Investigating the Cross Communication Between Motor Neurons and Muscle Using a Novel-Functional Human Neuromuscular Junction Model

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Dissertation submitted in part fulfilment of requirements for the degree of Master of Science in Biomedical Science

Date of Submission: 12/09/2019

Table of Contents

STUDENT DECLARATION: **3**

ABSTRACT **4**

1.0 INTRODUCTION **5**

1.1 Skeletal Muscle and Myogenesis 5
1.2 Motor Neurons 8
1.3 The Neuromuscular Junction 10
1.4 Cross-Communication 12
1.5 Aim and Objectives 15

2.0 MATERIALS AND METHODS **17**

2.1 Expansion of Neural Progenitor Cells 17
2.2 Preparation of Immortalised Human SkMC for Co-culture 17
2.3 Seeding of SkMCs for Culturing 18
2.4 Preparation of NPCs for seeding 20
2.5 Establishing the SkMC:NPC Co-Culture 20
2.6 Cell Count 21
2.7 Immunofluorescence Imaging 21
2.8 Human Growth Factor Array Analysis using a multiplex ELISA 22
2.9 Statistical Analysis 24

3.0 RESULTS **28**

3.1 Quantification/Analysis of Trophic Factors 28
3.2 Factors that Significantly Increased Between NPC only & CC Conditions 29
3.3 Factors that Significantly Increased Between Muscle only & CC Conditions 29
3.4 Factors that Significantly Increased Between NPC only & CC Conditions + Muscle only & CC Conditions 29

4.0 DISCUSSION **37**

4.1 BDNF 38
4.2 GDNF 39
4.3 NT-3 39
4.4 NGFR 40
4.5 bFGF 40
4.6 HGF 41
4.7 FGF-7 42

5.0 CONCLUSION **42**

6.0 REFERENCES **43**

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ABSTRACT

Background: The connection between skeletal muscle (SkM) and motor neurons (MN) is a crucial final-link in the human motor system controlling voluntary muscular movements. As co-dependent tissues that rely on cell-cell interactions and bi-directional communication via molecular-diffusible signals, the formation and maintenance of the neuromuscular junction (NMJ) and its components heavily depends on this cross talk. However, the bulk of information currently known in relation to these trophic factors was obtained through animal motor system models.

Aim: Employing a novel-functional and physiologically pertinent human NMJ model developed by Al-Samid et al., in 2018, this study aims to elucidate the cross-communication within this in-vitro model, which allowed the development of a functional human motor system in the absence of exogenously added trophic factors.

Methods: 40 trophic factors considered important in MN and SkM formation were measured in supernatants collected from MN and SkM monocultures as well as MN-SkM co-cultures, using an ELISA-based human growth factor microarray. The results obtained were subsequently compared between the two conditions.

Results: MN and SkM monocultures showed significant hindrances in their development whereas cells in co-culture conditions prospered, forming functional myotubes stimulated by MNs with typical characteristics of those found in-vivo. Additionally, evaluating the concentration of trophic factors between the two-culture settings revealed a significant increase ($P<0.05$) in 8 fundamental factors, namely BDNF, bFGF, FGF-7, GDNF, HGF, PIGF, VEGF and NT-3, in the nerve-muscle co-culture compared to monoculture conditions.

Conclusion: Collectively, this data revealed that the bi-directional interaction between the two tissues in co-culture induced the production of essential endogenous trophic factors, which established normal nerve, muscle and NMJ formation. The outcomes also stand testament to the suitability of this NMJ system as a powerful platform for studying the role of individual trophic factors involved in human NMJ formation as well as NMJ disorders such as diabetic neuropathy or myopathy

1.0 INTRODUCTION

1.1 Skeletal Muscle and Myogenesis

Skeletal muscle (SkM) is one of three types of muscles forms found in the human body, which accounts for up to 40% of an individuals total body weight (McKeon-Fischer and Freeman, 2011). As a multinucleated-striated muscle type it is found attached to the skeleton and is primarily responsible for converting chemical energy into mechanical energy. This mechanical energy generates the force integral for postural support and movement in vertebrates (Chen, 2017). SkM is mostly voluntary and similar to other muscle types are made up of a group of fascicles (bundle of muscle fibres) consisting of 100 to 1000s bodies of muscle fibres (see Fig 1.1) (Denoth et al., 2002). Also known as myofibres, these fibres are composed of even smaller fibres called myofibrils that contain repeating patterns of dark and light sequences produced by the overlapping of two proteins known as actin and myosin (Arnold et al., 2014). One dark and light banded structure creates the most basic level of construction in muscle called the sarcomere, the smallest contractile unit in a muscle fibre (Scott et al., 2001). The striation seen in SkM is a result of these inter-digitised thick (myosin proteins) and thin (actin proteins) filaments bounded by Z disks (stabilising and regulating protein) to hold them together. Thick filaments suspended in the middle, latch onto thin filaments and are able to move the Z disks closer/further apart during muscle contraction and relaxation allowing the muscle to shorten or lengthen correspondingly (Rayment et al., 1993).

Operating as a protein reservoir and one of the main tissues for glucose storage SkM is the largest metabolically active tissue in the human body (Yin et al. 2013). Playing an important role in governing other vital energy-dependant processes in addition to movement such as respiration, chewing, swallowing, and heat production, it acts as a regulator for glucose homeostasis and protein metabolism throughout the body, therefore balancing the metabolic requirements of all other organs (Argiles, et al, 2016). The equilibrium between the anabolic and catabolic status of human SkM is essential for muscle vitality (Chen, 2017). Any factor that upsets this balance for example age, disease, mechanical or nervous stimuli, hormonal changes and nutrient intake can cause muscle health degeneration (Pedersen and Febbraio, 2008). The deterioration of muscle quantity and quality can then lead to reduced movement

and a gradual decline in strength and power. Ultimately increasing the risk of morbidity, disability, mortality and a reduced quality of life in individuals (Pratesi et al., 2013).

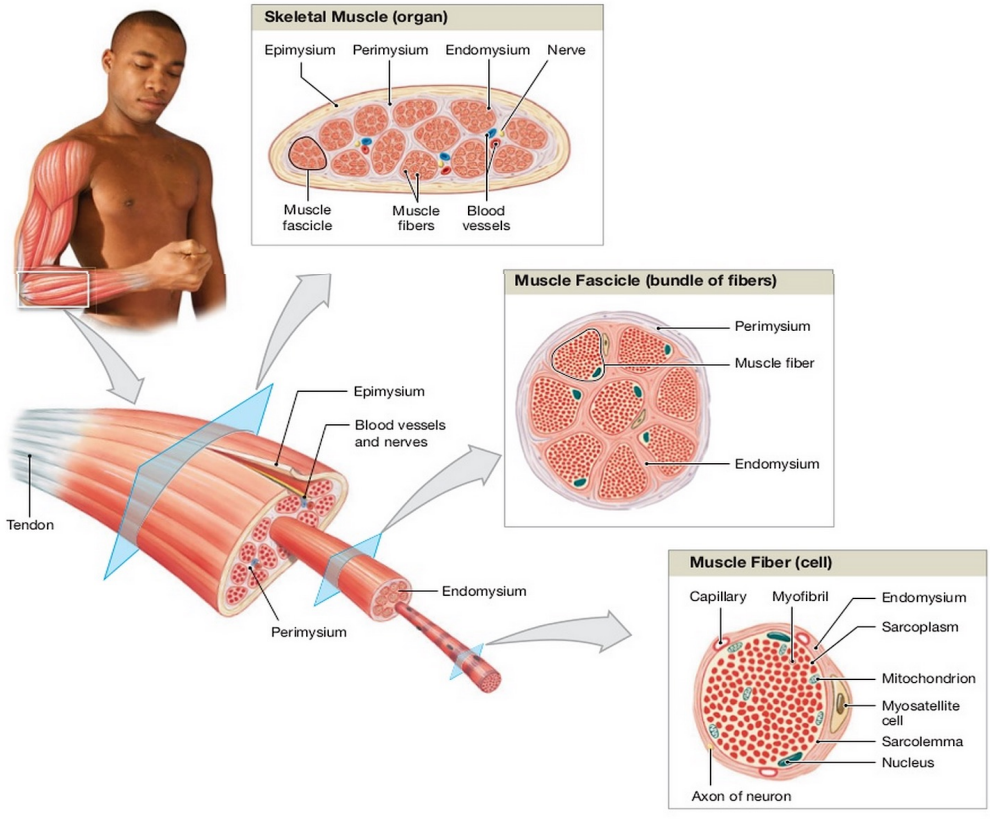


Fig 1.1 The anatomy of human SkM: SkM used primarily for moving bones is striated, voluntary, and is under the control of the somatic nervous system. SkMs are multinucleated elongated structures found attached to the skeleton and have numerous mitochondria in their cells. They are up to 0.3m long and each muscle is made up of a group of fascicles with each fascicle made up of 100-1000s bodies of fibers. Muscle fibres are cells composed of smaller fibres called myofibrils that contain repeating patterns of dark and light bands called striations. The dark bands have a constant length whilst the light bands change length as the muscle lengthens or shortens. Depending on the type of muscles and how often we use them different muscles have different concentrations of blood supply. Surrounding the muscle fibre is a sarcolemma which is the cell membrane for the muscle cells and within that membrane there are structures called the sarcoplasmic reticulum, transverse tubules (t-tubules) and terminal cisternae. The sarcoplasmic reticulum and terminal cisternae are used to store and release calcium. Whilst the t- tubules are responsible for passing on the electrical activity (so when an electrical impulse activates a muscle fibre it is transferred along these t-tubules and they cause the release of calcium) (Martini et al., 2014).

As a self-healing tissue SkM can regenerate through myogenesis, which is defined as the process of SkM formation and development (Wallace et al., 2016). This process, termed adult myogenesis, is what sanctions postnatal growth and repair of injured/damaged muscle. Typically activated following severe SkM damage adult myogenesis is fundamentally the process by which myofibres are maintained and regenerated to provide life-long muscle function (Wagers and Conboy, 2005). Since terminally differentiated myofibers lose their ability to proliferate following embryogenesis, the numbers of myofibers established prenatally are fixed and may reduce with age or disease. Thus SkM maintenance is achieved through undifferentiated myogenically specified progenitors called satellite cells (SCs) that are generated during foetal development (Le Grand and Rudnicki, 2007). These cells lie dormant within the basal lamina and sarcolemma of adult SkM to be triggered when needed for SkM repair, either in response to disease, trauma or injury (Bentizinger et al., 2012). Fig 1.2 illustrates the process by which SCs become activated for SkM repair.

Activation triggers of SCs remain unidentified however the production of intrinsic signals such as sphingosine-1-phosphate and extrinsic triggers such as the mechanical stretch of myofibres have been indicated to provoke the activation of SCs (Relaix and Zammit, 2010). Nonetheless several studies investigating Pax7 knockout mice have shown that SC activation and proliferation are contingent on the expression of the myogenic regulator Pax7 which initiates the successive activation of factors such as MRFs Myf5, MyoD, myogenin, and MRF4, all of which play an important role in the activation, proliferation, and differentiation of SCs from their state of quiescence to mature muscle fibres (Seale et al., 2000; Kaasik et al., 2012 and Wallace et al. 2016). In addition to the above-mentioned stimuli SC activation may also occur in response to specific microenvironment-secreted growth factors, which will be expanded on in a later section (Bentzinger et al., 2019). Upon activation SCs renter the cell cycle and proliferate, once they reach a certain protein synthesis threshold they divide asymmetrically to provide committed proliferative cells that will differentiate into skeletal myoblasts and a self-renewing daughter cell (Forcina et al., 2019). The committed cells ultimately fuse to one another or injured myofibers to restore the area of damage, warranting SkM repair (Wagers and Conboy, 2005).

Both embryonic and adult myogenesis, involve four fundamental stages, (1) the proliferation

of progenitors and their commitment into myoblasts, (2) the differentiation of myoblasts into post-mitotic mononuclear myocytes, (3) the fusion of myocytes to form multinucleated myofibers and lastly (4) the maturation of terminally differentiated myofibres (Bentzinger et al., 2012). This process occurs in two waves giving rise to two forms of myotubes, primary and secondary, depending on the population of pre-programmed myoblast types involved (Scott et al., 2001). Primary myotubes arise from the fusion of primary myoblasts that proliferate in the somites and later migrate to their final destination whereas distinct myoblast that only fuse following innervation or myoblasts that use primary myotubes as a framework and fuse with them, form secondary myotubes (Boncompagni et al., 2007). The majority of adult muscle consists of muscle fibres derived from secondary myotubes. Nonetheless, studies in rat and chick muscle have indicated that in order for complete differentiation to occur innervation is necessary in both myotube forms (Lee et al., 1988).

Relatedly, electrical stimulation stipulated through motor neuron (MN) innervation has also been proven essential in native human SkM in the formation of 'functional' myotubes (Wang and Ramakrishna, 2011). Several electrical stimulation experiments have supported this statement exposing that the lack of neuromotor activity can actually result in muscle atrophy (Cisterna et al., 2014). This is because the generation of large forces during muscle contraction and relaxation, which ultimately results in movement are only conceivable because of the unidirectional alignment of myoblasts, which can only be achieved through the presence of MN innervation during SkM formation (Argiles et al., 2016). Hence why non-terminally differentiated myotubes are observed as multiple myoblasts fused together forming a multinucleated construct however differentiated 'functional' myotubes are viewed as several myoblasts all fused together in the same orientation (McKeon-Fischer and Freeman, 2011).

1.2 Motor Neurons

For SkM to contract and ensue their function they must be activated by signals from alpha cells originating from the spinal cord called MNs (Scott et al., 2001). These cells consist of a soma, axon and special processes called dendrites. Unlike muscle development, growth and regeneration, which takes place throughout vertebrate life, neurogenesis, the process of neuron formation and development, is most active during embryology. Once the organism is

born, especially in higher evolutionary organisms such as humans, differentiated neurons lose their ability to divide and the same ones persist throughout life (Wu et al., 2010). When they are damaged, neurons have a very limited capacity for regeneration. Several studies have shown how the capacity of neuronal regeneration is dependent on their environment. Explaining why neurons of the peripheral nervous system can regenerate but neurons of the central nervous system cannot (Wang and Ramakrishna, 2011).

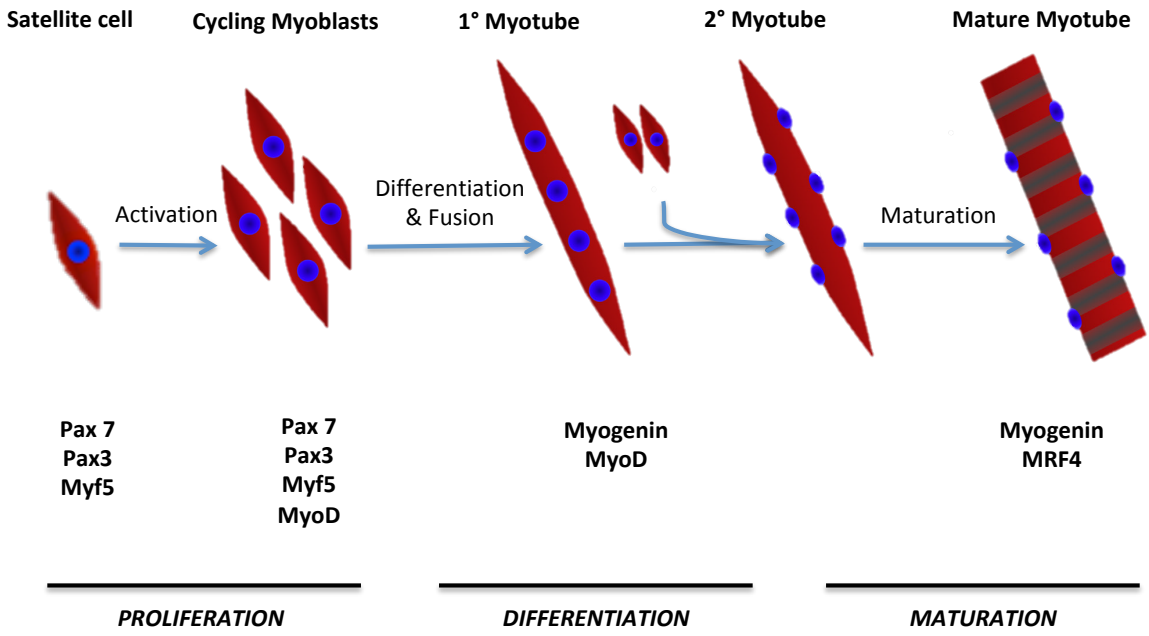


Fig 1.2 The activation of SCs following severe SkM damage/injury: The image illustrates the process by which SCs are activated in response to damaged SkM and hypertrophic stimuli. SCs become activated by specific mechanical and molecular signals leading to the expression of genes such as Pax7, Pax3 and Myf5. SCs that are induced to re-enter the cell cycle then undergo multiple divisions and have the ability to engage in both symmetric and asymmetric divisions, ensuring the maintenance of the SC pool and the production of committed cells to contribute to muscle repair and plasticity. Committed myoblasts undergo differentiation into myocytes that either fuse together to form primary, secondary and then mature myotubes or fuse to existing damaged myotubes for restoration (Adapted from Le Grand and Rudnicki, 2007 and Wallace et al., 2016).

Motor innervation plays an important role in the regulation of many properties of SkM, including neuromuscular activity. Though innervation is not needed for muscle formation, it is essential in the construction of nerve-muscle interactions, intramuscular nerve branching

and neuronal and muscle survival, which requires signals from both nerves and muscles (Witzemann, 2006). This occurs through a specialised interface known as the neuromuscular junction. Any defects to this nerve-muscle interface, to the MN or to the muscle fibre can adversely affect the normal function of SkM. This is due to the fact that the expression of many muscle specific proteins is reliant on the presence of MN innervation through these specialised synapses (Lee et al., 1988). In addition to neuromotor control the nervous system can also exert control over SkM via the production of soluble neurotrophic factors produced from the nerve terminals of MNs at the NMJ (Cisterna et al., 2014).

1.3 The Neuromuscular Junction

Neuromuscular Junctions (NMJs) are the highly specialised peripheral synapses that connect and form the bridge of communication between MNs and SkM, to essentially translate neural signals received from MNs to contractile activity in SkM (Witzemann, 2006). Highly important in the maintenance of normal muscle properties such as muscle size, elasticity, extensibility and excitability they are one of the first synapses to be formed during development and are responsible for the contractility of a large proportion of the body's muscle mass (Grinnel, 1995). NMJs consist of three main components (1) the highly branched terminal processes of the presynaptic MN, (2) the invaginated surface of the otherwise smooth SkM fibre and (3) the synaptic cleft (Wu et al., 2010). The architecture of the synapse also incorporates other specialised cells including modified Schwann cells and fibroblasts, which are known to cap motor nerve terminals and processes during NMJ formation. These specialised cells are thought to play a key role in motor end plate development and maintenance (Lee et al., 1988). The development of NMJs (synaptogenesis) is a multistep process involving coordinated cross communication between muscle and nerve terminals (Tintignac et al., 2015). However, fundamentally there are two basic stages (1) the establishment of nerve-muscle interactions, which commences early in synaptogenesis beginning with the guidance of motor axon cones towards the specific muscles to be innervated and (2) the stabilisation of the nerve-muscle contacts to generate mature NMJ structures (see Fig 1.3)(Bloch-Gallego, 2015).

Early in myogenesis when myoblasts undergo proliferation and segregation into dorsal and ventral muscle groups several MN cones infiltrate myoblasts masses and wait for instructions

from the developing muscle to instigate innervation (Sanes and Litchman, 1999). When myoblast fusion is initiated more than one MN forms contact with the centre of early myotubes to permit the continuation of myotube growth by fusion of additional myoblasts on either ends. During these initial nerve-muscle contacts a cascade of interactions are triggered eventually leading to subsequent regulatory events during postnatal development that results in the elimination of all but one MN terminal to innervate each muscle fibre (Bloch-Gallego, 2015). The MN fibre that is successful in stabilising and concentrating acetylcholine receptors (AChR) (an NMJ protein important in signal transmission) at sites of nerve-muscle contact remains while all other supernumerary nerve terminals are eliminated giving rise to mature and stabilised NMJs that will persist throughout an individuals lifespan (Wu et al., 2010).

This multifaceted journey displays the importance of bidirectional communication between muscle fibers and motor neurons in the development and maintenance of the neuromuscular apparatus as well as muscle and nerve vitality, which consequently, go hand in hand Cisterna et al. 2014). The same has been observed from *in-vitro* research using aneurally cultured SkMC that showed restricted development through the absence of a nerve element which resulted in restricted differentiation as well as non-contractile myotubes (Delaporte et al., 1986). The interdependence of these tissues is further apparent when considering neurodegenerative disorders where there is damage to peripheral nerves or in pathophysiological states where muscle wasting, atrophy and degeneration is observed as a consequence of nerve injury and/or muscle disuse. Failure in one or more of the components that contribute to NMJ structure and function could result in a hindrance in the cross-communication between the tissues involved and consequently lead to disorders such as amyotrophic lateral sclerosis (ALS) and spinal muscular dystrophy (SMD) (Tintignac et al., 2015).

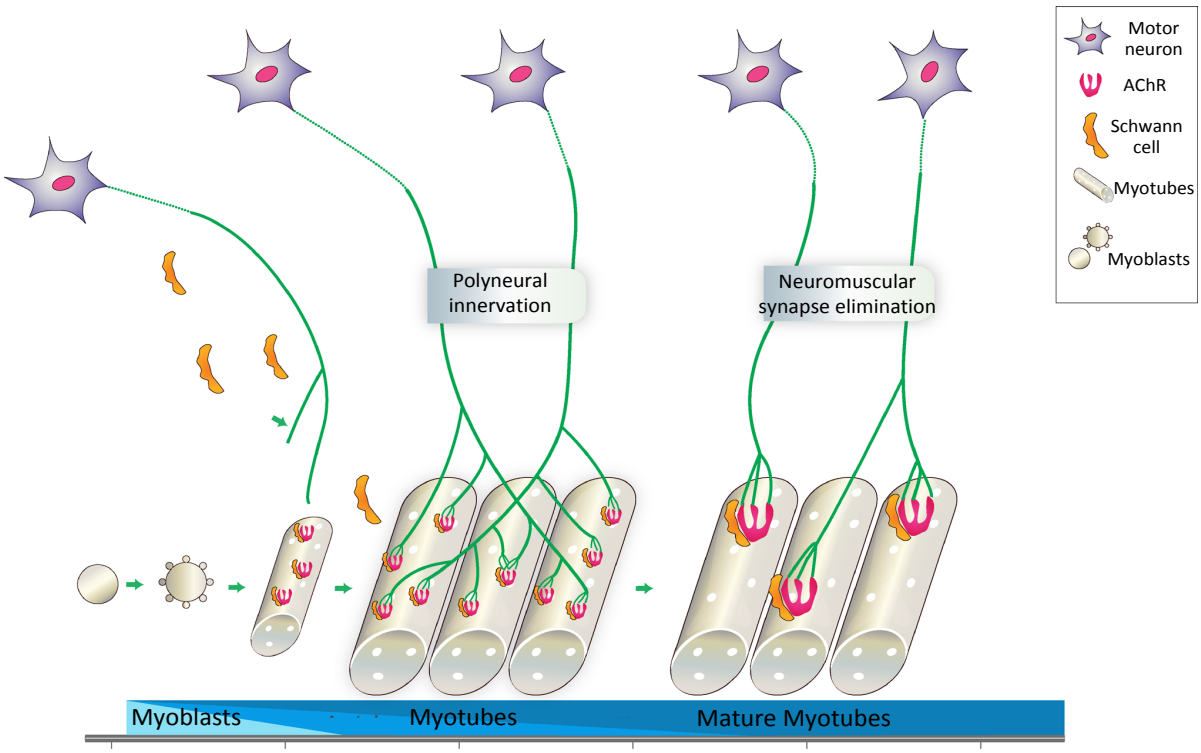


Fig 1.3 NMJ formation and Maturation/Stabilisation: illustrates critical stages of synaptogenesis with emphasis on the mechanism by multiple-innervation is eliminated as development progresses. MN extend axons towards myotubes and make initial contact with muscle cells. Schwan cells simultaneously migrate towards prospective NMJs and cap the terminal processes of MNs. As this is occurring the differentiation of myoblast into myotubes is also progressing in due course as demonstrated in the above figure. Eventually, dispersed ACh receptors found throughout the myotubes surface prior to the establishment of synapses begin to aggregate in the post-synaptic membrane of SkM. This altogether results in the transition from multiple to single innervation of each muscle fibre (Ferraro et al., 2012 and Bloch-Gallego, 2015).

1.4 Cross-Communication

In addition to cell-cell and cell-matrix interactions, NMJ assembly is also mediated by a series of molecular-diffusible signals that induce, regulate and support myogenesis, normal nerve state and nerve-muscle interactions (Wang and Ramakrishna, 2011). The connection between MN and SkM is the crucial final link in the human motor system controlling voluntary muscular movements (Barker et al., 1985). As co-dependent tissues, which rely on each other for trophic support and synaptic transmission/stimulation demonstrates that cross-communication

between these cells is essential for their development and formation. As a consequence, muscle denervation and reinnervation can dramatically alter muscle physiology and can cause muscle wasting (Cisterna et al., 2014). Conversely, there is also growing evidence that muscle-dependent trophic, cell adhesion, and axon-guidance signals play an essential role in the formation and maintenance of the neuromuscular junction and human motor system (Steinbeck et al., 2016).

In native NMJ development several factors hypothesized to be produced by MNs, SkM and other cell types (such as glial cells and fibroblasts) are involved in activities related to nerve, muscle and NMJ development and regeneration (Lee et al., 1998). This has been highlighted in several *in-vitro* and *in-vivo* experiments, where a large number of trophic factors are acknowledged for their vital functions in the development, plasticity, function, survival and death of neurons and myoblasts have been implicated (Oppenheim, 1991; Reichardt, 2006 and Syverud et al., 2016).

Amongst a few of the factors identified are the neurotrophic factors, a family of polypeptides required for the survival and differentiation of specific neuronal populations and acknowledged for their role in nerve regeneration (Sakuma and Yamaguchi, 2011). In 1982 the trophic factor brain-derived neurotrophic factor (BDNF) was shown to play a crucial role in promoting neuronal growth, survival and maintenance (Loeb et al., 2002). Since then recent studies have found its increase in human SkM following exercise and its reduction in subjects with Alzheimer's, major depressive disorders, coronary syndromes and type-2 diabetes mellitus, signifying its importance in maintaining normal muscle and nerve condition (Sakuma and Yamaguchi, 2011). Years later other neurotrophic factors called neurotrophins (NT) (i.e. NT-3/4/5) were described, each sustaining individual trophic effects on neurons of the peripheral and central nervous system (Pratesi et al., 2013). However recent evidence has suggested NT roles in a wider range of tissue systems including SkM (Giudice and Taylor, 2017). While SkM has been observed to provide neurotrophic support through the release of myokines it also expresses several neurotrophin receptors proposing a basis for neurotrophic signalling within muscle also (Sakuma and Yamaguchi, 2011). Studies conducted on rat gastrocnemius muscles propose a role for NT-3 in axonal rejuvenation that stimulates the differentiation of muscle fibres to prevent muscle degeneration (Hartmann et al., 2001). While

studies investigating neurotrophin k/o mice have suggested roles in muscle spindle formation for NT-3, muscle fibre transformation for NT-4/5 and dystrophic muscle pathology for nerve growth factor (NGF) (Ernfors et al., 1994 and Mantilla et al., 2008). Furthermore, glial-cell-line-derived neurotrophic factor (GDNF), another essential neurotrophin first discovered in glial cells and originally recognised for its function in inducing MN sprouting and muscle fibre innervation it is also assumed to be involved in maintaining normal MN structure and morphology (Lin et al., 1993). Similar to BDNF, GDNF expression has been found in a variety of tissues outside of the CNS such as SkM and Schwann cells (English, 2003). As a protective mechanism it has been seen to increase considerably in ageing muscle tissue operating to maintain cell body size and the cholinergic phenotype of MNs (Ulfhake et al., 2000 and Delezie and Handschin, 2018). Earlier studies have also denoted GDNFs role in regulating the transition from multiple innervation to single innervation of muscle fibres during synaptogenesis, which as mentioned earlier is a crucial step in NMJ maturation (Pratesi et al., 2013).

Other trophic factors indicated include members of the FGF, TGF-beta, IGF and HGF families all of which are known to maintain and regulate the growth and differentiation of SCs in muscle regeneration as well as MN survival and maintenance (Charge and Rudnicki, 2003). Of the FGF family the basic fibroblast growth factor (bFGF) and the heparin-binding growth factors (HBGF) are vital in the aggregation of AChR at sites of nerve-muscle contact (Ferraro et al., 2012). AChR aggregation in the postsynaptic muscle membrane being a central driving element in NMJ maturation and the first observable marker in NMJ formation expresses just how crucial these factors are to the development of functional myofibres and neuromuscular synapses (Kablar and Belliveau, 2005 and Pratesi et al., 2013). Evidence in chicken embryos where limb ablation resulted in the death of spinal MN also validates the need of trophic support for MN development from their target muscle (Kablar and Belliveau, 2005). Evidently the formation and maintenance of the NMJ relies heavily on the bidirectional communication elicited via these molecular diffusible signals (Fox et al. 2007). Damage to either one of these components can hinder the cross talk and result in adverse affects leading to diseases such as diabetic neuropathy and/or myopathy. Thus an understanding of the roles and mechanisms of these trophic factors in the human motor system can provide indispensible insights into disorders where such cross-communication is disabled (Chevrel et al., 2006). However, the

bulk of information currently known in relation to these trophic factors and cross communication within the motor system were obtained through animal models or models in which a concoction of up to 15 different growth/neural factors were supplemented to modulate and potentiate the development and function of the NMJ system (Belluardo et al., 2001; Chevrel et al., 2006; Das et al., 2010 and Garcia et al., 2010). Little is also known of their situation in-vivo and the relationship between MN, myofibres, trophic factors and their trophic actions on either of the tissues (Pratesi et al., 2013).

1.5 Aim and Objectives

Thus employing a novel-functional and physiologically pertinent human NMJ model, which was developed free of any exogenously added trophic factors by Al-Samid et al., in 2018, this study aims to elucidate the cross-communication i.e. the trophic factors secreted endogenously in this *in-vitro* model to allow the development of a functional human motor system in the absence of external trophic factors. The co-culture model to be utilized for the purpose of this study was engineered without the use of serum or the addition of external trophic factors as required by previously assembled nerve-muscle co-culture models. Implying that the nerve and muscle cells of the co-culture must endogenously produce and release all of the necessary factors needed to stimulate the propagation of MN axons and the formation of NMJs to yield operational myotubes. To clarify this simplified co-culture system's ability to generate robust NMJs without the addition of exogenous serum, growth and neurotrophic factors, ELISA-based microarray experiments were performed to investigate the concentration of endogenously secreted trophic factors in the *in-vitro* motor system. Additionally from comparing three culture situations the study will also explore the origin of these factors, whether they originate from nerve or muscle tissue and what impact they have on the development of these tissues.

The objective of the study was to determine the concentration of growth and neurotrophic factors endogenously occurring in the co-culture system. The aim is to:

1. Establish the co-culture model.
2. Culture myoblasts aneurally.
3. Culture neurons independently

4. Collect, analyse, and compare the supernatant of SkMCs co-cultured with NPCs against aneurally cultured SkMCs and solitary cultured NPCs using an ELISA-based human growth factor microarray.

2.0 MATERIALS AND METHODS

To investigate the cross-communication that occurs between motor neurons and skeletal muscle in the development and differentiation of functional NMJs and contractile myotubes in humans, the functional human motor unit platform established by Al-Samid et al., 2018 was reconstructed and employed for analysis. The human NMJ platform entailed cells acquired from a non-commercial human immortalised myoblast cell line (C25) and the Shef3 human embryonic stem cell (hESCs) line. The C25 cell line was generated at the institute of myology and established using primary myoblasts obtained from biopsies of the semi-tendinosus muscle from a 25-year-old male (acquired anonymously from Myobank, a tissue bank associated to EuroBank which is authorised by the French Ministry of Research). Whereas the hESC line was obtained from the UK StemCell Bank under the project SCSC10-48. Note the Shef3-hESC derived neural progenitor cells (NPCs) to be utilised were established previously following the protocol described in Al Samid et al., 2018.

2.1 Expansion of Neural Progenitor Cells

A 1ml cryovial containing $0.5x10^6$ green fluorescent protein (GFP) transfected NPCs suspended in 90% fetal bovine serum (FBS) and 10% (v/v) dimethyl sulfoxide (DMSO) was thawed and transferred onto a laminin coated T25 flask (prepared 72 hours earlier) and incubated at 37°C in a 5% CO_2 environment. Prior to the transfer, NPCs were integrated with 4ml neural expansion media (NEM) to initiate NPC expansion (Table 2.1). The NPC medium was renewed every 2 days for 6 days with 5ml NEM until a cell confluence of 80% was attained.

2.2 Preparation of Immortalised Human SkMC for Co-culture

On day 2 of NPC expansion a 1ml cryovial, containing a suspension of $1x10^6$ immortalised human skeletal myoblast cells (SkMC) (at passage 4) was rapidly defrosted. Similar to the NPCs the SkMCs were also immersed in 90% FBS and 10% DMSO when retrieved from liquid nitrogen. The thawed cells were then transferred into a 15ml conical tube, and re-suspended in 9ml of complete growth media (GM) (Table 2.2) to induce SkMC proliferation. The resultant 10ml suspension of SkMCs was homogenised and subsequently transferred into a T75 flask and incubated. To encourage SkMC proliferation the flask was sequentially placed on a leveling plate until they reached a cell confluence of 80%. An Axiovert 40C inverted

microscope was employed to evaluate the SkMC confluence every 24 hours until the desired confluence was attained. Cell confluence was assessed by the percentage of area covered by cells in any random microscopic field. This method of evaluating cell confluence was also exercised for NPCs.

2.3 Seeding of SkMCs for Culturing

Once SkMCs reached the required confluence the expended GM was aspirated and the cells washed twice, using 5ml of 1X Dulbecco's Phosphate Buffered Saline (DPBS) from Lonza (Nottingham, UK). DBPS was then removed from the cells, which were subsequently treated with 5ml recombinant cell-dissociation 1X TrypLE^{TM} express enzyme sourced at Gibco (Loughborough, UK). TrypLE^{TM} acts to disassociate the monolayer of cells from the flask and separate them from each other. To permit TrypLE^{TM} action the flask was immediately incubated for 2-5 minutes, checking the flask every 2 minutes to avoid overtreatment and the lysing of cells. After the cells had separated, 5ml of GM was added to the mixture to inhibit further TrypLE^{TM} action whilst simultaneously using high pressure pipetting to detach any cells that may have remained adherent to the flask. The dissociated cell suspension was successively centrifuged for 5 minutes at 300xg. Following centrifugation the supernatant was removed, leaving behind the desired SkMC pellet undisturbed. The pellet was sequentially re-suspended in 5ml fresh GM before aliquoting the cells for cell count. This step is necessary to determine the volume of the cell suspension required in obtaining the desired cell intensity for seeding; the method by which it was done is explained in a later section. The volume of cells taken was then diluted to the required concentration of $1.5x10^5$ cells/ml using GM and the SkMCs were plated.

For the establishment of the SkMC + NPC co-culture a ratio of 6:1 SkMCs to NPCs was predetermined as the ideal proportion for the development of a functional model by Al-Samid et al. in 2018 and thus recreated for this study. For a 6:1 ratio of SkMCs:NPCs 2ml of $1.5x10^5$ cell/ml of SkMC preparation was subsequently seeded onto 4 dishes of 0.5% gelatin coated 6-well plate. The plate was pre-coated in advance with gelatin and incubated for one hour, removing any excess gelatin prior to seeding of cells. As a result the concentration of SkMC in each well equaled $3x10^5$ cells. Similar to the NPCs the SkMC 6-well plate was re-incubated at

the aforementioned conditions for 24 hours prior to the addition of NPCs. The two remaining empty dishes were later to be plated with NPCs only.

Table 2.1: Neural Expansion Medium (NEM)

NEM Components	Volume/Concentration
Dulbecco's Modified Eagles Media (DMEM)- F12 (1:1) from Lonza (Nottingham, UK)	48.5 ml
MEM Non-essential amino acids (NEAA) (Life Technologies, UK)	0.5ml (1x)
Penicillin/Streptomycin (Sigma, UK) (100x)	0.5ml (1x)
bFGF (R&D systems, UK) (100ug/ml)	10uL (20ng/ml)
N2 supplement (Life Technologies, UK) (100x)	0.5 ml (1x)
Heparin (Sigma, UK) (2mg/ml)	50ul (2ug/ml)
B27 supplement (Life Technologies, UK) (50x)	1 ml (1x)

Table 2.2: Growth Media for SkMC Proliferation

Growth Media (GM) Components	Volume/Concentration
Dulbecco's Modified Eagles Media (DMEM) from Lonza (Nottingham, UK)	60% (v/v)
Medium 199 with Earle's BSS from Lonza (Nottingham, UK)	20% (v/v)
Heat inactivated fetal bovine serum (FBS) from Gibo (Loughborough, UK)	20% (v/v)
L-glutamine from Lonza (Nottingham, UK)	1% (v/v)
Fetuin from fetal bovine serum from Sigma-Aldrich (Dorset, UK)	25ug/ml
Recombinant human fibroblast growth factor-basic (FGFb) from Gibco (Loughborough, UK)	0.5ng/ml
Recombinant human epidermal growth factor (EGF) from Gibco (Loughborough, UK)	5ng/ml
Recombinant human hepatocyte growth factor (HGF) from Sino Biological Inc. (Suffolk, UK)	2.5ng/ml
Recombinant human insulin from Sigma-Aldrich (Dorset, UK)	5ug/ml
Dexamethasonne from Sigma-Aldrich (Dorset, UK)	0.2ug/ml
Penicillin/Streptomycin (Sigma, UK)	1% (v/v)
Plasmocin (InvivoGen, UK)	1:1000 (50ul)

Table 2.3: Differentiation Media

Differentiation media components	Volume/Concentration
Dulbecco's Modified Eagles Media (DMEM) from Lonza (Nottingham, UK)	500ml
L-glutamine from Lonza (Nottingham, UK)	1% (v/v)
Recombinant human insulin from Sigma-Aldrich (Dorset, UK)	10ug/ml
Penicillin/Streptomycin (Sigma, UK)	1% (v/v)
Plasmocin (InvivoGen, UK)	1:1000 (50ul)

2.4 Preparation of NPCs for seeding

Prior to the addition of NPCs the following procedure was implemented to prime NPCs for seeding. To begin with expended NPC media was aspirated from the T25 flask and the cells washed twice with 5ml DPBS. Following the removal of DPBS, cells were trypsinised and incubated for 2-5 minutes, once again observing the cells every 2 minutes. The detached cells were then supplemented with 6ml of GM, emulsified and transferred into a 5ml conical tube to be centrifuged (5 minutes at 300 x g). To obtain pure NPCs the supernatant was disposed of and the pellet was initially re-suspended in 1ml differentiation media (DM) (Table 2.3) mixed then consecutively topped up with 3ml DM. The NPCs were counted and seeded onto the 6-well plate as described below.

2.5 Establishing the SkMC:NPC Co-Culture

Following 24 hours of incubation with GM, the SkMCs were ready for co-culturing. Similarly ensuing the 6-day culturing period the NPCs were also ready for plating. GM was removed from the SkMC containing 6-well plate and washed twice with DPBS. NPCs at a concentration of $0.25x10^5$ cell/ml were added into two of the empty wells and two of the previously SkMC implanted wells to establish the previously cited ratio of 6:1 SkMC:NPC co-culture. Leaving two aneural SkMC dishes, which were supplemented with 2ml of DM following the removal of expended GM the cells were submersed in. Two identical plates were made permitting the execution of 4 simultaneous repeats of each experimental condition.

The growth and development of cultures in each of the wells was monitored for 7 days using the live cell imaging system, Leica CTR6000 equipped with a Leica DMI6000 B inverted microscope (Leica Microsystems, Milton Keynes, UK). Comparing both aneurally cultured and

co-cultured myotubes through visual assessment of phenotypic differentiation parameters i.e. alignment, elongation and fusion. Visualisation of GFP stained NPCs was achieved with fluorescence microscopy whilst visualisation of SkMCs was accomplished via phase contrast microscopy. ImageJ software was utilised to measure and compare the differentiation parameters. The plates were monitored every two days to ensure the correct establishment of the human NMJ from which the supernatants to be analysed were to be collected. Note the day of NPC addition to the 6-well plates was considered day 0 therefore the plates were cultured for 8 days in total.

Co-cultures and monocultures were maintained by changing half (1ml) of the DM every 2 days to ensure cells were supplemented adequately. Live cells i.e. differentiation of myocytes into immature myotubes and sprouting of NPCs in the formation of motor neurons were evaluated using the inverted microscope mentioned earlier.

2.6 Cell Count

Cell count of total viable cells in the SkMC and NPC cultures were quantified using established cell count protocols (Phelan and Lawler, 2001). The cells were counted by mixing a 1:1 ratio of cell suspension (50 µL) with Trypan Blue (50 µL), which labels non-viable cells blue, while viable cells remained transparent with a white nimbus around the membrane. The viable cells were counted using established techniques with a haemocytometer viewed under an A-Plan 10x / .25 Ph1 objective on the Axiovert 40C inverted microscope. The number of viable cells/mL of cell suspension was determined using the formula: [(Average number of live cells in eight large corner square) x (dilution factor) x (10^4)].

2.7 Immunofluorescence Imaging

The cells thawed were prepared for immunofluorescent microscopy prior to this study adhering to the following protocol. Firstly, DM was aspirated, and cells were washed twice in DPBS. Cells were then fixed in 4% paraformaldehyde and incubated at room temperature for 10 minutes. The paraformaldehyde was then removed, and the cells washed two times with DPBS. Next, the cells were permeabilized in 10% perm/wash buffer from BD Biosciences (Oxford, UK) and 10% goat serum diluted in distilled water, which was incubated at room

temperature for 1 hour. The solution was aspirated, and cells were washed twice. A blocking solution of 3% goat serum and 0.05% of Tween 20 all from Sigma Aldrich (Dorset, UK) diluted in DPBS was added to the wells. After incubation at room temperature for one hour, the solution was removed, and cells were once again washed twice. Subsequently, the following stains were added: Diamidino-2-phenylindole dihydrochloride (DAPI) from Sigma-Aldrich (Dorset, UK) at a concentration of 1:10,000 to stain nuclear material and Texas Red-X Phalloidin from Invitrogen (Paisley, UK) to stain actin filaments at a concentration of 1:200.

2.8 Human Growth Factor Array Analysis using a multiplex ELISA

Parallel evaluation of the NPC only, SkMC only and NPC + SkMC supernatants were performed to compare the concentrations of 40 human growth and neurotrophic factors (Table 2.5) believed to be involved in the formation and maintenance of NMJs and contractile myotubes. Both monocultures and co-cultures were prepared, seeded, and cultured for 7 days as described above. On day 7 supernatant samples were collected from all three conditions for assessment using the RayBiotech human growth factor array kit (QAH-GF-1). Day 7 is when spontaneous myotube contractions were first observed contracting in unison as a motor unit hence why supernatants were specifically collected on this day. Preparation of all standards and reagents in the array kit were completed using manufacturer guidelines.

Prior to the supernatants being subjected to a multiplex sandwich ELISA they all underwent standardisation to ensure the total protein content in all samples were equal. Thus any difference detected in the concentration of growth factors between the samples would be due to the environment of the supernatant prior to collection rather than the difference in total protein content. This was accomplished using the nanodrop 2000 (Thermofisher) spectrophotometer. To normalise protein concentrations the following steps were conducted. Initially the supernatant samples were centrifuged to separate the cells and obtain volumes of pure supernatants. A known concentration of BSA (2ug/ml) was then measured to standardise the spectrophotometer and ensure its acceptable performance. The concentration of all samples were consecutively measured followed by the measurement of the DM. Overly concentrated samples were diluted to 1.93ug/ml (the concentration of the DM) using DM and ready to be subjected the multiplex sandwich Elisa.

Initially cytokine standard dilutions (Std1 – Std7) were prepared. Subsequently, 100µl of sample diluents from the array kit was added to each well and incubated at room temperature (RT) for 30 minutes to block slides. The buffer was then decanted and 100µl of standard cytokines, controls and supernatant samples were added to the individually assigned wells. The slides were sealed and incubated overnight at 4°C on a microplate shaker set to 500 rpm. The following day samples were decanted from the wells and washed 5 times over 5 minutes for each wash with 150 µl of 1X wash buffer 1, then washed 2 times over 5 minutes each time with 150 µl of 1X wash buffer 2. In each washing step gentle swaying of the slide was performed to ensure suitable washing and the complete removal of wash buffer as per the manufactures instructions. Next, 80µl of detection antibody cocktail was added to each well and the slides incubated for 1-2.5 hours at RT. The detection antibody cocktail was decanted from the wells and any residual fluid aspirated. The wash steps with wash buffer 1 and wash buffer 2 were then repeated once again. After washing the slides, 80 µl of Cy3 equivalent dye-conjugated streptavidin was added to each well then sealed with aluminium and incubated in a dark room for 1 hour at RT. Following incubation, samples were decanted and the wash steps were repeated once more. The slides were then separated from the well gaskets and placed into the provided slide washer/dryer tube. The tube was filled with 30 mL of 1X wash buffer 1, enough to completely cover the slides and shaken gently for 15 minutes. The 1X wash buffer 1 was removed from the tube, which was washed again with 30 mL of 1X wash buffer 2 in the same manner used previously and gently shaken for 5 minutes. The slides were removed from the washer/dryer tube and dried with compressed N2 air to remove residual buffer solution.

Finally, the array was visualised with a GenePix 4000B laser scanner at 532nm wavelength. Raw data from the visualised array images was generated and processed with the GenePix Pro 4.1 Microarray Acquisition & Analysis Software, further statistical analysis of the data was performed with the RayBiotech Q-Analyzer® tool Software for QAH-GF-1. Figure 2.1 and 2.2 summarises the procedures followed as part of this study.

2.9 Statistical Analysis

The comparison of growth factor/cytokine concentrations in the monocultures and co-culture samples was performed using GraphPad Prism 8.0 statistical software (GraphPad Software, Inc., San Diego, CA). To determine the most suitable statistical tests to perform, the distribution of data was assessed using the Shapiro-Wilk test for normality alongside visual inspection of the associated histogram. A probability value of $P < 0.05$ indicates that the distribution of data does not deviate significantly from a normal distribution and as a consequence could be analysed using parametric tests. Contingent on the results from the Shapiro-Wilk normality test the differences between monoculture and co-culture results was analysed using the One-Way ANOVA. The data was also subjected to Tukey's multiple comparison tests to determine individual differences between each set of data and establish which of sets were actually significantly distinctive. Mean ± SD was also presented for each growth factor concentration along with the fold change for each group comparison.

Table 2.4: Descriptions of the human growth factors and neurotrophins quantified in NPC only, SkMC only and co-cultured myotube supernatants.

Target	Description
AR	Amphiregulin (AR)
BDNF	Brain-derived neurotrophic factor (BDNF) (Abrineurin)
bFGF	Basic fibroblast growth factor 2 (bFGF)
BMP-4	Bone morphogenetic protein 4 (BMP-4)
BMP-5	Bone morphogenetic protein 5 (BMP-5)
BMP-7	Bone morphogenetic protein 7 (BMP-7)
b-NGF	Beta-nerve growth factor (Beta-NGF)
EGF	Epidermal growth factor (EGF)
EGF R	Epidermal growth factor receptor
EG-VEGF	Prokineticin-1 (Endocrine-gland-derived vascular endothelial growth factor) (EG-VEGF)
FGF-4	Fibroblast growth factor 4 (FGF-4)
FGF-7	Fibroblast growth factor 7 (FGF-7)
GDF-15	Growth/differentiation factor 15 (GDF-15)
GDNF	Glial cell line-derived neurotrophic factor (hGDNF)
GH	Somatotropin (Growth hormone) (GH)
HB-EGF	Heparin-binding EGF-like growth factor (HB-EGF)
HGF	Hepatocyte growth factor
IGFBP-1	Insulin-like growth factor-binding protein 1 (IBP-1) (IGF-binding protein 1) (IGFBP-1)
IGFBP-2	Insulin-like growth factor-binding protein 2 (IBP-2) (IGF-binding protein 2) (IGFBP-2)
IGFBP-3	Insulin-like growth factor-binding protein 3 (IBP-3) (IGF-binding protein 3) (IGFBP-3)
IGFBP-4	Insulin-like growth factor-binding protein 4 (IBP-4) (IGF-binding protein 4) (IGFBP-4)
IGFBP-6	Insulin-like growth factor-binding protein 6 (IBP-6) (IGF-binding protein 6) (IGFBP-6)
IGF-1	Insulin-like growth factor I (IGF-I) (Mechano growth factor) (MGF) (Somatomedin-C)
Insulin	Insulin [Cleaved into: Insulin B chain; Insulin A chain]
MCSF R	Macrophage colony-stimulating factor 1 receptor (M-CSF-R)
NGF R	Low-affinity nerve growth factor receptor (NGF receptor)
NT-3	Neurotrophin-3 (NT-3) (HDNF) (Nerve growth factor 2) (NGF-2) (Neurotrophic factor)
NT-4	Neurotrophin-4 (NT-4) (Neurotrophin-5) (NT-5) (Neutrophic factor 4)
OPG	Tumor necrosis factor receptor superfamily member 11B
PDGF-AA	Platelet-derived growth factor subunit A (PDGF subunit A)
PlGF	Placenta growth factor (PlGF)
SCF	Stem cell factor (SCF)
SCF R	Mast/stem cell growth factor receptor Kit (SCFR)
TGFa	Protransforming growth factor alpha
TGFb1	Transforming growth factor beta-1 (TGF-beta-1)
TGFb3	Transforming growth factor beta-3 (TGF-beta-3)
VEGF	Vascular endothelial growth factor A (VEGF-A) (Vascular permeability factor) (VPF)
VEGF R2	Vascular endothelial growth factor receptor 2 (VEGFR-2)
VEGF R3	Vascular endothelial growth factor receptor 3 (VEGFR-3)
VEGF-D	Vascular endothelial growth factor D (VEGF-D)

Fig 2.1: Summary of Cell Culture Methods

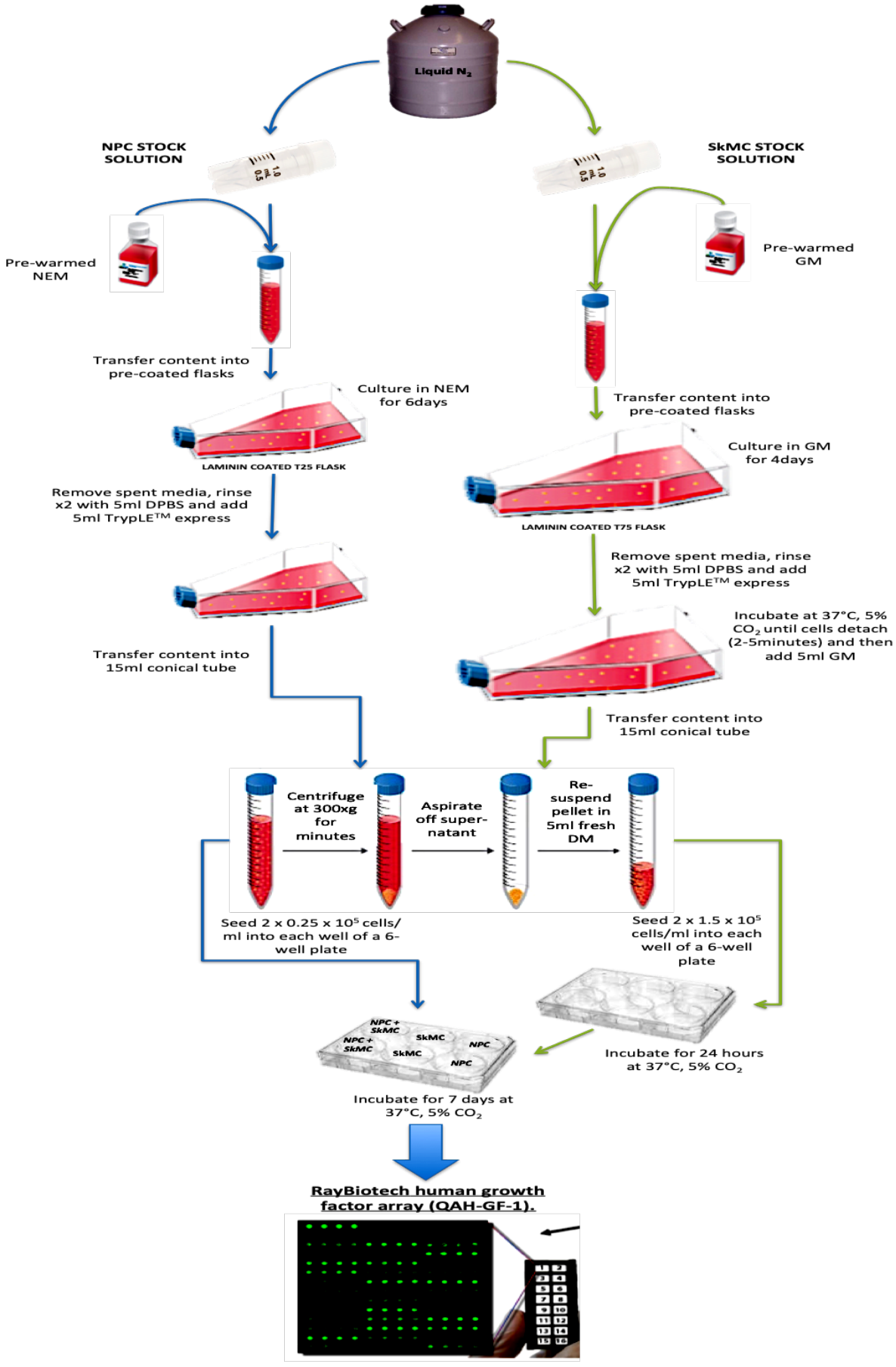
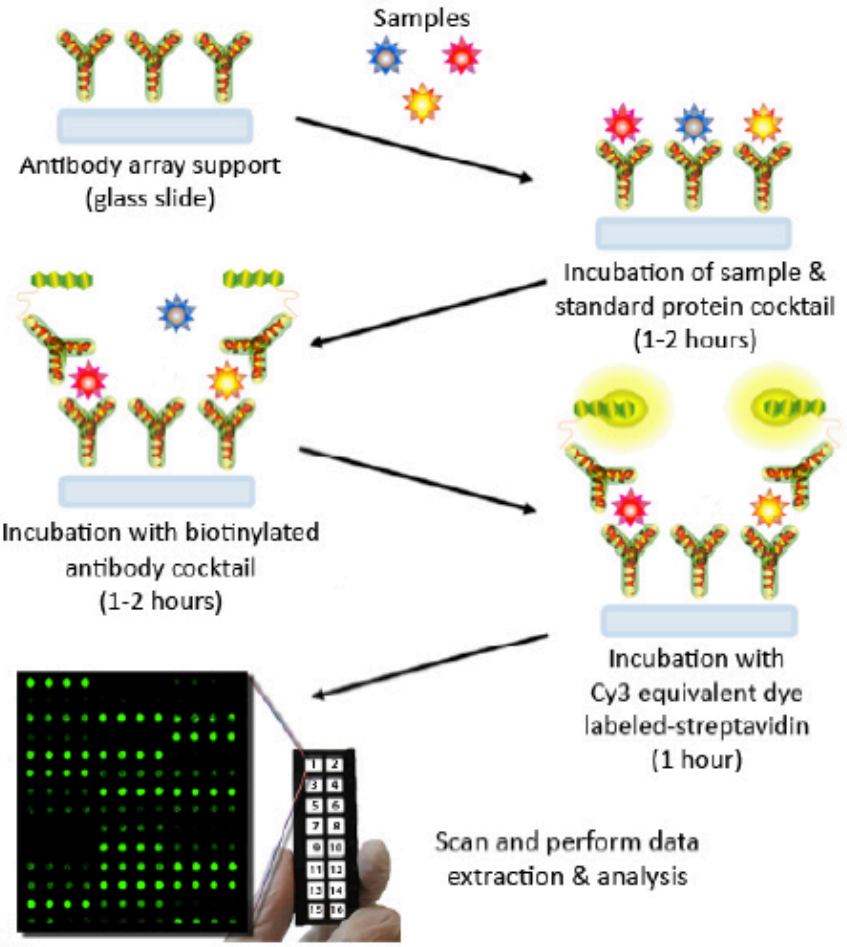


Fig 2.2: Summary of Human Growth Factor Array Analysis using a multiplex ELISA



3.0 RESULTS

The differentiation and development of myoblasts into myotubes and of NPCs into MNs in the presence and absence of one another were evaluated by assessing the morphologies of the three culture conditions over the 7 day-culturing period. Clear morphological variances between the *NPC only, Muscle only* and *Muscle + NPC* cultures were observed. These variances are illustrated in fig 3.1a-c, which demonstrates cells on day 7 of the culturing period. At 7 days NPC only cultures showed no growth or development, MN sprouting was absent and the majority of NPCs atrophied. Whereas in Muscle only cultures the myoblasts grew in size and fusion of myoblasts into primary myotubes was observed however their differentiation into functional myotubes, was hindered and not apparent. On the other hand co-cultured Muscle and NPCs prospered. Initially at 24 hours, myoblasts fusion was absent in the co-culture and the cells displayed similar characteristics to the myoblast only monoculture, indicating that cells were still in the initial stage of differentiation. Nevertheless by day 7 myoblasts had fused and NPCs sprouted neurites expanding over the myoblasts and eventually differentiated into motor neurons that formed neuronal connections with myotubes via axon terminals resulting in functional multinucleated myotubes. The first spontaneous contractions of individual myotubes in the co-culture were observed as early as day 6.

3.1 Quantification/Analysis of Trophic Factors

To analyse the secretion of growth/neurotrophic factors in the supernatant of co-cultures and monocultures, an ELISA-based microarray analysis of the trophic factor concentrations was performed and compared between the three culture conditions. The concentrations of 40 trophic factors were assessed in the supernatant collected on day 7 (Table 3.1). Results of the one-way ANOVA revealed that there was a statistically significant difference between the mean concentrations of 18 of the 40 factors explored ($P < 0.05$) (see Table 3.1). Specifically, the mean concentrations of BDNF, bFGF, EGF, EGFR, FGF-7, GDF-15, GDNF, HB-EGF, HGF, IGFBP -1, -2, -3, -4, -6, NGFR, NT-3, OPG, PDGF-AA, PIGF, SCF, SCFR, TGFb3 and VEGF all varied between the supernatant collected from the different culture conditions. Table 3.1 shows the mean concentration of each growth/neurotrophic factor in the three culture conditions alongside their P-values and the fold change in concentrations between monoculture and co-culture contrasts.

Post Hoc test results revealed where the differences occurred between the groups in regards to the concentration of the trophic factor under analysis. Fig 3.2 illustrates these comparisons and the groups between which the differences were actually significant ($p<0.05$). The factors found to significantly vary between monocultures and co-cultures (CC) include BDNF, bFGF, EGF, EGFR, FGF-7, GDNF, GDF-15, HB-EGF, HGF, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-6, NGF-R, NT-3, OPG, PDGF-AA, PIGF, SCF and VEGF.

3.2 Factors that Significantly Increased Between NPC only & CC Conditions

The levels of **GDF-15** (Fig3.2d) and **OPG** (Fig3.2n) were found to be significantly higher in CC condition compared to their concentrations in the NPC only cultures. The adjusted p-values after Tukey's multiple comparison test is as follows for each of the trophic factors mentioned respectively; $p= 0.0275$, $p=0.0002$ and $p=0.0002$.

3.3 Factors that Significantly Increased Between Muscle only & CC Conditions

On the other hand differences in **IGFBP-2** (Fig3.2h) and **PDGF-AA** (Fig3.2o) were only observed in comparisons between Muscle only vs. CC conditions. The adjusted p-values after Tukey's multiple comparison test is as follows for each of the trophic factors mentioned respectively; $p=0.0101$, $p= 0.0358$, $p<0.0001$, $p=0.0004$ respectively.

3.4 Factors that Significantly Increased Between NPC only & CC Conditions + Muscle only & CC Conditions

The levels of **BDNF** (Fig3.2a) were significantly different between the *NPC only vs. CC* conditions with a p-value of 0.0019 as well as *Muscle only vs. CC* conditions with a p-value of 0.0031. Levels of BDNF increased greatly in co-culture conditions compared to monoculture conditions. A similar pattern was observed with **bFGF** (Fig3.2b), **FGF-7** (Fig3.2c), **GDNF** (Fig3.2e), **HGF** (Fig3.f), **IGFBP-3** (Fig3.2i), and **IGFBP-6** (Fig3.2k) where differences in concentration of these trophic factors was observed between the *NPC only vs. CC* conditions and the *Muscle only vs. CC* conditions with an adjusted p-value of <0.0001 for both comparisons in relation to the respective factors. Additionally variances in **NT-3** (Fig3.2m) and **SCF** (Fig3.2q) concentrations were also significant between the *NPC only vs. CC* conditions

(p=0.007, 0.0006 respectively) and the *Muscle only vs. co-culture* conditions (p=0.007, 0.0002 respectively). Moreover, differences in **IGFBP-1** (Fig3.2g), **IGFBP-4** (Fig3.2j), **NGF-R** (Fig3.2l), **PIGF** (Fig3.2p) and **VEGF** (Fig3.2r) were also apparent and significant in both muscle only and NPC only monocultures vs. co-culture comparisons.

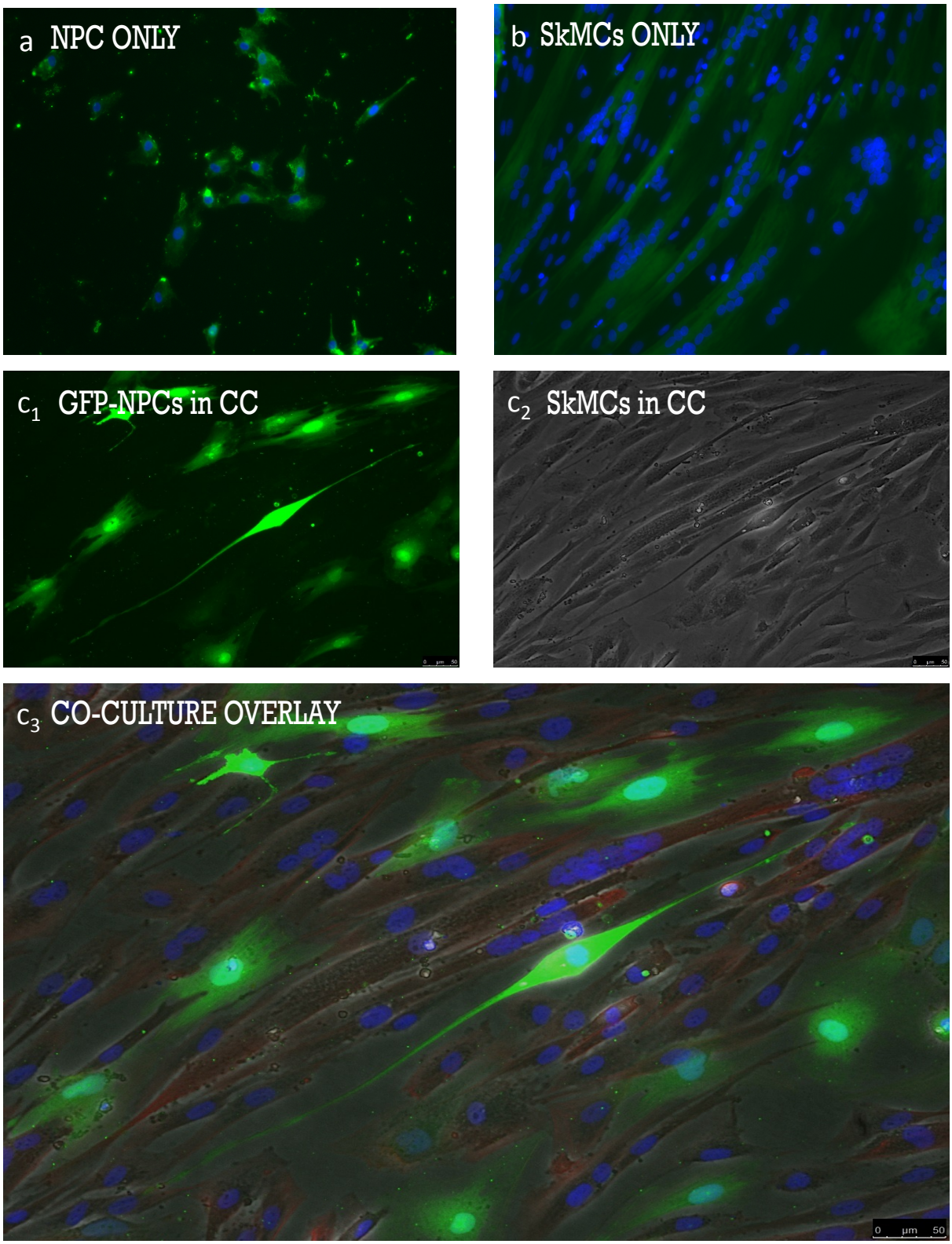
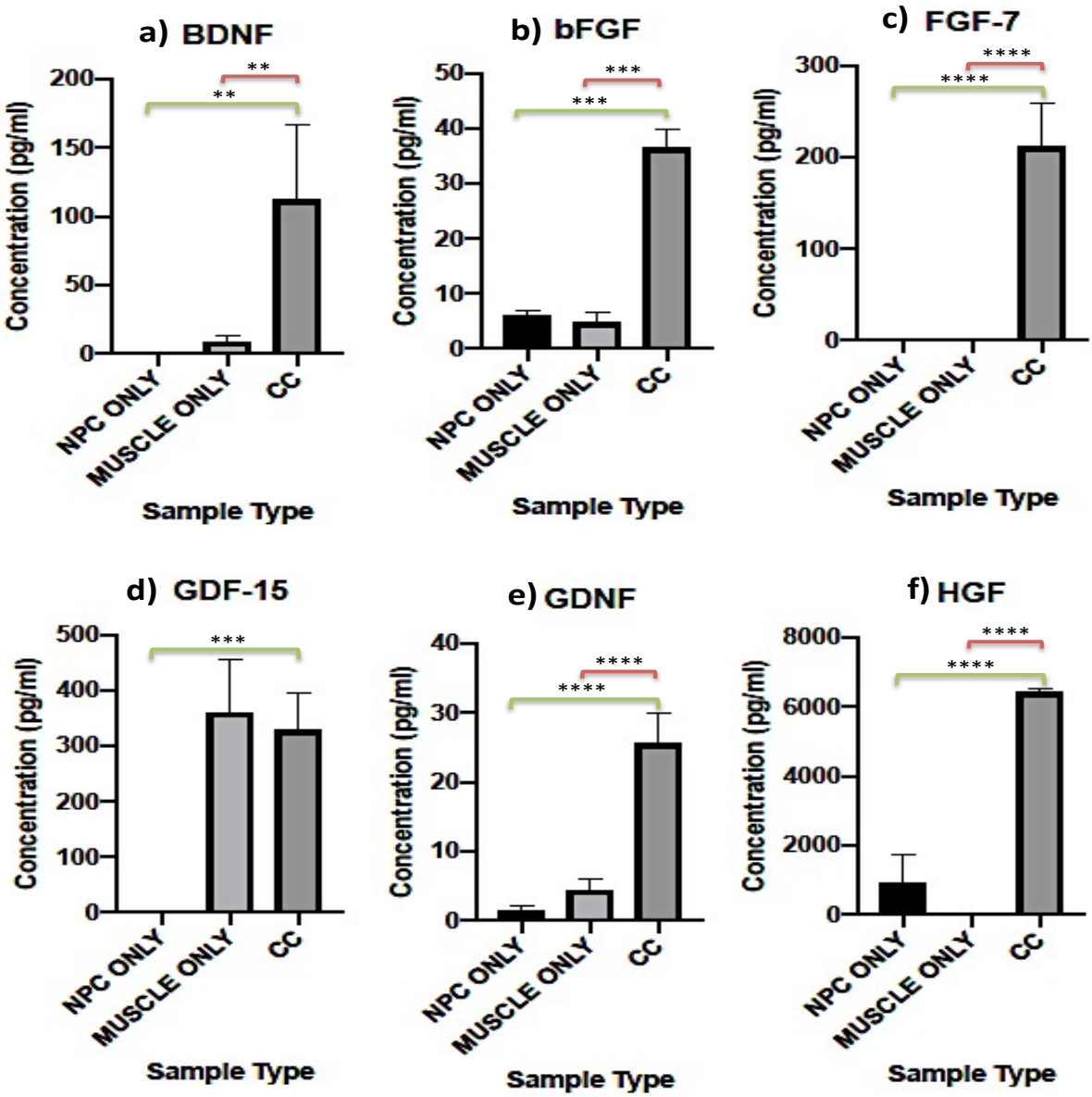
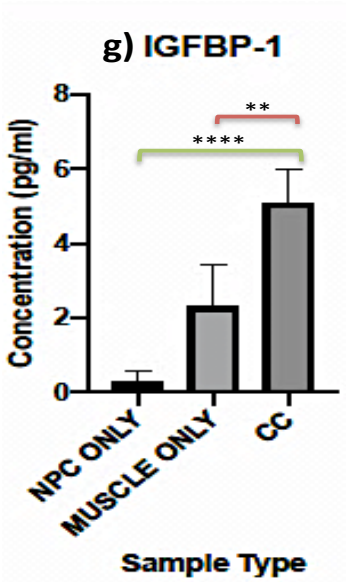
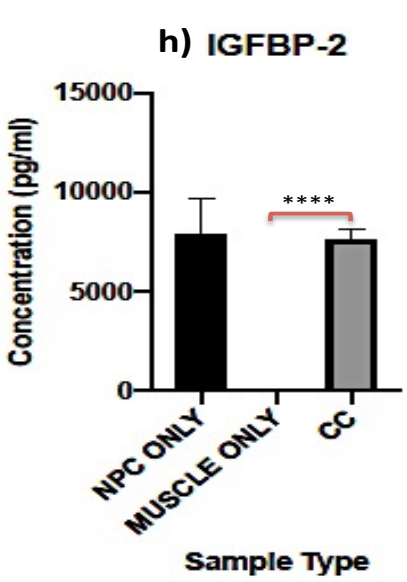


Fig 3.1a: Comparison of solitary cultured NPCs and SKMCs cultured and co-cultured NPCs & SkMCs on day 7. The images include phalloidin stained filamentous actin in muscle cells (red), nuclei (blue) and the GFP-NPCs (green). a) Image illustrating GFP tagged NPC only monoculture on day 7: NPCs did not develop fully and did not sprout neurites. The majority

atrophied by day 7 as shown. b) SkMC only monoculture on Day 7: myoblasts differentiated and fused to form multinucleated myotubes however these myotubes were not functional and contractions were seen. c) Immunofluorescence Microscopy of NPC + SkMC Co-Culture on Day 7: complete differentiation of both SKMC and NPCs occurred resulting in functional myotubes. Image c_1 demonstrates the morphology of NPCs in CC on day 7, image c_2 demonstrates the morphology of SkMCs in CC on day 7 and image c_3 is a overlaid image of both NPC (c_1) and SkMC (c_2) images in CC on day 7.




g) IGFBP-1
Concentration (pg/ml)
8
6
4
2
0
**
****
NPC ONLY
MUSCLE ONLY
CC
Sample Type


h) IGFBP-2
Concentration (pg/ml)
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10000
5000
0
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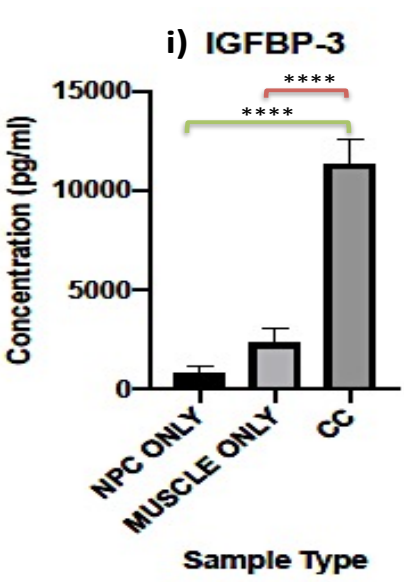
MUSCLE ONLY
CC
Sample Type


i) IGFBP-3
Concentration (pg/ml)
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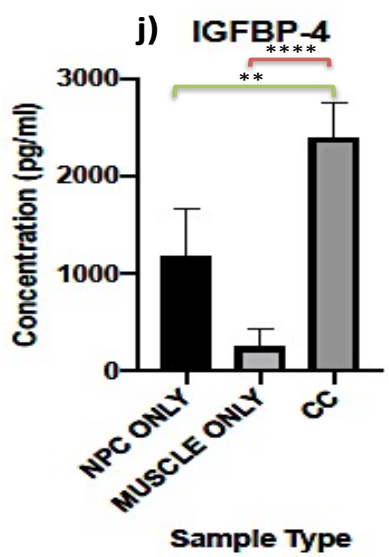
10000
5000
0
****
****
NPC ONLY
MUSCLE ONLY
CC
Sample Type


j) IGFBP-4
Concentration (pg/ml)
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2000
1000
0
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**
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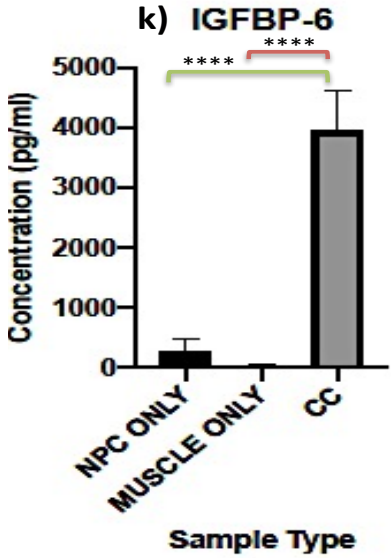
MUSCLE ONLY
CC
Sample Type


k) IGFBP-6
Concentration (pg/ml)
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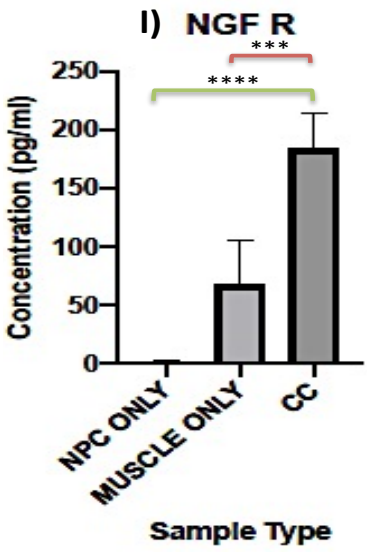
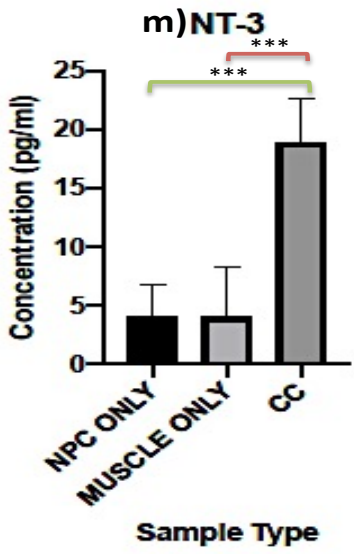
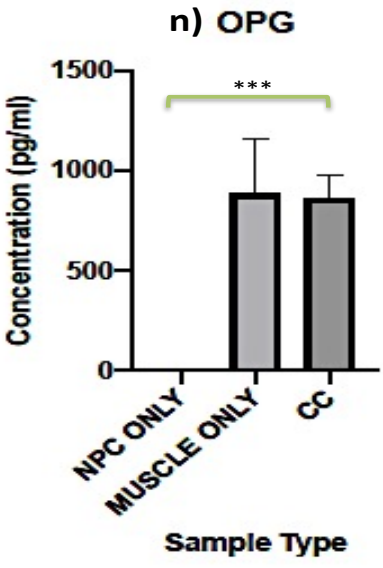
4000
3000
2000
1000
0
****
****
NPC ONLY
MUSCLE ONLY
CC
Sample Type


l) NGF R
Concentration (pg/ml)
250
200
150
100
50
0
***
****
NPC ONLY
MUSCLE ONLY
CC
Sample Type


m)NT-3
Concentration (pg/ml)
25
20
15
10
5
0
***
***
NPC ONLY
MUSCLE ONLY
CC
Sample Type


n) OPG
Concentration (pg/ml)
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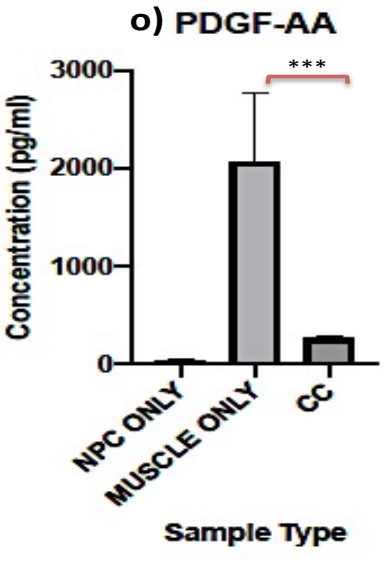
1000
500
0
***
NPC ONLY
MUSCLE ONLY
CC
Sample Type


o) PDGF-AA
Concentration (pg/ml)
3000
2000
1000
0
***
NPC ONLY
MUSCLE ONLY
CC
Sample Type

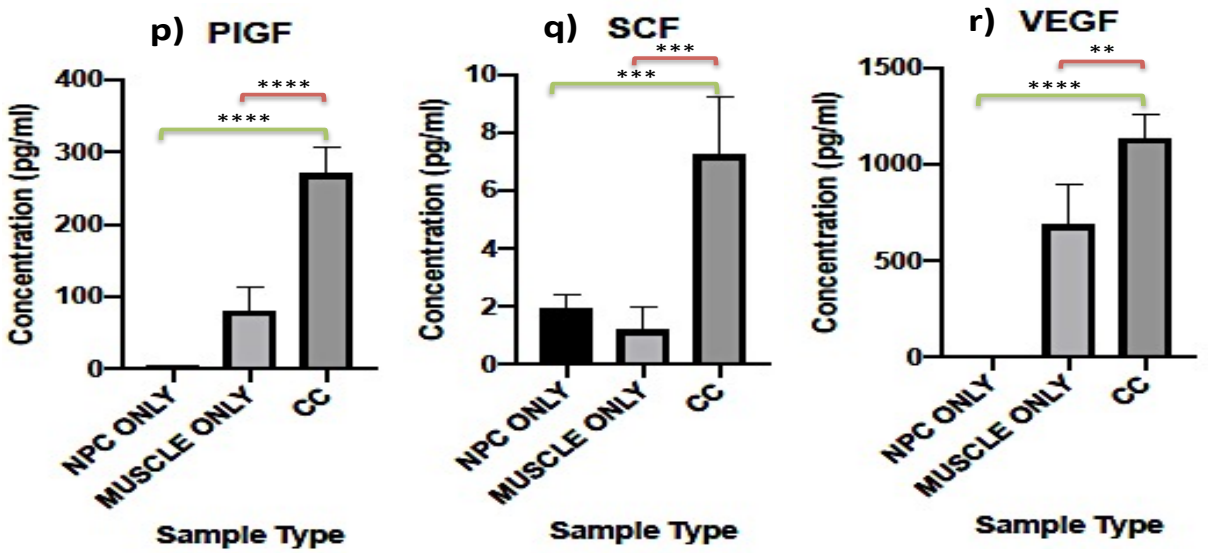


Fig 3.2 (a-u): Trophic factor quantification and comparison between different culture conditions. The above figures illustrate comparisons in the levels of the trophic factors between monoculture and CC conditions. Data is presented as a mean concentration of the trophic factor in each condition with error bars signifying ± SD. n=3 and represents the independent experiments/sample types from which the supernatant was collected. *Represents p=0.0101-0.0358, **represents p=0.0010-0.0089, ***represents p=0.0001-0.0007, ****represents p<0.0001. The red connectors indicate the comparisons between muscles only vs. co-culture conditions. The green connectors indicate the comparisons between NPC only vs. co-culture conditions.

Table 3.1: Summary of Elisa-based analysis of trophic factors in supernatant collected from Muscle only and NPC only monocultures as well as NPC & Muscle co-cultures on day 7.

Growth Factor	NPC ONLY ± SD (pg/ml)	MUSCLE ONLY ± SD (pg/ml)	COCULTURE ± SD (pg/ml)	Fold Change CC/NPC	Fold Change CC/M	P value	Standard curve R^2
AR	1.30 ± 1.84	0.00 ± 0.00	0.00 ± 0.00	0.0	0.0	0.1911	0.9966
BDNF	0.48 ± 0.15	8.55 ± 4.14	112.00 ± 54.50	233.3	13.1	0.0012**	0.997
bFGF	6.03 ± 0.74	4.63 ± 2.05	36.45 ± 3.31	6.04	7.87	<0.0001 ****	0.8267
BMP-4	6.50 ± 2.91	5.80 ± 4.91	5.80 ± 4.72	0.89	1.0	0.9650	0.9405
BMP-5	496.9 ± 278.1	481.60 ± 360.40	393.40 ± 285.60	0.79	0.82	0.8799	0.9986
BMP-7	7.75 ± 5.47	9.05 ± 3.31	3.78 ± 5.47	0.49	0.42	0.3243	0.9985
b-NGF	0.43 ± 0.33	0.18 ± 0.35	0.50 ± 0.42	1.16	2.78	0.4613	0.9406
EGF	0.00 ± 0.00	0.23 ± 0.10	0.05 ± 0.06	0.0	0.22	0.0020**	0.9673
EGF R	4.20 ± 1.12	47.40 ± 18.93	29.35 ± 4.05	7.0	0.62	0.0014**	0.998
EG-VEGF	0.35 ± 0.41	0.70 ± 0.22	0.13 ± 0.25	0.25	0.14	0.0707	0.9646
FGF-4	55.93 ± 45.63	30.83 ± 21.95	44.65 ± 31.21	0.80	1.44	0.6026	0.9564
FGF-7	0.05 ± 0.10	0.00 ± 0.00	211.70 ± 47.56	2117	0.0	<0.0001 ****	0.9983
GDF-15	0.88 ± 0.38	360.10 ± 95.80	328.20 ± 65.60	364.44	0.91	<0.0001 ****	0.9966
GDNF	1.38 ± 0.80	4.35 ± 1.70	25.50 ± 4.34	18.48	5.79	<0.0001 ****	0.998
GH	4.93 ± 1.28	5.83 ± 2.89	2.58 ± 1.67	0.53	0.44	0.1251	0.9673
HB-EGF	1.13 ± 0.73	1.58 ± 0.93	0.10 ± 0.20	0.09	0.06	0.0392*	0.865
HGF	908.80 ± 813.00	33.90 ± 24.18	6413.00 ± 93.10	7.06	189.17	<0.0001 ****	0.9956
IGFBP-1	0.30 ± 0.26	2.30 ± 1.12	5.08 ± 0.92	17	2.22	<0.0001 ****	0.9992
IGFBP-2	7909.00 ± 1822.00	34.53 ± 27.58	7640.00 ± 517.80	0.97	221.45	<0.0001 ****	0.9807
IGFBP-3	816.80 ± 371.60	2306.00 ± 805.70	11316.00 ± 1269.00	13.85	4.91	<0.0001 ****	0.9997
IGFBP-4	1168.00 ± 500.00	249.50 ± 189.20	2393.00 ± 361.10	2.05	9.58	<0.0001 ****	0.9364
IGFBP-6	268.00 ± 196.60	29.28 ± 20.32	3953.00 ± 674.40	14.75	134.91	<0.0001 ****	0.9993
IGF-1	96.68 ± 61.73	77.53 ± 74.38	102.70 ± 57.03	1.07	1.33	0.8511	0.9616
INSULIN	7462.00 ± 3199.00	9061.00 ± 8143.00	10240.00 ± 5951.00	1.37	1.130	0.8158	0.9995

MCSF R	5.98 ± 4.24	7.50 ± 7.81	2.13 ± 2.47	0.36	0.28	0.3789	0.9898
NGF R	2.00 ± 0.62	67.45 ± 38.49	184.00 ± 30.50	92	2.73	<0.0001 ****	0.9931
NT-3	4.03 ± 2.76	4.03 ± 4.34	18.90 ± 3.74	4.73	4.73	0.0003***	0.9839
NT-4	2.10 ± 1.54	6.38 ± 3.08	2.70 ± 2.27	1.29	0.42	0.0644	0.9961
OPG	4.93 ± 2.71	884.20 ± 277.00	864.50 ± 113.10	176.53	0.98	<0.0001 ****	0.9777
PDGF-AA	28.10 ± 18.70	2065.00 ± 703.80	260.70 ± 31.63	9.29	0.13	0.0001***	0.9996
PIGF	3.10 ± 0.59	79.75 ± 33.50	270.00 ± 37.27	87.10	3.38	<0.0001 ****	0.9354
SCF	1.90 ± 0.51	1.18 ± 0.84	7.23 ± 1.99	3.79	6	0.0002***	0.9776
SCF R	9.03 ± 1.01	2.60 ± 2.41	4.65 ± 3.05	0.52	1.81	0.0100*	0.9998
TGFa	0.18 ± 0.15	0.20 ± 0.18	0.10 ± 0.08	0.5	0.5	0.6113	0.7149
TGFb1	1520.00 ± 1475.00	3191.00 ± 2251.00	2525 ± 1682	16.61	0.79	0.4619	0.9906
TGFb3	9.78 ± 4.04	2.73 ± 2.03	6.65 ± 2.26	0.69	2.48	0.0235*	0.9615
VEGF	3.63 ± 3.46	685.00 ± 215.50	1132.00 ± 128.30	311.85	1.65	<0.0001 ****	0.9181
VEGF R2	15.68 ± 2.72	10.18 ± 6.20	6.45 ± 5.76	0.41	0.64	0.0855	0.996
VEGF R3	11.80 ± 1.27	9.55 ± 5.58	7.48 ± 4.37	0.64	0.78	0.3786	0.9947
VEGF-D	0.65 ± 0.41	0.98 ± 0.53	0.93 ± 0.21	1.29	0.9	0.4986	0.9993

4.0 DISCUSSION

Results revealed that the bi-directional interdependence of both MN and SkM tissue is indeed fundamental for the development of a functional motor system model, as it was seen to induce the production of essential endogenous trophic factors, either pre-synaptically from MNs or post-synaptically from SkM tissue. This was evident from assessing the morphology of cells in both co-culture and monoculture conditions, which demonstrated that without the supply of any trophic factors exogenously the lack of reciprocal interaction between the two tissues ensued abnormal/hindered development of both tissues when in monoculture. In contrast, SkM & MN co-cultures developed and formed a functional motor system model without growth and neurotrophic supplementation. This observation was consolidated by results of the multiplex ELISA, which showed a substantial variation, when compared between the different culture conditions, in the concentrations of several factors known to be involved in the regulation and support of normal nerve and muscle development and condition. Indicating that trophic factors fundamental to the development and formation of a functional human motor system must be secreted endogenously as consequence of muscle-nerve interaction within this physiologically pertinent human NMJ co-culture model.

The concentration of 18 trophic factors (see table 3.1) showed a significant variation when measured in monoculture compared to co-culture conditions. Nonetheless, focusing on the factors that have been utilized and applied in different tissue engineering studies as supplementation to aid the development of robust motor system models and enhance MN and myoblasts survival in culture, this section will largely have focused on seven primary trophic factors that are recurrently used and appear in the literature. Several studies investigating the development of NMJ models have optimized culture health by supplementing them with trophic factors such as brain derived and glial cell line derived neurotrophic factors (BDNF, GDNF), neurotrophin 3 (NT-3), nerve growth factor receptor (NGFR), basic fibroblast growth factors (bFGF), hepatocyte growth factor (HGF), and fibroblast growth factor 7 (FGF-7) (Omura et al., 2005; Das et al., 2010; Umbach et al., 2012; Puttonen et al., 2015; Vilmont et al., 2016 and Bakooshli et al., 2019). Reviewing the results from Table 3.1 the concentration of a number of factors especially those mentioned above displayed a

positive increase from the NPC only and SkMC only monocultures to nerve-muscle co-culture conditions.

4.1 BDNF

BDNF, a neurotrophic factor that has been the focus of numerous studies since it was discovered to be associated with various neurological disorders was observed to display a fold change of 233 in NPC only vs. CC and a fold change of 13 in Muscle only vs. CC comparisons. Studies investigating the function of BDNF were initially explored in relation to its role in the development and maintenance of the nervous system (Mousavi and Jasmin, 2006). However, the discovery of BDNF receptor expression in SkM and muscle SCs by Chevrel et al., 2006 has opened up interest to its postulated role in SkM development and regeneration. Reports exploring the role of BDNF have identified its supportive function in MN growth, survival, differentiation, regeneration and synaptic differentiation (Lee and Jun, 2019). Whilst studies specifically investigating muscle derived BDNF have demonstrated roles in regulating normal myogenic differentiation and regeneration following injury (Kolarow et al., 2007). Interestingly in SkM a knockout of BDNF was associated with increased myoblast differentiation, whilst low BDNF was concomitant with the maintenance of SC population (Mousavi et al., 2002). Which explains why an increase in BDNF expression has been observed following various peripheral nerve injuries denoting its function in facilitating SC response in muscle damage (Omura et al., 2005). Likewise findings from BDNF null and muscle specific BDNF KO mice studies conducted by Clow and Jasmin, 2010 exhibited the significance of muscle specific BDNF in the inhibition of abnormalities in myogenic differentiation and regeneration. BDNF signaling has also been shown to promote the withdrawal of weaker MN contacts whilst leaving active MN terminals intact regulating synaptic density in synaptogenesis (Je et al., 2012 and Garcia et al., 2010). Jointly this could possibly justify how the endogenously regulated concentration of BDNF secreted in the muscle-nerve co-culture system allowed for the appropriate formation and development of a human motor system model, which is representative of the *in-vivo* situation.

4.2 GDNF

In addition to BDNF, results also showed elevated levels of GDNF, one of the most potent factors for motor neuron survival in-vitro (Wang et al., 2002 and Lee and Jun, 2019). Expressed by SkM and eliciting its actions via its receptor Ret Tyrosine kinase expressed in MNs, GDNF enhances the survival and morphological differentiation of MNs and sensory neurons (Kingham and Terenghi. 2006). In contrast to BDNF the over expression of GDNF in transgenic mice lead to a marked increase in the number of MN contacts innervating myotubes at a time, when redundant MNs would normally undergo elimination (Nguyen et al., 1998) suggesting a role for GDNF in rescuing and preventing the atrophy of MNs (Suzuki, et al., 2007). Exogenous GDNF in embryonic chicks studies as well as neonatal rodents displayed GNFs ability to rescue MNs from pre-programmed cell death and following limb ablation (Yuen and Mobley, 1995). Furthermore Keller-Peck et al., 2001 reports that GDNF may be more specifically involved in axonal growth, branching, maturation and synapse formation rather than synapse elimination. As the conditional ablation of **Ret** in cranial MNs of mice resulted in obvious disruptions to MN terminal maturation and reduced motor end plate size at NMJs (Baudet et al., 2008). This could explain the absence of MN branching and growth spotted in the NPC monoculture where NPCs atrophied compared to the normal axonal growth and branching observed in the co-culture system described in this project. Further indicating the physiologically pertinent interaction between pre and postsynaptic components of NMJs in the system.

4.3 NT-3

NT-3 another member of the neurotrophin family was similarly found elevated in the co-culture system when compared to monocultures. NT-3 the amplest neurotrophin communication in muscle is transported to MN cell bodies during synaptic development to support the survival and maturation of MNs as well as drive the differentiation of muscle precursor cells through tyrosine kinase receptors (Henderson, 1996). Research in NT-3 deficient mice showed declined SkM innervation and a reduction in the number of SkM fibers, observations which were supported by Sheard et al., 2010 who presented a reduction in SkM fibers associated with induced NT-3 insufficiency in mice. In addition to its involvement in the formation and maintenance of muscle spindles (Copray and Brouwer, 1994) NT-3 also has the ability to rescue, protect and repair MNs in the absence of other target-derived factors (Yuen

and Mobley, 1995; Oakley et al., 1997; Wiese et al., 2004 and Henriques et al., 2010). These NT-3 functions were further supported via a study conducted on rat gastrocnemius muscles that found applied NT-3 to induce axonal revival by encouraging the differentiation of muscle fibres and preventing muscle atrophy in NMJs (Sterne, et al.,1997). Being the most sufficient neurotrophic factor in muscle it was also shown to be more effective than both BDNF and nerve growth factor (NGF) in ensuring the survival of afferent neurons branching from muscles (Yuen and Mobley, 1995). Reflecting on these studies it can be appreciated that the significantly elevated NT-3 expression detected in the co-culture system must have supported NMJ formation and development.

4.4 NGFR

Additionally, NGFR a receptor for the neurotrophic factor - NGF was also elevated in co-culture. NGFR, transiently expressed in MN during development and briefly re-expressed following nerve injury during regeneration, is not generally expressed in normal adult animals thus appearing to be associated with axonal growth and maturation (Wood et al., 1990 and Matusica et al., 2016). In contrast other researchers have identified NGFR to mediate MN death during development and following nerve injury (Seeburger et al., 1993; Sedel et al., 1999; Ernfors, 2001; Lory et al., 2001; Dechant and Barde, 2002 and Ibanez and Simi, 2012). However, researchers have recognized NGFR as a multifunctional receptor with capabilities in regulating different biological effects depending on the ligand bound (Roux and Barker, 2002; Blochl and Blochl, 2007; Underwood and Coulson, 2008). Again given the functions of NGFR one could associate the aptitude, in part, for the development of the functional in-vitro human motor system to the significantly raised levels of NGFR expression in this co-culture system.

4.5 bFGF

ELISA results also revealed a raised bFGF concentration in co-culture in comparison to monocultures. As established previously SkM development, repair and regeneration is a complex process that requires the release of essential growth factors and a particular microenvironment to elicit an effective myogenic response (Rushton et al., 2009). Amongst the molecules thought to be involved in this process is bFGF a member of the FGF family, which are widely known for their range of roles in cell proliferation, differentiation and

migration in cells originating from the mesoderm and ectoderm (Shi et al., 2016). Investigation into the effect of bFGF *in-vitro* has elucidated a role in stimulating proliferation of SCs and muscle precursor cells known as myoblasts (Guthridge et al., 1992). bFGF receptors have consequently been identified in myoblasts but not in differentiated myotubes suggesting a physiological role in early muscle development *in-vivo* as well as in the muscle repair response ensuing muscle injury. Hence why bFGF has been extensively used in the treatment of diabetic ulcers, gastric ulcers, surgical wounds, burns and even spinal cord injury (Zhang et al., 2013). Shi et al., 2013 showed bFGF effects in action in response to endoplasmic reticulum stress where bFGF was observed to promote wound healing and reduce scar formation. Whilst further study by Shi et al., 2016 showed bFGF to improve skeletal muscle recovery following skeletal muscle degradation induced by deep tissue injuries, accordingly proposing roles for bFGF not only in promoting myoblast proliferation but also in encouraging angiogenesis.

4.6 HGF

HGF production is substantially up regulated following SkM injury and normally during embryonic development. It is thus believed to be essential for muscle repair and regeneration (Yang et al., 1998). HGF acts by stimulating trk receptors expressed widely in SCs through which it can trigger their transition from a quiescent state to a proliferative state (i.e. enter the cell cycle). HGF also acts by inhibiting cell differentiation until cells are ready to exit the cell cycle (Karalaki et al., 2009). This notion is supported through several studies with transgenic mice. One such study reported inappropriate muscle formation as a result of HGF ectopic expression in the CNS. Whilst another study examining HGF receptor null mice described a failure to develop and form limb and body wall muscle as a result (Wong et al., 1997). Yamamato et al., 1997 and Novak et al., 2000 additionally demonstrated the transient expression of the HGF receptor in lumbar MNs, specifically during periods of programmed cell death signifying its responsibility in the survival of lumbar MNs. HGF has also been implicated in controlling axonal growth and survival in mammalian neuron development acting simultaneously with NGF (Maina et al., 1997 and Wong et al., 1997). Taken together these observations indicate the importance of HGF in the development and maintenance of the neuromuscular system and could explain the elevated concentrations witnessed in co-culture.

4.7 FGF-7

In addition to HGF several members of the FGF family have also been previously indicated to promote the shift of SCs from their quiescent states to proliferative states whilst repressing differentiation in the primary SkM cells generated. Interestingly a study analysing the affect of different members of the FGF family discovered that although many members such as FGF-1, -2, -4 and -6 enhance satellite cell proliferation FGF-7 had no such effect (Kastner et al., 2000). Yet results from the microarray analysis disclosed FGF-7 amongst the factors with the most significant variation ($p<0.0001$) in terms of concentration when comparisons between monocultures and co-culture were made. Thus, although FGF-7 might not play a role in enhancing myoblast proliferation it must have some role in NMJ development. Experiments looking into the expression of FGFs throughout SkM development noticed that FGF-7 is in fact expressed in proliferating skeletal cell myoblasts (Hannon et al., 1996). Other researchers found a synergistic function of FGF-7 and agrin in SkM for regulating presynaptic differentiation at the NMJ (An et al., 2010). Thus possibly explaining the elevated FGF-7 observed in the co-culture system.

5.0 CONCLUSION

Collectively, the data indicated that the absence of MN or SkM from culture results in abnormal development of the tissues without the external addition of trophic factors, which suggests that SkM to MN cell-cell interaction is fundamental for the production of necessary trophic factors in optimum concentrations to elicit normal NMJ development and hence the formation of a functional motor system. Since trophic factors were not supplemented in the co-culture system it also implies that the trophic factors were secreted endogenously as consequence of muscle-nerve interaction and hence proving the suitability of this co-culture system as an authentic and valid platform for investigating the individual trophic factors involved in the development and maintenance of a human motor system and NMJ disorders were this communication is disabled such as ALS, muscular dystrophy, diabetic neuropathy and/or myopathy, which could be a direction for future study. Elucidation of the roles and mechanisms of these factors would significantly contribute to an understanding of their physiological role in the normal myogenic program, in the pathogenesis of diseases such as those mentioned above and, in the formulation, or their inclusion into treatment regimes.

6.0 REFERENCES

Al-Samid, M. A., McPhee, J. S., Saini, J., McKay, T. R., Fitzpatrick, L. M., Mamchaoui, K., Bigot, A., Mouly, V., Butler-Browne, G. and Al-Shanti, N. (2018) 'A Functional Human Motor Unit Platform Engineered From Human Embryonic Stem Cells and Immortalized Skeletal Myoblasts.' *Stem Cells Cloning: Advances and Applications*, 11 pp. 85–93.

An, M. C., Lin, W., Yang, J., Dominguez, B., Padgett, D., Sugiura, Y., Aryal, P., Gould, T. W., Oppenheim, R. W., Hester, M. E., Kaspar, B. K., Ko, C.-P. and Lee, K.-F. (2010) 'Acetylcholine negatively regulates development of the neuromuscular junction through distinct cellular mechanisms.' *Proceedings of the National Academy of Sciences*, 107(23) pp. 10702-10707.

Argilés, J. M., Campos, N., Lopez-Pedrosa, J. M., Rueda, R. and Rodriguez-Mañas, L. (2016) 'Skeletal Muscle Regulates Metabolism via Interorgan Crosstalk:Roles in Health and Disease.' *JAMDA*, 17(9) pp. 789-796.

Arnold, A.-S., Gill, J., Christe, M., Ruiz, R. o., McGuirk, S., St-Pierre, J., Tabares, L. a. and Handschin, C. (2014) 'Morphological and Functional Remodelling of the Neuromuscular Junction by Skeletal Muscle PGC-1 a.' *Nature Communications*, 5(3569) pp. 1-11.

Barker, A.T., Jalinous, R., Freeston, I.L. (1985) 'Non-Invasive Magnetic Stimulation of Human Motor Cortex.' *The Lancet.* 325(8437) pp. 1106-1107.

Bakooshli, M. A., Lippmann, E. S., Mulcahy, B., Iyer, N., Nguyen, C. T., Tung, K., Stewart, B. A., Dorpel, H. v. d., Fuehrmann, T., Shoichet, M., Bigot, A., Pegoraro, E., Ahn, H., Ginsberg, H., Zhen, M., Ashton, R. S. and Gilbert, P. M. (2019) 'A 3D Culture Model of Innervated Human S keletal Muscle Enables Studies of the A dult Neuromuscular Junction.' *eLife*, 8(1) pp. 1-29.

Baudet, C., Pozas, E., Adameyko, I., Andersson, E., Ericson, J. and Ernfors, P. (2008) 'Retrograde Signaling onto Ret during Motor Nerve Terminal Maturation.' *The Journal of Neuroscience*, 28(4) pp. 963-975.

Belluardo, N., Westerblad, H., Mudo, G., Casabona, A., Bruton, J., Caniglia, G., Pastoris, O., Grassi, F. and Ibanez, C. F. (2001) 'Neuromuscular junction disassembly and muscle fatigue in mice lacking neurotrophin-4.' *Mol Cell Neurosci*, 18(1), pp. 56-67.

Bentzinger, C. F., Wang, Y. X. and Rudnicki, M. A. (2012) 'Building Muscle: Molecular Regulation of Myogenesis 'Cold *Spring Harbor Laboratory Press*, 4(2) pp. 1-17.

Blochl, A. and Blochl, R. (2007). 'A cell-biological model of p75NTR signaling.' *Journal of Neurochemistry.* 102(2), pp. 289-305.

Bloch-Gallego, E. (2015) 'Mechanisms Controlling Neuromuscular Junction Stability.' *Cellular and Molecular Life Sciences*, 72(6) pp. 10299-11043.

Boncompagni, S., Kern, H., Rossini, K., Hofer, C., Mayr, W., Carraro, U. and Protasi, F. (2007) 'Structural Differentiation of Skeletal Muscle Fibers in the Absence of Innervation in Humans.' *PNAS*, 104(49) pp. 19339-19344.

Charge, S. B. P. and Rudnicki, M. A. (2003) 'Cellular and Molecular Regulation of Muscle Regeneration ' *Physiological Review*, 84(1) pp. 209-238.

Chen, C.-n. (2017) Nutrition and Functional Foods for Healthy Aging- Late-Onset Caloric Restriction Alters Skeletal Muscle Metabolism: Mechanisms from Animal and Human Studies In: Watson, R. R., pp. 337-344. Academic Press.

Chevrel, G., Hohlfeld, R. and Sendtner, M. (2006) 'The role of neurotrophins in muscle under physiological and pathological conditions.' *Muscle Nerve*, 33(4), pp. 462-476.

Cisterna, B. A., Cardozo, C. and Sáez, J. C. (2014) 'Neuronal Involvement in Muscular Atrophy.' *Frontiers in Cellular Neuroscience*, 8(405) pp. 1-11.

Copray, J.C. and Brouwer N. (1994) 'Selective Expression of Neurotrophin-3 Messenger RNA in Muscle Spindles of the Rat.' *Neuroscience,* 63(4) pp.1125-1135.

Das, M., Rumsey, J. W., Bhargava, N. e., Stancescu, M. and Hickman, J. J. (2010) 'A Defined Long-term In-v*itro* Tissue Engineered Model of Neuromuscular Junctions.' *Biomaterials*, 31(18) pp. 4880-4888.

Dechant, G. and Barde, Y.-A. (2002). 'The neurotrophin receptor p75NTR: novel functions and implications for diseases of the nervous system'. *Nature Neuroscience*. 5(1), pp. 1131-1136.

Delaporte, C., Dautreaux, B. and Fardeau, M. (1986) 'Human myotube differentiation in vitro in different culture conditions.' *Biology Cell*, 57(1) pp. 17-22.

Delezie, J. and Handschin, C. (2018) 'Endocrine Crosstalk Between Skeletal Muscle and the Brain.' *Frontiers in Neurology*, 9(698) pp. 1-14.

Denoth, J., Stussi, E., Csucs, G. and Danuser, G. (2002) 'Single Muscle Diber Contraction is Dictated by Inter-Sarcomere Dynamics.' *Journal of Theoretical Biology*, 216(1) pp. 101-122.

Ernfors, P., Lee, K. F., Kucera, J. and Jaenisch, R. (1994) 'Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents.' *Cell*, 77(4), pp. 503-512.

Ernfors, P. (2001). 'Local and target-derived actions of neurotrophins during peripheral nervous system development.' *Cellular and Molecular Life Sciences.* 58(8), pp.1036-1044.

English, A. W. (2003) 'Cytokines, Growth Factors and Sprouting at the Neuromuscular Junction.' *Journal of Neurocytology*, 32(5-8) pp. 943-960.

Ferraro, E., Molinari, F. and Berghella, L. (2012) 'Molecular Control of Neuromuscular Junction Development.' *Journal Cachexia Sarcopenia Muscle*, 3(1) pp. 13–23.

Forcina, L., Miano, C., Pelosi, L. and Musarò, A. (2019) 'An Overview about the Biology of Skeletal Muscle Satellite Cells.' *Current Genomics*, 20(1) pp. 24-37.

Fox, M. A., Sanes, J. R., Borza, D. B., Eswarakumar, V. P., Fassler, R., Hudson, B. G., John, S. W., Ninomiya, Y., Pedchenko, V., Pfaff, S. L., Rheault, M. N., Sado, Y., Segal, Y., Werle, M. J. and Umemori, H. (2007) ''Distinct target-derived signals organize formation, maturation, and maintenance of motor nerve terminals.' *Cell*, 129(1) pp. 179-193.

Garcia, N., Santafe, M. M., Tomàs, M., Lanuza, M. A., Besalduch, N. and Tomàs, J. (2010) 'Involvement of brain-derived neurotrophic factor (BDNF) in the functional elimination of synaptic contacts at polyinnervated neuromuscular synapses during development.' *Journal of Neuroscience Research*, 88(7) pp. 1406-1419.

Giudice, J. and Taylor, J. M. (2017) 'Muscle as a Paracrine and Endocrine Organ.' *Current Opinion in Pharmacology*, 34(1) pp. 49-55.

Grinnell, A. D. (1995) 'Dynamics of Nerve-Muscle Interaction in Developing and Mature Neuromuscular Junctions ' *Physiolgical Reviews*, 75(4) pp. 789-834.

Guthridge, M., Wilson, M., Cowling, J., Bertolini, J. and Hearn, M. T. W. (1992) 'The Role of Basic Fibroblast Growth Factor in Skeletal Muscle Regeneration ' *Growth Factors*, 6(1) pp. 53-63.

Hannon, K., Kudla, A. J., McAvoy, M. J., Clase, K. L. and Olwin, B. B. (1996) 'Differentially Expressed Fibroblast Growth Factors Regulate Skeletal Muscle Development through Autocrine and Paracrine Mechanisms.' *The Journal of Cell Biology*, 132(6) pp. 1151-1159.

Hartmann, M., Heumann, R. and Lessmann, V. (2001) 'Synaptic Secretion of BDNF After High-Frequency Stimulation of Glutamatergic Synapses.' *The EMBO Journal*, 20(21) pp. 5887-5897.

Henderson, C. E. (1996) 'Role of neurotrophic factors in neuronal development.' *Current Opinion in Neurobiology*, 6(1) pp. 64-70.

Henriques, A., Pitzer, C. and Schneider, A. (2010). 'Neurotrophic growth factors for the treatment of amyotrophic lateral sclerosis: where do we stand?' *Frontiers in Neuroscience,* 4(32) pp. 1-14.

Ibanez, C. F. and Simi, A. (2012). 'p75 neurotrophin receptor signaling in nervous system injury and degeneration: paradox and opportunity.' *Trends Neuroscience*. 35(7), pp. 431-440

Kaasik, P., Aru, M., Alev, K. and Seene, T. (2012) 'Aging and Regenerative Capacity of Skeletal Muscle in Rats.' *Current Ageing Science*, 5(2) pp. 126-130.

Kablar, B. and Belliveau, A. C. (2005) 'Presence of Neurotrophic Factors in Skeletal Muscle Correlates With Survival of Spinal Cord Motor Neurons.' *Developmental Dynamics*, 234(3) pp. 659-669.

Karalaki, M., Fili, S., Philippou, A. and Koutsilieris, M. (2009) 'Muscle Regeneration: Cellular and Molecular Events.' *In vivo*, 23(5) pp. 779-796.

Kästner, S., Elias, M. C., Rivera, A. J. and Yablonka-Reuveni, Z. (2000) 'Gene expression patterns of the fibroblast growth factors and their receptors during myogenesis of rat satellite cells.' *Journal of Histocytochemistry*, 48(8) pp. 1079-1096.

Keller-Peck, C. R., Feng, G., Sanes, J. R., Yan, Q., Lichtman, J. W. and Snider, W. D. (2001) 'Glial Cell Line-Derived Neurotrophic Factor Administration in Postnatal Life Results in Motor Unit Enlargement and Continuous Synaptic Remodeling at the Neuromuscular Junction.' *Journal of Neuroscience*, 21(16 6136-6146)

Kolarow, R., Brigadski, T. and Lessmann, V. (2007) 'Postsynaptic Secreaton of BDNF and NT-3 from Hippocampal Neurons Depends on Calcium-Calmodulin Kinase II Signaling and Proceeds via Delayed Fusion Pore Opening.' *The Journal of Neuroscience*, 27(39) pp. 10359-10364.

Le Grand, F. and Rudnicki, M. A. (2007) 'Skeletal Muscle Satellite Cells and Adult Myogenesis.' *Current Opinion Cell Biology*, 19(6) pp. 628-633.

Lee, J. H. and Jun, H.-S. (2019) 'Role of Myokines in Regulating Skeletal Muscle Mass and Function.' *Frontiers in Physiology*, 10(42) pp. 1-9.

Lee, J. F., Shneidman, P. S., Skuntz, S. F., Carden, M. J. and Lazzarini, R. A. (1988) 'The Structure and Organisation of the Human Heavy Neurofilament Subunit (NF-H) and the Gene Encoding It.' *Embo Journal*, 7(7) pp. 1947-1955.

Loeb, J. A., Hmadcha, A., Fischbach, G. D., Land, S. J. and Zakarian, V. L. (2002) 'Neuregulin Expression at Neuromuscular Synapses Is Modulated by Synaptic Activity and Neurotrophic Factors.' *Journal of Neuroscience* 22(6) pp. 2206-2214.

Lowry, K. S., Murray, S. S., McLean, C. A., Talman, P., Mathers, S., Lopes, E. C. and Cheema, S. S. (2001). 'A potential role for the p75 low-affinity neurotrophin receptor in spinal motor neuron degeneration in murine and human amyotrophic lateral sclerosis.' *Amyotroph. Lateral Sclerosis and Other Motor Neuron Disorders*. 2(3), pp. 127-134.

Lu, B. and Je, H.-S. (2003) 'Neurotrophic regulation of the development and function of the neuromuscular synapses.' *Journal of Neurocytology*, 32(5-8), pp. 931-941.

Maina, F., Hilton, M. C., Ponzetto, C., Davies, A. M. and Klein, R. d. (1997) 'Met receptor signaling is required for sensory nerve development and HGF promotes axonal growth and survival of sensory neurons ' *Cold Spring Harbor Laboratory Press*, 11(1) pp. 3341-3350.

Mantilla, C. B. and Sieck, G. C. (2008) 'Trophic Factor Expression in Phrenic Motor Neurons.' *Respiratory Physiology & Neurobiology*, 164(1-2) pp. 252–262.

Matusica, D., Alfonsi, F., Turner, B. J., Butler, T. J., Shepheard, S. R., Rogers, M.-L., Skeldal, S., Underwood, C. K., Mangelsdorf, M. and Coulson, E. J. (2016) 'Inhibition of motorneuron death in vitro and in vivo by a p75 neurotrophin receptor intracellular domain fragment' *Journal of Cell Science,* 129(1), pp.517-530.

Mc Keon-Fischer, K. D. and Freeman, J. W. (2011) Electrospinning for Tissue Regeneration - Muscle Tissue Regeneration. In: Bosworth, L. A. and Downes, S., pp. 127-147. In Woodhead Publishing Limited.

Nguyen, Q. T., Parsadanian, A. S., Snider, W. D. and Lichtman, J. W. (1998) 'Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle.' *Science*, 279(5357), pp. 1725-1729.

Novak, K. D., Prevette, D., Wang, S., Gould, T. W. and Oppenheim, R. W. (2000) 'Hepatocyte Growth Factor/Scatter Factor Is a Neurotrophic Survival Factor for Lumbar But Not for Other Somatic Motoneurons in the Chick Embryo ' *The Journal of Neuroscience*, 20(1) pp. 326-337.

Oakley, R.A., Lefcort, F.B., Clary, D.O., Reichardt, L.F., Prevette, D., Oppenheim, R.W. and Frank, E. (1997) 'Neurotrophin-3 Promotes the Differentiation of Muscle Spindle Afferents in the Absence of Peripheral Targets' *Journal of Neuroscience,* 17(11) pp.4262-4272.

Pedersen, B. K. and Febbraio, M. A. (2008) 'Muscle as an endocrine organ: focus on muscle-derived interleukin-6.' *Physiol Rev*, 88(4), pp. 1379-1406.

Phelan, M. C. and Lawler, G. (2001) 'Cell counting.' *Current Protocols in Cytometry*, 00(1) pp. A.3A.1–A.3A.4.

Pratesi, A., Tarantini, F. and Bari, M. D. (2013) 'Skeletal Muscle: An Endocrine Organ.' *Clinical Cases in Mineral and Bone Metabolism*, 10(1) pp. 11-14.

Puttonen, K. A., Ruponen, M., Naumenko, N., Hovatta, O. H., Tavi, P. and Koistinaho, J. (2015) 'Generation of Functional Neuromuscular Junctions from Human Pluripotent Stem Cell Lines.' *Frontiers in Cellular Neuroscience*, 9(473) pp. 1-9.

Rayment, I., Holden, H. M., Whittaker, M., Yohn, C. B., Lorenz, M., Holmes, K. C. and Milligan, R. A. (1993) 'Structure of the Actin-Myosin Complex and its Implications for Muscle-Contraction.' *Science*, 261(5117) pp. 58-65.

Reichardt, L. F. (2006) 'Neurotrophin-regulated signalling pathways.' *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 361(1473) pp. 1545-1564.

Relaix, F. and Zammit, P. S. (2012) 'Satellite Cells are Essential for Skeletal Muscle Regeneration: The Cell on the Edge Returns Centre Stage.' *Development*, 139(16) pp. 2845-2856.

Roux, P. P. and Barker, P. A. (2002). 'Neurotrophin signaling through the p75 neurotrophin receptor.' *Progress in Neurobiology,* 67(3), pp. 203-233.

Rushton, E., Rohrbough, J. and Broadie, K. (2009) 'Presynaptic Secretion of Mind-the-Gap Organizes the Synaptic Extracellular Matrix-Integrin Interface and Postsynaptic Environments.' *Developmental Dynamics*, 238(3) pp. 554–571.

Sakuma, K. and Yamaguchi, A. (2011) 'The Recent Understanding of the Neurotrophin's Role in SkeletalMuscle Adaptation.' *Journal of Biomedicine and Biotechnology*, 2011(1) pp. 1-12.

Sanes, J. R. and Lichtman, J. W. (1999) 'Development of the Vertebrate Neuromuscular Junction.' *Annual Reviews*, 22(1) pp. 389-442.

Scott, W., Stevens, J. and Binder-Macleod, S. (2001) 'Human Skeletal Muscle Fiber Type Classifications ' *Physical Therapy*, 81(11) pp. 1810-1816.

Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P. and Rudnicki, M. A. (2000) 'Pax7 is Required for the Specification of Myogenic Satellite Cells.' *Cell*, 102(6) pp. 777-786.

Seeburger, J. L., Tarras, S., Natter, H. and Springer, J. E. (1993). 'Spinal cord motoneurons express p75NGFR and p145trkB mRNA in amyotrophic lateral sclerosis'. *Brain Research*. 621(1), pp.111-115.

Sedel, F., Béchade, C. and Triller, A. (1999). 'Nerve growth factor (NGF) induces motoneuron apoptosis in rat embryonic spinal cord in vitro.' *European Journal of Neuroscience*. 11(11), pp. 3904-3912.

Shi, H., Xie, H., Zhao, Y., Lin, C., Cui, F., Pan, Y., Wang, X., Zhu, J., Cai, P., Zhang, H., Fu, X., Xiao, J. and Jiang, L. (2016) 'Myoprotective effects of bFGF on skeletal muscle injury in pressure-related deep tissue injury in rats.' *Burns and Trauma*, 4(26) pp. 1-10.

Shi M., Li J., Maruyama R., Inoue J. and., S. R. (2013) 'FGF19 (fibroblast growth factor 19) as a novel target gene for activating transcription factor 4 in response to endoplasmic reticulum stress.' *The Biochemical Journal*, 450(1) pp. 221-229.

Suzuki, M., McHugh, J., Tork, C., Shelley, B., Klein, S.M., Aebischer, P. and Svendsen, C.N., (2007) 'GDNF Secreting Human Neural Progenitor Cells Protect Dying Motor Neurons, but Not Their Projection to Muscle, in a Rat Model of Familial ALS.' *PLoS ONE* 2(8): e689 pp.1-14.

Steinbeck, J. A., Jaiswal, M. K., Calder, E. L., Kishinevsky, S., Weishaupt, A., Toyka, K. V., Goldstein, P. A. and Studer, L. (2016) 'Functional Connectivity under Optogenetic Control Allows Modeling of Human Neuromuscular Disease.' *Cell Stem Cell*, 18(1), pp. 134-143.

Sterne, G. D., Coulton, G. R., Brown, R. A., Green, C. J. and Terenghi, G. (1997) 'Neurotrophin-3-enhanced nerve regeneration selectively improves recovery of muscle fibers expressing myosin heavy chains 2b.' *J Cell Biol*, 139(3), pp. 709-715.

Tintignac, L. A., Brenner, H.-R. and Rüegg, M. A. (2015) 'Mechanisms Regulating Neuromuscular Junction Development and Function and Causes of Muscle Wasting.' *Physiological Reviews*, 95(3) pp. 809-852.

Ulfhake, B., Bergman, E., Edstrom, E., Fundin, B. T., Johnson, H., Kullberg, S. and Ming, Y. (2000) 'Regulation of neurotrophin signaling in aging sensory and motoneurons: dissipation of target support?' *Molecular Neurobiology*, 21(3), pp. 109-135.

Underwood, C. K. and Coulson, E. J. (2008). 'The p75 neurotrophin receptor.' The International Journal of Biochemistry & Cell Biology, 40(9), pp. 1664-1668.

Vilmont, V., Cadot, B., Ouanounou, G. and Gomes, E. R. (2016) 'A system for studying mechanisms of neuromuscular junction development and maintenance.' *Development*, 143(1) pp. 2464-2477.

Wagers, A. J. and Conboy, I. M. (2005) 'Cellular and Molecular Signatures of Muscle Regeneration: Current Concepts and Controversies in Adult Myogenesis.' *Cell*, 122(5) pp. 659-667.

Wallace, M. A., Hughes, D. C. and Baar, K. (2016) mTORC1 in the Control of Myogenesis and Adult Skeletal Muscle Mass. in Molecules *to Medicine with mTOR: Translating Critical Pathways into Novel Therapeutic Strategies.*, pp. 37-56. Elsevier Inc.

Wang, C., Koh, H. and Ramakrishna, S. (2011) Electrospinning for tissue regeneration - Nerve tissue regeneration. In: Bosworth, L. A. and Downes, S., pp. 168-201. In Woodhead Publishing Series in Biomaterials.

Wang, C.-Y., Yang, F., He, X.-P., Je , H.-S., Zhou , J.-Z., Eckermann , K., Kawamura , D., Feng , L., Shen, L. and Lu, B. (2002) 'Regulation of Neuromuscular Synapse Development by Glial Cell Line-derived Neurotrophic Factor and Neurturin.' *Journal of Biological Chemistry*, 277(12) pp. 10614-10625.

Witzemann, V. (2006) 'Development of the Neuromuscular Junction.' *Cell Tissue Research*, 326(2) pp. 263-271.

Wong, V., Glass, D. J., Arriaga, R., Yancopoulos, G. D., Lindsay, R. M. and Conn, G. (1997) 'Hepatocyte Growth Factor Promotes Motor Neuron Survival and Synergizes with Ciliary Neurotrophic Factor.' *The Journal of Biological Chemistry*, 272(8) pp. 5187–5191.

Wood, S.J., Pritchard, J., and Sofroniew, M.V. (1990), 'Re-expression of Nerve Growth Factor Receptor after Axonal Injury Recapitulates a Developmental Event in Motor Neurons: Differential Regulation when Regeneration is Allowed or Prevented' *European Journal of Neuroscience,* 2(1) pp.650-657.

Wu, H., Xiong, W. C. and Mei, L. (2010) 'To Build a Synapse: Signaling Pathways in Neuromuscular Junction Assembly.' *Development*, 137(7) pp. 1017–1033.

Yamamoto, Y., Livet, J., Pollock, R. A., Garces, A., Arce, V., deLapeyrière, O. and Henderson, C. E. (1997) 'Hepatocyte growth factor (HGF/SF) is a muscle-derived survival factor for a subpopulation of embryonic motoneurons ' *Development*, 124(1) pp. 2903-2913.

Yang, X.-M., Toma, J. G., Bamji, S. X., Belliveau, D. J., Kohn, J., Park, M. and Miller, F. D. (1998) 'Autocrine Hepatocyte Growth Factor Provides a Local Mechanism for Promoting Axonal Growth 'The *Journal of Neuroscience*, 18(20) pp. 8369-8381.

Yin, H., Price, F. and Rudnicki, M. A. (2013) 'Satellite Cells and the Muscle Stem Cell Niche.' *Physiological Review*, 93(1) pp. 23-67.

Yuen, E.C. and Mobley,W. C. (1995).'Therapeutic applications of neurotrophic factors in disorders of motor neurons and peripheral nerves'. *Molecular Medecine Today,* 1(6), 278-286.

Zhang H.Y., Wang Z.G., Wu F.Z., Kong X.X., Yang J., Lin B.B., Zhu S.P., Lin L., Gan C.S, Fu X.B., Li X.K., Xu H.Z. and., X. J. (2013) 'Regulation of autophagy and ubiquitinated protein accumulation by bFGF promotes functional recovery and neural protection in a rat model of spinal cord injury.' *Molecular Neurobiology*, 48(3) pp. 452-464.