The Characterisation of Three-Dimensional Constructs of Muscle Progenitor Cells

Submitted by

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Statement of Authorship

I certify that the attached document is my original work. Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution

Sabrina Erdossy 4 January 2022

Impact Statement

This degree was completed during the COVID-19 pandemic. Ongoing lockdowns and restriction of movement limited access to the laboratory. The reduced time in which the experimental portion of the project could be conducted resulted in changes to the study plan and some experiments needed to be excluded entirely due to time constraints.

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The completion of this Masters project would have been made much harder if it were not for the continued guidance and support.

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Abbreviations

ANOVA - Analysis of Variance

ATP - Adenosine Triphosphate

C2C12 - Mouse Myoblast Cell Line

cDNA - Complimentary Deoxyribonucleic Acid

Col1a1 - Collagen Type I Alpha 1

COPD - Chronic Obstructive Pulmonary Disease

DMEM - Dulbecco's Modified Eagle Medium

DNA - Deoxyribonucleic Acid

dpi - Days Post Injury

DPX - Dibutylphthalate Polystyrene Xylene

FBS - Foetal Bovine Serum

H&E - Haematoxylin and Eosin

HGF - Hepatocyte Growth Factor

IGF - Insulin-like Growth Factor

IL-6 - Interleukin-6

MyHC - Myosin Heavy Chain

NSAID - Non-Steroidal Anti-Inflammatory Drug

OCT - Optimal Cutting Temperature

PBS - Phosphate Buffered Saline

PureZOL - PureZOL RNA Isolation Reagent

qRT-PCR - Quantitative Real-Time Polymerase Chain Reaction

RNA - Ribonucleic Acid

SC - Satellite Cell

SEM - Standard Error of the Mean

TA - Tibialis Anterior

TGF-β - Transforming Growth Factor-β

Tm - Melting Temperatures

TNF-α - Tumour Necrosis Factor-α

VEGF - Vascular Endothelial Growth Factor

WGA - Wheat Germ Agglutinin

$\Delta\Delta C_T$ - Delta Delta Cycle Threshold

Tables and Figures

Abstract

Spheroids are three-dimensional cell aggregates, the implantation of which can improve regeneration in a number of tissues. Previous studies have found that implanting myoblast spheroids into regenerating skeletal muscle in mice improved functional recovery when compared to implanting an equivalent number of 2D-cultured myoblasts. The aim of this study was to determine if forming C2C12 myoblasts into spheroids altered cellular characteristics, and if spheroids had any direct effect on *in vitro* myogenesis. It was hypothesised that the aggregation of C2C12 myoblasts into a spheroid alters the secretome, ultimately upregulating myogenesis in surrounding cells. Spheroids of various sizes (10,000, 25,000 and 100,000 cells) were formed by seeding C2C12 myoblasts into low-attachment plates. Spheroids were incubated in normal growth conditions (DMEM + 10% FBS, 95% O_2/5% CO_2) or a hypoxic environment with glucose- and serum-free media (to replicate conditions in damaged skeletal muscle) for 24 or 48 hours. Cell viability assays found that cells from hypoxic spheroids were less viable than those from normoxic spheroids ($P<0.05$), and that in both cases cell viability decreased as spheroid size increased ($P<0.05$). Real-time PCR to examine changes in expression of key genes involved in muscle repair and regeneration found no difference between spheroids and monolayer cells. Co-culturing spheroids or an equivalent number of monolayer cells with differentiating myoblasts produced myotubes of greater size however there were no changes in gene expression of either Myogenin or myosin heavy chain, two characteristic markers of myogenesis. The findings suggest that forming myoblasts into spheroids has little effect on cell function and that spheroids have little direct influence on myoblast function. Previously observed results *in vivo* may therefore be due to spheroid implantation causing altered inflammatory and/or immune responses in host mice rather than a direct effect on regenerating myoblasts.

CHAPTER 1:

INTRODUCTION

1.1 Introduction

Skeletal muscle is one of the most active and abundant types of tissue in the human body, accounting for over a third of the total body weight [1]. There are a number of key functions that skeletal muscle is responsible for that are essential to human survival including; movement of the diaphragm for breathing, aiding with posture through support of the spine and the provision of movement through skeletal muscles in limbs [2, 3]. In addition good skeletal muscle health is required for overall health due to the role skeletal muscle plays in thermoregulation, metabolic health and bone strength [4]. Defects in skeletal muscle through poor nutrition and a lack of exercise can lead to the development of diseases such as diabetes and heart disease [4]. The highly mobile nature of skeletal muscle means it is susceptible to varying sources of damage such as muscle tears, strains and fibre loss that occur due to trauma, however injury can also occur as a result of disease processes such as muscular dystrophy [5].

1.1.1 Structure of Skeletal Muscle

Skeletal muscle is a complex structure that is comprised of a network of muscle fibres (also known as myofibers) that are interconnected with nerves, blood vessels and other connective tissue, all contained within the epimysium (Figure 1.1). Skeletal muscle attaches to tendons which permit the connection to bone [2]. Each myofiber is made up of multiple myofibrils which allows muscle to produce force necessary for movement [6]. These myofibers are surround by a thin sheath called the endomysium. The endomysium is comprised of collagen that aids in transferring force produced within the myofibers to tendons. Within each skeletal muscle, the muscle fibres are separated into units called fascicles that are surrounded by another sheath called the perimysium [6, 7]. The network of blood vessels and nerves are critical to the health and function of skeletal muscle. Vascularisation of tissue occurs primarily through arteries with muscle fibres sourced via secondary arterioles and capillaries which surround the fibres [8]. Neuronal innervation of skeletal muscle is multifaceted. Sensory and motor nerve fibres work in conjunction with the neuromuscular junction to innervate multiple myofibers and produce an action [9].

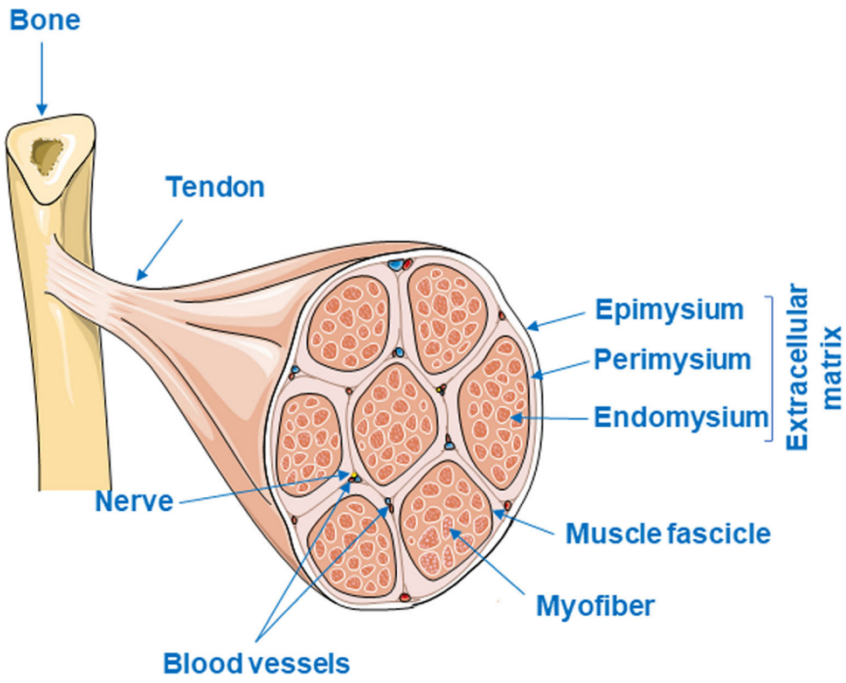

Figure 1.1: Structure of skeletal muscle. Skeletal muscle is composed of several muscle fibres that are interconnected with other connective tissues, blood vessels and nerves. These all play a unique role in ensuring skeletal muscle can carry out its functions. (From: Mahdy [10] Fig 1.)

1.2 Skeletal Muscle Repair Pathway

Skeletal muscle has an innate response that occurs post injury. This response is highly organised and follows a predictable route each time it is activated. There are four stages to skeletal muscle healing: injury/degeneration, inflammation, regeneration and remodelling. Each stage is important to ensure that full function is restored [11]. An injury will initiate the healing process through damage to muscle tissue resulting in muscle degeneration. Muscle degeneration can be characterised by complete or partial rupture or tearing of the myofibers, development of necrosis within the myofibers and the formation of a haematoma. All of these will trigger a disruption to the homeostasis within the skeletal muscle leading to a response from other cells and tissues. As all can impede the ability for complete mobility and function of skeletal muscle it is necessary that the innate system responds quickly to prevent permanent injury [12]. The inflammation stage sees inflammatory mediators (histamine, leukocytes, monocytes etc) flood to the area and aid in the control of any ongoing haemorrhage and protect the system from any foreign bodies that may have entered during injury. Inflammatory mediators will also remove any necrotic tissue that has

developed by phagocytosis by macrophages. The damage will also result in the activation of skeletal muscle progenitor cells which will help to regenerate the lost cell population that will assist at the next stage rebuilding myofibers [13]. Skeletal muscle regeneration is essential for repopulating the myofiber population that was lost due to injury. This is achieved through progenitor cells that act similar to stem cells and proliferate to fill in the gaps where myofibers previously existed. Not all myofibers will be repopulated as some areas will experience scar tissue formation during the remodelling stage [12]. Skeletal muscle remodelling occurs in two phases: the maturation and revascularisation of myofibers and the functional repair of the tissue. The first phase involves regenerated myofibers maturing into a functional form that will enable movement upon completion of tissue repair. Muscle fibres are revascularized during this stage as existing vessels damaged at the time of injury need to be replaced to allow for best possible outcome. The second phase of remodelling represents the stage where function is restored within the muscle. Regenerated myofibers regain essential innervation required for movement and scar tissue is deposited surrounding the injury where myofibers cannot form. Levels of scar tissue formation will depend on the severity of the injury and if the tissue has been damaged before [14].

1.2.1 Skeletal Muscle Progenitor Cells

Skeletal muscle contains a high abundance of undifferentiated muscle progenitor cells which contribute to muscle regeneration. More commonly known as satellite cells (SCs), these cells can be found residing between the basal lamina and sarcolemma (plasma membrane of the myofiber) [10]. Satellite cells are activated when skeletal muscle is injured when there is an increase in inflammatory mediators [15–17]. The ability of SCs to act in response to such markers ensures that skeletal muscle repair and regeneration can be initiated soon after an injury, providing the best chance of a full repair and in turn restoring function to the greatest degree [15]. Satellite cells form an essential part of the skeletal muscle repair and regeneration pathway being one of the primary cell types involved [18]. How well muscle fibres can be repaired is largely dependent on the presence of SCs within skeletal muscle [19]. These structures are in muscle all throughout the body reducing the distance that must be travelled to reach the damaged tissue [20].

Satellite cells are activated when they detect damage to muscle fibres which causes the normal structure of the tissue to which they attach to be disrupted [21]. The process by which muscle regenerates through the proliferation and differentiation of SCs is myogenesis (Figure 1.2) [22]. A SC cannot spontaneously produce new cells to integrate into the damaged muscle and requires a trigger to initiate this cascade. No matter what causes the damage, degeneration partnered with a haematoma to the tissue and necrosis of the myofibers triggers the repair cascade [17]. The natural response when injury occurs is for neutrophils to infiltrate the site causing inflammation. Once adequately saturated with neutrophils, there is a release of pro-inflammatory molecules such as cytokines (TNF-α, IL-6) and growth factors (VEGF, FGF, TGF-β1) creating an environment that is attractive to macrophages and monocytes which are required for regeneration to be viable [12]. M1 macrophages remove cellular debris that develop and stimulate myoblast proliferation and in contrast where as M2 macrophages are responsible for activating the SCs, up to 4 days after an injury has occurred. Both have very differently roles to play but assist in regeneration [23]. After the activation of SCs, they start to proliferate into myoblasts. There is a limited supply of SCs and it is at this stage that they undergo both symmetric and asymmetric division to regenerate lost muscle fibres and replenish stem cells used in this process respectively [24]. Newly proliferated myoblasts are differentiated into myocytes which fuse to form myotubes and become vascularised through angiogenesis triggered upon injury. Myotubes mature into myofibers and undergo innervation completing the process [12]. In some cases, there is an unfavourable result with the production of non-contractile scar tissue resulting in reduced mobility for an individual. A response by macrophages that is unregulated causes the void that is haematoma to be filled with non-contractile fibrotic scar tissue such as collagen instead of the intended myofibers. The deposition of fibrotic scar tissue also occurs when an area has been injured repeatedly. A fine balance of all the steps is required in order to regain full functionality with minimal scarring [25].

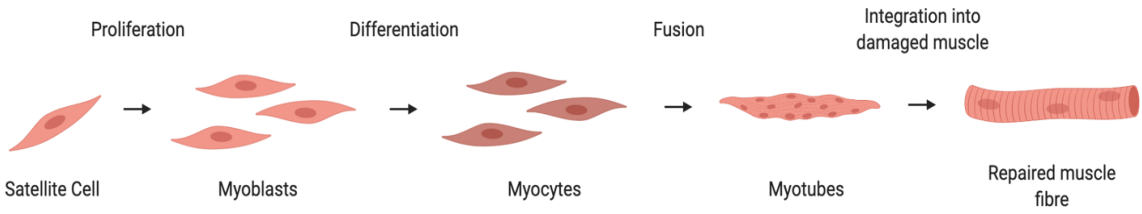

Figure 1.2: Process of myogenesis. Satellite cells proliferate into myoblasts which continue down the myogenic pathway differentiating into myocytes, fusing into myotubes and integrating into the damaged muscle fibre.

1.2.2 Quiescence in Satellite Cells

Cells will expend large amounts of energy through many of their cellular processes such as the cell cycle, protein maintenance and packaging and transport of waste products [26]. To avoid constantly expending large amounts of energy, SCs predominantly reside in a proliferatively inactive state which can be readily reversed. This state of proliferative inactivity is known as quiescence and is vital to the efficient functioning of these cells [27, 28]. Quiescence is a feature of SCs that allows the opportunity to rest when new cells and muscle fibres are not required, preventing the chances of an over-production of differentiated cells [27]. Remaining quiescent also ensures that the cells do not age too fast or prematurely and can remain readily available for the life of the host [29].

1.2.3 Homeostasis in Skeletal Muscle

Homeostasis is the ability of cells, tissues and organisms to regulate physiological processes to maintain a stable state needed for proper functioning. This stable state is achieved through constant regulation and adjustment of biochemical and physiological pathways. When homeostasis of a physiological process is not maintained, there can be serious implications including death. When a change is detected outside of the set limits for the stable homeostatic state, this sets off a chain reaction to address the imbalance [30].

Skeletal muscle repair is often overlooked as a physiological mechanism kept under homeostatic control. Ensuring that there is a balance between proliferation and differentiation is critical for satellite cell function and thus the cellular environment and the overall success of skeletal muscle repair [31]. A disruption in this balance could lead to an under- or over-production of

differentiating cells which could limit the ability for skeletal muscle repair to occur after an injury due to lack of proliferative cells [32]. Asymmetric division allows for the division of a primary cell to have different fates i.e., one differentiated cell and one stem cell. It most often occurs in stem cell lines however can be seen in other cell lines. The asymmetric division that occurs within SCs ensures that there are always cells available to repair damaged skeletal muscle (Figure 1.3) [33]. If this division does not occur in such a manner, cells that participate in muscle repair may not be available and thus returning the damaged muscle to its uninjured state may be unachievable. Asymmetric division also ensures that only some of the cell population differentiates into the muscle fibre, whilst the rest remain as stem cells with the potential to differentiate later. Maintenance of this state of homeostasis is important for preparing and enabling muscle for future repair as necessary [28, 34].

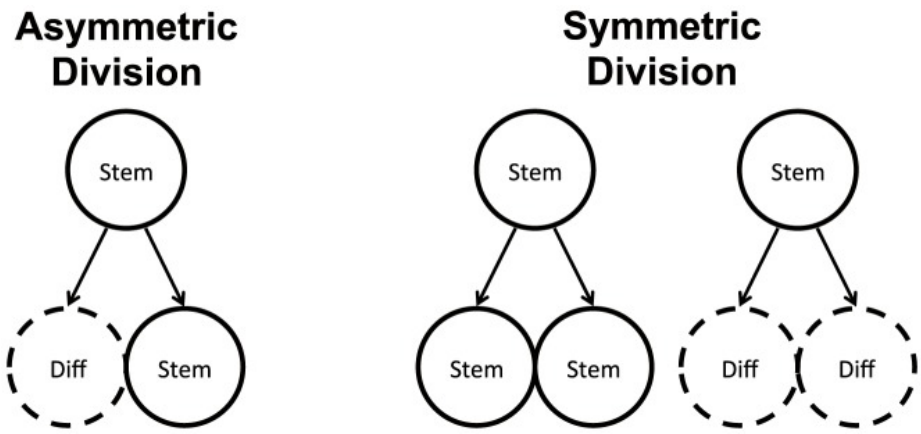

Figure 1.3: Asymmetric and symmetric cell division. In asymmetric division, the stem cell produces one differentiated cell and one stem cell. In symmetric division, the stem cell produces either two stem cells or two differentiated cells. (From: Shahriyari et al. [35] Fig 1.)

1.2.4 Existing Approaches to Skeletal Muscle Repair

Despite the primary function of SCs being to repair damage, they are not always able to fully repair as the degree of damage may be too great and therefore function may not be restored. In these cases, clinical interventions may be required to either assist in speeding up the repair time or restoring the function altogether. Upon experiencing a skeletal muscle injury, most patients will utilise a first-line treatment. This could include seeking out non-steroidal anti-inflammatory drug (NSAID) such as ibuprofen or aspirin to help reduce the pain and inflammation. NSAIDs are not

intended to treat the injury but to provide relief during repair and regeneration that occurs naturally [36]. When injury occurs, the cyclooxygenase (COX) enzymes (COX-1 and COX-2) produce inflammation and pain promoting prostaglandins. NSAIDs are useful in instances where inflammation and pain occur as they selectively inhibit these COX enzymes thereby reducing prostaglandin production [36, 37]. Questions have been raised in recent years as to the effectiveness of NSAIDs and whether they are worsening muscle regeneration by limiting inflammation. Multiple studies have been conducted and shown that mild NSAID dosing does not worsen regeneration however there are limited reports using higher doses and no conclusions have been made on these as yet [38, 39]. Engaging in physical therapy shortly after an injury will provide the least invasive approach to recovery. Using exercises that engage the injured muscle to prevent loss of muscle mass, physical therapy strengthens surrounding muscles which has been shown to promote tissue repair and regeneration [40]. Physical therapy can be used in conjunction with NSAID medication therapy. If an injury is severe resulting in severe muscle volume loss or structural changes have occurred, surgery can prove helpful through the transfer healthy tissue to the area of damage, reinnervation or revascularization of the tissue, or repairs to any breaks in the tissue itself [41]. However, surgery is not suitable for everyone and is generally avoided due to the potential for further scar tissue development [13].

Second-line treatments for muscle repair have been developed for cases where first-line treatments were unsuccessful. Current first-line treatments can be very invasive, have a long recovery and rehabilitation time and may not be suited for every injury. So far, these treatments have had only limited success and are used in only a few countries. These techniques are intended to work alongside the muscle repair process that occurs naturally within the body after damage, with the hope of producing a result that occurs at a faster rate and with less scar tissue formation. These second-line approaches include; autologous stem cell transplantation and injections of growth factors [42, 43].

Stem cell implantation sees satellite cell derived myoblasts injected intramuscularly into the area of injured or diseased muscle. Once implanted these give rise to a large number of cells that can

integrate with the injured region. It has been shown to improve contractile function and even repopulate the depleted SC population within the injured muscle [41]. Growth factor injections are also delivered intramuscularly directly into the area of damage or disease. These injections most often are comprised of a mixture of hepatocyte growth factor (HGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). They will act to assist with muscle repair by stimulating quiescent SCs, stimulating hypertrophy and myogenic differentiation, promoting innervation and reperfusion and self-renewal of satellite cells [41, 44].

A variety of cellular techniques have been studied and used extensively to repair several tissues in the body including skeletal muscle. Single cell implantation is the only approach that has been used to date with success on a limited number of conditions, with *in vivo* models being utilised for muscle wasting [45], dystrophin-deficient mice [46], complete muscle regeneration [47] and formation of new muscle fibres [48]. The implantation of multicellular constructs is also being explored for the potential of increased clinical effects. Most multicellular constructs that have been implanted to date are constructed from cells, such as endothelial and cardiac muscle cells [49, 50] with very few having being formed from skeletal muscle cells [51, 52]. There are limitations with the current cell implantation techniques available. The short lifespan of the cells restricts implantation time frames and results in tissue wastage if the implant cannot take place [53]. Skeletal muscle stem cells proliferate slower than other stem cell lines and may not repopulate an area at a speed that is sufficient for the muscle to restore [54]. These limitations have created the need for the development of a new technique to aid skeletal muscle repair.

1.3 Spheroids

As it has been discussed in 1.2.1, when a tissue is damaged SCs are recruited to the injury site to repair the damaged tissue [55]. Due to the small size of these cells, contact cannot be guaranteed to all cells within an area of damaged tissue. [56]. A recent approach to improve the reparative effects of implantation is to use cells that have been cultured into multicellular structures known

as spheroids. Spheroids are three-dimensional spherical constructs that are formed by the aggregation of single cells to each other (Figure 1.4) [51, 57]. They are formed and implanted into tissues to increase the degree of cell-cell contact that is experienced between the immune cells recruited to repair the damage and the damaged tissues themselves [58]. This technique has been previously successful in wound healing models in other cell types. The number of cells that can aggregate to form a spheroid can vary, with some containing up to 100,000 cells within the one structure [58]. The variation in size enables a variety of tissues to be treated with spheroids with an appropriate size or 'dose' spheroid according to the need [59].

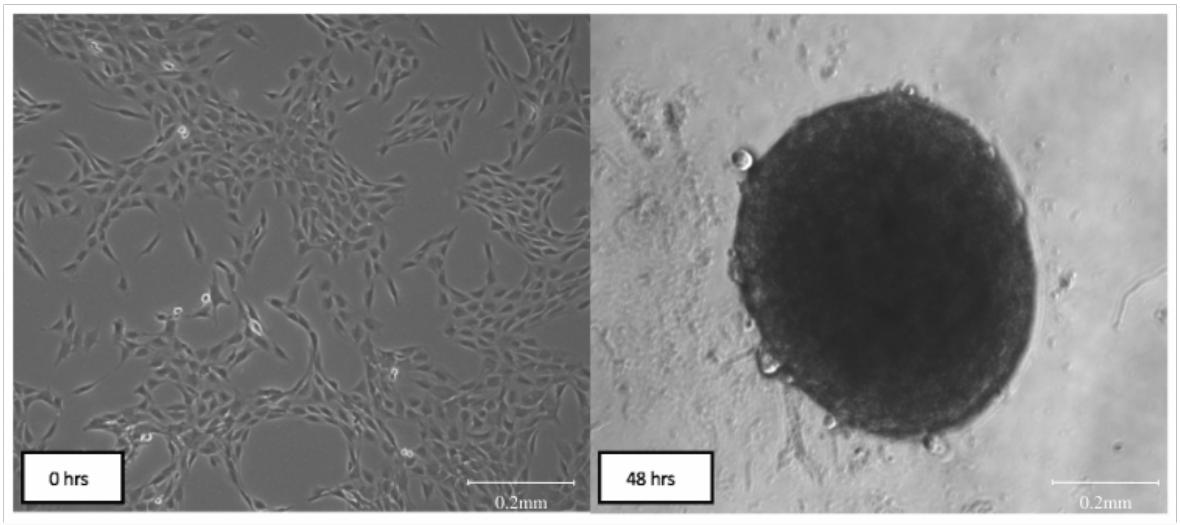

Figure 1.4: Formation of myoblast spheroids. At 0 hours monolayer cells are seeded in growth media. At 48 hours myoblast monolayer cells from a suspension have aggregated together and formed a spheroid.

1.3.1 Structure of a Spheroid

All cells require nutrients and oxygen to perform critical functions and survive. Despite being part of the spheroid all cells still require an equal distribution of nutrients and oxygen to ensure continual growth and survival [60]. Due to the relatively vast size of the structures and overall distance between cells, it is believed however that there may be an imbalance in this distribution. It has been hypothesised on several occasions and in several spheroids of different cell lines that they feature a hypoxic or necrotic core. This is a feature that is commonly observed in tumours, making spheroids ideal for studying tumours. Whilst the use of spheroids to study tumours has been greatly explored, it remains to be understood whether spheroids of a non-tumour cell line

could spontaneously form a tumour. It is believed that the greater the diameter is, the larger the necrotic core may be. As a result of the increased diameter key nutrients have to travel further to reach cells, however the result is in fact more cells are starving [61]. A spheroid is thought to be made up of three zones with decreasing availability of oxygen and nutrient that produce different cellular characteristics, similar to a tumour. The outermost layer features a layer of cells that continue to proliferate, increasing the size of the spheroid over its lifetime. Cells in this layer have the greatest availability of nutrients and oxygen and are the healthiest [62, 63]. The second layer, also known as the quiescent layer, is where it is believed hypoxia begins. Access to oxygen and nutrients begins to deplete and as a result, cells within the spheroid alter the secretome – the set of proteins released into the extracellular space. Finally, the innermost layer is known as the necrotic core. An almost complete lack of oxygen and nutrients has led to cell death due to starvation and resulted in necrosis of cells [62, 63] (Figure 1.5). Alterations in the secretions within the quiescent layer could have a large impact to both the immune and skeletal muscle repair response once implanted.

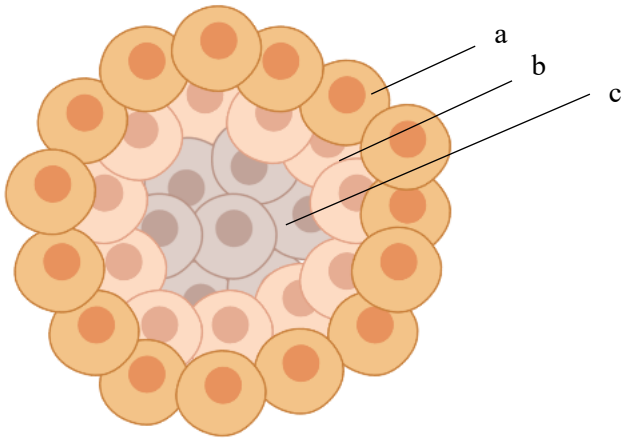

Figure 1.5: Functional zones of a spheroid. Spheroids are believed to be composed of three layers; (a) the outermost proliferative layer, (b) a middle quiescent layer and a (c) necrotic core.

1.3.2 The Formation of Spheroids

Spheroids can be produced in several different ways that vary in the amount of time and cost required. The most commonly used methods include low adherence plates and hanging drop techniques, however there are other techniques including suspension cultures and microfluidic

methods [64–67]. Low adherence plates have round bottom wells and a hydrophilic hydrogel surface coating that prevents cells attaching and utilise a growth media that is suitable for the cell line. Due to the plates shape, cells aggregate and assemble to form a spheroid. This method is more likely to produce spheroids of similar size due to limiting space for multiple small spheroids to form or large blebbing to occur off a main spheroid, however if a lower cell number is initially seeded it is still possible for this to occur [68]. In comparison, the hanging drop method has cells placed into drops of growth media on a dish, inverted over a plate of PBS to prevent drying and allowed to aggregate. This method is more cost effective than the low attachment plates however the lack of a scaffold-like structure means that size and shape is not always as uniform as can be produced when using low adherence plates [68]. All methods that can be used to form spheroids have both positives and negatives however the most commonly observed among all is that no matter how well the technique and conditions are controlled, the shape of the spheroid will begin to distort the larger it gets [51].

1.3.3 Evidence for Use of Spheroids in Tissue Repair

The use of spheroids is a new area of research rapidly expanding in both research and clinical applications. To date, spheroids derived from tumours, kidney and endothelial cells have been used to develop and study disease models [69–71]. Spheroids have also been widely studied as an aid in tissue regeneration [72]. The implantation of spheroids into mice with damaged muscle has shown to result in an enhanced repair response [51]. A previous study using spheroids made from adipose derived stem cells demonstrated enhanced cutaneous wound healing when compared with monolayer cells [73]. It has also been previously shown that implantation of myoblast spheroids into mice who had sustained a muscle injury resulted in increased signs of muscle recovery and regeneration compared with mice that were implanted with monolayer myoblast cells [52]. These studies all indicate that spheroid implantation may prove beneficial in enhancing the recovery process in disease and injury.

It is not yet fully understood what is producing the enhanced response seen in the study completed by Brooks [52]. A possible mechanism could be that the spheroids are secreting growth factors,

cytokines or myokines into the damaged muscle. The study conducted by Cheng et al (2013) [73] noted that spheroids exhibited an increase growth factor production and secretion in comparison to monolayer cells, potentially indicating a significant change in phenotype. A similar response may be occurring in spheroids formed from skeletal muscle like those used by Brooks [52].

The concept of tissue repair and spheroids is still in its infancy and there is still much to be learnt. From the limited studies that have been conducted to date there are indications that spheroids will assist with tissue repair however much further study will be required on specific cell lines and tissues of interest as necessary.

1.3.4 Possible Mechanism of Action in Spheroids

Cytokine is a general term for small proteins that are secreted by cells that have an effect on cell-to-cell communications and actions [74]. Myokines are a specific group of cytokines that are secreted in skeletal muscle by myocytes in response to muscular contractions [75]. One of the most common cytokines are growth factors. Growth factors exist in almost all tissues and control the way cells respond to a biological stimuli evoking responses such as formation of new vasculature, initiating repair pathways and the development of cells [76–79]. An increase in expression of growth factors may be itself responsible for repair occurring more effectively in models implanted with spheroids or the increase in growth factors may be triggering another response. Another possibility is that implantation of spheroids could produce an immune response that itself results in tissue repair that is more effective. Whenever an object is implanted, no matter the size or origin of it, there is the risk that the body will identify it as a foreign body or invader and initiate the inflammatory cascade [80]. This cascade brings with it a multitude of circulating immune and inflammatory cells to combat the foreign body and protect the host [81]. The cells that were initially recruited to repair the muscle damage may therefore be added to by the inflammatory and immune cells activated upon detection of the spheroid, and these cells may work together to produce muscle repair that is greater compared to muscle that has not had spheroids implanted. The implantation of spheroids could also alter the immune or inflammatory response that occurs within the host producing a response.

1.4 Oxygen Demands of Cells

1.4.1 Hypoxia within Cells

The human body relies on oxygen to maintain the actions of all tissues. Tissues usually sit in a state known as normoxia when there is enough circulating oxygen to maintain the function of organs and tissues. When a cell or tissue is receiving lower levels of oxygen than is needed to function it is considered to be hypoxic or experiencing hypoxia [82]. It is common for most cells and tissues to periodically experience a degree of hypoxia due to an event resulting in reduced exposure to oxygen or as a result of insufficient functioning of the respiratory and/or cardiovascular system [83]. The level of oxygen within blood and tissues is recorded as partial pressure of oxygen ($[O_2]$). The expected $[O_2]$ within normoxic blood can range from 90-100mmHg and this number decreases among tissues e.g. normoxic skeletal muscle has an expected $[O_2]$ of 25-31mmHg [84]. When blood or tissue becomes hypoxic the $[O_2]$ value decreases however there are no defined criteria for these.

Hypoxia can occur in any tissue at any time and can be both an acute and chronic pathology. There are many causes of hypoxia including high altitude, pathological conditions such as chronic obstructive pulmonary disease (COPD) and sleep apnoea as well as trauma and injury [85, 86]. Different tissues have different oxygen demands therefore the responses that tissues will present also differ. A lack of oxygen can result in tissue death, which in turn may cause apoptosis of hypoxic cells and apoptosis of cells in nearby tissues. Apoptosis can occur due to upregulation of hypoxia inducible factors (HIF) which can cause activation of the apoptosis cascade through induction of proapoptotic proteins such as Bcl-2 [87, 88]. It is not fully understood why some cells will undergo apoptosis as it is known that HIF are critical in signalling cells to switch metabolic pathways [89].

1.4.2 Energy Requirements in Cells

The requirement for energy is protected by a pathway that ensures cells always have a form of cellular energy. For most cells and tissues in the body the primary form of cellular energy is adenosine triphosphate (ATP) [90]. Energy that is formed as ATP most commonly occurs during

the process of oxidative phosphorylation and approximately 5% of energy is generated through glycolysis [91]. When tissues become hypoxic, the ability to produce energy through the aerobic pathways and thus via ATP is significantly reduced. As a result, depending on the duration of hypoxia and inability to produce energy aerobically, tissues will progressively switch from producing their energy through oxidative phosphorylation to glycolysis in order to meet their energy demands. This form of energy production is not sustainable for a prolonged period of time, as a much smaller amount of energy is produced through glycolysis [92]. Glycolysis can also result in the production of by-products that can have toxic effects to muscles when produced in large enough amounts, in addition to affecting the pH levels within the muscles [93]. The hypoxic environment is one that occurs most commonly in muscle immediately after an injury and whilst it is undergoing repair. This may be due to the usually traumatic nature of injuries which can cause damage to the vasculature within the body of the muscle, resulting in a decrease in blood and hence oxygen supply to the muscles. The occurrence of hypoxia within these muscles results in cells becoming apoptotic due to the upregulation of genes such as HIF [87]. It can be beneficial for tissues to have a degree of HIF upregulation as these factors induce upregulation of genes that are involved in the production of new blood vessels via angiogenesis [94]. Angiogenesis is a critical function that occurs when the existing vascular network has become damaged and can no longer sufficiently exchange nutrients and gasses such as oxygen [95]. Angiogenesis is induced by an increase in the expression of vascular endothelial growth factor (VEGF) which occurs when HIF is upregulated. When this occurs, damaged vessels are repaired as well as possible and new ones are formed to carry these nutrients and gasses around tissues [95]. An increased expression of VEGF by spheroids may result in more efficient repair of the damaged muscle via more rapid reintroduction of nutrients and gasses to the tissues.

1.5 Secretome

Not all proteins that are needed for cellular activities will be produced by the cell performing the action. Other cells can secrete the necessary proteins and hormones and secrete into the extracellular space where they travel to a target tissue. A set of proteins secreted by the cell at any given time is known as the secretome. Secreted proteins account for approximately 10-20% of

proteins in the human proteome [96]. Molecules that are secreted can act in a variety of physiological processes such as cell communication, immune responses, and cell repair. Pathological processes including cancer angiogenesis, metastasis and invasion also heavily rely on proteins that are secreted [97, 98]. The importance of the secretome in these roles has created a clinical relevance as it can provide therapeutic targets as well as provide as source of biomarkers [99].

Molecules that are secreted by cells can be found either as free entities or bound within extracellular vesicles [100]. With a protein that is secreted and transported to its target, the secretory cell does not need to be in the direct vicinity. The ability of proteins to be transported to sites distant from the secretory cell enable different methods of signalling including endocrine, paracrine and autocrine signalling. These different forms of signalling differ on how far the secreted proteins must travel to act on a target and the types of responses they elicit. Endocrine signalling features the secretion of hormones by specialised cells that are transported through blood vessels until they reach their target at a distant body site. An example of this is oestrogen, it is produced and secreted from the ovaries but has several different actions on cells in organs such as the uterus, breast, skin, and brain [101]. Unlike hormones which travel to act at a distant site, some secreted molecules will be released and act in the local area in a paracrine manner. Neurotransmitters such as dopamine must only cross the short distance of the synapse between nerve cells to reach their target and initiate a response [102]. In some cases, cells will respond to the molecules they themselves had produced. This is known as autocrine signalling and is very important for immunity. If a cell becomes infected, it can release a signal to itself to undergo apoptosis, reducing the spread of the infection [103]. As the secreted molecule generally works in the immediate vicinity from where it was secreted in both autocrine and paracrine signalling, it will be present and act at higher concentrations. In contrast hormones that are conveyed via the bloodstream will act at low concentrations on the target cells [104]. If cells are kept in stable conditions, the secretome will not differ greatly as it maintains the ideal state of the organism [2]. The development of a pathological process such as cancer or an infection can greatly affect the secretome, as cells respond to new signals being produced by foreign cells [105, 106]. Upon the

development of cancerous cells, the cellular environment immediately changes, and cells begin to produce responses that would not normally be occurring. Changes in the secretome are necessary for cancerous cells to survive as some proteins normally secreted may hinder survival of cells, whilst upregulating others could be beneficial, even aiding in proliferation and the progression of the cancer [105]. Alterations such as these occur in the secretome with most pathological processes, and these changes are what provide biomarkers for disease detection [106].

The study of secretomes is useful as it can help diagnose disease through identifying certain proteins, identify potential targets that could be used for therapeutics as well as identify proteins required for particular functions e.g., muscle repair. The way the secretome will be examined will depend on whether the protein targets are known. If looking for a known protein of interest or biomarker, the task is considerably easier as a complete secretome analysis is not required. When there is a protein of interest, methods that can be used include qPCR, Western blot and DNA microarray [107, 108]. When the proteins present within a particular secretome are not known, it is not possible to use the aforementioned techniques. In these instances, the aim would be to identify all the secreted proteins. To determine the identity of proteins within the secretome, there are a four commonly used techniques available; mass spectrometry, Serial Analysis of Gene Expression (SAGE), secretion traps and various types of arrays [109]. When working in this manner, results are not controlled for as tightly as one cannot restrict the type of protein they will find using these methods, and any proteins existing within a media solution will interfere with results [110].

1.5.1 Potential Differences Between Spheroid and Monolayer Cell Secretomes

Between 2000 and 2021, the number of secretome studies rose significantly from only one paper published on the topic in 2000 to over 4776 in 2021. Many of these studies were observing the secretome in different pathologies e.g., cancer, diabetes, or infectious diseases on cultured cells. Whilst a significant advancement in secretome knowledge in pathologies has taken place we have yet to see many studies explore the changes that occur through changing the form of monolayer cells to spheroids. It has been suggested that the secretome does differ between these two states, however few have explored this. This idea arose when spheroids were initially used in a wound

healing project. It was seen that the introduction of the spheroid cell clusters formed from mesenchymal stem cells would enhance paracrine induction of wound healing than when the same number of monolayer cells were introduced to tissue that had undergone that same damage in addition to the inflammatory response [69].

1.6 C2C12 Cell Line

The C2C12 cell line is commonly used as an *in vitro* model of myoblast proliferation, differentiation and fusion [111]. The C2C12 cell line was developed as a subclone of the C2 cell line that was initially developed as a control line in 1977 from thigh muscle of C3H adult mice that had experienced a crushing-type injury seventy-two hours prior [112]. Since its development, the C2C12 line has become widely used in skeletal muscle studies. The cells are ideal for use in skeletal muscle research as they are easy to culture and highly proliferative. They can be readily used in both 2D and 3D culture methods and mimic several critical processes in cell formation and development [113]. For successful proliferation of the cell line to take place, conditions must closely mimic physiological conditions in which temperature, oxygen and pH are maintained within certain limits [114]. Proliferation also requires a growth medium (GM), which will generally consist of Dulbecco's Modified Eagle Medium (DMEM) supplemented with foetal bovine serum and if required glutamine. The GM will provide necessary nutrients, hormones and growth factors required for cell proliferation. The hormones and growth factors present within the GM will trigger proliferation of the myoblasts, whilst the inhibitor of differentiation (Id) proteins will upregulate proliferation, preventing the myoblasts from differentiating into myotubes allowing cells to be kept for further passages. Changing the media every 2-3 days ensures cells always maintain a source of nutrients limiting the likelihood of cell death. Maintaining tight incubation conditions such as oxygen (95%), carbon dioxide (5%) and humidity levels is also essential to maintaining cellular survival. Due to the sensitive nature of this cell line, changing the foetal bovine serum to horse serum will cause C2C12 cells to differentiate. The use of horse serum will withdraw cells from proliferation as there is no longer a sufficient supply of

proliferation triggering factors [115]. There is also less insulin within horse serum which is believed to supply cells during proliferation.

Whilst differentiation is most often induced through changing the media, C2C12 cells can also differentiate into myotubes when the plate within which they were seeded becomes overly confluent. Unlike most cell types, the C2C12 cell line will detect when there is little space left to proliferate and will switch to differentiating due to a depletion of growth factors. A large number of cells within the culture plate has an increased nutrient demand reducing factors available within the growth media and therefore can prevent sufficient access to nutrients. The switch to differentiation is a protective mechanism ensuring that there are the highest chances for cell survival [116].

1.7 Skeletal Muscle Models of Regeneration

When studying skeletal muscle physiology and disease, both *in vitro* and *in vivo* models are valid and suitable options to utilise. In most cases using multiple models will provide a more robust result if cellular characteristics seen *in vitro* can account for observations *in vivo*. Selecting which model to use will often depend on funding available and at what stage in a study the model is being used. It is widely accepted to use a cell model if it is a proof-of-concept experiment or in early stages of a study. In comparison it is expected that an animal model would be used in later stages of a study to confirm results obtained using the cell model are valid in animals. It is necessary to utilise both models as not all conclusions can be made using just one and as they are preclinical certainty is needed before use in human studies.

1.7.1 Cell Based Model

The use of cells when undertaking any form of research provides an opportunity to answer questions a research topic may be asking, whilst reducing costs and minimising the impact to animals and humans. It is unnecessary to inflict harm on animals when the effect of a treatment is still unknown demonstrating the critical need to include a cell model in a study. Cell models are low cost and allow large populations of cells to be treated with desired experimental conditions in

preclinical settings. There are limitations when using cell models as some cell lines will not grow in standard culture systems. Selecting the correct cell line is critical to the success and if incorrectly chosen, an experiment can incorrectly reflect behaviour *in vivo* [117].

1.7.2 Animal Based Model

Once an understanding of the cellular behaviour in response to a treatment or the effect of proteins has been established, this knowledge can be used to develop a model in animals to observe whether the results can be replicated. There is a much higher cost associated with the use of animals and due to this is it not always possible to have sample sizes that were as large as with a cell model. Results obtained using this model will however better replicate what will occur in human models and provide critical data for adverse events that can arise. Cell models only observe what is happening within that primary cell line of interest. Animal models are helpful as systems could respond differently to disease and subsequently treatment, and a holistic understanding of a response is required to progress to human studies [117, 118].

1.8 Aim and Hypothesis

The aim of this study was to determine if forming C2C12 myoblasts into spheroids altered cellular characteristics and if spheroids had any direct effect on *in vitro* myogenesis. It was hypothesised that the aggregation of C2C12 myoblasts into a spheroid alters the secretome, ultimately upregulating myogenesis in surrounding cells.

CHAPTER 2:

METHODOLOGY

2.1 Cell Culture

C2C12 skeletal mouse muscle myoblasts were provided by ATCC (Manassas, Virginia, USA). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco Life Technologies, Mulgrave, Vic) which was supplemented with 10% foetal bovine serum (FBS) (Gibco Life Technologies, Mulgrave, Vic) and 1% GlutaMAX (Gibco Life Technologies, Mulgrave, Vic). Cells were incubated in a humidified atmosphere at 37°C with 5% carbon dioxide and 95% oxygen. Cells were grown until ~75% confluence, after which they were washed with 2ml phosphate buffered saline (PBS) (SIGMA-ALDRICH Life Science, Castle Hill, NSW), dissociated with 2ml TryPLE^{TM} (Gibco Life Technologies, Mulgrave, Vic), and either used for experiments (see below) or subcultured at 1:10 dilution into a 75cm^2 flask for future use. All cell culture procedures and experiments were conducted in a class II biosafety cabinet and all associated equipment was sterile.

2.1.1 C2C12 Myoblast Spheroid Generation

Spheroids were formed using C2C12 myoblast cells that had reached approximately 75% confluency. After dissociation with TryPLE^{TM} cells were counted using a haemocytometer and the required number of cells for spheroids with 10,000, 25,000 and 100,000 cells were isolated and seeded in growth media at a total volume of 1mL into 96 well, low attachment plates that featured a hydrophilic non-ionic hydrogel coating (SIGMA-ALDRICH Life Science, Castle Hill, NSW). Plates were placed in a humidified atmosphere at 37°C with 5% carbon dioxide and 95% oxygen and incubated for 48 hours. During this initial time cells naturally aggregated together to form spheroids.

2.1.2 Spheroid Histology

Spheroids were harvested using a pipette with a tip that had been cut to enlarge the hole and sterilised. Spheroids were then washed with PBS and transferred into a small parafilm mould. Spheroids were covered with optimal cutting temperature compound (OCT) (Thermo Fisher Scientific, Scoresby, VIC) and frozen at -80°C. Using a cryostat (Leica CM1850, Leica

Biosystems, Wetzlar, Germany), 10μm sections of spheroids were cut, which were then mounted onto glass slides. A single slide from each sized spheroid was stained using toluidine blue to localise the spheroids and ensure adequate area of the spheroid had been sectioned. All slides were stored at -80ºC for later use.

2.1.3 Haematoxylin and Eosin Staining

Haematoxylin and Eosin (H&E) staining was used to determine the structure of the spheroid samples that had previously been mounted onto glass slides and frozen at -80 ºC. Slides were initially immersed in haematoxylin for 2 minutes which was followed by rinsing 5 minutes in running water. 70% ethanol was applied for 3 minutes which was followed by application of eosin for a further 1 minute. Slides were then immersed in 100% ethanol for 3 minutes x3 and histolene for 3 minutes x3. A drop of dibutylphthalate polystyrene xylene (DPX) was applied and then a cover slip applied and slides allowed to dry. Once slides were dried, sections were examined using an Olympus DP73 camera (Olympus Australia, Notting Hill, VIC).

2.2 Spheroid Conditioning

After cells had aggregated into spheroids in growth media for 48 hours, the media was carefully removed and replaced with fresh growth media (for normal growth conditions) or glucose- and serum-free DMEM (for hypoxic conditions). Cells intended for hypoxic treatment were then placed into a GENbox Jar 2.5L hypoxia box (bioMerieux, Baulkham Hills, NSW) for a further 24 or 48 hours in an incubator at 37 ºC with 0% Oxygen. Hypoxia conditioning was induced through the use of GENbox anaer sachets (bioMerieux, Baulkham Hills, NSW) which absorb the oxygen present in the GENbox Jar to create the required hypoxic environment. It was confirmed that hypoxia had been achieved using anaerobic indicators that change colour upon exposure to oxygen (Thermo Fisher Scientific, Scoresby, VIC). A second spheroid plate that consisted of growth media was returned to the incubator for 24 or 48 hours as a control. For each condition, a 60mm dish plated with C2C12 cells was used as a monolayer control. After the allotted time had elapsed, plates were removed from incubators and spheroids were pooled together according to

their condition. Media was collected from the pooled spheroids and stored at -80ºC for future experiments. Spheroids were prepared for qRT-PCR via RNA isolation. Samples were stored at -80°C for use in qRT-PCR.

2.3 Effect of Preconditioned Media on C2C12 Myoblast Cells

C2C12 myoblast cells were split and seeded in growth media in a 12-well plate. Cells were left until they had adhered to the plate, after which growth media was removed and replaced with media that had been previously collected from spheroids in preconditioning experiments (see above). Cell were then placed in an incubator for 24 hours before being prepared qRT-PCR via RNA isolation.

2.4 Effect of Time and Oxygen Availability on Size of Spheroids

Prior to the media being changed on spheroids images were taken at x100 magnification on an AxioCam ICM1 (Zeiss^{TM}) microscope. After the spheroids were conditioned for 24 or 48 hours in both normal cell culture conditions and hypoxic conditions images were retaken. The size of the spheroids (in pixels) of the spheroid images obtained before and after conditioning were measured using the program ImageJ. Size was recorded in area with the unit of measurement set to μm^2.

2.5 Cell Viability Assay

2.5.1 Generating a Monolayer Control

C2C12 myoblast cells were counted using a haemocytomer and 1,500 cells were seeded onto 96 well plates in growth media. After 48 hours the growth media was removed from each plate and cells were treated with either growth media or glucose- and serum-free media. The plates were then placed into a GENbox Jar 2.5L hypoxia box in the incubator (for hypoxic treatment group) or directly into the incubator (normoxic treatment group) for 24 hours. After 24 hours elapsed, plates were removed and cell viability was assessed.

2.5.2 Cell Viability Assay

The assay was conducted on the monolayer controls developed above and 5 spheroids of each size and condition that had been incubated for a total of 72 hours. Initially the CellTiter-Glo® 3D Reagent (Promega, Auburn, Victoria) was thawed overnight at 4°C. Approximately 30 minutes prior to using the CellTiter-Glo® 3D Reagent it was placed at room temperature to equilibrate. Plates containing spheroids and monolayer cells were also equilibrated to room temperature for approximately 30 minutes. To each well, 100µl of CellTiter-Glo® 3D Reagent was added and contents were mixed vigorously for 5 minutes using an electronic plate shaker. Plates were incubated in a dark room at room temperature for a further 25 minutes to stabilize the luminescent signal. Using ATP free filter tips, contents were transferred to white opaque wall plates with clear bottoms (Sigma-Aldrich Life Sciences, Castle Hill, NSW) that had been equilibrated to room temperature as well. After this time luminescence in each well was measured for 5 seconds using a TRIAD Multimode Detector plate reader (Dynex Technologies, Denkendorf, Germany).

2.6 Effect of Co-culture on Myoblast Differentiation

C2C12 myoblast cells were used to form 25,00 cell spheroids following the protocol described above, and maintained for 24 hours, at which point a separate passage of C2C12 myoblast cell were split and sparsely seeded with growth media in a 24-well plate. After waiting 24 hours for the cells to adhere, the growth media was removed and replaced with differentiation media (DMEM supplemented with 2% horse serum) (Gibco Life Technologies, Mulgrave, Vic) or fresh growth media. Nunc™ Polycarbonate Cell Culture Inserts (Thermo Fisher Scientific, Scoresby, VIC) were placed into each of the wells and either 10 of the 25,000 cell spheroids or 250,000 monolayer cells were added to an insert. Some wells were left without an insert to act as controls. The plate was returned to the incubator for a further 3 days, after which the wells were imaged using ZEN microscope imaging software (Zeiss, Oberkochen, Germany). Upon obtaining all images, all media was removed and 500µl of PureZOL™ RNA Isolation Reagent (PureZOL) (BioRad Laboratories, Oakleigh East, VIC) was applied to each well for RNA isolation. Samples were stored at -80°C for use in qRT-PCR.

2.6.1 Myotube Size Analysis

Using the images obtained with the ZEN microscope imaging software, the sizes of myotubes were analysed using the ImageJ image processing and analysis software (National Institutes of Health, Bethesda, Maryland, USA). Size was recorded in area with the unit of measurement set to μm^2.

2.7 RNA Isolation

RNA was isolated from samples by adding 500µl of PureZOL™ RNA Isolation Reagent (PureZOL) (BioRad Laboratories, Oakleigh East, VIC). Samples were left to incubate on rocker at low speed for 5 minutes. PureZOL containing cells was collected and placed in a 1.5ml Eppendorf tube. 100µl of chloroform (VWR International, Murarrie, QLD) was added for every 500µl of PureZOL originally used and samples were mixed by shaking for 15 seconds. Samples were left at room temperature to incubate for 5 minutes and then centrifuged (12,000 x g, 15 minutes, 4°C) to separate the phases of the mixture. The aqueous phase was removed and placed in an RNase-free tube where 250µl of isopropyl alcohol (Chem-Supply, Gillman, SA) was added per 500µl of PureZOL originally added and the sample mixed using the pipette. Samples were incubated at room temperature for 5 minutes and then centrifuged (12,000 x g, 10 minutes, 4°C). The supernatant was carefully discarded and the RNA pellet was washed with 500µl of 75% ethanol for every 500µl of PureZOL originally added. Samples were then briefly vortexed and centrifuged (7,500 x g, 5 minutes, 4°C). The supernatant was again carefully discarded and the pellet was air-dried for 5 minutes and resuspended in 30µl RNase-free water. Quantification of RNA levels and purity was completed using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Scoresby, VIC) measuring the absorbance at wavelengths of 260 nm and 280 nm. RNA was stored at -80°C for future use.

2.8 cDNA Synthesis

Synthesis of cDNA was performed using an iScript cDNA Synthesis Kit (BioRad Laboratories, Oakleigh East, VIC). Each reaction mix consisted of 2µl 5x iScript reaction mix, 0.5µl iScript

reverse transcriptase, and 7.5µl of RNA template and H_2O for 500ng, for a total sample volume of 10µl. Once combined, samples were run on a BioRad C1000 Touch Thermal Cycler (BioRad Laboratories, Oakleigh East, VIC) (5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, then held at 4°C). Samples were diluted with 190µl nuclease free water to a final volume of 200µl and stored at -20°C for future use.

2.9 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

In a 96 well PCR plate, 2µl nuclease free water, 0.5µl of each primer mix at a stock concentration of 10µM, 5µl Sso Fast EvaGreen Supermix (BioRad Laboratories, Oakleigh East, VIC) and 2µl of cDNA sample was added per reaction. Reactions were performed in triplicate. The plate was incubated for 30 seconds at 95°C in a BioRad C1000 Touch Thermal Cycler (BioRad Laboratories, Oakleigh East, VIC) followed by the cycling parameters; 95°C for 5 seconds, 55°C for 5 seconds for a total of 40 cycles. This was followed by a melt curve analysis for 5 minutes with the temperature increasing from 65°C to 95°C in 0.5°C increments. Gene expression was quantified by normalising the gene expression to the reference gene 18S or β2M, using the $\Delta\Delta C_T$ method. Primers used are listed in Table 2.1.

2.9.1 Primer Design

Primers were obtained from Sigma-Aldrich Life Sciences (Castle Hill, NSW) and GeneWorks Pty Ltd (Thebarton, SA) (Table 2.1).

Table 2.1: Primer sequence and melting temperatures (Tm) for gene analysis in qRT-PCR

Target Gene		**Sequence 5'-3'**	**Tm (°C)**
18S	Forward	GGTGCATGGCCGTTCTTA	50.0
(Housekeeper)	Reverse	TCGTTCGTTATCGGAATTAACC	51.0
β2M	Forward	GTATGCTATCCAGAAAACCC	57.6
(Housekeeper)	Reverse	CTGAAGGACATATCTGCATC	56.1
VEGFA	Forward	TAGAGTACATCTTCAAGCCG	57.1
	Reverse	TCTTTCTTTGGTCTGCATTC	59.4
IL-6	Forward	AAGAAATGATGGATGCTACC	58.0
	Reverse	GAGTTTCTGTATCTCTCTGAAG	53.9
Col1a1	Forward	GATCTGTATCTGCCACAATG	58.1
	Reverse	TGGTGATACGTATTCTTCCG	60.0
Bax	Forward	CCTTTTTGCTACAGGGTTTC	60.2
	Reverse	ATATTGCTGTCCAGTTCATC	56.5
Bcl2	Forward	ATGACTGAGTACCTGAACC	53.6
	Reverse	ATATAGTTCCACAAAGGCATC	57.1
TNFα	Forward	AGTTGGGGAGGGAGACCTT	53.0
	Reverse	CATCCACCCAAGGATGTTTAG	52.0
TGFβ	Forward	ATTCCTGGCGTTACCTTGG	51.0
	Reverse	CCTGTATTCCGTCTCCTTGG	54.0
Myogenin	Forward	AGTACATTGAGCGCCTAC	55.3
	Reverse	CAAATGATCTCCTGGGTTG	60.5
MyHC	Forward	TCCAAACCGTCTCTGCACTGTT	55.0
	Reverse	AGCGTACAAAGTGTGGGTGTGT	55.0

2.10 Immunohistochemistry

2.10.1 Tissue Preparation and Histological Analysis

The Tibialis Anterior (TA) muscle was previously harvested from mice treated with spheroids. The mice underwent a myotoxic muscle injury at d0 of the study and at d3 were treated with an intramuscular injection of 20 x 25,000 cell spheroids or equivalent number of monolayer cells or saline for the control. At d21 post injury the TA was harvested [52]. Using a cryostat, 10μm sections of TA muscle were cut, which were then mounted onto glass slides. A single slide from each condition was stained using toluidine blue to localise the TA and ensure adequate area of the TA had been sectioned as well as the direction of the fibres was correct. All slides were stored at -80ºC for later use. Utilising the protocol previously described in 2.1.3, all samples underwent a H&E stain prior to any immunohistochemistry.

2.10.2 Wheat Germ Agglutinin Stain

A Wheat Germ Agglutinin (WGA) stain was used to stain fibrotic tissue within the Tibialis Anterior muscle. Sections were removed from -80ºC and examined. Any samples that were no longer intact or showed damage from the freezer were discarded. Sections were allowed to thaw for 15 minutes at room temperature. After this time sections were fixed in 4% cold paraformaldehyde (PFA) (SIGMA-ALDRICH Life Science, Castle Hill, NSW) in PBS (SIGMA-ALDRICH Life Science, Castle Hill, NSW) for 10 minutes. Slides were then washed in PBS. They were then incubated in WGA-FITC (1:50) (Thermo Fisher Scientific, Scoresby, VIC) in PBS for 15 minutes whilst being protected from light. The slides were washed again with PBS, dried and a coverslip was mounted with DAPI free mounting medium (Biotium, California, USA) before being viewed under the fluorescent microscope (Leica, DMRBE, Leica Microsystems, Wetzlar, Germany).

2.10.3 Fibrosis Analysis

Using the ImageJ image processing and analysis software (National Institutes of Health, Bethesda, Maryland, USA), images obtained during the WGA stain were analysed for the

development of fibrotic tissue in samples from d14 and d21. Samples from d7 were excluded as fibrotic tissue formation does not occur until approximately 10 days after an injury.

2.11 Statistical Analysis

All data were expressed as mean ± standard error of the mean (SEM). Statistics were performed using either paired t-tests, one-way ANOVAs with a Tukey's post-hoc test or Kruskal-Wallis test as appropriate.

All data and statistics were collated and analysed using GraphPad Prism 9.0 Software. For all analyses a P value of < 0.05 was considered statistically significant.

CHAPTER 3: RESULTS

3.1 Spheroid Histology

Examination of C2C12 myoblast spheroid sections under the microscope showed the effects of time and condition on the appearance of the spheroids (Figure 3.1 and Figure 3.2). In spheroids that were exposed to normal cell culture conditions (DMEM with 10% FBS and 1% GlutaMAX added incubated in a humidified atmosphere at 37°C with 5% carbon dioxide and 95% oxygen), there were no distinct changes between the 24- and 48-hour time groups (Figure 3.1). When examining the spheroids that underwent hypoxic conditioning (glucose- and serum-free DMEM and a hypoxia chamber), a similar observation was noted with the 10,000 and 100,000 cell spheroids. It was observed that in the 25,000 cell spheroids there was a change in the shape (Figure 3.2).

Examination of spheroid sections that had been stained with H&E demonstrated that the spheroids formed uniformly after 48 hours (Figure 3.3). It was also seen that there appeared to be no large areas within the spheroid that formed without cells. The most notable finding from the H&E staining was the presence of a distinct capsule that had formed around the outside of the spheroid during cell aggregation (Figure 3.3). As seen in Figure 3.3, only spheroids that were kept in normal cell culture conditions were harvested and stained. This was due to the fragility of hypoxic spheroids preventing sectioning required for staining.

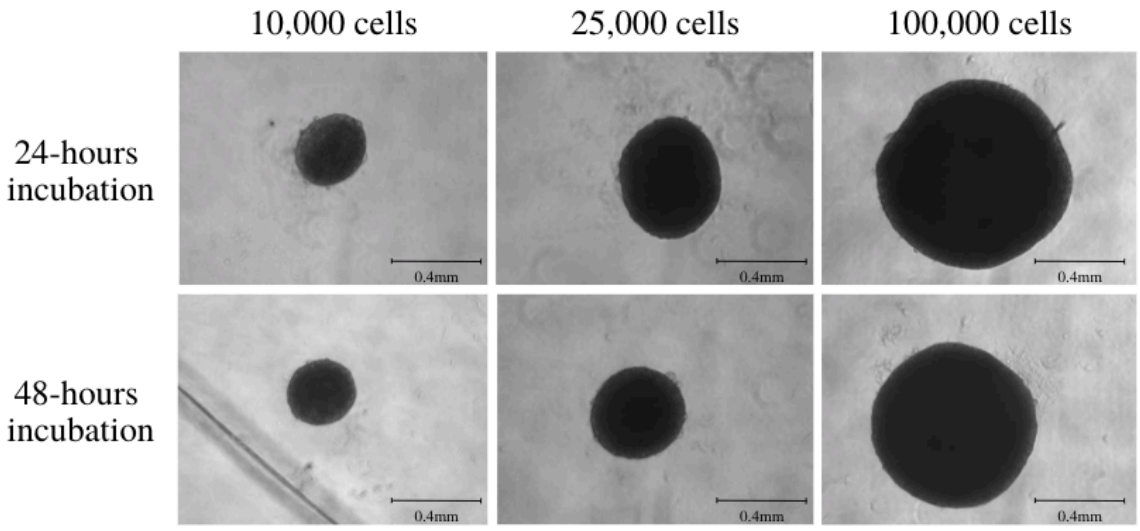

Figure 3.1 Microscopy of spheroids 24- and 48-hours post normal cell culture conditions.

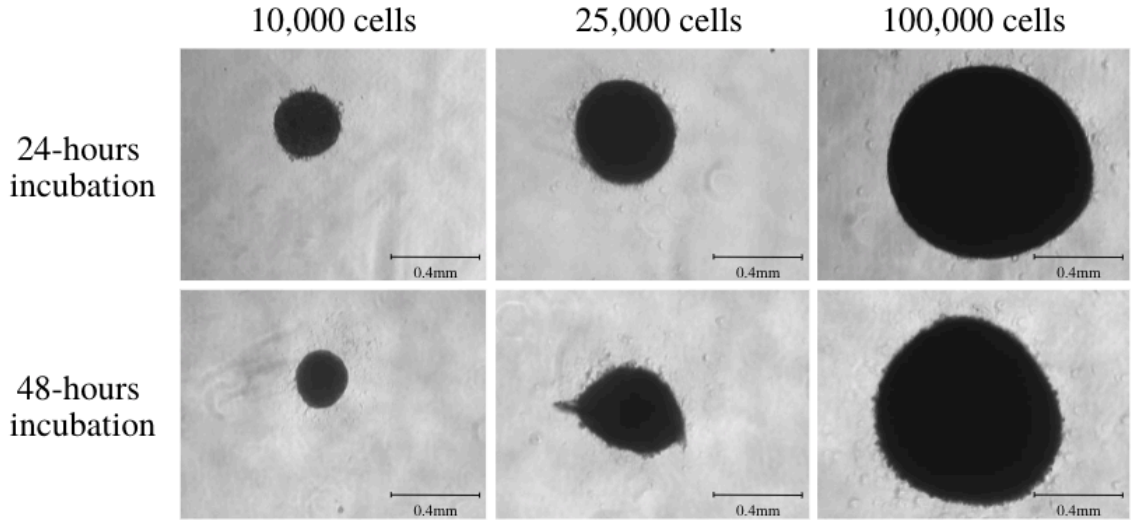

Figure 3.2 Microscopy of hypoxic spheroids 24- and 48- hours post conditioning.

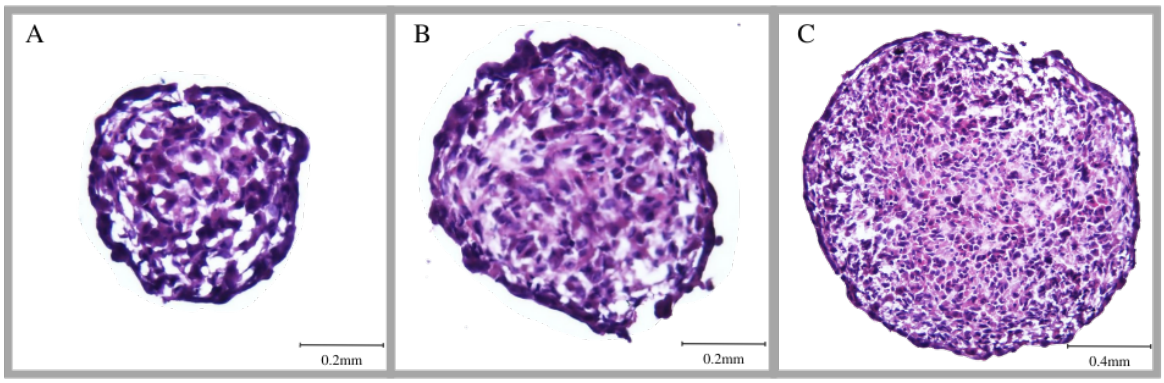

Figure 3.3: Microscopy of H&E-stained spheroids that were exposed to normal cell culture conditions for 48 hours. (A) 10,000 cells, (B) 25,000 cells and (C) 100,000 cells.

3.2 Effect of Time and Culture Conditions on the Size of C2C12 Myoblast Spheroids

Many cell types continue to proliferate after spheroid formation, which could lead to an increase in spheroid size [119]. There is no information on whether this is the case in spheroids formed from C2C12 myoblasts as so few studies have been conducted using this cell line in this application. When the same spheroids were measured pre- and post-hypoxia or normal cell culture conditioning (Figure 3.4 and Figure 3.5); a significant decrease in size was observed for all of the spheroids that had exposure to normal cell culture conditions for 24 hours ($p<0.05$, Figure 3.6). A significant decrease in size was also observed between pre- and post-conditioning of each of the spheroids of all sizes at the 48-hour time point ($p<0.05$, Figure 3.7).

The same comparison was performed in spheroids that had been placed in hypoxic conditions and glucose and serum free media to mimic repairing muscle and a similar result was noted. A significant decrease in size was observed between pre- and post-conditioning of each of the spheroids that had undergone hypoxia for 24 hours ($p<0.05$, Figure 3.8). A significant decrease in spheroid size was also observed between pre- and post-conditioning of each of the spheroids that had undergone hypoxic conditioning for 48 hours ($p<0.05$, Figure 3.9).

The effect of culture conditions on size was analysed to determine whether one condition would have a greater effect on size than the other. The analysis demonstrated that when the size of spheroids of the 24-hour conditioning group and same size from different conditions were compared there was no change in the size of the spheroids despite the differing nutrient and oxygen availability (Figure 3.10). This comparison was repeated on spheroids that were conditioned for 48 hours and the same result was observed (Figure 3.11).

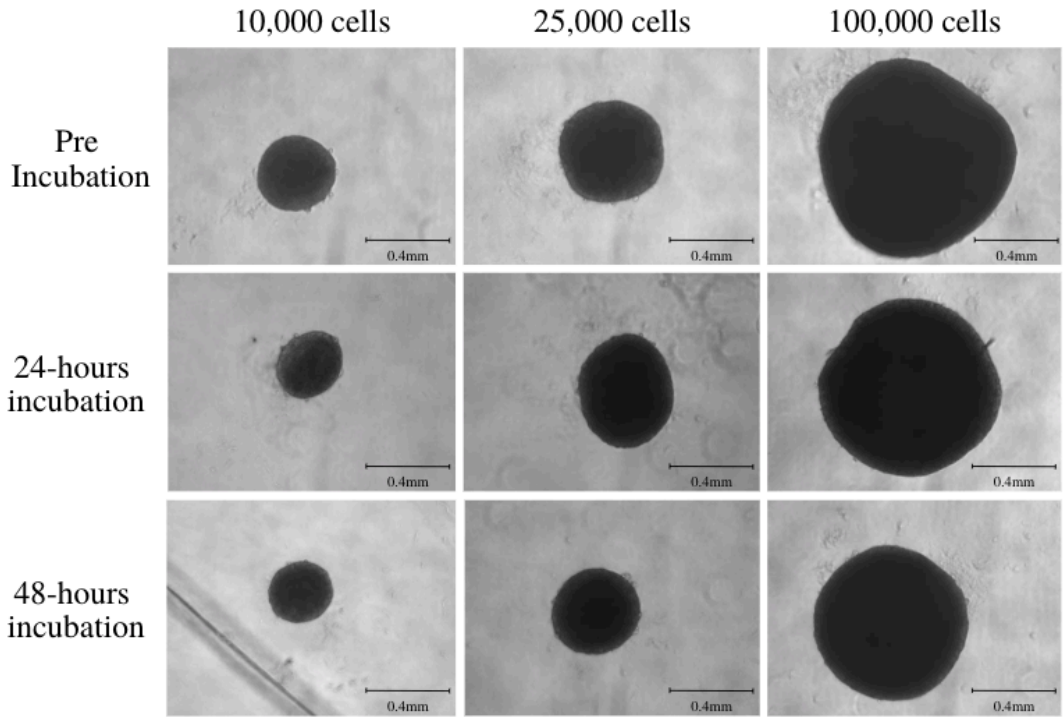

Figure 3.4: Effect of time on the size of spheroids exposed to normal cell culture conditions. Spheroids made up of 10,000, 25,000 or 100,000 C2C12 myoblast cells were incubated in growth media and normal growth conditions for either 24 or 48 hours.

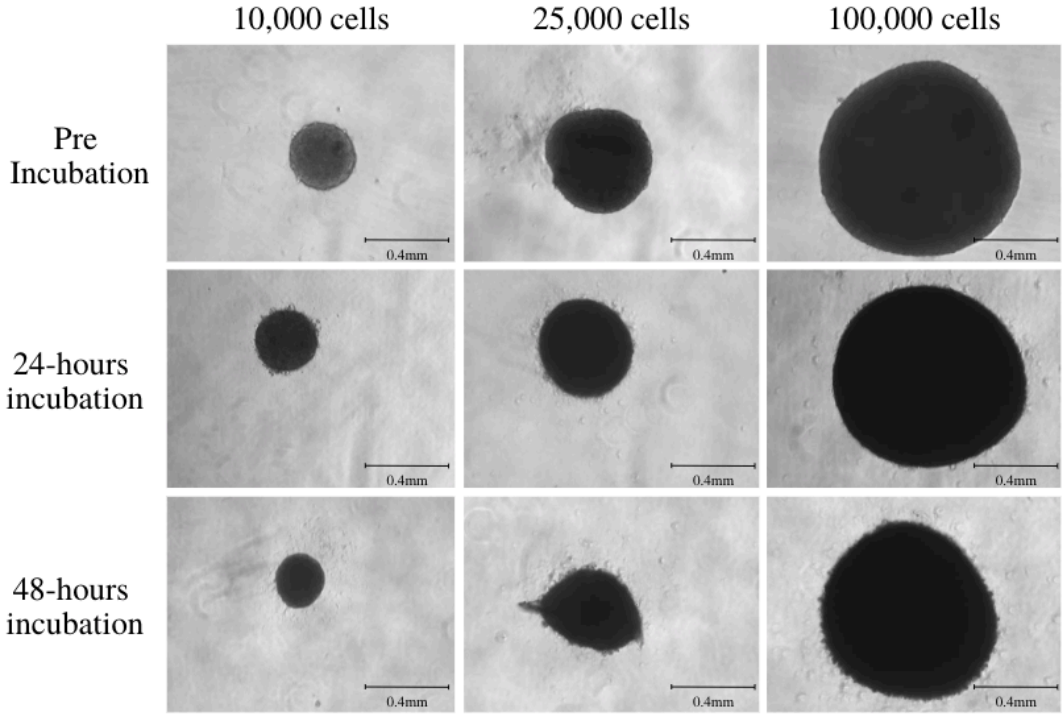

Figure 3.5: Effect of time on the size of hypoxic spheroids. Spheroids made up of 10,000, 25,000 or 100,000 C2C12 myoblast cells were conditioned in glucose and serum free media and hypoxic conditions for either 24 or 48 hours.

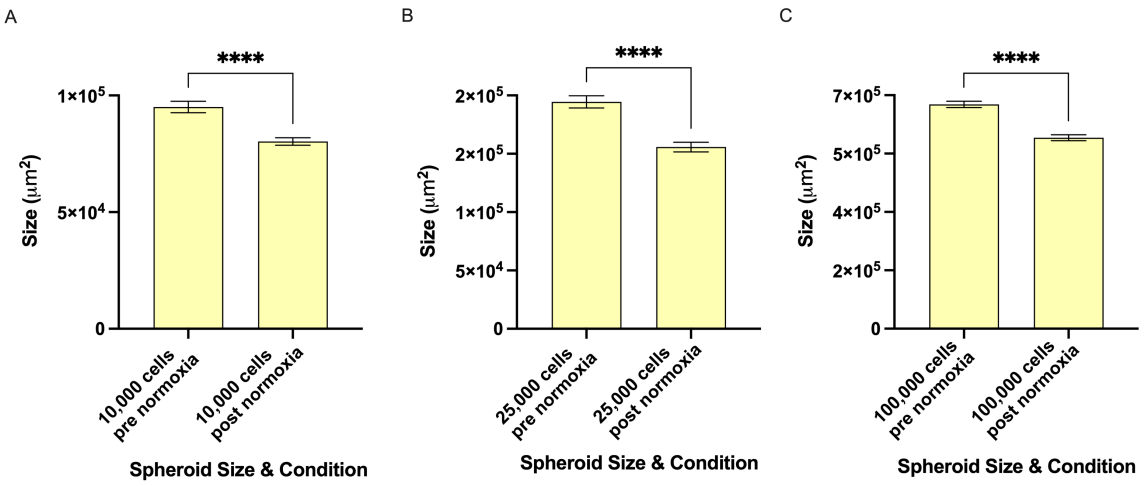

Figure 3.6: Effect of time on the size of spheroids exposed to normal cell culture conditions for 24 hours. Spheroids made up of 10,000 (A), 25,000 (B) or 100,000 (C) C2C12 myoblast cells were conditioned in growth media and normal growth conditions for 24 hours. *p<0.05 pre vs post condition using paired t-test. Data expressed as mean ± SEM, (n=4 with 10 replicates).

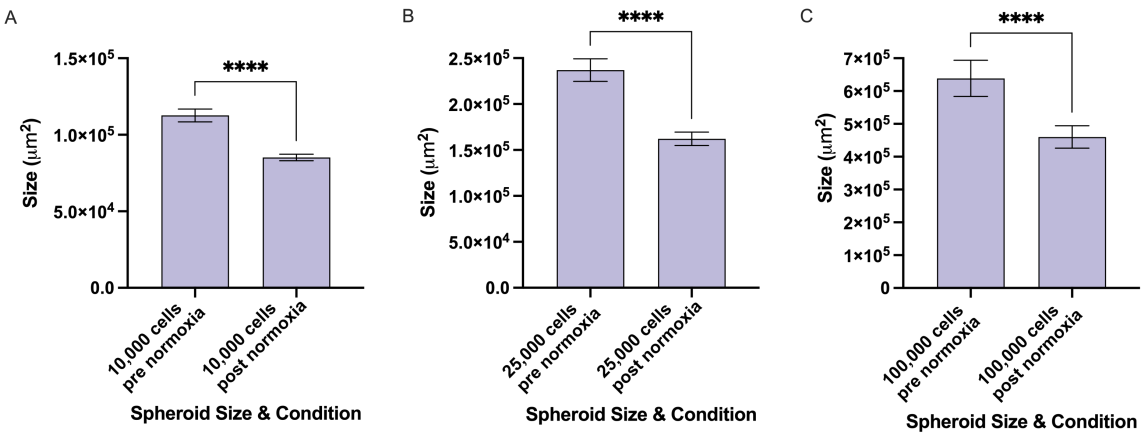

Figure 3.7: Effect of time on the size of spheroids exposed to normal cell culture conditions for 48 hours. Spheroids made up of 10,000 (A), 25,000 (B) or 100,000 (C) C2C12 myoblast cells were conditioned in growth media and normal growth conditions for 48 hours. *p<0.05 pre vs post condition using paired t-test. Data expressed as mean ± SEM, (n=4 with 10 replicates).

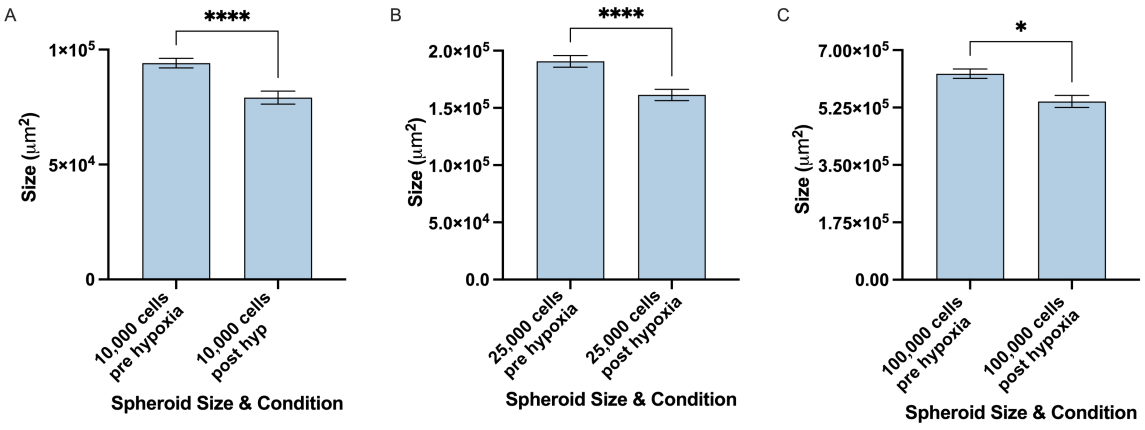

Figure 3.8: Effect of time on the size of spheroids exposed to hypoxic conditions for 24 hours. Spheroids made up of 10,000 (A), 25,000 (B) or 100,000 (C) C2C12 myoblast cells were conditioned in glucose and serum free media and hypoxic conditions for 24 hours. *p<0.05 pre vs post condition using paired t-test. Data expressed as mean ± SEM, (n=4 with 10 replicates).

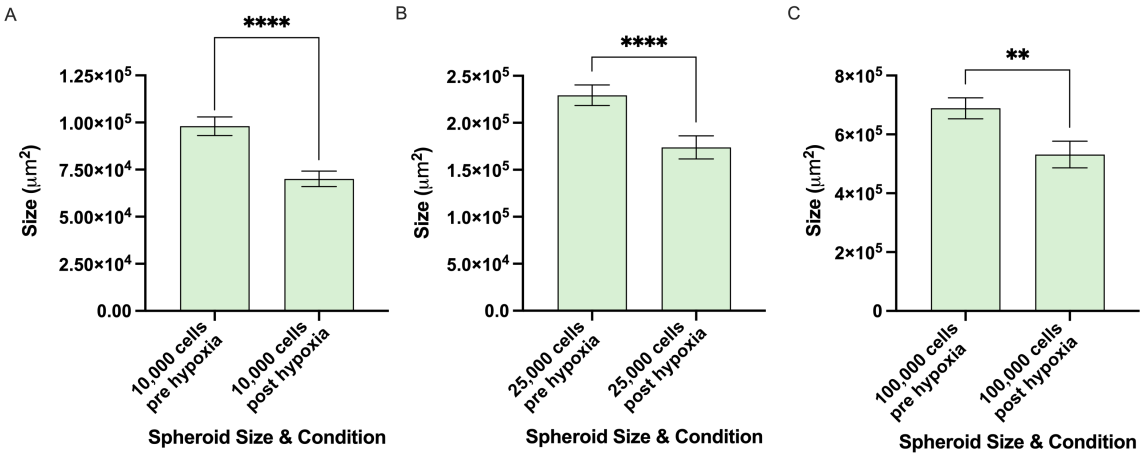

Figure 3.9: Effect of time on the size of spheroids exposed to hypoxic conditions for 48 hours. Spheroids made up of 10,000 (A), 25,000 (B) or 100,000 (C) C2C12 myoblast cells were conditioned in glucose and serum free media and hypoxic conditions for 48 hours. *p<0.05 pre vs post condition using paired t-test. Data expressed as mean ± SEM, (n=4 with 10 replicates).

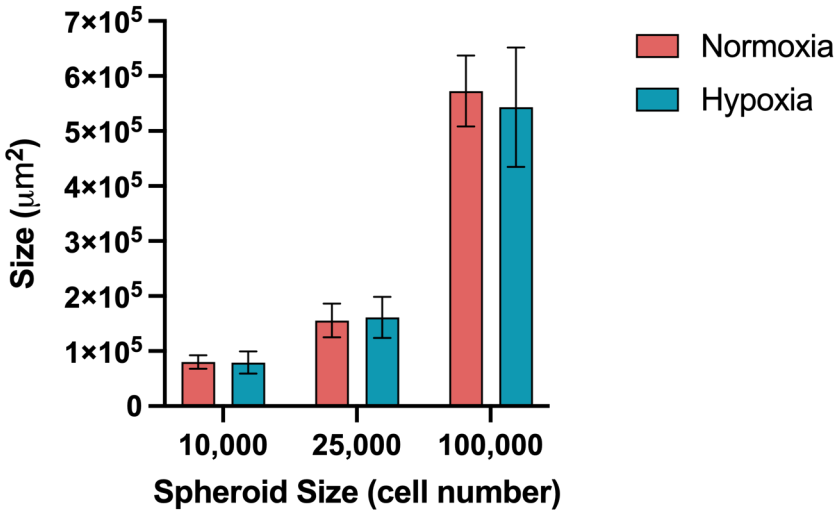

Figure 3.10: Effect of 24 hours conditioning on the size of spheroids. Spheroids made up of 10,000, 25,000 or 100,000 C2C12 myoblast cells were conditioned in normal growth media and normoxic conditions or glucose and serum free media and hypoxic conditions for 24 hours. $p>0.05$ using two-way ANOVA. Data expressed as mean ± SEM, (n=4 with 10 replicates).

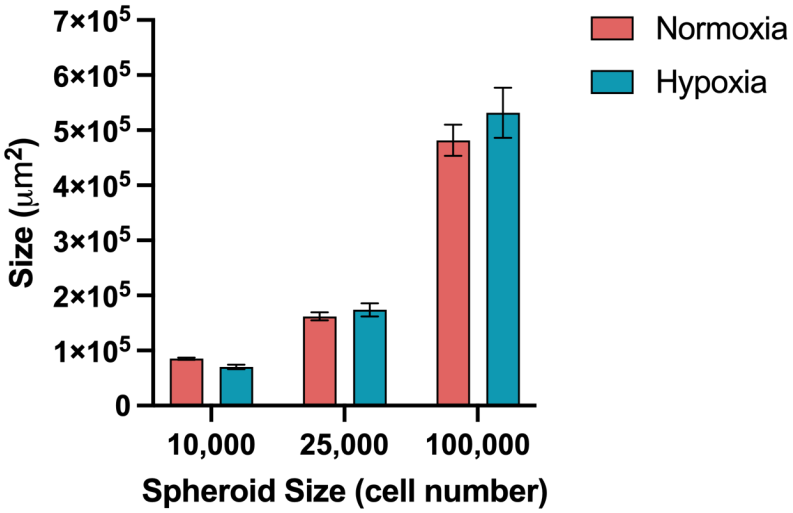

Figure 3.11: Effect of 48 hours conditioning on the size of spheroids. Spheroids made up of 10,000, 25,000 or 100,000 C2C12 myoblast cells were conditioned in normal growth media and normoxic conditions or glucose and serum free media and hypoxic conditions for 48 hours. $p>0.05$ using two-way ANOVA. Data expressed as mean ± SEM, (n=4 with 10 replicates).

3.3 Effect of Time and Oxygen Availability on Cell Viability of C2C12 Myoblasts in Spheroids

Due to the nature of the previous study involving implantation of spheroids into damaged muscle, examining the viability of C2C12 myoblast spheroids provided insight into how well they potentially survived post implantation. We also wanted to determine if there was a difference in viability in spheroids that had been conditioned in either normal culture conditions or hypoxia. When measuring cell viability of the spheroids via the CellTiter-Glo assay, it was observed that there was a significant decrease in viability among the spheroids that had undergone normal cell culture conditions as the size of the spheroid decreased ($p<0.05$, Figure 3.12). In comparison to spheroids that were kept in normal growth culture conditions, spheroids that had been conditioned in hypoxia to mimic repairing muscle showed much lower viability. Viability did not increase in this group following a linear type of relationship as seen in normoxic conditioned spheroids (Figure 3.12).

Due to the varying sizes of the spheroids, we normalised the cell viability to cell number. This resulted in cell viability decreasing overall as the size of the spheroid increased (Figure 3.13). To determine which spheroid size was most viable in each condition, spheroids were compared and in both the spheroid groups that had been conditioned with hypoxia and normoxia, the spheroids with 100,000 cells had the lowest viability (Figure 3.13). In spheroids that had undergone normoxic conditioning it was found that there was a significant decrease in viability between the 10,000 cell spheroids and the 25,000 and 100,000 ($p<0.05$, Figure 3.13). It was observed in spheroids that underwent hypoxic conditioning there was a significant decrease in viability between the 10,000 cell and 100,000 spheroids ($p<0.05$, Figure 3.13). When viability was normalised to spheroid size, we observed the greatest viability in the smallest spheroids.

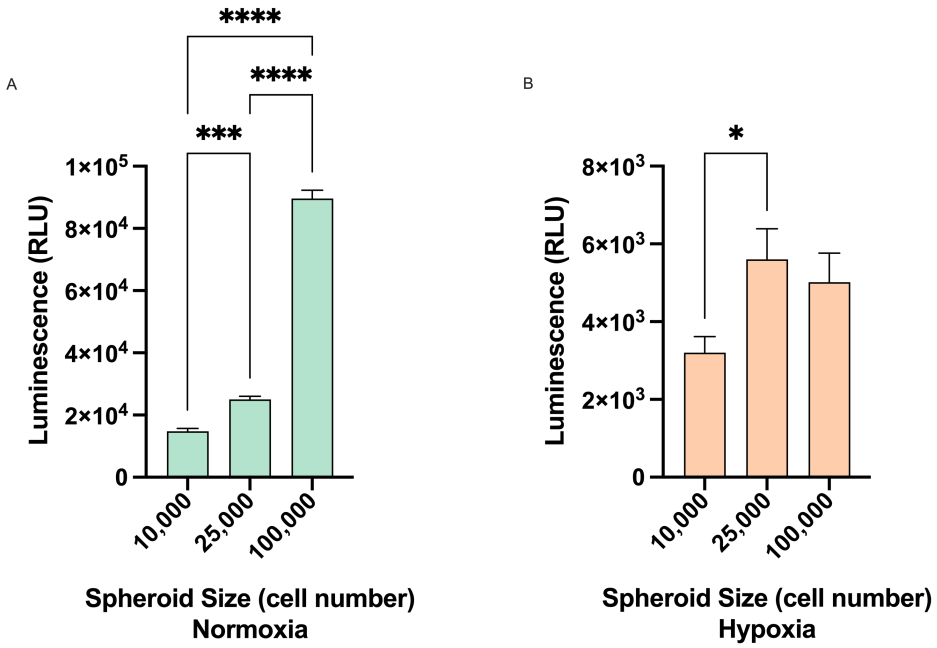

Figure 3.12: Effects of normoxic and hypoxic conditioning on the cell viability of spheroids. Spheroids made up of 10,000, 25,000 or 100,000 C2C12 myoblast cells were conditioned in growth media (A) or glucose and serum free media (B) for 24 hours. After this time, cell viability was determined using the CellTiter-Glo reagent. *p<0.05 using one-way ANOVA with a Tukey post-hoc test. Data expressed as mean ± SEM, (n=4 with 5 replicates).

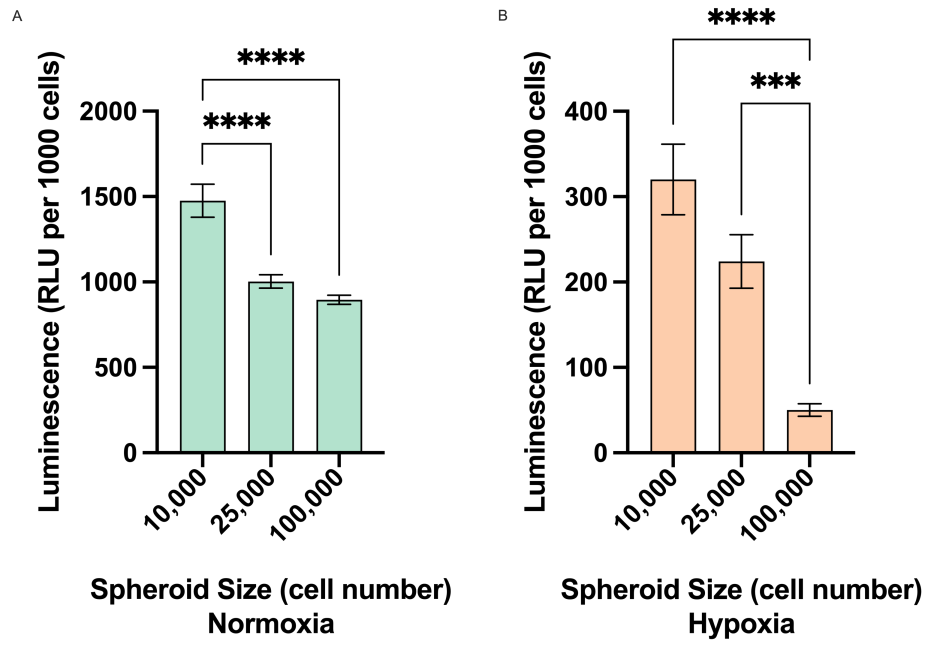

Figure 3.13: Effects of normoxic and hypoxic conditioning on the normalised cell viability of spheroids. Spheroids made up of 10,000, 25,000 or 100,000 C2C12 myoblast cells were exposed to normoxic (A) or hypoxic (B) conditions for 24 hours. After this time, cell viability was determined using the CellTiter-Glo reagent. Cell viability was normalised per 1000 cells of the original cell number of the spheroid to account for large variance in size. *p<0.05 using one-way ANOVA with a Tukey post-hoc test. Data expressed as mean ± SEM, (n=4 with 5 replicates).

3.4 Effect of Oxygen Deprivation on Gene Expression in Spheroids

There are several key genes that are involved in muscle repair and regeneration via a number of different pathways. The expression of these genes was measured using real-time PCR to determine whether forming monolayer cells into spheroids alters the expression of these genes. This was repeated comparing hypoxia conditioned monolayer cells and spheroids, to determine whether spheroids that are in an environment that replicates the repairing muscle have any differences in expression. Gene expression was normalised to the reference gene 18S.

Spheroids and monolayer cells were kept in normal cell culture conditions for 24 hours before RNA analysis. RNA expression of interleukin-6 (IL-6) (Figure 3.14A), collagen type I, alpha 1 (Col1a1) (Figure 3.14B), vascular endothelial growth factor A (VEGFA) (Figure 3.14C), tumour necrosis factor α (TNFα) (Figure 3.14D), transforming growth factor β (TGFβ) (Figure 3.14E), Bax (Figure 3.14F) and Bcl2 (Figure 3.14G) were examined. There were no statistically significant differences observed in the expression of cytokines and myokines between monolayers and spheroids in cultured in normal growth conditions ($p>0.05$, Figure 3.14).

Spheroids and monolayer cells were also conditioned in hypoxia for 24 hours before RNA analysis. Following this RNA expression of interleukin-6 (IL-6) (Figure 3.15A), collagen type I, alpha 1 (Col1a1) (Figure 3.15B), vascular endothelial growth factor A (VEGFA) (Figure 3.15C), tumour necrosis factor α (TNFα) (Figure 3.15D), transforming growth factor β (TGFβ) (Figure 3.15E), Bax (Figure 3.15F) and Bcl2 (Figure 3.15G) were examined. There were no statistically significant differences observed in the expression of cytokines and myokines between hypoxia conditioned monolayers and spheroids ($p>0.05$, Figure 3.15).

Apoptosis was measured through the expression of pro- and anti- apoptotic genes, Bax and Bcl2 and the overall change in susceptibility apoptosis was measured using the Bax:Bcl2 ratio. This provides an indication of the susceptibility of the cells within the spheroid to apoptosis. In both normal cell growth and hypoxia conditions there was no statistically significant change in the

Bax:Bcl2 ratio observed, suggesting that neither conditions the spheroids were exposed to cause a greater risk of apoptosis than monolayer cells ($p>0.05$, Figure 3.16A and 3.16B).

The experiment was repeated on spheroids and monolayer cells that had been conditioned for 48 hours and there was no significant change in the expression of the gene previously mentioned.

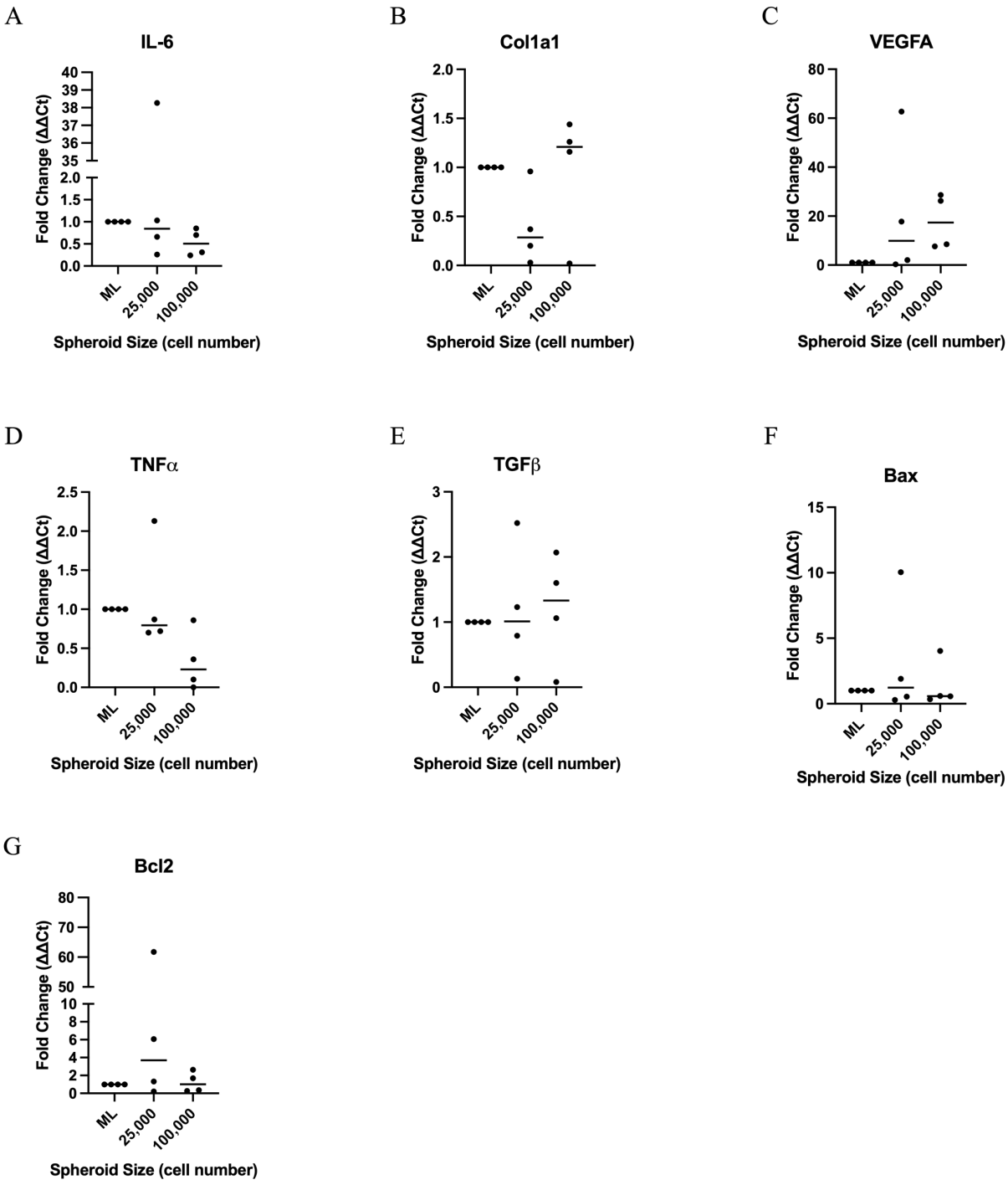

Figure 3.14: Effect of 24-hour in normal cell culture conditions on the gene expression of *IL-6, Col1a1, VEGFA, TNF-α, TGF-β, Bax and Bcl2.* Spheroids made up of 25,000 or 100,000 C2C12 myoblast cells were incubated in normal cell growth conditions for 24 hours. After this time spheroids were harvested and RNA was isolated for PCR analysis. $p>0.05$ using one-way ANOVA (A-G). Data expressed as median, (n=4 with 10 replicates).

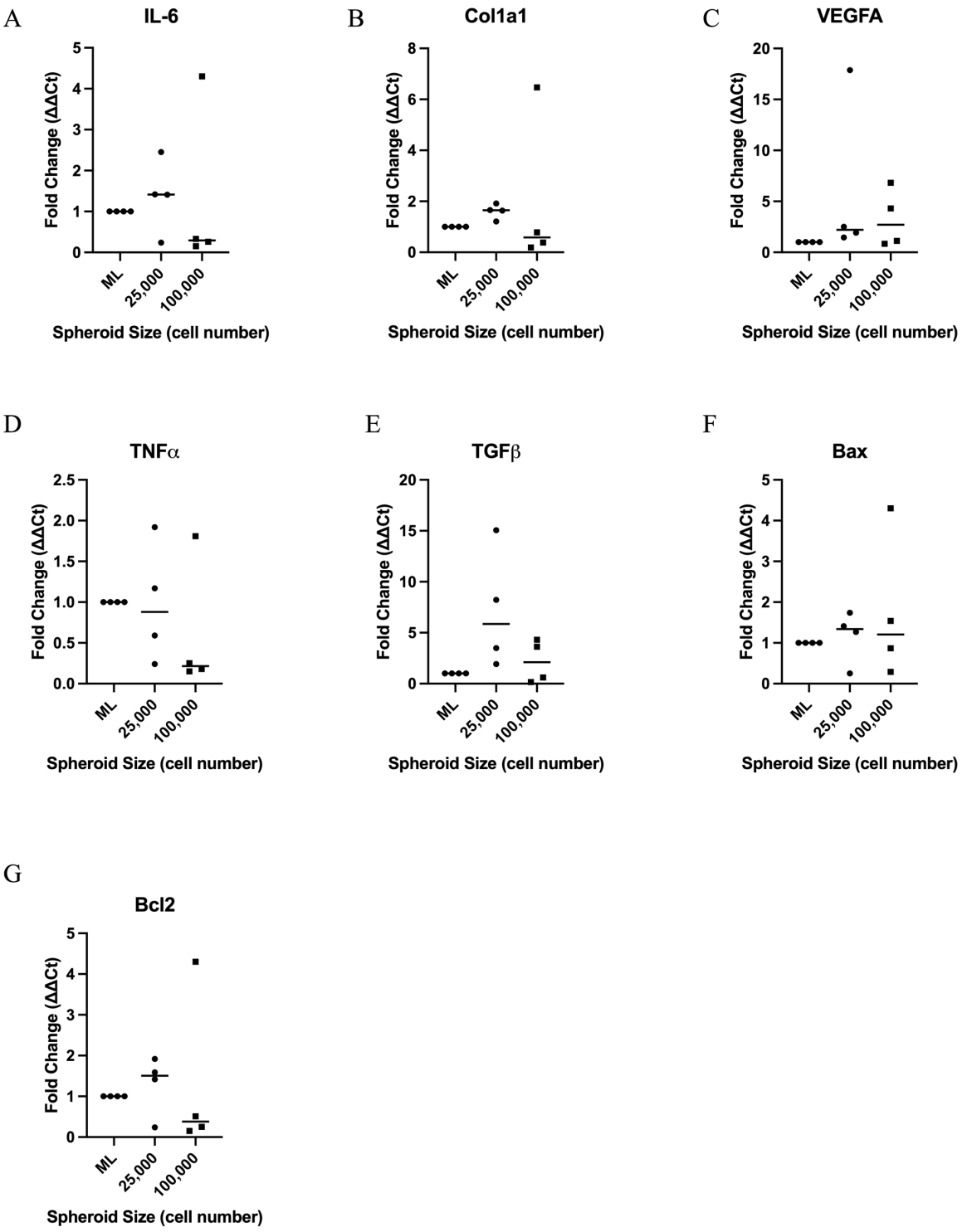

Figure 3.15: Effect of 24-hour conditioning in hypoxia on the gene expression of *IL-6, Col1a1, VEGFA, TNF-α, TGF-β, Bax and Bcl2.* Spheroids made up of 25,000 or 100,000 C2C12 myoblast cells were conditioned in glucose and serum free media and hypoxia for 24 hours. After this time spheroids were harvested, and RNA was isolated for PCR analysis. $p>0.05$ using one-way ANOVA (A-G). Data expressed as median, (n=4 with 10 replicates).

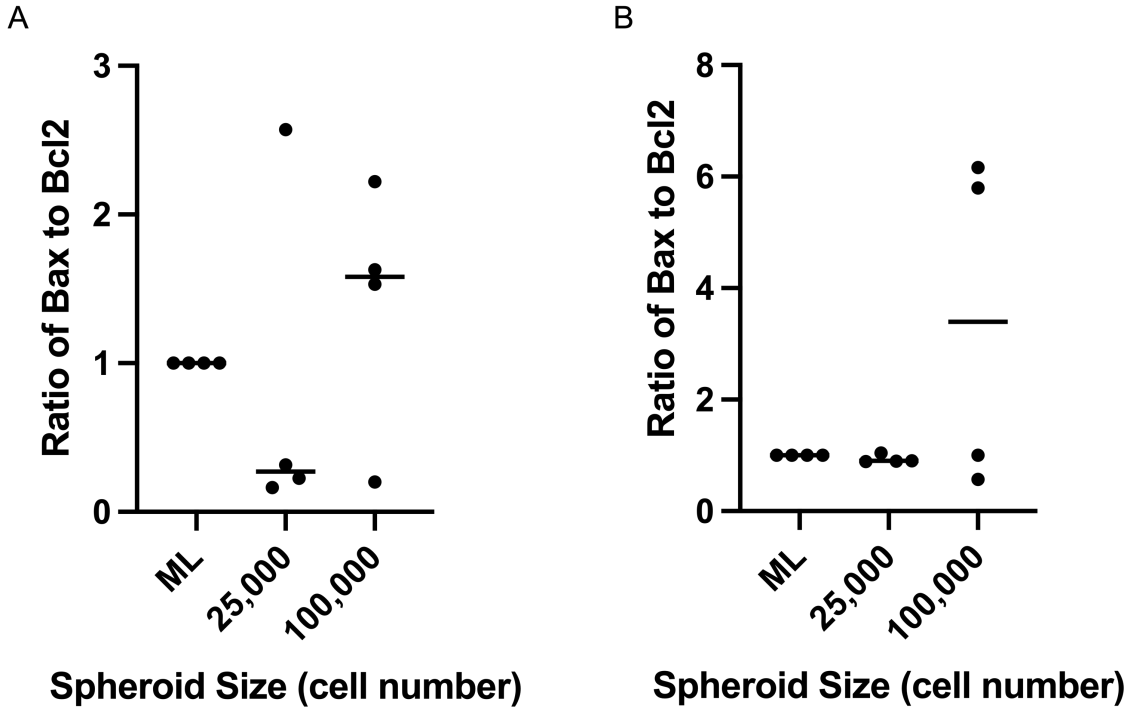

Figure 3.16: Effect of 24-hour conditioning on the *Bax:Bcl2* ratio. Spheroids made up of 25,000 or 100,000 C2C12 myoblast cells were conditioned in (A) normal growth conditions or (B) glucose and serum free media and hypoxia for 24 hours. After this time spheroids were harvested, and RNA was isolated for PCR analysis. $p>0.05$ using one-way ANOVA. Data expressed as median, (n=4 with 10 replicates).

3.5 Effect of Preconditioned Media on C2C12 Myoblast Cells

As the spheroids were conditioned for 48 hours in either hypoxia or normal growth conditions, they may have been releasing a variety of growth factors, cytokines or myokines that could impact cell function. The effect of placing media preconditioned by spheroids on C2C12 myoblast cells was examined. It appeared that the preconditioned media was resulting in the death of the C2C12 myoblast cells with no cells surviving the 24-hour incubation. These experiments were not pursued further as the protocol was adjusted to become the co-culture experiments (see section 3.6).

3.6 Effect of Co-culture on Myogenesis

It has previously been observed that treating injured tibialis anterior muscle with spheroids produced an increase in function. This was believed to be a result to changes of myogenesis within the spheroid treated muscle tissue [52].

3.6.1 Effect of Co-culture on Myoblast Differentiation

The diameter of myotubes were analysed using images taken after 3 days of differentiation and measured on the ImageJ software. Analysis showed the effect co-culture exposure to spheroids can have on the size of myotubes during differentiation. Upon examination it was seen that there was a significant increase in the size of myotubes that had been exposed to spheroids when compared with the group exposed to the monolayer cells and the control ($p<0.0001$, Figure 3.18). The group treated with monolayer cells also demonstrated a significant increase in size compared with the control ($p<0.05$, Figure 3.18). As seen in the images obtained of the myotubes (Figure 3.17), the difference in size between the groups after exposure to the conditions is clearly evident. When looking at these images it was also seen that the control and monolayer groups appeared to have a greater number of cells that were still myoblasts.

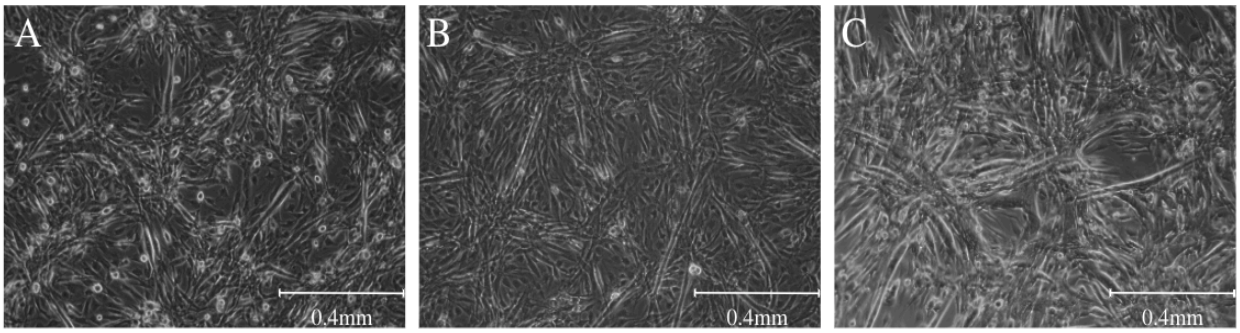

Figure 3.17: Effects of co-culturing on myotube size. C2C12 myotubes were co-cultured with spheroids made up of 25,000 C2C12 myoblast cells for 3 days. (A) Control (B) Monolayer co-culture and (C) Spheroid co-culture.

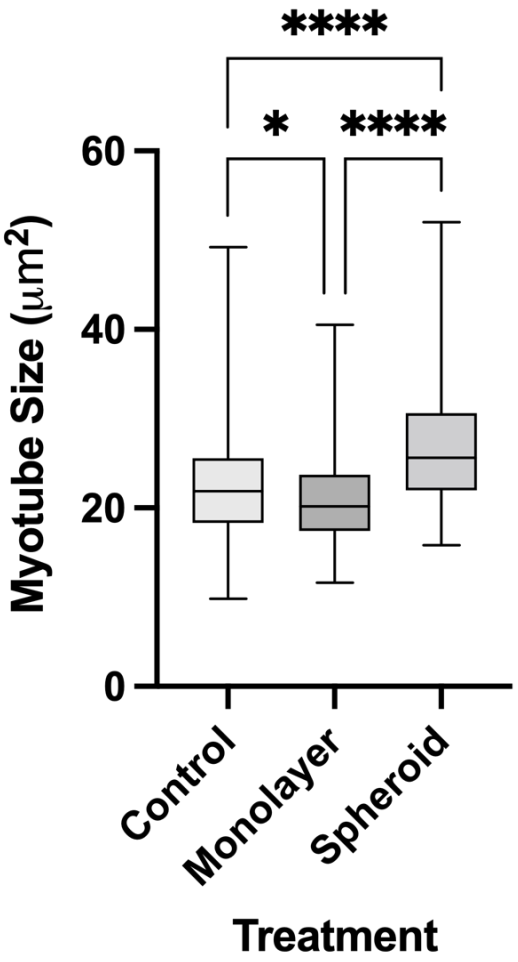

Figure 3.18: Effects of co-culturing myotubes with spheroids on size. C2C12 myotubes were co-cultured with spheroids made up of 25,000 C2C12 myoblast cells for 3 days. After this time, the size (area) of each myotube was measured. *$p<0.05$, comparing size of each condition using a one-way ANOVA. Data expressed as mean ± SEM, (n=150 myotubes per treatment taken from 4 independent experiments).

3.6.2 Effect of Spheroid Co-culture on Differentiation Linked Gene Expression

There are several genes that can be used as key indicators for skeletal muscle differentiation. To confirm whether co-culturing myoblasts with spheroids resulted in increased expression and subsequent changes in differentiation we assessed gene expression levels. The expression for two of the genes normally abundant during differentiation, Myogenin and Myosin heavy chain (MyHC) were measured using real-time PCR. Gene expression was normalised to the reference gene β2M. Analysis showed that whilst there was expression of Myogenin and MyHC greater than the control there were no significant changes ($p>0.05$, Figure 3.19).

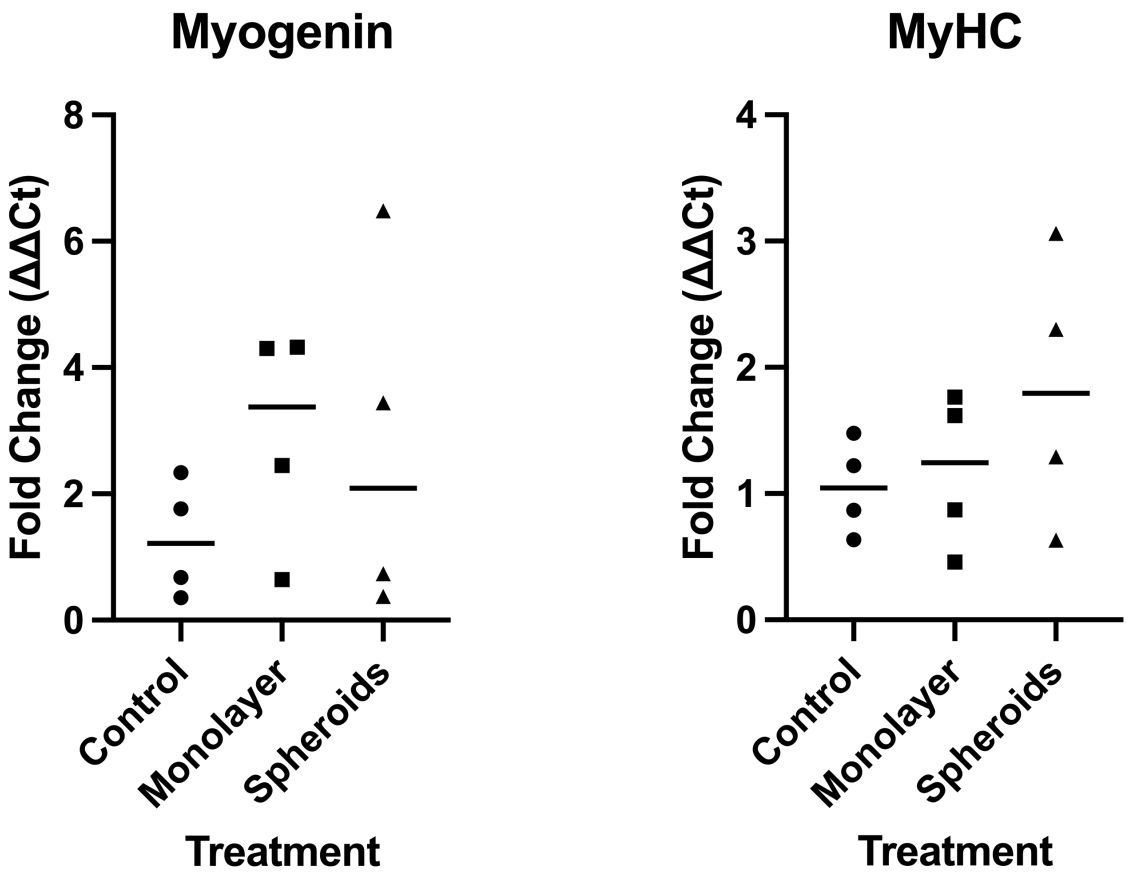

Figure 3.19: Effect of 3-day co-culture with spheroids on the gene expression of *Myogenin* and *Myosin heavy chain (MyHC)*. Myotubes were co-cultured with spheroids made up of 25,000 C2C12 myoblast cells for 3 days. After this time myotubes were harvested, and RNA was isolated for PCR analysis. Data expressed as median, (n=4).

3.7 Immunohistochemistry

3.7.1 Histological Analysis

Examination of injured tibialis anterior (TA) muscle sections stained with H&E under the microscope showed the effect of myoblast spheroid treatment on the number of nuclei within muscle fibres.

Muscle sections were stained to show the nuclei as a blue and extracellular matrix and cytoplasm as pink. As seen in Figure 3.20 the appearance of centralised nuclei is greatest at day 7 post injury due to cellular proliferation and muscle regeneration. It was observed that at days 14 and 21, the nuclei return to the periphery of the fibres indicating muscle regeneration is coming to an end. Both the monolayer and spheroid treated groups show a greater number of nuclei at day 7, however these numbers reduced by day 14 and again at day 21. Muscle cells (bright pink rounded structures) appeared to not have undergone any significant changes across treatment groups and time points as a result of the treatment however we did see some cell damage. When examining changes in connective tissue deposition (identified as light pink structures between muscle cells), there did not appear to be a vast difference in this between timepoints and treatments. It should be noted that these slides were only analysed visually to confirm viability of samples and no statistical analysis took place on these.

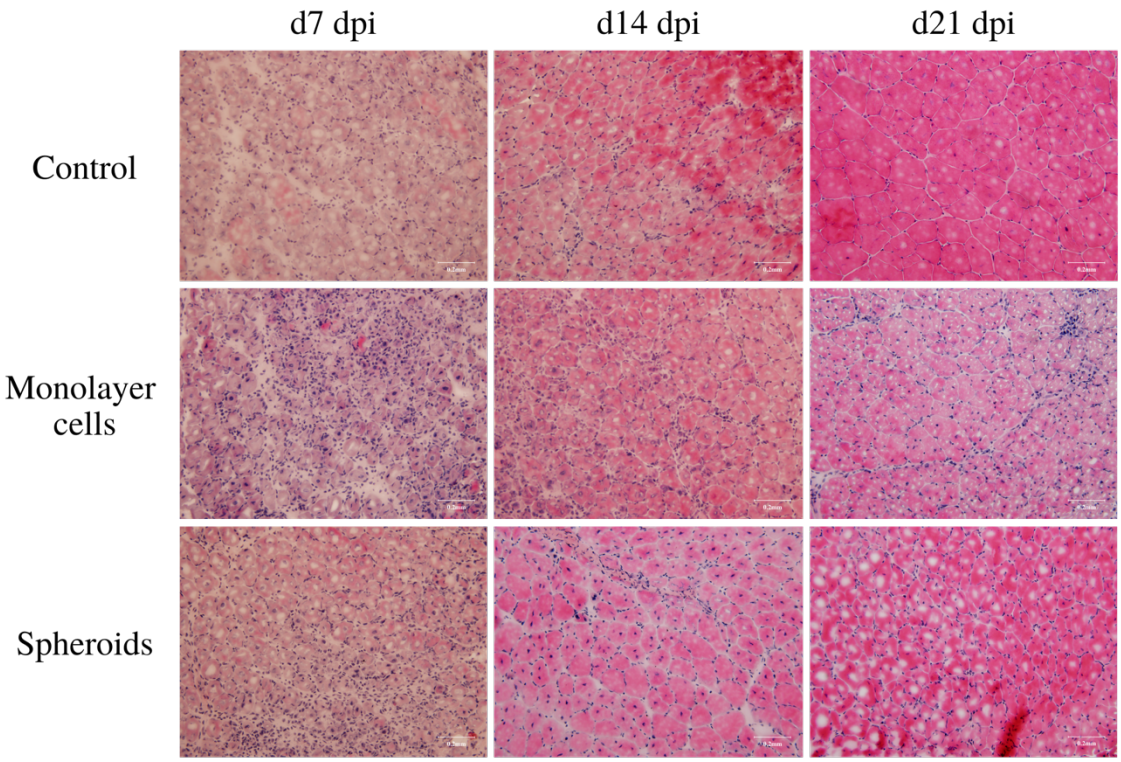

Figure 3.20: Microscopy of H&E-stained tibialis anterior muscle post injury. Injured TA muscle was treated with spheroids or monolayer cells to observe what change in morphology was produced at 7, 14 and 21 days post injury (dpi).

3.7.2 Wheat Germ Agglutinin Stain

Examination of injured tibialis anterior (TA) muscle sections stained with FITC labelled WGA under the microscope showed the effect of treatment with myoblast spheroids on the development of fibrosis within the skeletal muscle tissue. Upon examination of the WGA stained sections it was noted that samples from 7 days post injury (dpi) had too little fibrosis to accurately measure and these were omitted from any further analysis. D14 and D21 dpi demonstrated a trend where muscle that had been treated with spheroids had the greatest percentage of fibrosis followed by those treated by monolayer with control having the lowest percentage of fibrosis. It is seen that there is little change ($p>0.05$, Figure 3.22) between d14 and d21 control groups however there is a decrease ($p>0.05$, Figure 3.22) from d14 to d21 for both the spheroid and monolayer treated groups. At both timepoints the control group appeared to have developed to least fibrotic scar tissue whereas the monolayer and spheroid treated groups had developed more. At d21 dpi there appeared to be greater fibrotic scar tissue deposition within the spheroid treated group however this is not reflected in the results (Figure 3.21).

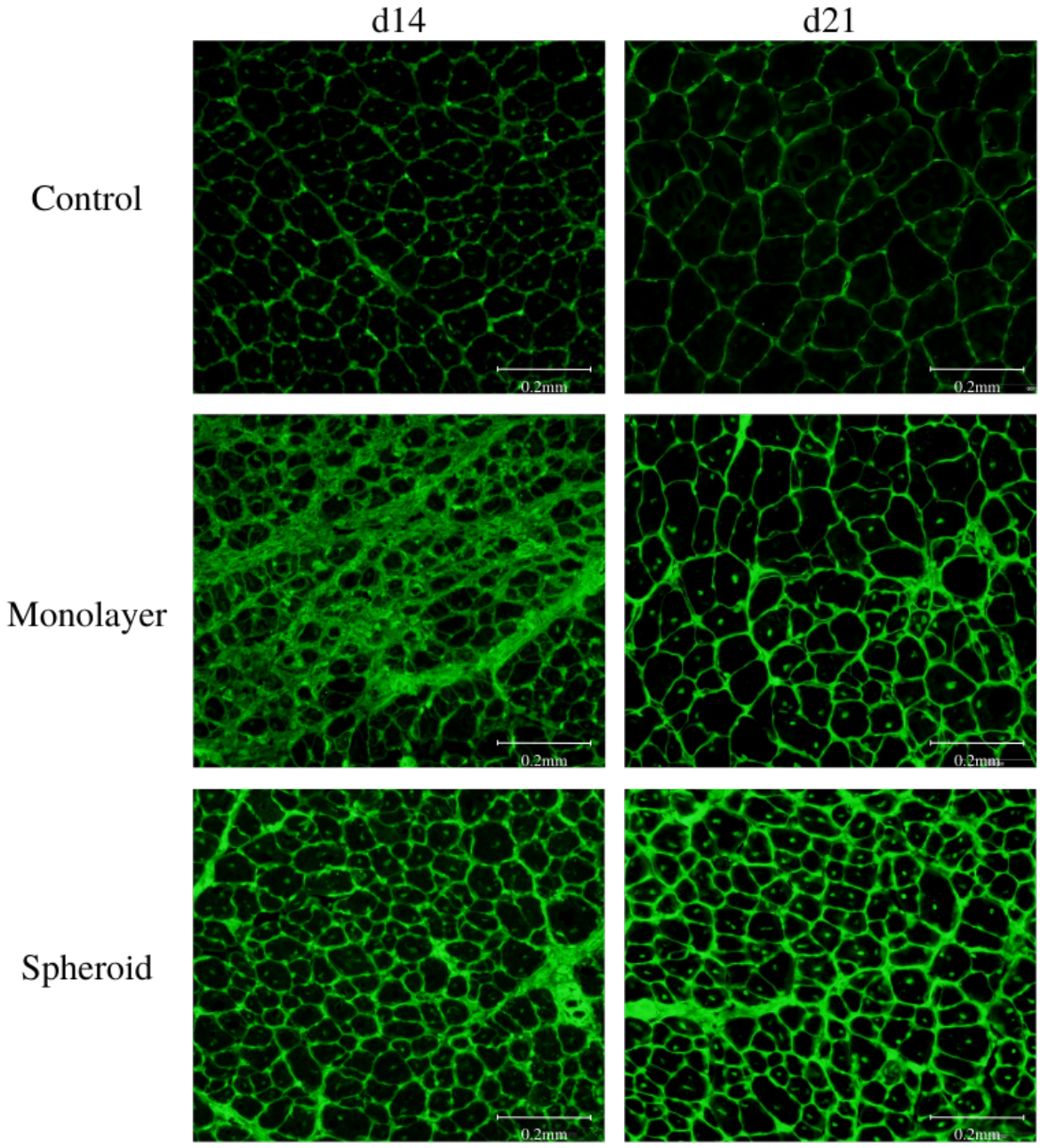

Figure 3.21: Microscopy of FITC labelled WGA-stained tibialis anterior muscle post injury. Injured TA muscle was treated with C2C12 myoblast spheroids *in vivo*. 21 days post injury the TA was harvested, prepared and a WGA stain was later applied. Images taken at 20x magnification.

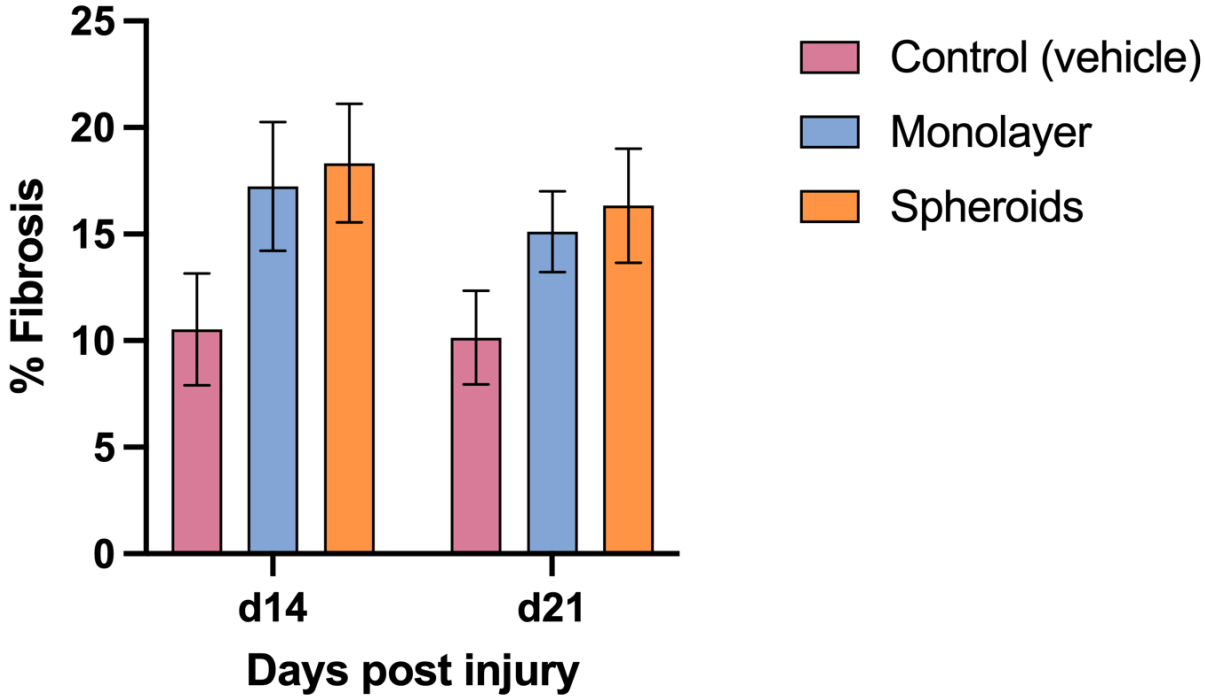

Figure 3.22: Effect of spheroid treatment in injured muscle on the development of fibrosis. Injured TA muscle was treated with C2C12 myoblast spheroids *in vivo*. 21 days post injury the TA was harvested, prepared and a WGA stain was later applied. $p>0.05$ using two-way ANOVA. Data expressed as mean ± SEM, (n=7, with 70 data points per time point/treatment).

CHAPTER 4:

DISCUSSION

4.1 Summary

Whilst the research area of spheroids itself is not new, exploring C2C12 spheroids *in vitro* and *in vivo* is relatively new. There have been many studies on spheroids using other cell lines, however whether these findings translate to myoblast-derived spheroids remains to be confirmed. Basic characterisation of myoblast-derived spheroids will enable future research to be developed into applying these spheroids for skeletal muscle injury repair. This would not be a new application with spheroids currently used as an adjunct to normal system healing in the cardiac, renal and integumentary systems [69]. Use of spheroids *in vitro* in cell lines other than skeletal muscle has been explored vastly, however we do not yet know the effect they have on differentiating myotubes. If spheroids developed using skeletal muscle cell lines demonstrate a change in outcome this will further justify the need to use myoblast spheroids in applications post skeletal muscle injury.

The primary aim of this study was to investigate the effect that forming C2C12 myoblast cells into spheroids of different sizes has on certain cellular phenotypic characteristics. It was hypothesised that when myoblasts are formed into spheroids, they produce cytokines and/or myokines in response to the hypoxic conditions of the interior of the spheroid construct. We found that the normoxic or hypoxic experimental conditions that were used for spheroids did not result in a change in the expression of cytokines or myokines. It was found however that being formed into a spheroid has a decreased the viability of cells as the size of the spheroid increased and that the condition of the spheroid (normoxia or hypoxia) affected the size of the spheroid. It was also found that co-culturing myotubes with spheroids had an effect on the size of myotubes with an increase in size although did not change gene expression in myotubes. The secondary aim of this study was to investigate the effect injecting spheroids and monolayer cells into damaged mouse muscle would have on the formation of fibrosis. It was hypothesised that spheroid treatment would result in the greatest levels of fibrosis. We found that there were no significant differences between the levels of fibrosis among the groups. The results obtained do not support that spheroids directly act on myogenesis and there may be other physiological processes being altered.

4.2 Spheroid Development Resulted in Capsule Formation

Traditionally in cell culture, monolayer cells are seeded into standard tissue culture treated polystyrene plates. These plates facilitate cell adhesion, which encourages cell proliferation and allows for further cell passages to be obtained and used downstream [120]. However, when these monolayer cells are seeded into an environment in which it is not possible or very difficult to attach to the plate (such as culture plates covered in a hydrophilic, neutrally charged coating) over time the cells will aggregate, and this results in the formation of spheroids [121].

This study found that giving C2C12 myoblasts cells 48 hours in growth media using the low attachment plates mentioned above was sufficient time for spheroids to develop uniformly with most cells incorporated into a single spheroid unit. We did observe that there was on occasion some single cells that were not incorporated within the spheroid however we cannot be sure if these did form into the spheroid and had since broken off or never joined the spheroid at all. This time frame is in line with most cells lines reported that have used these plates except for cancer cell lines which can form a structure within 24 hours [122]. It was also observed in this study that the formation of spheroids resulted in the development of a capsule like structure around spheroids of each size in the normoxia spheroids as seen in Fig. 3.3. This experiment was also conducted in the spheroids that had been conditioned in hypoxia, however these spheroids began to fall apart when they were removed from their wells and had lost a lot of the spherical structure upon imaging, most likely a result of cell death due to a lack of oxygen.

An increase in the cellular density does not result in a structural change in many cell types, however when myoblast cells come in close contact with each other such as in the case with the spheroids, they switch from a proliferative state to one of differentiating into myocytes as cellular sensors detect that there is limited space to continuing proliferating [123]. This switch to early-stage differentiation may be resulting in a change in the cell type that is sitting on the outer most layer of the spheroid, giving it an appearance of having a capsule, when it is in fact just a different cell type to much of the spheroid. A similar capsule has been observed in a previous study using a tumour spheroid model however there have been no studies identifying this capsule where cell

lines other than cancer were used. This study did not clearly identify how the capsule may have been formed, what it was formed from or whether or not it provided any significant benefit to the spheroid [124].

When performing RNA isolation, it was more difficult to break up spheroids that had been conditioned with normoxia than hypoxia. It was also much harder for the spheroids that had been kept in normoxia to be agitated and broken up during the cell viability assay. The difficulty that was experienced may have been encountered because of this intact capsule we observed and therefore understanding this capsule like structure would be beneficial for repetitions of this study. As spheroids that were examined had been conditioned for only 48 hours in this case, examining further time points could provide insight into the development of the capsule and observe earlier time points to identify when capsule formation begins.

As previously mentioned, there was difficulty handling hypoxic spheroids after conditioning, and therefore we were unable to complete microscopic analysis on these. Ideally, we would have sectioned these spheroids and completed H&E stains on them similarly to the normoxic spheroids and compared the effects of conditioning on histology. It was also not ideal that the spheroids were stained and examined were only from one timepoint. Future research examination of further timepoints in both hypoxic and normoxic spheroids could lead to a greater understanding of the development of the spheroids themselves and the capsule that surrounds them.

4.3 Spheroid Size is Impacted by Time and Not Oxygen Availability

Many cell types will proliferate no matter how compact and dense the cellular environment around them becomes [125]. In contrast, myoblasts will only continue to proliferate and increase cell number until they reach a proliferative limit. Once this limit is hit the myoblasts switch from a proliferative state to a state of differentiation to prevent overcrowding and ensure nutrient demand can still be met [123]. Therefore, there is a limit as to how many cells can be present at any one time in a spheroid and this can potentially affect the size as spheroid can ever achieve.

Considering the size of a spheroid may be important in future applications as some sizes may be unachievable and unsuitable for cell survival.

The present study found that when spheroids were conditioned in both hypoxia and normoxia, their size decreased in all spheroid groups. Conditioning for 24 and 48 hours resulted in a significant decrease in the size of spheroids when measured pre and post normoxia and hypoxia exposure ($p>0.05$). These findings are in contrast to previous studies using other cell types which have demonstrated quite significant spheroid growth over time, with some spheroids diameter increasing in size almost 5 fold in these studies [124]. As previously mentioned, myoblast cells are unlike other cell types and their behaviour is uncommon. When there is an area of high cellular density, they stop proliferating and, become compacted and if allowed enough time differentiate into myotubes. This is most likely to explain why spheroids of other cell lines will continue to grow whereas the myoblast spheroids generated in this study, significantly decreased size in both normoxic and hypoxic environments. Whilst a change in size was observed when comparing time, no differences were observed when spheroids of the same size and different conditions were compared. This suggest that despite the condition, the spheroids size will not change significantly and any changes that do occur will not result in a spheroid to shrink or become larger.

Hypoxia was utilised in this experiment as a condition to mimic the *in vivo* state muscles present during repair after an injury. After an injury, hypoxia is triggered by a disruption in the vasculature from the severing or trauma of blood vessel of the muscle [126]. Devascularisation of the tissue leads to the activation of hypoxia-inducible factor (HIF) mediating functions within cells to limit oxygen uptake requirements ensuring that survival can be maintained for as long as possible. Despite seeming undesirable, hypoxia is beneficial to the tissues as it promotes the formation of new blood vessels and reformation of epithelium, essential to the complete repair and regeneration of skeletal muscle [127].

The duration of time a spheroid is conditioned for appears to be related to its size. The longer that a spheroid was conditioned for in either full oxygen or in hypoxia, the smaller it became. Prolonging conditioning of the spheroids could have allowed for a longer proliferative phase and pushed cells into differentiation due to the compactness of the environment. It may be that the longer a spheroid sits for, it enables the differentiation of cells within the spheroid and thus causing a more densely compact environment. Therefore overtime, more cells will differentiate resulting in shrinkage or compacting of the spheroid which causes a visible decrease in the diameter of the spheroid. As it is not clear whether the size of the spheroid has a dramatic impact on the function of the spheroid, further research could be useful in determining this factor.

4.4 Spheroid Viability and Spheroid Size are Inversely Proportional

Cells are exposed to a number of different stressors when being formed into spheroids that could potentially affect their viability in turn altering their ability to perform optimally [128]. Cell survival and viability can be influenced by a lack of oxygen, excess carbon dioxide, infectious agents, immune response, exposure to drugs and chemicals, extreme temperature, exposure to radiation and certain disease processes [129].

In this study we observed what happened to the viability of the cells contained in the spheroid as the number of cells used and overall size of the structure changed. It was observed that as the size of the spheroid increased, there was a significant decrease in cell viability when normalised to spheroid size ($p<0.05$). Changes in the growth media and oxygen availability are potential stressors encountered that may have caused this. The formation of the spheroid itself could have also produced such a result with previous studies identifying that cells have become hypoxic when formed into a spheroid which can lead to cell death [130]. The technique used in the current study was a mitochondrial luminescence assay based on quantitation of ATP. ATP is a known marker that is suitable for detecting the presence of metabolically active cells [131]. Another assay that could have been utilised include a trypan blue stain assay or a mitochondria assay [132]. The trypan blue stain has often been used as a go to for assessing cell viability. Unlike

many other stains, trypan blue can only stain permeable dead cells and not live cells [133]. A trypan blue stain has been documented to over-estimate viability and was not chosen for this reason. Utilising this assay could confirm results obtained from the mitochondrial luminescence assay as measuring metabolic activity can be misleading if cells suddenly alter their metabolic activity such as in skeletal muscle cells [132].

Measuring cell viability *in vitro* provided a general understanding of how well monolayer cells survive once they are formed into spheroids [128]. As seen in Figs. 3.12 and 3.13, it was evident that spheroids that had been conditioned to mimic repairing muscle by exposure to hypoxia had a much lower viability than those that were kept in normal growth conditions. This difference in viability may be due to the previously mentioned three layers of a spheroid [62, 63]. The second layer is believed to be comprised of cells that are beginning to enter hypoxia, whilst the third is thought to be made up of necrotic cells that have starved from a lack of nutrients and oxygen. As these layers already exist within the spheroid, exposing the entire spheroid to a hypoxic environment may speed up the hypoxic and necrotic layer development [62, 63]. As the necrotic and hypoxic layers have reached these points sooner, the cells will continue to deteriorate leading to an increased number of cells becoming necrotic. In addition to this, as the outermost layer which is normally rich in oxygen and nutrients can no longer access oxygen, it also becomes hypoxic, with cells eventually starving and turning necrotic. As it was observed that viability did decrease as size increased size selection would become more important when using spheroids to limit the development of hypoxia and necrosis within the spheroid and avoid reducing the viability of the spheroid overall.

A limitation of working with the CellTiter-Glo Cell Viability Assay kit is that it only measures mitochondrial activity. From this we inferred a reduction of mitochondrial activity as reduction in the viability of the spheroid. There may have been cases where spheroids were in fact still alive, present in a quiescent state, with less detectable mitochondrial activity. To verify viability of the spheroids found in this present study, other measures of cell viability could be tested such as completing an apoptosis assay to detect dead or dying cells. Using this in conjunction with the

results that are obtained in the CellTiter-Glo viability assay kit, a clearer idea of cell viability will be reached.

4.5 Forming Monolayer Cells into Spheroids Does Not Influence Gene Expression

In some instances, altering the conditions of a cell can in turn alter the expression of key cytokines and myokines. This occurs as cells become stressed and their secretome changes [134]. The present study found that when myoblast cells were formed into spheroids, the expression of cytokines and myokines that are a key for muscle repair and regeneration were not altered.

These findings are in contrast to previous studies which have observed changes in spheroid gene expression [135]. These studies did however predominantly use stem cells which may also be a reason for these findings as in this study we used myoblast cells. The cell lines greatly vary as stem cells will specialise into a cell line and cannot perform specific functions within the body. In comparison the myoblast cells are differentiated cells that can only perform specific functions and respond to certain signals. In the normoxic environment there likely would have been few cues to signal for muscle repair or regeneration. As myoblast cells require certain signals such as the influx of inflammatory or immune cells to activate and begin the repair and regeneration process, the signal being produced may not have been significant to trigger a change in gene expression within the spheroids. The growth media used and oxygen availability provided supports this as the environment is used to grow cells and any factors that could trigger a change would not be ideal. It therefore is likely that there was no need for the cells within the spheroids in normoxia to change what they were expressing.

Similar results were observed in the spheroids that were conditioned in hypoxia with no significant changes in myokine and cytokine expression occurring. Unlike the normoxic spheroids, the hypoxic spheroids did experience a stressor which could potentially cause a change in gene expression. Whilst there were no significant changes in gene expression in hypoxic

spheroids when comparing sizes, there were some variations in levels of gene expression in spheroids compared to monolayer cells, most notably in VEGFA. This change may have been as a result of alterations in expression within the spheroid as it is known that VEGFA levels can be increased when cells are in a hypoxic environment [136].

In this study we found that there was no significant change in the Bax:Bcl2 ratio in either normoxic or hypoxic spheroids. Bax and Bcl2 are genes that can increase or decrease a cells susceptibility to undergo apoptosis. Changes in this ratio would indicate that the spheroids have become either less or more apoptotic than their monolayer cells, and that the process of forming into spheroid may have an impact on apoptotic susceptibility [137]. Lower Bax:Bcl2 ratios may indicate that cells are resistant to apoptosis however higher levels may indicate that cells have an increased susceptibility to apoptosis [137]. Normoxic spheroids showed little changes in this ratio compared with the control, however the hypoxic spheroids of 100,000 cells had an almost 2-fold increase in the ratio when compared with the control. This may mean that the cells within these spheroids were slightly more susceptible to undergoing apoptosis than the spheroids that showed no change in the Bax:Bcl2 ratio. An increased susceptibility to undergo apoptosis may be plausible as these spheroids also had the lowest viability of any of the spheroids measured. The decreased viability that was observed may have also been because of cells turning necrotic. Necrosis may have been caused because of the cells within the spheroid starving and dying. To confirm whether these spheroids were in fact apoptotic or necrotic, running an apoptosis assay and comparing with other spheroids of the same size but different condition in conjunction with using these results could aid in confirming whether hypoxia resulted in an increased risk of apoptosis and as such reduced viability. As there is no definitive test for necrosis, an apoptosis assay with negative results and a reduction in cell number have previously been used as an indicator of necrotic death [138].

Future research in gene expression could also including running an array to measure all the cytokines and myokines that are within the media of spheroids. This could provide a greater

understanding of any changes that occur within the spheroids and provide a direction for future research.

4.6 Exposure to Preconditioned Media Kills C2C12 Myoblast Spheroids

The environment which a cell is exposed to can change how it will behave and ultimately determine its survival. Exposure to hypoxia, a lack of nutrients, inflammation and tissue repair all play a part and can result in changing metabolic activity and at a wider level a change in gene expression [139]. In this case when the myoblasts are exposed to differing media and oxygen concentrations, gene expression can change due to internal signals that trigger the need for proteins due to stress [140].

The present study aimed to expose C2C12 myoblast cells to preconditioned media from spheroids and observe how the myoblasts developed. It was important to note if there were any changes in appearance of the myoblasts or progression of their regenerative development as this could signify that the secretome of the spheroids contained a protein that was affected by myoblast proliferation and differentiation. We also wished to determine if there were any changes in gene expression as a result of the myoblasts exposure to the preconditioned media. This technique has been carried out successfully previously [141, 142], however when we attempted to replicate it, the preconditioned media appeared to result in cell death. Cell death was identified after the incubation had been complete and cells were visualised under a microscope.

Due to time constraints, we were unable to investigate potential causes for the outcomes observed further or reattempt the experiment, although it is anticipated that this experiment will be optimised and repeated in the future in an attempt to determine if there is an effect of media conditioned from C2C12 myoblast spheroids on C2C12 myoblast cells.

4.7 Myotube Development is Influenced by Extrinsic Factors

Myoblasts are exposed to a number of intrinsic and extrinsic factors during development that can impact the outcome of myotubes and differentiation both positively and negatively [143].

Observing the size and appearance of cells as they progress through skeletal muscle repair and regeneration is important for identifying differentiation and ultimately overall success. In its healthy maturing form, myotubes will appear as elongated cylindrical structures free of any irregular masses, or multi-branched formations [144]. A myotube that does not appear in the classical elongated cylindrical shape could indicate that the preceding myoblast experienced replicative senescence – the irreversible arrest of cell proliferation resulting in an altered cell function or exposure to extrinsic or intrinsic factors that altered the resulting myotube [145]. Replicative senescence most commonly occurs due to the accumulation of reactive oxygen species with aging [146]. If this were identified, the passage of that cell line would be deemed unsuitable for use as they could produce undesirable results.

This study showed that when myoblasts underwent differentiation and were co-cultured with spheroids, the resulting myotubes developed significantly larger than myotubes that were either co-cultured with monolayer cells or with no cells at all ($p<0.05$). The myotubes observed maintained the expected elongated cylindrical shape indicating that no structural changes of concern had occurred. An increase in the size of the myotubes demonstrates that differentiation is being upregulated within the cells due to either extrinsic or intrinsic factors or even a combination of both [146]. This finding suggests that the spheroids could be secreting a substance into the media of the myoblasts that is a positive regulator of differentiation. The proteins most likely to be producing this result are growth factors [43]. An alternative theory to this is that the presence of the spheroids themselves were resulting in activation of fusion of small myotube clusters to form mature myofibers [147]. Similar results have been observed in studies that explored the effect of a treating differentiating cells with extrinsic agents [148].

Altering myotube development is only beneficial if it results in an increase in the number of myotubes and functional myofibers. Decreasing myotube number and size is an undesired outcome, which can lead to decreased muscle strength, atrophy, abnormal fibre structure [149]. In comparison, increasing the myotube number has the opposite effects, increased muscle strength, hypertrophy and reduced morbidity without compromising the structure of the fibres and the

skeletal muscle tissue [150]. The treatment of damaged or diseased muscle with myoblast spheroids would be advantageous as cells could be harvested from the individual. Utilising an individual's own cells reduces the chances of an immune response causing the rejection of the treatment or an allergic response occurring due to the introduction of foreign materials into the damaged tissue. This experiment would need to be repeated *in vivo* examining myofibers harvested from muscle tissue that was treated with spheroids. If the results observed are similar it would further support that spheroids positively regulate differentiation promoting the growth of myotubes and subsequently muscle growth.

4.8 Co-culturing Myotubes with Spheroids Did Not Change Gene Expression

Exposing cells to each other and the factors they secrete can result in a change in gene expression and subsequent protein secretion. This experiment co-cultured myotubes with spheroids using co-culture buckets to determine whether the any of the spheroids secretions would have such an effect on the myotubes. The buckets allowed the secretome of the spheroids to travel through a one-way filter to the media feeding the myotubes without any direct contact. This prevented the myotubes from attaching to the spheroids and compromising the experiment. The study found that when spheroids and myotubes were co-cultured, there was not a significant change in the expression of Myogenin or MyHC in the myotubes.

Myogenin and MyHC are two proteins that exhibit increased expression during skeletal muscle differentiation [151]. Myogenin belongs to a group of myogenic regulatory factors (MRFs) and its expression along with other MRFs determines cellular commitment and is essential for differentiation into skeletal muscle [152, 153]. Myogenin is required in order for differentiation to occur and levels of Myogenin can correlate to how much the cells are differentiating. MyHC is a protein complex that is responsible for driving movement through muscle contraction. The expression of this complex is upregulated during muscle regulation providing a specific identifying marker of fibres that are regenerating [154]. An increase in the expression of the genes

responsible for these two proteins was expected as during differentiation the requirement for such increases, causing an increase in the gene expression [155]. Whilst differentiation was observed to occur in myotubes, and it was believed that differentiation was upregulated in myotubes co-cultured with spheroids, it did not appear that Myogenin or MyHC were the proteins responsible for this change. As previously mentioned, there are a number of proteins and genes that are at play during differentiation. This could account for why we did not observe an increase in Myogenin or MyHC. Other MRFs such as myogen termination gene (MyoD), myogenic factor 5 (Myf5) or myogenic regulatory factor 4 (MRF4) [156] could be the proteins that were upregulated during co-culturing with spheroids. These all play a similar role to Myogenin and MyHC to push cells to differentiating or as specific marker for differentiation. As we did not assess for expression of these proteins, we would not have been able to identify if there was an upregulation of there, nor whether these were the factors at play in differentiation. Future experiments exploring the expression of other proteins would be important to evaluating and conclusively understanding whether co-culturing myotubes with spheroids would change expression of genes key to skeletal muscle differentiation.

Whilst we were unable to find any previously documented studies that had C2C12 myotubes co-cultured with C2C12 spheroids, multiple studies have co-cultured C2C12 with monolayer cells of different cell types. These studies demonstrated that co-culturing C2C12 myotubes with monolayer cells improved skeletal muscle tissue formation and function. Ostrovidov et al. (2014), co-cultured C2C12 myotubes with PC12 neural cells with the aim to improve the formation of mature muscle tissue that demonstrated greater function [157]. Upon co-culturing they found that there was improved differentiation with enhanced formation of myotubes. Measurements of the myotubes showed significant increases, with myotube alignment, length and coverage area all increasing after co-culturing. Muscle differentiation markers, muscle maturation markers and neuromuscular markers were all upregulated [157]. As we only assessed the cells themselves it would be beneficial to examine how the media changed after co-culturing. The result from this study further supports the need to reconduct the PCR on additional markers as upregulation of Myogenin and MyHC was not significant enough.

Further exploration of the co-culturing effects of spheroids on myotubes would be necessary as we only explored the expression of two markers of differentiation and we did not see any significant changes. Limiting ourselves to just the two has prevented us from fully understanding how well co-culturing these two cell types together works and would need to be repeated broadening the gene expression analysis. As we did not look further into this due to time constraints, we cannot conclusively determine how effective co-culturing these together is.

4.9 Treating Injured TA Muscle with Spheroids Did Not Alter Skeletal Muscle Tissue Appearance

Visually observing and accounting for general changes within skeletal muscle architecture can help to account for positive and negative effects of treatments. The haematoxylin and eosin (H&E) stain enables this observative as structures can be easily identified. Under the microscope, samples that have undergone a H&E stain will present with purple-blue nuclei, the extracellular matrix and cytoplasm appearing pink and other structures taking on colours that are a combination of these colours.

When examining the H&E-stained tibialis anterior (TA) muscle sections it was seen that some (not pictured) samples experienced freezer burn. The freezer burn was identified when there was any deviation from parallel muscle cells [158]. Freezer burn most likely resulted from storage in an ultra-low temperature (ULT) freezer at -80°C for an extended time.

The myotoxic injury that had been inflicted upon the TA muscle would have triggered degeneration of the muscle fibres, a haematoma to form and necrosis of myofibers. In 7 days post-injury (dpi) samples, both the monolayer and spheroid treated groups had greater centralised nuclei than the control. At this time in muscle regeneration, satellite cell proliferation is at its greatest and we see this with a larger number of cells represented through more nuclei [159]. These nuclei account for almost all of the myofibers that are regenerating to restore any lost myofibers as well as the nuclei of the spheroids or monolayer cells that were injected into the

muscle, however it is difficult to identify how many of these cells were resident in the tissue prior to treatment using H&E [159].

At day 14 dpi we see that the number of centralised nuclei had reduced with a majority returning to the periphery of the fibres. There still appeared to be more nuclei within the spheroid- and monolayer-treated samples however this difference was much harder to identify than at day 7. Day 14 dpi is identified as the time point when myofibers that were injured are regenerating, and new myofibers are maturing from the cells that were previously formed [12, 160]. There is no longer a need for a large number of new cells generated at rapid speed therefore we no longer observe the large number of centralised nuclei, however there are still some centrally located.

In all samples at day 21 dpi, we see that there is a complete return of nuclei to the periphery of the fibres. This signifies skeletal muscle regeneration is almost complete. Scar tissue is formed, and regenerated fibres are innervated. Skeletal muscle tissue that had been injured will continue to repair until it achieves an endpoint of either complete or partial muscle maturation. It is only at this time that it will be known whether function will be fully restored or if some deficits will remain [161].

4.10 Treating Injured TA Muscle with Spheroids Did Not Change Fibrosis Formation

In minor skeletal muscle injuries, the muscles are able to easily regenerate to pre-injury state up to a certain threshold and no functional deficits result. However when the injury is severe surpassing the threshold, the muscle is unable to repair and fibrotic scar tissue forms in place of previously functional tissue [14]. Such a change in tissue type can change how functional a muscle group is and overall result in a change in mobility. Once fibrosis does develop the muscle cannot be fully regenerated, even with external support [40]. Fibrosis is a potential result of tissue injury as an end result of inflammatory reactions such as those induced by Brooks (2017) in the mice used in this study due to repeated or severe injury [52, 162]. Fibrotic scar tissue deposition can also be as

a result of disease processes such as; persistent infections, allergic responses, radiation and autoimmune reactions [162]. It is not uncommon to see fibrosis forming in athletes that experience repeated injuries as the muscle that once could respond and repair loses its ability with each additional injury to the area [14].

Upon examination of sections taken from Brooks (2017), it was seen that the percentage of fibrotic scar tissue deposition did not change in regenerating TA muscles that were treated with spheroids [52]. We did see a trend that demonstrated the control group produced the least fibrotic tissue post injury despite experiencing the same cardiotoxin injury as the spheroid and monolayer groups. The monolayer treated group showed greater fibrosis deposition than control and the spheroid group even more. This trend was seen in both d14 and d21 samples, however fibrosis was higher in samples at d14 than d21.

In the study, Brooks (2017) analysed the force production of the injured TA muscle to observe if there was a loss of function within the groups. They observed that at d14 and d21 force production within the spheroid and monolayer groups was significantly lower than the control. She also measured the specific force output which was force produced normalised to the muscle mass and cross-sectional area. A similar result at d14 and d21 was observed here when she compared the control with the spheroid and monolayer groups. At d21 she also observed that the spheroid group produced a significantly greater force than the monolayer group [52].

The fibrosis stain results that were observed in this study do not reflect the results Brooks (2017) obtained as we did not see a significant difference in fibrosis deposition in muscles from mice that had been treated with spheroids compared to a monolayer cell suspension. It had been previously thought that we would observe a significant increase in collagen deposition correlating with the results retrieved in 2017.

While a picrosirius red stain is often used to observe collagen build up within a paraffin embedded stain, our use of tissue samples that had been frozen in OCT presented problems as

staining such tissues results in samples with poor contrast between collagen and muscle tissue. This led us to the use of the FITC-labelled Wheat Germ Agglutinin (WGA). WGA binds to connective tissue and when bound to FITC allows for visualisation, imaging and quantification [163]. It has not been reported that the use of WGA-FITC over a picrosirius red stain is less reliable for quantification of fibrosis [163].

Repeating this study using the protocol described above and embedding sections in paraffin would be important as previously discussed these samples did experience freezer damage. The use of embedded sections would also allow for staining using other techniques that could stain for inflammatory cells, immune cells or further fibrosis. Analysing inflammation within the regenerating muscle would indicate whether levels of inflammatory cells fall within the standard range for regeneration or if these levels fall outside of this. An increase on inflammatory cells could be reflected in Brooks (2017) results [164].

Freezer burn was a significant issue encountered with TA muscle samples stained for H&E and WGA-FITC. As we were unable to assess the entire sample pool there is likely to have been conclusions missed. Repeating the *in vivo* experiment and harvesting the TA muscle for immediate or soon after staining will likely reduce any issues encountered due to freezer burn and limit results missed.

4.11 Limitations and Future Prospects

Due to the time constraints of the Masters year, there were limitations of this study that should be addressed in future research.

This project was completed during the COVID-19 pandemic. This had a significant impact on the study as lockdowns limited access to the lab preventing experiments from being undertaken as well as the loss of cell lines due to the sudden calling of these lockdowns. The repeated lockdowns and stress around the COVID-19 pandemic limited what could be undertaken, ultimately limiting the outcome of this study.

One of the main limitations that were encountered during this study was that to produce the number of spheroids required for this study. Our C2C12 myoblast cells were generally achieving a confluency of approximately 75% in 48 hours however this was not yielding enough cells for the initially desired study design. Initially it had been planned that when conditioning spheroids and performing analysis of gene expression 3 sizes would be created and then analysed, 10,000, 25,000 and 100,000. Following through with this became very difficult as the required cell numbers were only just being achieved so there was greater focus on achieving the 25,000 and 100,000 cell spheroids for PCR experiments.

Future research for this topic may include further study into the effects of longer incubation on cytokine and myokine expression. Preliminary findings indicated that there were no significant changes in either of the test groups. We were restricted in this project by how long our spheroids could be conditioned for, however longer conditioning, like those that spheroids are expected to survive implantation may yield different results. Other studies have conditioned spheroids for up to a week so a future study could include conditioning lasting this long. A limitation that may be encountered from this is the GENbox hypoxic environment used, only guarantees hypoxia for 48 hours, therefore it would need to be investigated as to whether other systems could be used to guarantee hypoxia for a longer period. A longer incubation would also require media changes and therefore, the conditions that the spheroids were being exposed to would not be kept constant. If a longer incubation was undertaken this would need to be considered as exposure to oxygen would impact the results that are obtained in the hypoxia condition group.

The results obtained do not reflect those that have been obtained in previous studies exploring the use of spheroids in wound healing [69]. The C2C12 cell line used in this study could have been the cause for this and these experiments should be repeated using other skeletal muscle cell lines such as primary human cell lines to observe if the results obtained favour use in aiding wound healing.

4.12 Conclusion

In summary, the present study demonstrated that forming C2C12 myoblast monolayer cells into spheroids has no significant impact on the expression of key cytokines and myokines that are involved in muscle repair and regeneration. As a proof-of-concept study it has managed to answer many previously unknown questions and also raise new ones. It would be beneficial to repeat many of these experiments using optimised protocols to help solidify this concept. Preliminary findings of other characteristics have shown however that there is a significant difference in cell viability dependent on spheroid size. In addition to this spheroid size is significantly altered dependent on condition. We saw that spheroids do change the outcome for myoblast differentiation however the cause of this is still unclear. As such, the use of spheroids is recommended in further research to investigate the states of the cells within the spheroids, and the potential use of spheroids within the field of tissue engineering and the benefits it may provide in repairing damaged skeletal muscle through potential changes to inflammatory, immune or angiogenic responses.

CHAPTER 5:

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