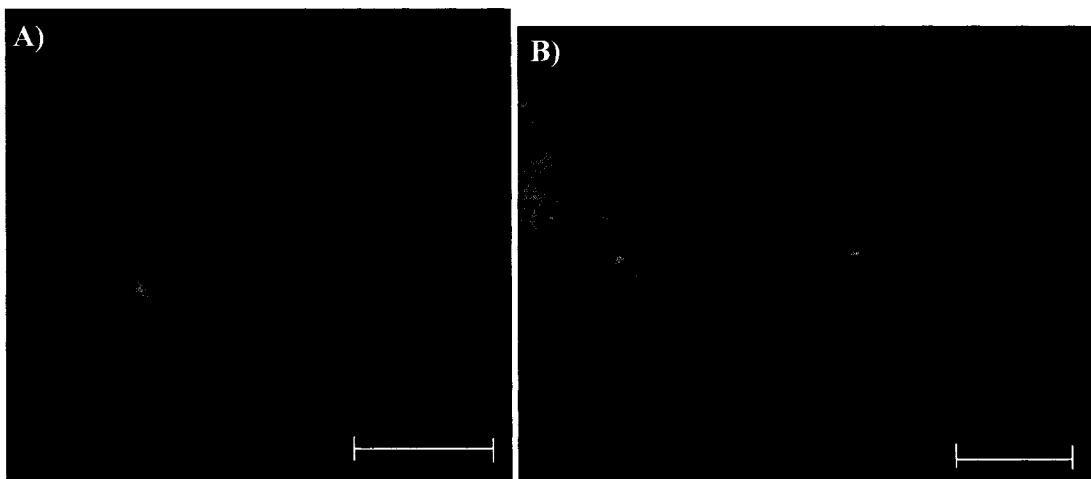


Figure 17. Formation of neuromuscular junction between ND-Cs and muscle targets. A) Clustering of acetylcholine receptors (red) at the point of contact between GFP-ND-C (green) and C2C12. B) Acetylcholine receptor clustering at point of contact between NF-200 stained ND-Cs (green) and P19 cells. DAPI nuclear staining in blue. Scale bar, (A) 20 μ m and (B) 100 μ m.

NDC cultured in the trilaminar co-culture system with targets cells, extended neurites through collagen gel (approximately 300 μm thick) to contact target cells (Fig. 18).

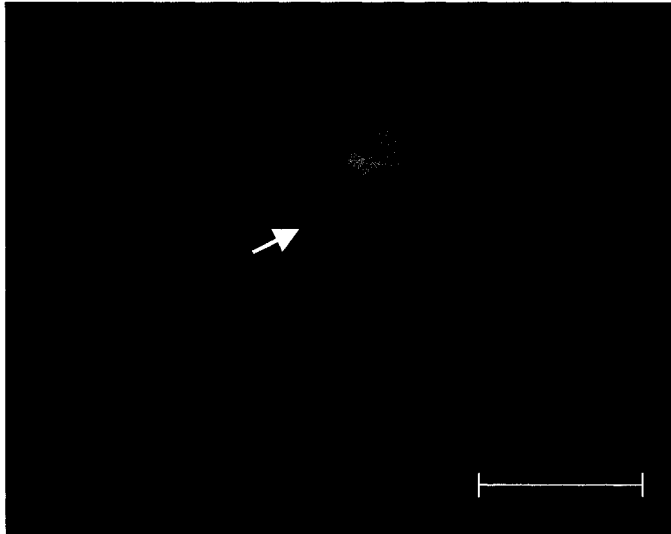


Figure 18. Immunocytochemical staining of NDC extending through collagen gel to contact C2C12 targets. Neurite indicated by arrow. Myosin (red), Nf-200 (green), DAPI (blue). Scale bar, 20 μm .

3.7. Effect of Optimized Media on Neurite Extension of Alternate Nerve Source

Dorsal root ganglia (DRG) cultured in the optimized media showed an increase in neurite extension compared to DRG cultured in KSFM without the added dexamethasone, DMSO, cAMP and NGF (Fig.19). There was a significant increase in the number of neurites that were present at 750-1500 μm from the center of the DRG. When the optimized supplements (DMSO, cAMP, dexamethasone and NGF) were added to the standard DRG media a significant increase in neurite extension was observed (Fig. 20). The increase was seen in the number of neurites that were present at 750-1500 μm from the center of the DRG.

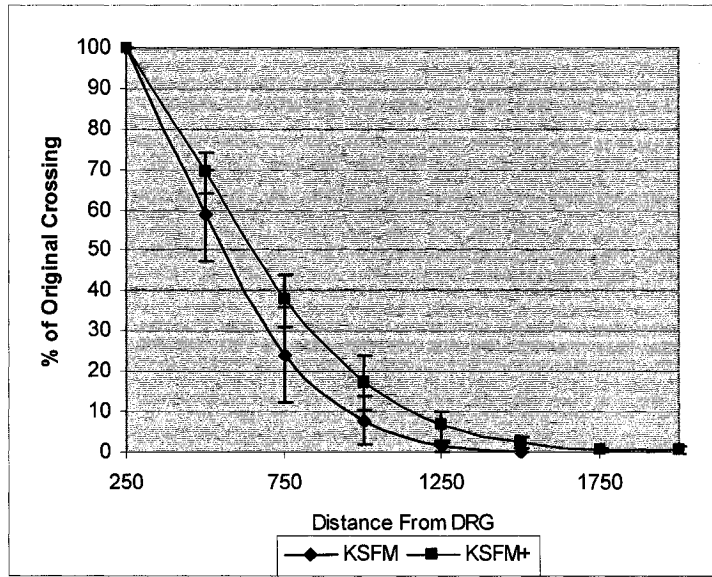


Figure 19. Distribution of neurites at increasing radial distances from the center of the dorsal root ganglion (DRG) culture in either in KSFM base media (KSFM) and optimized media (KSFM +). In the optimized media, there was a significantly more neurites present at radial distances of 750-1500 μm from the center of the DRG . * indicates $p < 0.05$ by ANOVA.

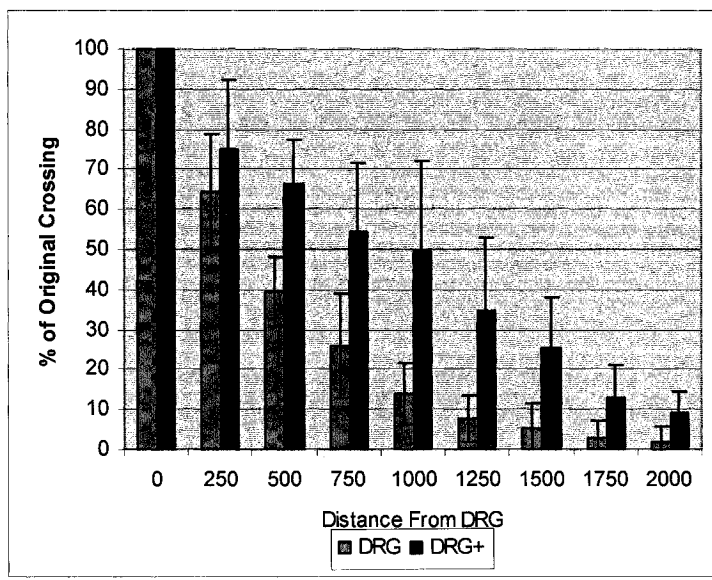


Figure 20. Distribution of neurites at increasing radial distances from the center of DRG cultured in either standard DRG media (DRG) or DRG media with optimized media supplements added (DRG+). In the DRG media with optimized supplements, there were significantly more neurites present at radial distances of 750-1500 μm from the center of the DRG . * indicates $p < 0.05$, by ANOVA.

3.8 Effect of Optimized Media on Neuronal Differentiation of Alternate Nerve Source

SH-SY5Ys cultured in KSFM base media or the optimized media displayed a neuronal morphology, characterized by a round cell body and long neurite extensions (longer than two times the diameter of the cell body) and stained positive with a monoclonal NF-200 antibody. NF-200 staining was more intense in the SH-SY5Ys cultured in the optimized media. The SH-SY5Ys culture in retinoic acid (RA) supplemented media either with or without the added optimized supplements showed only weak staining with the NF-200 antibody. Further, SH-SY5Y cells cultured in the RA media with or without supplements displayed fibroblast-like, elongated cell bodies and formed aggregates. These results suggest that KSFM promotes a neuronal morphology and increased the expression of NF-200 in SH-SY5Y cells and this expression is enhanced by the addition of the optimized supplements (Fig. 21).

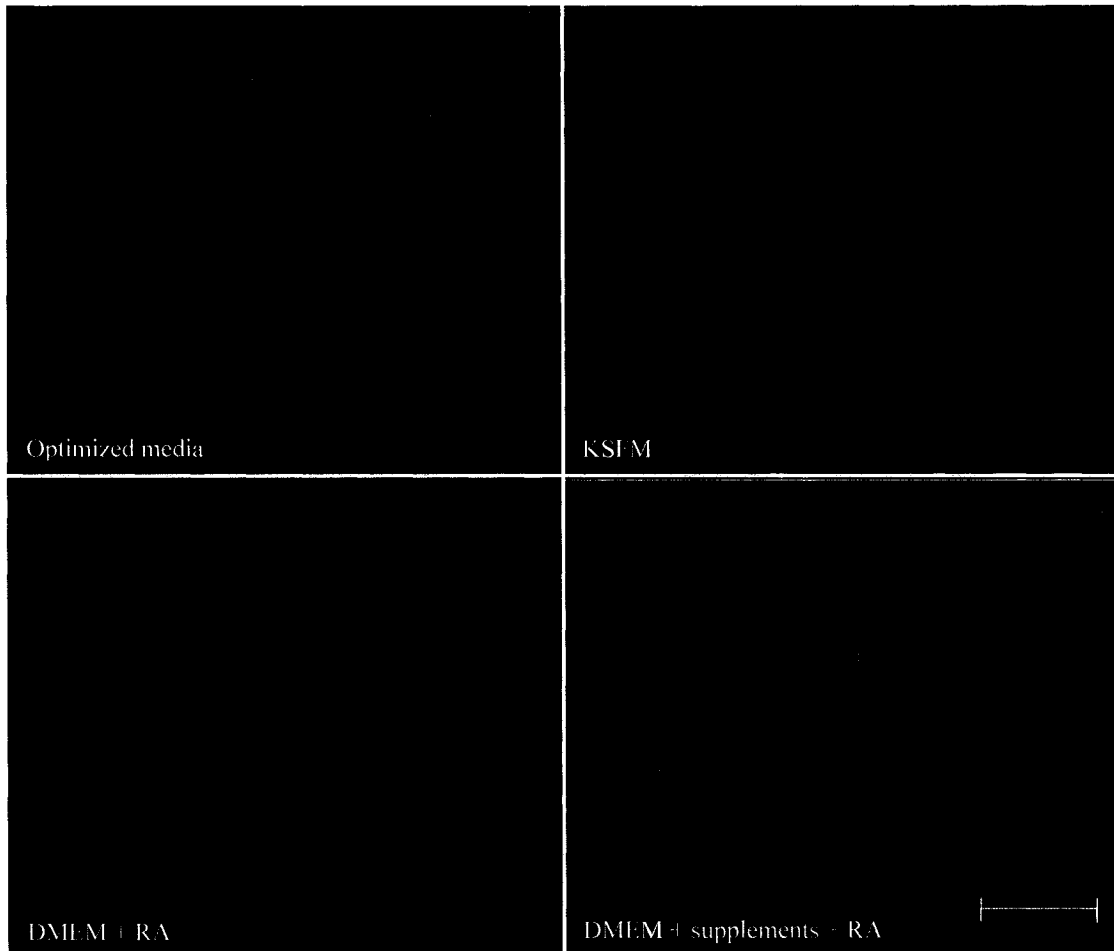


Figure 21. SH-SY5Y cells cultured in KSFM base media, optimized media, DMEM supplemented with RA, and DMEM supplemented with RA with optimized media supplements added. Immunostaining with NF-200 (green) and DAPI nuclear stain (blue). Scale bar 100 μ m.

4. DISCUSSION

4.1 Differentiation of ND-Cs

4.1.1 Unsupplemented KSFM induces Neuronal Differentiation of ND-Cs

28% of ND-Cs cultured in KSFM media demonstrated a neuronal morphology.

Recently, Moore et al. (2005) also reported successful neuronal differentiation of ND-Cs in keratinocyte media. Neurobasal media was the only other media that induced a considerable proportion of ND-Cs to differentiate into neurons. However, at best, only a 11% yield of neurons was achieved. Neurobasal is supplemented with N2, a proprietary mixture of insulin transferrin, progesterone, selenium and putrescine that was developed specifically to support the survival of neurons in serum-free media. N2 has been shown to both stimulate the proliferation and enhance the differentiated properties of neuronal cell lines (Bottenstein et al. 1979).

KSFM is a serum free media that is supplemented with bovine pituitary extract and EGF. SHEM media, another medium used in the culture of keratinocytes and corneal epithelial cells, is also supplemented with EGF but did not induce a significant degree of differentiation of ND-Cs. This suggests that EGF is not the active component in KSFM that was inducing neuronal differentiation. The combinatorial assay performed to assess each component of KSFM separately and identify the active ingredient showed that pituitary extract and EGF added to the KSFM base media alone or in combination did not generate more neurons than the KSFM base media on its own. This showed that it is most likely the unsupplemented KSFM base media itself which was inducing differentiation.

Included in the KSFM base media (complete base media formulation in given in the Appendix) are various vitamins, salts, trace elements and minerals that may stimulate differentiation. The lack of serum in KSFM may also be responsible for the observed differentiation of ND-Cs. Many neuronal cell lines, including neuroblastoma cells are differentiated by serum starvation (Howard et al. 1993, Evangelopoulos et al. 2005). Neurobasal media is also serum free, which may account for the differentiation of ND-Cs in this media.

When cultured in KSFM, a proportion of the ND-Cs also differentiated into GFAP-positive glial cells. The presence of glial cells in the culture may be beneficial for the neurons, as glial-neuron interaction has been shown to regulate neurogenesis and synapse formation (Ma et al 2005, Mars et al, 2001). As mentioned, in the ND-C system, EGF did not have ($p < 0.05$ by ANOVA) an effect on the neuronal differentiation, however the addition of EGF to KSFM increased the proportion of ND-C differentiating into glial cells. Pruss et al. (1982) and Raff et al. (1983) have shown that type-I fibroblast-like astrocytes will proliferate in the presence of EGF.

4.1.2 Effect of ECM substrate on differentiation and neurite extension

The use of different ECM molecules (type I collagen, fibronectin or laminin) as culture substrates did not affect the proportion of cells that differentiated into neurons in KSFM media. However, fibronectin and laminin substrates were found to result in significant increases in the proportion of ND-Cs with longer neurites. Compared to uncoated controls, fibronectin and laminin substrates significantly increased the proportion of neurites with lengths ranging between 100 and 200 μm . On laminin, neurons also extended a greater proportion of neurites with lengths exceeding 200 μm .

The observed difference in the distribution of neurite lengths on fibronectin and laminin substrates is supported by studies that show fibronectin to be less effective than laminin in promoting growth cone advancement (Gomes et al. 1994, Kuhn et al. 1995). Thus, among the substrates evaluated, laminin was identified as the best substrate for achieving maximal neurite extension from ND-Cs in 2D.

Further investigation was not performed to determine the optimal concentration of laminin. Studies have shown that laminin acts as a permissive substrate rather than an instructional substrate. This means, “the magnitude of behavioural response does not match the magnitude of change in concentration of laminin” (Lukenbill-Edds, 1997). Rat superior cervical ganglia neurons exhibit accelerated neurite extension on laminin but there is no change in neurite initiation, outgrowth or branching when the concentration of laminin ranges from 0.01 to 1 $\mu\text{g}/\text{cm}^2$ (Buettner et al. 1991). Similar results have been obtained for the NG108-15 neuroblastoma cell line (Smalheiser, 1991).

4.1.3 Media supplements enhance neuronal differentiation and extension

The addition of dexamethasone, DMSO, db cAMP or NGF to the KSFM base media resulted in a significant increase in the proportion of neurons generated. As outlined in the introduction, DMSO and cAMP are well recognized for their stimulatory effect on the differentiation of neuroblastoma-derived cell lines. However, most established neuroblastoma cell lines are unresponsive to NGF. This is likely due to the decreased expression of TrkA in neuroblastoma cells. The introduction of exogenous TrkA can restore the ability of NB cell to morphologically differentiate in response to NGF (Matsushima and Bogenmann 1993). Due to the hybrid nature of ND-Cs,

expression of TrkA is retained thus preserving NGF-responsiveness. Dexamethasone has been shown to stimulate neuronal differentiation in a limited number of neuroblastoma cell lines. ND-Cs cultured in chondrogenic media containing dexamethasone will differentiate into chondrocytes as well as neurons (unpublished data) This data demonstrates the pluripotent nature of ND-C and their responsiveness to dexamthasone as an inducer of neuronal differentiation.

The presence of db cAMP in the culture media also resulted in a greater proportion of long neurites. This increase was not significant due to variability among experimental trials. However, the general trend among trials suggests that cAMP stimulates neurite outgrowth. This inference is supported by theories implicating cAMP in the elongation of neuritic processes (Sanchez et al 2004).

ND-Cs were cultured in a range of concentrations of dexamethasone, DMSO, db cAMP or NGF to determine the concentration that produces the greatest proportion of neurons. For Dex, cAMP and NGF, no substantial difference in neuronal differentiation was observed among the different concentrations evaluated. These supplements may act within a broad concentration range to stimulate differentiation of ND-Cs. The concentration curve for DMSO is a bell shaped curve that peaks at a concentration of 15 μ M. This data is consistent with results from other studies showing that DMSO-induced differentiation is optimal within a very narrow concentration range (Clean 1996, Morley 1993).

Selected concentrations of dexamethasone, DMSO, cAMP and NGF were added to the KSFM base media to create an 'optimized neuronal differentiation medium". The combination of supplements (see appendix) in the optimized media allows for maximum

neuronal differentiation and neurite extension of ND-Cs. In combination, the supplements do not exhibit an additive effect. This is likely due to overlapping signaling pathways mediating differentiation. The NGF receptor (TrkA) and cAMP both activate the PI3K-AKT and MAPK signaling pathways. Laminin also acts through these pathways to stimulate neurite extension. This may explain why the laminin substrate did not increase neurite extension of ND-Cs cultured in optimized media. Laminin activates integrin receptors that signal via PI3K-AKT and MAPK pathways to stimulate neurite outgrowth.

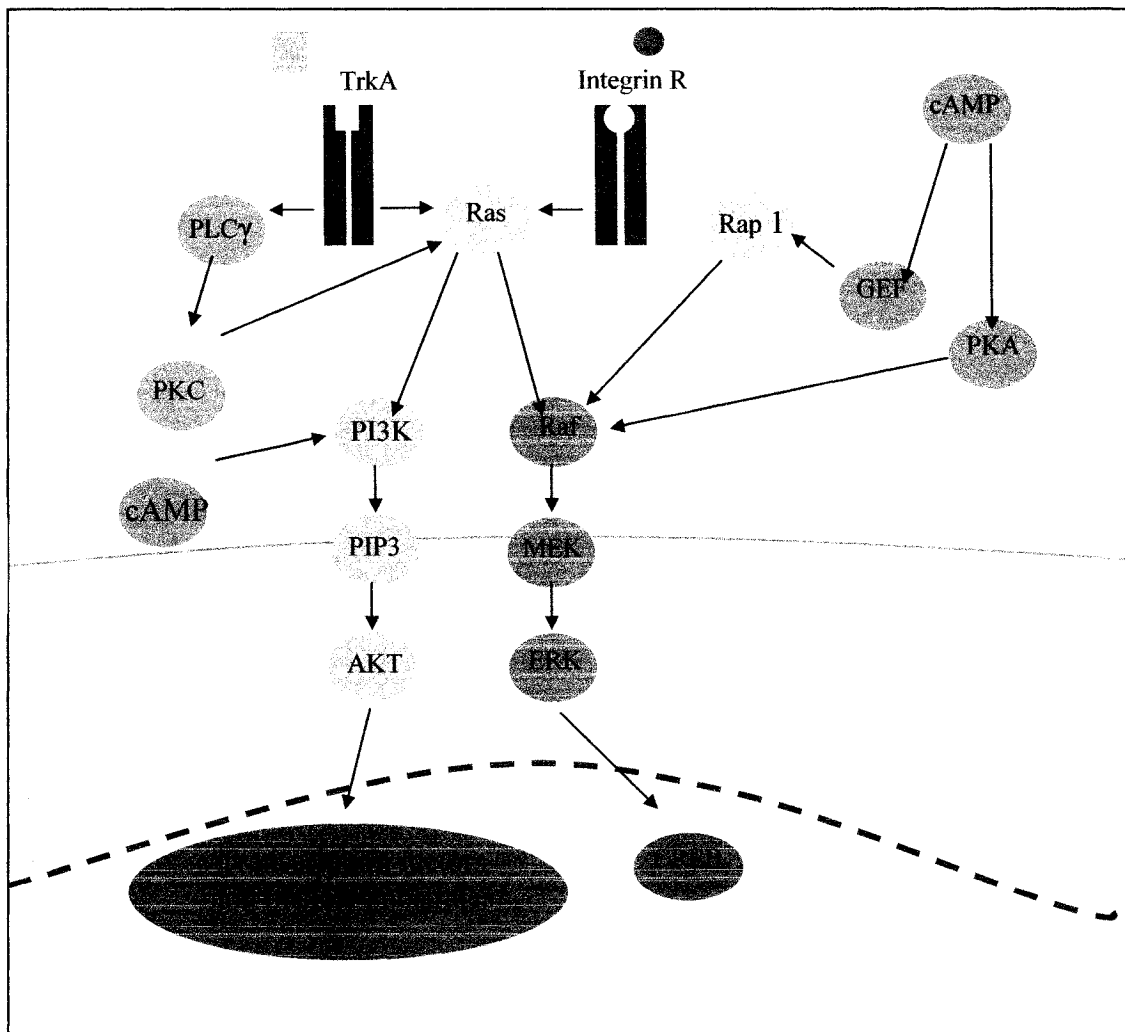


Figure 22. TrkA (NGF receptor), Integrin receptor and cAMP have overlapping signaling pathways

4.2 Extension of ND-Cs in 3D

ND-Cs seeded within a glutaraldehyde-crosslinked collagen-based 3D matrix were able to extend long neurites in optimized media. Glutaraldehyde is a commonly used crosslinker for the fabrication of collagen-based biomaterials because it generates chemically, biologically and thermally stable crosslinks. However, glutaraldehyde is a potentially toxic reagent and has been identified as the cause of poor cell attachment and growth on collagen-based biomaterials (Gough et al. 2002). Indeed, cells have been shown to undergo apoptosis on glutaraldehyde-crosslinked films. We have also found that a higher collagen content resulted in higher levels of apoptosis with poor cell attachment and spreading of remaining cells.

In this investigation, neurite extension from ND-Cs was greatest in the 0.06 % (w/v) collagen gel, which had the lowest concentration of collagen. The observed increase in neurite extension in the 0.06 % gel may not be due to the superior physical properties of the gel but simply due to decreased amounts of cytotoxic glutaraldehyde. Alternative crosslinkers are currently being evaluated. Also, studies have shown that the cytotoxic effects of glutaraldehyde can be reduced by glutamic acid and insulin-like growth factor-1 (Gough et al. 2002).

4.3 Neuron target-cell interaction

4.3.1 NDC-s form contacts with target cells

Differentiated ND-Cs formed contacts with both epithelial cells and myotubes. Connectivity between neurons and target cells was pronounced in the co-cultures. The presence of NGF in the culture media may have enhanced the formation of these

contacts. NGF has been shown to act in conjunction with target-derived factors to increase the number of neurites establishing target contact (Lockhart et al. 2000).

Some of the contacts in the ND-C-myotube co-cultures displayed the morphological characteristics of a synaptic connection. Most striking was the presence of the presynaptic bouton, which is a small swelling that buds from the tip of the neurite contacting the target cell. When the growth cone encounters its target, it stops advancing, spreads out and develops into the presynaptic bouton.

4.3.2 Neuromuscular junction formation between ND-Cs and muscle target

An accumulation of acetylcholine (ACh) receptors was observed at the site of contact between differentiated ND-Cs and myotube targets. ACh receptor clustering is indicative of a neuromuscular junction formation (Anderson et al. 1977). Prior to contact, ACh receptors are distributed diffusely over the entire surface of the myotube. Contact with motor neurons induces ACh receptor migration and clustering in order to facilitate synaptic transmission. Local accumulation of ACh receptors is not necessarily indicative of a functional synapse (Harris, 1971). However, it does prove that there is an interaction between ND-Cs and myotubes. To truly determine the functionality of the synapse electrophysiology studies must be done.

4.3.3 Can the neuron-target interaction be modeled using two clonal cell lines?

An immortalized corneal epithelial cell line, HCEC, was used as the epithelial target for the ND-Cs. HCECs are similar to normal corneal epithelial cells in their structural and functional properties. Therefore, they have been used as a substitute for

primary corneal cells in various research and toxicology applications (McNamara et al. 2005, Huhtala et al. 2002). Recently, HCECs have been combined with ND-7/23 sensory neurons (subcloned from the same parental cell as ND-Cs) to successfully develop an innervated epithelial barrier model (Moore et al. 2005).

The C2C12 myoblast cell line was used as the source of myotubes for the targeting experiments. Although they lack some of the properties of primary myotubes, C2C12s have been shown to form functional neuromuscular junctions with primary neurons (Miles et al. 2004). Other studies have demonstrated functional neuromuscular junctions between a similar myogenic clonal line and a neuronal hybrid cell line (Christian et al 1977).

There is sufficient evidence to validate the use of clonal cell lines in this investigation. Clonal cell lines have been shown to accurately model the interaction between neurons and target cells. Both HCECs and C2C12 are similar to their normal cell counterpart. In addition, they have been used in conjunction with neuronal cell lines to successfully mimic the neuron-target interactions seen in vivo.

4.3.4 Plasticity of ND-C

ND-Cs were able to form contacts with both epithelial and muscle targets, demonstrating the plasticity of the cell line. In addition, ND-Cs cultured with muscle targets were induced to express acetylcholine whereas ND-Cs cultured with epithelial targets did not. These results suggest that target-derived factors are influencing the differentiated phenotype of ND-Cs. Recent studies have shown that acquisition of cellular

identity, including neurotransmitter phenotype, can be mediated through retrograde signals from the target (Hippenmeyer et al. 2004).

ND-Cs in their undifferentiated state display some of the properties of sensory neurons, including the expression of substance P (Wood et al. 1990). It seems that ND-Cs, although not fully differentiated, have advanced along a sensory neuron developmental program. Therefore it is not surprising that ND-Cs are responsive to signals from sensory tissues, such as epithelial cells. However, ND-Cs still retain the plasticity to respond to muscle targets. As mentioned, it has been shown that neurons can switch neurotransmitter and neuropeptide phenotype based on environmental cues. (McMahon et al.1989).

ND-Cs were shown to express acetylcholine. Therefore, the hybridization of neurotransmitter inactive N18TG2 cells with neonatal DRG neurons resulted in the production of a cell line capable of synthesizing acetylcholine. However, it seems that production is only stimulated by the presence of myocyte-derived factors.

4.4 Increased Extension of DRG neurites in optimized media

The optimized media supplements (dexamethasone, DMSO, cAMP and NGF) stimulated and increased neurite extension from the DRG. Whether the supplements were added to KSFM media or standard DRG media, the proportion of long neurites that were generated was greater in the supplemented media compared to unsupplemented controls.

There are two possible explanations for the observed results. First, that the optimized supplements may have stimulated neurite extension directly. NGF and cAMP have both been implicated in growth cone advancement (Toman et al. 2004, Sanchez et

al. 2004). The second explanation is that the optimized supplements may have induced differentiation of neuronal precursors present in the DRG. NGF has been shown to induce neurogenesis in DRG (Namaka et al., 2001), thus affecting the distribution of neurite lengths extending from the DRG. Even with careful dissection, DRG will contain contaminating fibroblasts that extend filopodia that can be mistaken for short neurites. Therefore, if neurogenesis is occurring there will be an increase in the number of longer neurites but the number of shorter neurites remains relatively the same. This distribution of neurite lengths was observed.

The optimized media or the optimized supplements alone can be used in the culture of DRG and most likely other nerve sources to increase neurite extension and possibly promote differentiation.

4.5 Differentiation of Neuroblastoma cell in optimized media

SH-SY5Y neuroblastoma cultured in the optimized media adopted a neuron-like morphology and stained positive with anti-NF-200 antibody. However, neurite extension and NF-200 expression were less than what was observed in ND-Cs. Like most neuroblastoma cell lines, the SH-SY5Y cells are unresponsive to NGF (Matsushima and Bogenmann, 1993). This may account for the less prominent morphological response to the optimized media compared to ND-Cs which are NGF-responsive.

The optimized media and a retinoic acid (RA) supplemented media were compared in their ability to induce neuronal differentiation of the SH-SY5Y cells. The cells cultured in the two different media showed a marked difference in morphology and neurofilament expression. The cells cultured in the optimized media showed considerable

neurite outgrowth and strong neurofilament expression. Cells cultured in the RA media were smaller in size. They formed aggregates with only a few short neurites and showed very weak staining for neurofilament. Based on these results, the optimized media was more effective than the RA media in inducing neuronal characteristics in the SH-SY5Y cells, confirming the efficacy of the formulation developed.

The unsupplemented KSFM also induced the expression of neurofilament in SH-SY5Y but to a lesser degree compared to the optimized media. These results suggest that the KSFM base media itself is contributing to the differentiation effect. The RA media did not seem to induce neuronal differentiation. When the optimized supplements were added to the RA media no noticeable phenotypic change was observed. This is evidence that the optimized supplements on their own cannot induce neuronal characteristics in the SH-SY5Ys. Therefore, KSFM induces a neuron-like phenotype in the SH-SY5Ys and the optimized supplements enhance this effect but cannot produce it on their own.

4.6 ND-Cs as a nerve source for in-vitro models

The results show that ND-Cs can be induced to differentiate into neurons within hydrated 3-D matrices. The differentiation in turn appears to be influenced by the target cells. The plasticity confers ND-Cs cells “progenitor” cell properties and therefore makes them a useful cell source for developing in vitro nerve models.

It should be noted that ND-Cs do not differentiate into nerves alone but also glial cells. As well, a proportion of cells remain undifferentiated. In order to obtain a pure population of neural cells, undifferentiated cells could be removed by treatment with Arabinosylcytosine, a cytotoxic drug that has been shown to destroy all proliferating cells

(Phillips et al. 1975). Glial cells, however, have been shown to be beneficial to neuronal development and should not be removed from the population. Glial-neuron interaction has been shown to regulate neurogenesis and synapse formation (Ma et al. 2005, Mars et al. 2001).

The neurons generated from ND-Cs displayed morphological differentiation, illustrated by the extension of long neurites. The neurons also demonstrated biochemical differentiation. ND-Cs cultured with myotube targets produced the neurotransmitter acetylcholine. The neurons were not assessed for electrical activity. However, studies have shown that subclones derived from the same parental cells as ND-Cs are electrically excitable (Wood et al. 1990).

There are some limitations to using ND-Cs as a nerve source. First, immortalized cell lines often display genetic instability that may cause cells to lose certain characteristics when passaged. The aneuploid nature of the ND-Cs increases this risk of genetic instability. However, in this investigation ND-Cs were passaged up to 30 times without any indication of instability.

Secondly, ND-Cs should only be used as a nerve source in certain experimental systems. As discussed in the introduction, there are many different types of neurons and each type responds differently to guidance cues in the body. Therefore, when attempting to model a biological process such as nerve regeneration or neuron targeting in vitro, it is important to choose a nerve source that has the same properties as the neurons involved in the process in vivo and will accurately mimic their behavior. For example, if an investigator wants to use an in vitro model to test the ability of an artificial cornea construct to promote innervation, he/she must choose a nerve source that will accurately

predict the responses of nociceptive sensory neurons in the eye. Otherwise, the results obtained from the in vitro model will not necessarily be relevant in vivo. ND-Cs co-cultured with myotubes acquire the characteristics of motor neurons upon differentiation. There is speculation that ND-Cs cultured with epithelial targets differentiate into a type of sensory neuron. Thus, ND-Cs have potential for use as a nerve source to model sensory or motor neurons.

4.7 Applications of proposed in vitro model

The ultimate goal is to use this model to; 1) identify molecules regulating neurite outgrowth and axonal guidance; 2) study neuron-target cell interaction; 3) identify mediators of the regeneration process; and 4) test scaffolds designs for peripheral nerve repair. This model may also be used for in vitro toxicology testing. Nerves play a role in the toxic response of the tissue they innervate. Current in vitro toxicology models do not include components aimed at modeling sensory nerve stimulation. As a result the toxicity of many chemicals, such as nicotine, have been under predicted (Ekwall, 1999). The proposed model provides an innervated tissue layer that may more accurately predict the toxicity of chemicals.

5. CONCLUSION AND FUTURE DIRECTIONS

ND-C cells are a viable source of neuronal precursor cells for use in *in vitro* models of neurite extension and nerve-target interaction. They can be efficiently differentiated into neurons when cultured in the optimized neuronal differentiation media that I developed (consisting of KSFM base medium, dexamethsone, DMSO, dibutryl cAMP and NGF), generating long neurites that were able to extend within a 3D collagen matrix. Neurites from ND-Cs formed contacts with both epithelial and muscle target cells (i.e., sensory and motor targets), indicating their plasticity. Connections made between ND-Cs and the latter targets show evidence of a neuromuscular junction formation.

The optimized neuronal differentiation media may also be used to enhance neurite extension and neuronal differentiation in other nerve sources. For, example, Sh-sy5y neuroblastoma cells were induced to differentiate when cultured in the optimized media. Increased neurite extension from DRG was observed in the optimised media compared to other standard differentiation media.

It is anticipated that, with further development, *in vitro* models comprising ND-Cs with epithelial or muscle targets, can be used for neurotoxicity testing as alternatives to animal testing. They may also be used to study neurite extension and targeting and provide useful information for the development of nerve scaffold for peripheral nerve repair.

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APPENDIX

Complete Media Formulations

Media	Ingredients
Keratinocyte Serum Free Media (KSFM)	<ul style="list-style-type: none"> ▪ 0.1-0.2 ng/ml recombinant epidermal growth factor (Invitrogen, ON, Canada) ▪ 20-30 µg/ml of bovine pituitary extract (Invitrogen, ON, Canada) ▪ Inorganic salts ▪ Sugars ▪ Organic salts ▪ Trace elements ▪ Thiamine ▪ B12 ▪ Folic acid ▪ I-inositol ▪ Pyridoxine riboflavin ▪ Biotin ▪ Choline chloride ▪ D-pentothenate ▪ Niacinamide
StemPro-34	<ul style="list-style-type: none"> ▪ Inorganic salts ▪ Sugars ▪ Organic salts ▪ Human serum albumin ▪ Soluble human lipids for serum free media ▪ Ethanolamine ▪ Sodium selenite ▪ Hydrocortisone ▪ D, L-Tocopherol ▪ Iron Saturated Human Transferrin ▪ Human Zinc Insulin ▪ N-acetyl-L-cysteine ▪ 2-Mercaptoethanol ▪ Biotin ▪ Folic Acid ▪ I-Inositol ▪ Niacinamide ▪ Thiamine ▪ B12 ▪ Riboflavin ▪ Phenol red ▪ HEPES

Optimized neuronal differentiation media	<ul style="list-style-type: none">▪ KSFM media▪ 0.1 μM Dexamethasone▪ 1.5 μM DMSO▪ 1.5 μM NGF▪ 20 μM cAMP▪ KSFM media
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