Cell culture models of insulin signalling and glucose uptake.

By

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A Doctoral Thesis

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Acknowledgements

Abstract

Insulin maintains glucose homeostasis through its binding of the insulin receptor and activation of the insulin signalling cascade in insulin sensitive tissues. Skeletal muscle is a major endocrine organ, and is responsible for the majority of post-prandial glucose disposal. The maintenance of glucose homeostasis is a delicate balance and impairments in glucose disposal can have significant physiological effects, resulting in the onset of metabolic diseases such as diabetes mellitus.

Insulin stimulated glucose uptake involves a number of signalling proteins to enable uptake to occur. In order to understand the complexities associated with the insulin signalling cascade, cell culture models have provided a controlled and easily manipulated environment in which to investigate insulin stimulated glucose uptake in skeletal muscle. While the majority of these experiments have been conducted in conventional monolayer cultures, the growing field of three-dimensional tissue engineering provides an alternative environment in which skeletal muscle cells can be grown to investigate their physiological function.

The purpose of this thesis was to investigate the use of different cell culture models for investigating the effects of acute and chronic insulin exposure on skeletal muscle.

Initial investigations aimed to establish glucose uptake in tissue engineering skeletal muscle constructs using tritium labelled (H^3) 2-deoxy-d-glucose. Monolayer cultures were used to developed base line conditions. In these cultures, concentrations greater than 0.5 µCi for 15 minutes of insulin stimulation suggested an initial assay window for investigating insulin stimulated glucose uptake. However, the duration of insulin stimulation was not effective in measuring uptake in tissue engineered skeletal muscle constructs based upon western blot experiments of Akt phosphorylation, therefore insulin stimulation in skeletal muscle tissue engineered constructs was increased to 30 minutes.

Glucose uptake is mediated via specific glucose transporter protein, GLUT1 and GLUT4. Therefore, the transcriptional profile of these transporters was elucidated in monolayer culture and tissue engineered skeletal muscle constructs. Time course experiments showed an increase in GLUT4 transcription in tissue engineered and monolayer culture systems which is associated with an increase in the transcription of skeletal muscle development and myogenic genes.

In two dimensional culture, skeletal muscle cells were exposed to insulin during differentiation and in post-mitotic skeletal muscle myotubes to investigating the potential effects upon metabolic genes and proteins involved in insulin signalling. Chronic exposure to insulin during skeletal muscle differentiation reduced insulin signalling and resulted in an increase in basal glucose uptake and ablated insulin stimulated glucose uptake. In contrast, post-mitotic skeletal muscle myotubes did not shown similar changes and were not as responsive to acute insulin exposure. Therefore future experiments exposed skeletal muscle to insulin during differentiation.

Using the previous findings as a basis for experimentation, the effects of chronic and acute insulin exposure upon three dimensional skeletal muscle constructs were investigated. Fibrin and collagen constructs were grown for a total period of 14 days. Constructs were exposed to insulin during differentiation and acutely stimulated for 30 minutes at day 14. Although there was a mean increase in Akt protein phosphorylation in both types of tissue-engineered constructs, these changes were not significant following acute insulin stimulation. In addition, glucose uptake in fibrin skeletal muscle constructs increased as a result of acute insulin stimulation however was not significantly difference to unstimulated constructs.

The work presented in this thesis provides initial experimental data of the use of different skeletal muscle cell culture models for investigating insulin signalling and glucose uptake. Further research should further characterise these *in vitro* models for investigating skeletal muscle metabolism.

Published Work

The work conducted in this thesis has been presented at the following conferences or published in peer reviewed journals.

Conference Proceedings:

Turner, M.C., Player, D.J., Martin, N.R.W., Lewis, M.P. (2014). Tissue engineering skeletal muscle to investigate glucose uptake. Tissue, Cell and Engineering Society Annual Conference, Newcastle.

Turner, M.C., Player, D.J., Martin, N.R.W., Lewis, M.P. (2014). Chronic insulin exposure results in transcriptional alternations in metabolic and myogenic genes in *in vitro* skeletal muscle. Advances in Skeletal Muscle Biology in Health & Disease Conference. Gainesville, FL.

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Abbreviations

AA	Aminocaproic acid
Akt	Protein Kinase B
BSA	Bovine Serum Albumin
Δ	Change
Ci	Curie
C^t	Cycle Threshold
DAPI	4',6-diamidino-2-phenylindole
°C	Degrees Centigrade
DM	Differentiation Media
dH_2O	Distilled Water
dpm	Disintegrations Per Minute
DNA	Deoxyribonucleic Acid
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular Matrix
FBS	Foetal Bovine Serum
g	Gram
GLUT1	Glucose Transporter 1
GLUT4	Glucose Transporter 4
GS	Goat Serum
GM	Growth Media
HKII	Hexokinase II
HCl	Hydrochloric Acid
HS	Horse Serum
IGF-1	Insulin-like Growth Factor 1
IR	Insulin Receptor
IRS-1	Insulin Receptor Substrate 1
IT	Insulin Treatment
kDa	kilodalton
L	Litre

MgCl$_2$	Magnesium Chloride
MM	Maintenance Media
mRNA	Messenger Ribonucleic acid
μCi	Micro curie
μl	microliter
μg	microgram
mg	milligram
mM	Millimole
mmol·L	Millimoles per Litre
mL	millilitre
M	Molar
MEF	Myocyte enhancer factor
MyoD	MyoDesmin
MyoG	Myogeninn
nM	Nanomole
NIDDM	Non-Insulin Dependent Diabetes Mellitus
OGTT	Oral glucose tolerance test
PBS	Phosphate Buffered Saline
PGC-1α	Peroxisome proliferator-activated receptor coactivator – 1α
PI3-Kinase	Phosphatidylinositol-3-Kinase
PIP2	Phosphatidylinositol di-phosphate
PIP3	Phosphatidylinositol tri-phosphate
PCR	Polymerase Chain Reaction
KCl	Potassium Chloride
KOH	Potassium Hydroxide
pH	Potential Hydrogen
RIPA	Radio-Immunoprecipitation Assay
rRAD	Ras Associated Diabetes
RPIIβ	RNA polymerase II

RT-qPCR	Real time quantitative polymerase chain reaction
RNA	Ribonucleic acid
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
Na_3VO_4	Sodium Orthovanadate
SA	Specific Activity
3D	Three-dimensional
^3H	Tritium
^3H-2DG	Tritium labelled 2-deoxy-d-glucose
TBS	Tris Buffered Saline
2D	Two-dimensional
UV	Ultra violet
2-NBDG	2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino] – D-glucose

1 Introduction

1.1 Introduction

Since the time of Hippocrates, the promotion of a healthy diet and sufficient exercise in the treatment of metabolic disease has been a major priority (Hawley & Gibala, 2012). Despite health guidelines and the associated benefits of changes in habitual routines in order to attenuate the prevalence of chronic disease, poor diet and inadequate levels of physical activity remain a major health concern in the modern era (Blair, 2009). Connected to this, and despite advancements in modern medicine, the increase in prevalence of non-communicable diseases such as diabetes mellitus is escalating on a pandemic scale and it is estimated that 5.4% of the global population will be affected by 2025 (King, Aubert, & Herman, 1998).

In a healthy individual, circulated blood glucose is maintained at approximately 3-5mmol/L^{-1}, or approximately four grams of blood glucose (Wasserman, 2009). Although the amount of circulating blood glucose is small, a delicate balance exists between hypo and hyperglycaemia and both can have adverse effects upon an organisms function (individual circulated blood glucose is maintained at approximately 3-5mmol/L^{-1}, *Figure 1-1*) (Wasserman, 2009). Glucose provides the primary source of cellular energy through its conversion to ATP by anaerobic and aerobic pathways (Bouché, Serdy, Kahn, and Goldfine, 2004). The hydrophilic nature of glucose means that it is obtained up into the cell via facilitated diffusion. This process occurs following a series of signalling cascade events mediated by the hormone, insulin. Insulin stimulates the rapid recruitment of glucose transporters to the cell membrane (Pessin, Thurmond, Elmendorf, Coker, and Okada, 1999) upon which, glucose is obtained up and rapidly phosphorylated into glucose-6-phosphate by hexokinase the initial step in the process of glycolysis (Bouché et al., 2004).

In order to maintain euglycemia in the blood, several tissues are involved in controlling preserving blood glucose. While insulin sensitive tissues such as adipose tissue and skeletal muscle play a crucial role in glucose disposal (DeFronzo, Gunnarsson, Bjorkman, Olsson, & Wahren, 1985), tissues including skeletal muscle but also the liver, which store glucose in the form of glycogen, breakdown glycogen stores by glycogenolysis, at equal rates to glucose disposal by insulin sensitive tissues to maintain blood glucose concentrations (Wasserman, 2009). In contrast, during periods of hypoglycaemia, the release of glucagon from alpha cells in the pancreases increases endogenous glucose production release from the liver in order to maintain blood glucose concentrations (Wasserman, 2009). Hepatic clearance of glucagon is approximately 20-30% (D'Alessio, 2011). Administration of glucagon increases blood glucose concentrations in healthy individuals (Del Prato, Castellino, Simonson, and

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DeFronzo, 1987), however, in addition to impaired β-cell function and insulin secretion, there is some evidence to suggest that impaired α-cell function could contribute to the pathogenesis of NIDDM (Dunning and Gerich, 2007). Insulin resistance, which is characterised as the inability of a target cell or organ to respond to a normal physiological concentration to which it has been exposed can result in detrimental effects upon glucose homeostasis, leading the onset of metabolic diseases such as, diabetes mellitus , tissues including skeletal muscle but also the liver, which store glucose in the form of glycogen, breakdown glycogen stores by glycogenolysis, at equal rates to glucose disposal by insulin sensitive tissues to maintain blood glucose concentrations (Wasserman, 2009) In contrast, during periods of hypoglycaemia, the release of glucagon from alpha cells in the pancreases increases endogenous glucose production release from the liver in order to maintain blood glucose concentrations (Wasserman, 2009). Hepatic clearance of glucagon is approximately 20-30% (D'Alessio, 2011). Administration of glucagon increases blood glucose concentrations in healthy individuals (Del Prato et al., 1987), however, in addition to impaired β-cell function and insulin secretion, there is some evidence to suggest that impaired α-cell function could contribute to the pathogenesis of NIDDM (Dunning and Gerich, 2007). The cellular mechanisms required for effective insulin stimulated glucose uptake are highly complex and require several signalling protein kinases working in unison to enable cellular glucose uptake. As a result, any impairment within the signalling cascade can significantly abate glucose uptake (Shulman, 2000). Therefore, due to the increase in prevalence of diabetes globally (Amos, McCarty, & Zimmet, 1997), research investigating the molecular mechanisms of the disease is of utmost interest.
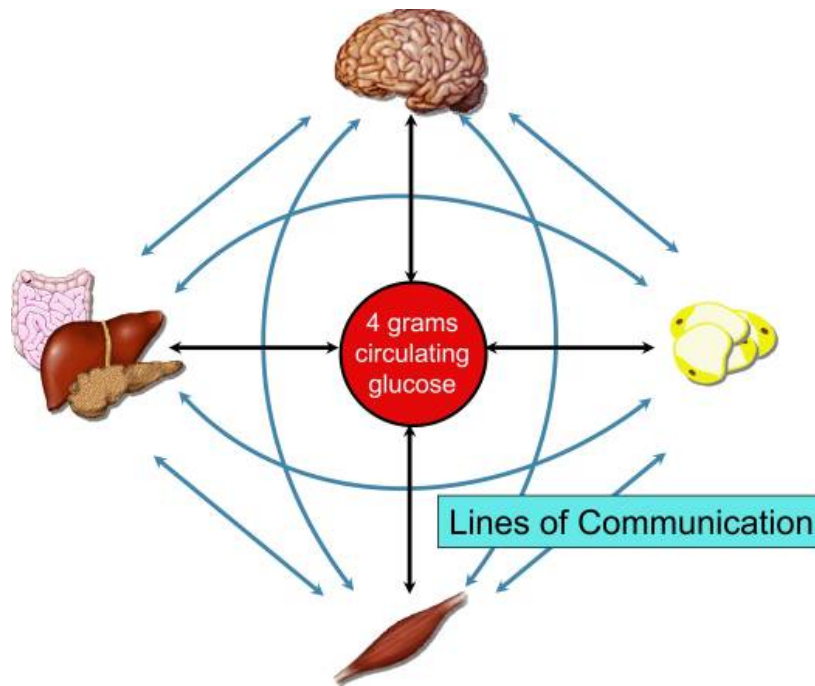
Figure 1-1 The maintenance of glucose homeostasis. Several lines of communication between organs maintain euglycemia. Skeletal muscle and adipose tissue contribute to a large proportion of glucose disposal. Image from Wasserman (2009).

Skeletal muscle has been described as a major endocrine organ, with ~80% of glucose uptake occurring within skeletal muscle under hyperinsulineamic-euglycemic conditions (DeFronzo, Gunnarsson, Bjorkman, Olsson, & Wahren, 1985). Glucose uptake is regulated by both insulin dependent and independent mechanisms (Pereira & Lancha, 2004). Regulation of glucose uptake by insulin dependent mechanisms involves activation of the insulin signalling pathway through binding of the hormone, insulin to its receptor, leading to downstream activation of a number of proteins kinases which lead to GLUT4 translocation to the membrane (Taniguchi, Emanuelli, and Kahn, 2006)(Taniguchi et al., 2006). while the prior accounts for the majority of post-prandial glucose uptake, insulin independent mechanisms, which is often associated with skeletal muscle contraction, results in GLUT4 mediated glucose uptake, independent of insulin (Holloszy, 2003).(Holloszy, 2003) Both *in vitro* (Zierath et al., 1996), and *in vivo* work (DeFronzo et al., 1985), have shown that type II diabetes mellitus (NIDDM) patients' present marked decreases in insulin stimulated glucose uptake. Furthermore, both lean and obese populations exhibit the defects associated with skeletal muscle insulin resistance (Kelley, Mokan, & Mandarino, 1993; Zierath et al., 1996).

While *in vivo* research has begun to elucidate some of the mechanisms associated with insulin resistance in skeletal muscle (Karlsson et al., 2005; Krook et al., 2000), *in vitro* research can provide further insight into the effects of insulin resistance upon the cellular signalling

pathways involved in glucose uptake (Lambernd et al., 2012; Sell et al., 2008). The use of cell culture models to investigate this phenomenon has been, and continues to be, a well-established scientific method. An additional approach to cell culture has been through the use of three-dimensional (3D) skeletal muscle tissue engineering. Skeletal muscle tissue engineering is a rapidly growing field with regards to regenerative medicine (Mertens, Sugg, Lee, and Larkin, 2014), and has recently been used for implantation to replace damaged or degenerative tissue (Juhas, Engelmayr, Fontanella, Palmer, and Bursac, 2014; Williams, Kostrominova, Arruda, and Larkin, 2013). What is more, the use of these models for research purposes could provide a more replicable and effective model both morphologically and physiologically to investigate metabolic and physiological functions (Khodabukus, Paxton, Donnelly, and Baar, 2007).

The advancement in the use of skeletal muscle tissue engineered constructs has resulted in a number of methods using various extracellular matrices (Hinds, Bian, Dennis, and Bursac, 2011). Such models have produced constructs, which result in the formation of uniaxial myotubes under tension (Dennis & Kosnik, 2000), and develop cylindrical muscle bundles as found *in vivo* (Huang, Dennis, Larkin, & Baar, 2005). Constructs of this nature have already been used to characterise early muscle development, (Cheema, Yang, Mudera, Goldspink, & Brown, 2003; Smith et al., 2012), as well as the molecular responses to exercise (Passey, Martin, Player, & Lewis, 2011; Player et al., 2014). However, despite a number of challenges currently within the field (Cheng, Davis, Madden, Bursac, and Truskey, 2014), little research has explored the role of glucose uptake (Baker, Dennis, and Larkin, 2003) or the responses of tissue engineered constructs to insulin and the associated proteins implicated in this signalling pathway. Research into the development of a culture model could provide further in vitro model with which to investigate some of the mechanisms associated with insulin signalling and glucose uptake in skeletal muscle. Therefore, the aim of this thesis is to develop a skeletal muscle model of insulin resistance through exposure of skeletal muscle cells to chronically high levels of insulin, in monolayer culture and in three dimensional tissue engineered cultures.

The objectives of this thesis are described through the following experimental objectives:

  I.   *To investigate the suitability of tissue engineered constructs to investigate glucose uptake.*

II. *To determine if insulin signalling and insulin stimulated glucose uptake can be observed in skeletal muscle tissue engineered constructs.*

III. *To determine if insulin sensitivity can be manipulated in vitro in 2D and 3D cultures.*

2   Literature Review

2.1 Skeletal Muscle Structure and function

2.1.1 Skeletal muscle structure

Skeletal muscle fibres are surrounded by layers of extracellular matrix (ECM), which maintains their structure and function (Kjaer, 2004; Lewis, Machell, Hunt, Sinanan, and Tippett, 2001). Individual fibres are contained by the endomysium, which are grouped in bundles to form the perimysium, with the epimysium surrounding the whole muscle (*Figure 2-1*) (Gillies and Lieber, 2011).

2.1.2 Extracellular matrix

The ECM is comprised of four components, collagenous and non-collagenous glycoproteins, proteoglycans and elastin (Lewis et al., 2001). A number of adhesion molecules are involved in cell-cell and cell-ECM interaction the dystrophin-dystroglycan complex and integrins, (Lewis et al., 2001). Collagen is a highly abundant protein accounting for approximately one quarter of protein mass in skeletal muscle (Alberts, Bray, and Lewis, 1986). It is a major component of intramuscular connective tissue, and although several types of collagen have been identified, type I collagen represents the highest proportion of intramuscular collagen content (Kjaer, 2004).
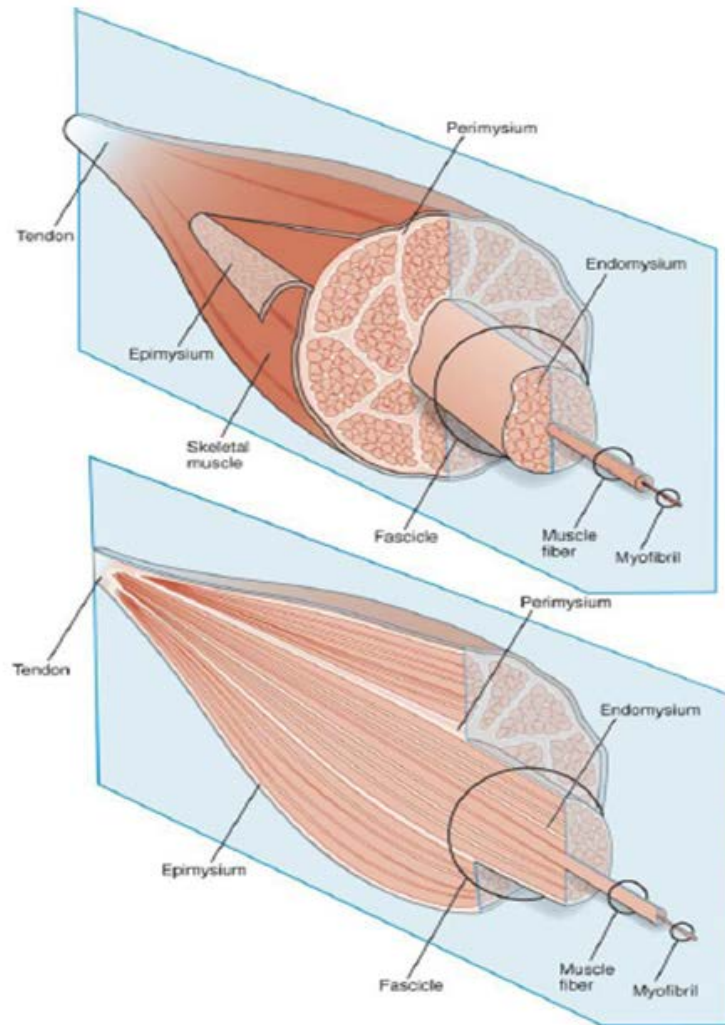
Figure 2-1 The structure of skeletal muscle. The whole muscle is encompassed by the epimysium with muscle bundles surrounded by the perimysium. Each muscle fibre is surrounded by the endomysium. Image from Gillies and Leiber, (2011).

2.2 Skeletal Muscle adaption and maladaptation

Skeletal muscle is very adaptable in its response to external stimuli resulting in transcriptional and translation changes following exercise (Egan and Zierath, 2013), nutritional intake (Greenhaff et al., 2008) and environmental exposures, such as hypoxia (D'Hulst et al., 2013). Likewise, skeletal muscle also responds rapidly both morphologically and physiologically as a result of disuse atrophy (Brooks and Myburgh, 2014), ageing, and to severe illness like cancer cachexia (Fearon, Glass, and Guttridge, 2012). The aforementioned problems can have major effects upon skeletal muscle metabolism, often leading to further complications (Egan and Zierath, 2013).

Skeletal muscle is major endocrine organ, accounting for the majority of glucose disposal (DeFronzo, Ferrannini, Hendler, Felig, & Wahren, 1983). Interventions such as exercise training have shown to improve insulin sensitivity and glycaemic control (Kasumov et al., 2015; Little et al., 2011). In contrast, physical inactivity and poor nutritional intake can have negative effects upon skeletal muscle metabolism. Metabolic diseases such as metabolic syndrome X and diabetes *mellitus* are a result of changes in glucose metabolism and insulin signalling (Roberts, Hevener, and Barnard, 2013). Although, genetics can contribute to the risk of non-communicable disease development, poor dietary intake and physical inactivity contribute to the onset and potential development of these metabolic diseases which result in molecular changes at the transcriptional (Rome et al., 2009), and translational (Bouzakri et al., 2003; Krook et al., 2000) level, which attenuate skeletal muscle glucose metabolism.

2.3 Activation, proliferation and differentiation of skeletal muscle cells.

Skeletal muscle cells can be described as syncytial as they are comprised of several nuclei within the cytoplasm (Alberts et al., 1986). More commonly referred to as muscle fibres, these structures can be up to two to three centimetres in length and 100µm in diameter. As well as this variation in size, skeletal muscle also has the ability to regenerate (Zammit et al., 2002). Satellite cells, or skeletal muscle stem cells, are quiescent muscle precursor cells, located below the basal lamina (Scharner & Zammit, 2011, Mauro, 1961) and are activated by a variety of stimuli such as chemical damage or mechanical loading (Cosgrove, Sacco, Gilbert, and Helen, 2010). Activated skeletal muscle cells proliferate extensively before committing to the myogenic lineage and terminally differentiating to form new muscle fibres, fuse to regenerate existing fibres. Cells that do not commit to the myogenic linage return to the satellite cell niche and replenish the satellite cell pool (*Figure 2-2*). The development of skeletal muscle cells from activation to differentiation is regulated by a number of

transcriptional factors (Buckingham et al., 2006). Proliferating cells express paired box transcription factors, Pax 3 and Pax 7 which maintain these cells in the cell cycle (Zammit et al., 2004). During differentiation, these cells show a reduction in the expression of Pax genes, increasing the expression of basic helix loop helix (b-HLH) transcription factors which are imperative for skeletal muscle development (Rudnicki et al., 1993). The b-HLH transcription factors such as, desmin (*MyoD*), myogenic regulatory factor 5 (*Myf 5*), Myogenin (*MyoG*) and myogenic regulatory factor 4 (*MRF4*), are important in the commitment of these cells to the myogenic lineage and the regulation of differentiation into mature skeletal muscle fibres (Buckingham et al., 2003).
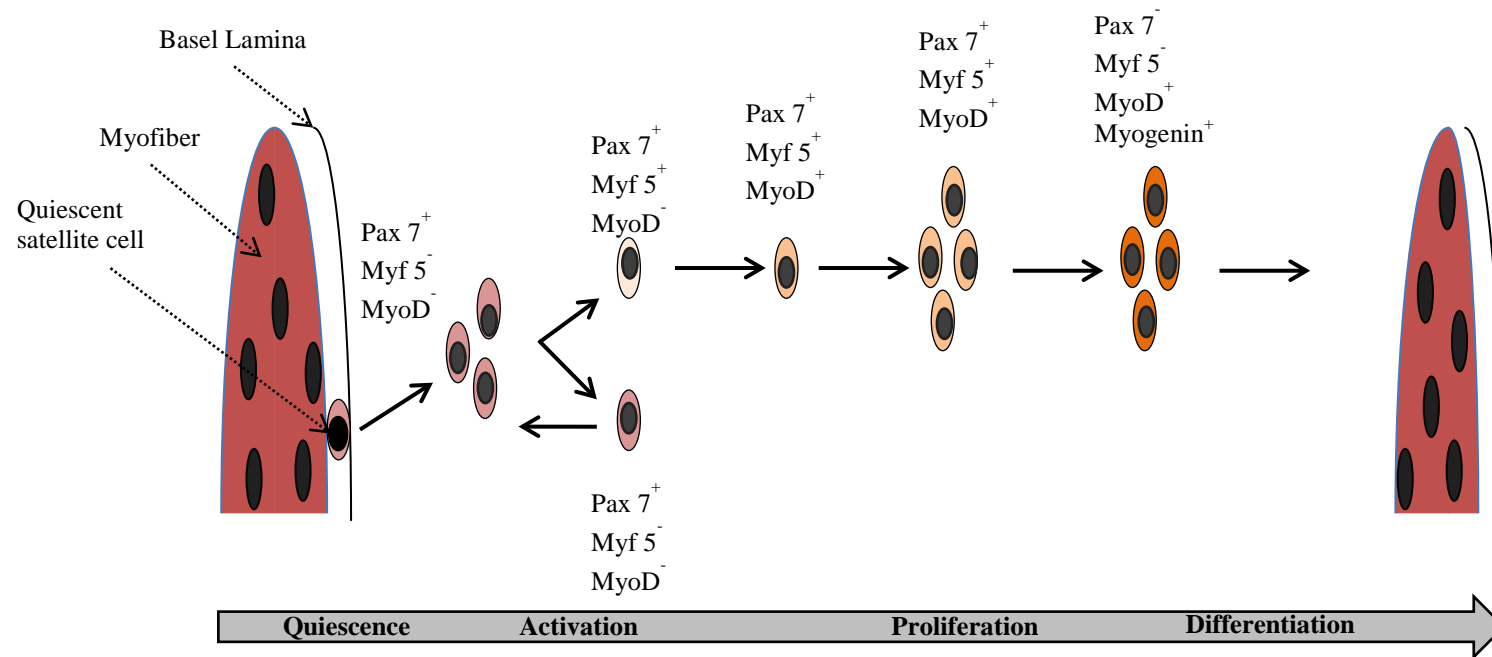
Figure 2-2 The role of skeletal muscle satellite cells in skeletal muscle regeneration. Satellite cells (SC) are in a state of quiescence located below the basal lamina of the myofiber. Upon activation by external stimuli, satellite cells undergo asymmetric division and either commits to the myogenic lineage through the expression of myo-regulatory factors (Myf5, MyoD), differentiating into new myofibres, or alternatively maintain expression of Pax7 and aid replenishment of the SC pool. Figure adapted from Perdiguero et al., (2009).

2.4 Culture of skeletal muscle cells in monolayer

The development and regeneration of skeletal muscle cells can be recapitulated *in vitro* (Cosgrove et al., 2010). Isolation of skeletal muscle cells for *in vitro* culture has been established for several decades (Blau & Webster, 1981; Yaffe & Saxel, 1977), and has subsequently furthered the understanding of the mechanisms behind skeletal muscle development (Zammit et al., 2004). Extraction of muscle derived cells from rodents (Smith, Passey, Greensmith, Mudera, and Lewis, 2012) and humans (Blau and Webster, 1981; Brady, Lewis, and Mudera, 2008; Mudera, Smith, Brady, and Lewis, 2010) amongst many species has successfully been performed. Primary skeletal muscle cells have been found to replicate their physiological characteristics *in vivo* (Aas et al., 2013), and have been cultured *in vitro* in a manner which recapitulates their native environment (Brady et al., 2008; Martin et al., 2013).

In addition to primary cell sources, the establishment of myogenic cells lines such as C2C12 and L6 rodent cells lines are alternative cell source that have been characterised and used to investigate skeletal muscle physiology. These cell lines, which are homogenous in population, have been used to develop an *in vitro* system. Both C2C12 and L6 cells are well established within the literature for investigating the molecular basis of growth and development (Burattini et al., 2004; Cheema, Yang, Mudera, Goldspink, & Brown, 2003), skeletal muscle hypertrophy and atrophy (Sharples, Al-Shanti, and Stewart, 2010), as well as insulin signalling and glucose uptake (del Aguila, Claffey, and Kirwan, 2011; Nedachi and Kanzaki, 2006).insulin

2.5 The culture of skeletal muscle cells in three dimensional (3D) constructs

A plethora of research relives on 2D culture of skeletal muscle cells on tissue culture plastic. However, removal of cells from a 3D environment into 2D, or monolayer environment can affect cell behaviour (Tibbitt and Anseth, 2009). As a result researchers have begun to employ the use of artificial and biological matrices in which cells can be grown in a 3D environment (Hinds et al., 2011). The culture of cells in a 3D environment has been shown to result in differential gene expression compared to 2D cultures (Vergani, Grattarola, and Nicolini, 2004) and improve functional capacity of cells (Weber, Hayda, and Anseth, 2008). As with many cell types including skeletal muscle, 2D culture models provide a basic platform for investigating mechanistic responses without the confounding systemic factors found *in vivo*. Furthermore, the morphological differences between 2D and 3D cell cultures, mean that the development of 3D cultures provides a systems which better recapitulates *in*

vivo skeletal muscle (Huang, Dennis, Larkin, & Baar, 2005; Vandenburgh, Karlisch, & Lynee, 2012).

2.5.1   Skeletal muscle cell types in tissue engineering

The development of skeletal muscle tissue engineered constructs was arguably started with the work Vandenburgh and colleagues who seeded skeletal muscle cells within a collagen gel fixed between two anchor points (Vandenburgh, Karlisch, & Farr, 1988). The resulting tension from the fixation of the constructs results in spontaneous contraction (Vandenburgh, Hatfaludy, Karlisch, & Shansky, 1991; Vandenburgh et al., 1988), as well as enhancing myogenesis (Vandenburgh, Hatfaludy, Karlisch, & Shansky, 1991). Differences in metabolism, phenotype and development lead to alterations in the excitability and contractility of established cell lines and primary cultures differs within a 3D environment (Dennis, Kosnik, Gilbert, & Faulkner, 2001; Kosnik, Faulkner, & Dennis, 2001). These differences in excitability and contractual responses are potentially due to the role of the ECM (Brady et al., 2008; Kosnik et al., 2001; Mudera et al., 2010). In addition, the use of C2C12 skeletal muscle cells have been utilised and characterised within the 3D environment (Khodabukus & Baar, 2009; Sharples et al., 2012; Player et al., 2014) and have subsequently provided a replicable system to further investigate the skeletal muscle physiology and function.

2.5.2   The use of extracellular matrices in tissue engineering.

Optimisation of tissue-engineered constructs has used varying biological organic matrices such as laminin (Dennis & Kosnik, 2000; Dennis et al., 2001), collagen (Cheema et al., 2003; Okano & Matsuda, 1997; Smith et al., 2012) or fibrin (Huang et al., 2005; Martin et al., 2013) as a surrogate ECM. This is supported by data which has shown that the ECM plays an important role in the generation of force in 3D tissue engineering constructs (Hinds et al., 2011).

2.5.2.1.   *Fibrin*

Later work by Strohman and colleagues observed the formation of three dimensional skeletal muscle constructs using a self-assembly model (Strohman et al., 1990). Later work using fibrin as a ECM has subsequently been used (Huang et al., 2005), which developed formation of connective tissue and expression of mature skeletal muscle characteristics (Strohman et al., 1990). More recent work has substituted theses matrices with a major structural protein involved in blood clotting, fibrin (Grassl, Oegema, and Tranquillo, 2002). Fibrinogen is the monomeric form of fibrin which undergoes fibrinogensis by thrombin enzyme which cleaves

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fibro-peptides, enabling for lateral formation of linear fibrils (Grassl et al., 2002). Fibrin enables for free migration and proliferation of cells on the gel, but also the development of an ECM, which upon formation results in degradation of the excess fibrin (Ross and Tranquillo, 2003). The ding of primary myoblast cells of rats in fibrin coated gels has been found to lead the formation of unidirectional, multinucleated myotubes, (Huang et al., 2005; Khodabukus & Baar, 2009). In addition, recent research by Martin and colleagues has further characterised the phenotype and optimal myogenic cell population for growth of human muscle derived cells in a fibrin ECM (Martin et al., 2013).

2.5.2.2. *Collagen*

The use of collagen as an ECM provides a physiologically relevant matrix with which skeletal muscle cells reside *in vivo* (Cheema et al., 2005; Powell, Smiley, Mills, and Vandenburgh, 2002). Similar to earlier 3D tissue engineered models, cells grown within this environment differentiate into uniaxial, multi-nucleated myotubes, which is a result of the mechanical signals that are present within these models. The use of collagen gels provide repeatable cellular architecture of culturing primary skeletal muscle cells in three-dimensional culture (Smith et al., 2012). Similar to other models of tissue engineering, primary MDC's require an optimal population of myogenic cells in order to form into multinucleated myotubes (Brady et al., 2008; Mudera et al., 2010). In addition, C2C12 skeletal muscle cells in this system has been shown to alter their transcriptional expression of genes involved in myogenic development (Cheema et al., 2005), Furthermore, mechanical loading upon these constructs has been shown to induce a transcriptional change in splice variants of IGF-I (mechano growth factor (MGF) and insulin like growth factor -1 (IGF-1Ea)) (Cheema et al., 2005; Cheema, Yang, Mudera, Goldspink, and Brown, 2003). In addition to investigation of skeletal muscle development within these constructs (Cheema et al., 2003), more recent work by Sharples and collages has investigated skeletal muscle cell development of multiple populations doubled C2C12 myoblast cells as a model of skeletal muscle hypertrophy and atrophy (Sharples et al., 2012). Previously used in two dimensional culture (Sharples, Al-Shanti, Lewis, and Stewart, 2011), MPD cells within collagen based tissue engineered constructs showed to have impaired mRNA expression of genes involved in matrix remodelling (MMP9) as well expression of IGF and IGF binding protein expression (Sharples et al., 2012). Previous work by Player and colleagues had briefly elucidated to the metabolic response of these constructs by the measurement of extracellular lactate production

following mechanical load and mRNA expression of genes implicated in matrix remodelling and hypertrophy (Player et al., 2014).

2.6 *In vitro* investigation of insulin signalling and glucose uptake

2.6.1 Investigating insulin signalling and glucose uptake using skeletal muscle cell lines

Skeletal muscle cell lines (*Table 2-2*) have been used to investigate insulin signalling and glucose uptake (Sarabia, Ramlal, and Klip, 1990), and to elucidate the mechanisms of GLUT4 exocytosis to the plasma membrane (Ishikura, Antonescu, and Klip, 2010). The work by Klip and colleagues has extensively investigated the roles of glucose transporters within skeletal muscle culture through transfection of a *myc*-tagged GLUT4 protein into L6 myoblast cells (L6GLUT4*myc*) (Antonescu, Randhawa, and Klip, 2008). Work with the L6GLUT4*myc* has enabled visualisation of GLUT4 vesicle trafficking within skeletal muscle and the associated mechanisms of mechanical remodelling which occurs during GLUT4 translocation (Klip, Sun, Chiu, & Foley, 2014; Sun, Chiu, Foley, Bilan, & Klip, 2014).

However, C2C12 muscle cells have been used to investigate insulin signalling and have been reported to respond to insulin stimulation (Nedachi and Kanzaki, 2006) and manipulated through hormonal and pro-inflammatory cytokine exposure (del Aguila et al., 2011; Kumar and Dey, 2003). In addition, C2C12 cells have been used as a model to investigate insulin independent mechanisms of glucose uptake, due to their contractile activity and ability to generate force production (Nedachi, Fujita, and Kanzaki, 2008). Through the use of electrical pulse stimulation (EPS) as a method for inducing skeletal muscle contraction, Nedachi and colleagues have shown both AMPK and p38 MAPK signalling to be activated following EPS (Nedachi et al., 2008). In addition, uniaxial stimulation has be used has shown calcium dependent mechanism of glucose uptake in C2C12 skeletal muscle cells (Iwata et al., 2007; Iwata, Suzuki, Hayakawa, Inoue, and Naruse, 2009). These studies provide evidence that this cell line has been used to investigate cellular signalling through insulin stimulation and EPS to investigate the mechanisms of glucose uptake and GLUT4 recycling (Nedachi et al., 2008; Nedachi and Kanzaki, 2006). Similar to previous research using the L6GLUT4*myc*, transfection of the myc-tagged GLUT4 has been successfully transfected into C2C12 skeletal muscle cells (Niu et al., 2010). The use of the C2C12GLUT4*myc* line has helped to further elucidate the mechanisms of contraction mediated GLUT4 translocation to the cell membrane through a both AMPK and Calcium mediated signalling pathways (Niu et al., 2010).

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Table 2.1 the use of skeletal muscle cells lines to investigate insulin signalling and glucose uptake

Rodent Skeletal Muscle Cell Lines			
L6 Skeletal Muscle Cells		**C2C12 Skeletal Muscle Cells**	
Reference	**Summary**	**Reference**	**Summary**
Sarabia et al., (1990), Ishikura et al., (2010) Antonescu et al., (2008) Michael et al., (2001)	• Initially used to investigate insulin stimulated glucose uptake • Used to investigate GLUT4 vesicle trafficking • Used to investigate myogenic development and glucose transporter expression • Have been successfully transfected to further elicited the mechanism of glucose transporter vesicle trafficking	Nedachi et al., (2009) Nedachi et al., (2006); Kumar & Day, (2003) Philp et al., (2011) Iwata et al., (2009) Niu et al., (2010)	• Investigate Insulin dependent and independent glucose uptake • Effects of metabolic stressors upon glucose uptake • Have been successfully transfected to further elicited the mechanism of glucose transporter vesicle trafficking

2.6.2 *Human skeletal muscle cells,* insulin signalling and glucose uptake

In addition to the use of cell lines, the culture of human primary cells is a well-established technique (Blau and Webster, 1981). These cells, derived from biopsies, proliferate and differentiate into multi nucleated myotubes and provide an in vitro model for investigating the mechanisms of a number of skeletal muscle diseases, such as skeletal muscle insulin resistance. In addition to the use of cell lines, the culture of human primary cells is a well-established technique (Blau and Webster, 1981). These cells, derived from biopsies, proliferate and differentiate into multi nucleated myotubes and provide an in vitro model for investigating the mechanisms of a number of skeletal muscle diseases, including insulin resistance (*Table 2-1*) (Aas et al., 2013). The use of skeletal muscle cells has offered some insight into such questions independently of the systemic environment with which they normally inhabit (Cosgrove et al., 2010). The extraction of skeletal muscle cells from NIDDM populations has been successfully achieved since the mid-nineties (Henry, Abrams, Nikoulina, and Ciaraldi, 1995). While skeletal muscle cells from both healthy and diseased populations express the fundamental genes of the myogenic linage, characterising these cells as skeletal muscle (Thompson, Pratley, and Ossowski, 1996), importantly, the characteristics of skeletal muscle from NIDDM patients is conserved in culture, with impaired glucose uptake and reduced glycogen synthase activity compared to muscle cells from healthy donors (Ciaraldi, Abrams, Nikoulina, Mudaliar, & Henry, 1995; Gaster, Petersen, Højlund, Poulsen, & Beck-nielsen, 2002; Thompson et al., 1996). More recent research by Feng and colleagues has shown that skeletal muscle cells from NIDDM populations in addition to impaired insulin stimulated glucose uptake, skeletal muscle cells from this population retain the impaired insulin signalling that is associated NIDDM (Feng et al., 2015). Additionally, experiments investigating the cross talk between skeletal muscle cells and adipocytes have been performed by culturing healthy human MDCs in adipocyte-conditioned media (Eckardt, Sell, and Eckel, 2008; Sell et al., 2008). Eckardt and colleagues reported impaired insulin signalling through impaired Akt protein phosphorylation and subsequently insulin stimulated glucose uptake in healthy human skeletal muscle cells incubated with adipocyte conditioned media (Eckardt et al., 2008).

Further attenuation in metabolic flexibility has been observed in cultured skeletal muscle cells from NIDDM (Gaster, 2007). One hypothesis for the differences in free fatty acid (FFA) oxidation between healthy and insulin resistant individuals is resultant of differences in

mitochondrial lipid oxidation, which has been found to be attenuated in skeletal muscle cells from both obese and NIDDM individuals (Boyle, Zheng, Anderson, Neufer, and Houmard, 2012; Cha et al., 2005). Boyle and colleagues reported a reduction in mitochondrial DNA (mtDNA) copy number in skeletal muscle cells from obese individuals as well as a reduction in respiration content (Boyle et al., 2012). More recently the investigation of adipocyte conditioned medium upon healthy skeletal muscle cells have shown to have negative effects upon distal and proximal insulin signalling proteins (Dietze et al., 2002; Sell et al., 2008) and impaired mRNA expression of myogenin, a key marker of skeletal muscle myogenisis (Sell et al., 2008). Further attenuation in metabolic flexibility has been observed in cultured skeletal muscle cells from NIDDM insulin adipocyte conditioned medium upon insulin and impaired mRNA expression of myogenin, a key marker of skeletal muscle myogenisis

The increased circulation of free fatty acids (FFA), and pro- inflammatory cytokines such as, tumor-necorsis factor- alpha (TNF-α) has been implicated in the alterations in insulin signalling and subsequently glucose utilisation. Animal models of obesity have shown an increase in expression of TNF-α, and the elevations in plasma TNF-α is correlated with an increase in fasting plasma and insulin concentrations (Hotamisligil, Arner, Caro, Atkinson, and Spiegelman, 1995). Furthermore, *in vitro* experiments have linked an increase in TNF-α, impairing insulin signalling through serine induced phosphorylation of the insulin receptor and insulin receptor substrate (Hotamisligil et al., 1996; Hotamisligil, Budavari, Murray, and Spiegelman, 1994). Later work using murine myoblast cells confirmed the earlier findings of the effects of TNF-α incubation upon insulin signalling by reducing IRS-1 associated PI-3 kinase activity phosphorylation, in addition to the impairment in glucose uptake by ~20% (del Aguila et al., 2011).

Table 2.2 Subject characteristics of human skeletal muscle cell donors to investigating in vitro insulin signalling and glucose uptake

Reference	Subject No.	Age (yr.)	BMI (kg/M^2)	Fasting Plasma Glucose (mmol/l)	Oral Glucose Tolerance Test (OGTT)		Insulin (pmol/l) Concentrations	Other Measures
					Glucose (mmol/l)	Insulin (pmol/l)		
Henry et al., (1995)	ND = 14 NIDDM= 11	ND = 42 ± 2 NIDDM = 50 ± 2*	ND 27.5 ± 1.0 NIDDM = 30.4 ± 1.2*	ND 5.2 ± 0.1 NIDDM = 8.8 ± 0.9*	ND 6.0 ± 0.1 NIDDM = 14.9 ± 1.2*	ND 382 ± 93 NIDDM = 293 ± 71*	ND 36 ± 12 NIDDM = 102 ± 3*	HEC
Ciaraldi et al., (1995)	ND = 15 NIDDM= 11	ND = 42 ± 2 NIDDM = 50 ± 2*	ND 27.5 ± 1.0 NIDDM = 30.4 ± 1.2*	ND 5.2 ± 0.1 NIDDM = 10.2 ± 1.6*	ND 6.1 ± 0.3 NIDDM = 16.8 ± 2.1*	ND 424 ± 84 NIDDM = 371 ± 106*	NS 75 ± 14 NIDDM = 137 ± 17*	
Henry et al., (1996)	ND = 8 NIDDM = 11	ND = 42 ± 2 NIDDM = 50 ± 2**	ND 23.9 ± 1.0 NIDDM = 30.6 ± 0.8**	ND 5.5 ± 0.1 NIDDM = 10.7 ± 1.4**	ND 6.3 ± 0.5 NIDDM = 16.0 ± 2.5‡	ND 254 ± 84 NIDDM = 271 ± 85	ND 32 ± 12 NIDDM = 108 ± 15**	
Thompson et al., (1996)	IR = 11	29 ± 7		85 ± 7 mg/dl	117 ± 33 mg/dl			HEC IMGD % Fat

Reference	Subject No.	Age (yr.)	BMI (kg/M^2)	Fasting Plasma Glucose (mmol/l)	Oral Glucose Tolerance Test (OGTT)		Insulin (pmol/l) Concentrations	Other Measures
Gaster et al., (2002)	ND = 8 NIDDM = 8	ND = 26.9 ± 0.2 NIDDM = 49.5 ± 1.8‡	ND 23.6 ± 0.7 NIDDM = 30.4 ± 1.1‡	ND 5.3 ± 0.1 NIDDM = 9.9 ± 0.1‡			ND 42.0 ± 8.4 NIDDM = 71.8 ± 10.6*	
Dietze et al., (2002)	ND = 3	M= 9, 28 F= 10						
Bouzakri et al., (2003)	ND = 9 NIDDM = 9	ND = 42 ± 6 NIDDM = 56 ± 4	ND = 24 ± 1 NIDDM 32 ± 2	ND = 5.0 ± 0.2 NIDDM = 9.6 ± 0.9			31 ± 4	HbA1c %
Al-Khalili et al., (2003)	ND = 8	55 ± 5	25.6 ± 1.5	5.4 ± 0.3				
Dietze et al., (2004)	ND = 4	M = 9, 5 F = 10, 48						
Dietze-Schroeder et al., (2005)	ND = 4	M = 9, 5 F = 10, 48						

Reference	Subject No.	Age (yr.)	BMI (kg/M^2)	Fasting Plasma Glucose (mmol/l)	Oral Glucose Tolerance Test (OGTT)		Insulin (pmol/l) Concentrations	Other Measures
Cha et al., (2005)	ND = 39 NIDDM = 30	ND = 44 ± 2 NIDDM = 53 ± 2	ND 29.0 ± 1.1 NIDDM = 34.7 ± 1.5†	ND 5.1 ± 0.1 NIDDM = 9.6 ± 0.6†			NS 68 ± 6 NIDDM = 208 ± 33*	FFA† TG† GDR† HbA1c %†
Pender et al., (2005)	NO = 9 Ob = 16	NO = 44 ± 1 Ob = 38 ± 3	NO 25.5 ± 0.9 Ob = 54 ± 4.2‡	NO = 83 ± 6 mg/dl Ob = 91 ± 3 mg/dl			NO 3.6 ± 1.7 Ob = 20.1 ± 3.2 (μU/mL)*	HOMA
Sell et al., (2008)	ND = 4	M= 9, 47 F= 10, 48						
Lambernd et al., (2012)	ND = 5	M= 16, 21, 47 F= 33, 37						

ND, Non-diabetic; NIDDM, Non-insulin dependent diabetes mellitus; IR, Insulin resistant; NO, Non-Obese; Ob, Obese; M, Males; F, Females; BMI, body mass index; HEC, Hyperinsulineamic euglycemic clamp; FFA, free fatty acid; TG, triglycerides; GDR, glucose disposal rate; HbA1c, haemoglobin A1 glycosylate; IMGD, Insulin mediated glucose disposal; HOMA, Homeostasis model assessment; Significantly difference vs. ND *p <0.05, †p<0.025, **p<0.01, ‡p<0.001

2.7 Glucose metabolism in skeletal muscle

As previously eluded too (*Section 1.1*), the balance of blood glucose is carefully maintained (Wasserman, 2009). All cells require glucose in order to function; although some tissues are greater than others. The brain accounts for a large proportion of glucose disposal along with liver and insulin sensitive tissues such as adipose tissue and skeletal muscle. Skeletal muscle is a major endocrine organ, accounting for approximately 30% of the body's metabolic turnover at rest (Zurlo, Larson, Bogardus, and Ravussin, 1990), and the majority of insulin stimulated glucose uptake (DeFronzo et al., 1983). Changes in metabolism either through diet or physical activity result in the alteration in blood glucose concentrations (Barnard and Youngren, 1992). The breakdown of glucose is mediated by aerobic and anaerobic pathways (Egan and Zierath, 2013), where it is metabolised for ATP resynthesis (Bouché et al., 2004). Upon entry to the cells glucose is rapidly phosphorylated by hexokinase to form glucose-6-phosphoate, which is further phosphorylated by phosphofructokinase to generate fructose 1,6 diphosphate, the oxidation of glucose to pyruvate enables for the production of ATP (Bouché et al., 2004). While anaerobic glycolysis yields only a small proportion of ATP, aerobic glycolysis by which pyruvate is utilised within the tricarboxylic acid cycle within the mitochondria results in a net production of 38 molecules of ATP per molecule of glucose (Bouché et al., 2004).

2.8 Insulin and Insulin Receptor Signalling

2.8.1 Insulin as a hormone

The discovery of insulin by Banting, Best and Colleagues in the early 1920s provided an instant cure for patients with diabetes *mellitus* and began a long journey to understanding the regulation of glucose by insulin in humans (Roth et al., 2012). The original experiments, involving the injection of pancreatic extracts into pancreatectomized dogs, gave light to the role of the secretions of the pancreas in glycaemic control (Banting, Best, Collip, Campbell, and Fletcher, 1922). Prior to, and leading up to the announcement of the discovery of insulin on May 3rd 1922, the successful extraction of insulin was inconsistent. Yet despite this, the successful injection of insulin into a human in 1922, and its subsequent use in the treatment of diabetes *mellitus* saw Banting Best and McCloud recipients of the Nobel Prize for physiology and medicine in 1923. In addition to its therapeutic use, insulin was the first protein to be sequenced (Sanger and Tuppy, 1951a, 1951b) for which, Fredrick Sanger received the Nobel prize in 1958. Since these major contributions to science and medicine, it could be argued that insulin continues to have an influence upon the understanding of metabolism.

Insulin is developed by proteolysis from preproinsulin in pancreatic β-cells (*Figure-2-3*). Following synthesis, preproinsulin is released into the rough endoplasmic reticulum where it is cleaved by photolytic enzymes. The conversion into insulin is mediated through the action of prohormone converts 2 and 3 as well as carboxy peptidase H (Joshi, Parikh, and Das, 2007). Insulin, a peptide hormone, consists of two chains, an A and B chain comprising of 21 and 30 amino acids respectively, bound by disulphide bonds. Its basic structure of three helices and three disulphide bonds is existent in all members of the insulin peptide family (De Meyts and Whittaker, 2002). Two surfaces of the insulin hormone are reported to interact with the insulin receptor, the first which contacts with the primary binding site on the insulin receptor comprises of hormone-dimerising residues, the second, containing hormone-hexamerising residues, is suggested to interact with the secondary insulin receptor site (Menting et al., 2013).

At physiological concentrations plasma insulin has a short half-life of approximately 4-6 minutes, with the majority of insulin degradation occurring as a result of binding to its

receptor tyrosine kinase, the insulin receptor, and by insulin degrading enzyme (IDE) where it is internalised and degraded (Duckworth, Bennett, & Hamel, 1998).

2.8.2 Insulin resistance and hyperinsulinemia

By definition, insulin resistance is the attenuated response of a target cell or organ to a normal physiological concentration of insulin to which it has been exposed. It is suggested that the symptom of hyperinsulinemia is implicated in alternations of metabolic homeostasis (Shanik et al., 2008), which characterise in part, metabolic syndrome X, but also pre-cede the onset of diabetes *mellitus* (Roberts et al., 2013).

Although acute insulin exposure has a stimulatory effect upon several transcription factors (Coletta et al., 2008; Wu et al., 2007), as well as its role in metabolic signalling (Taniguchi et al., 2006), chronic exposure to insulin can result in desensitising effects between itself, its binding domain and downstream proteins (Roth et al., 2012). While short periods of hyperinsulinemia promote insulin sensitizing effects, chronic exposure results in abrogation of this effect in skeletal muscle (Cusin, Terrettaz, Rohner-Jeanrenaud, and Jeanrenaud, 1990). A suggested mechanism of impaired insulin signalling as a result of hyperinsulinemia is via a negative feedback loop through which activation of the mTOR/p70 S6 kinase pathways leads to serine phosphorylation of IRS-1 (Ueno et al., 2005). Ueno and colleagues reported a significant reduction in IRS-1 tyrosine phosphorylation and IRS-1/PI-3 kinase associated activity in skeletal muscle of rat under hyperinsulineamic conditions (Ueno et al., 2005). The effects of hyperinsulineamia upon skeletal muscle have been recapitulated *in vitro*, which when exposed to chronic insulin within the culture media, has resulted in attenuation in both insulin receptor (IR) and insulin receptor substrate 1 (IRS-1) phosphorylation and subsequently impaired insulin stimulated glucose uptake (Kumar and Dey, 2003).
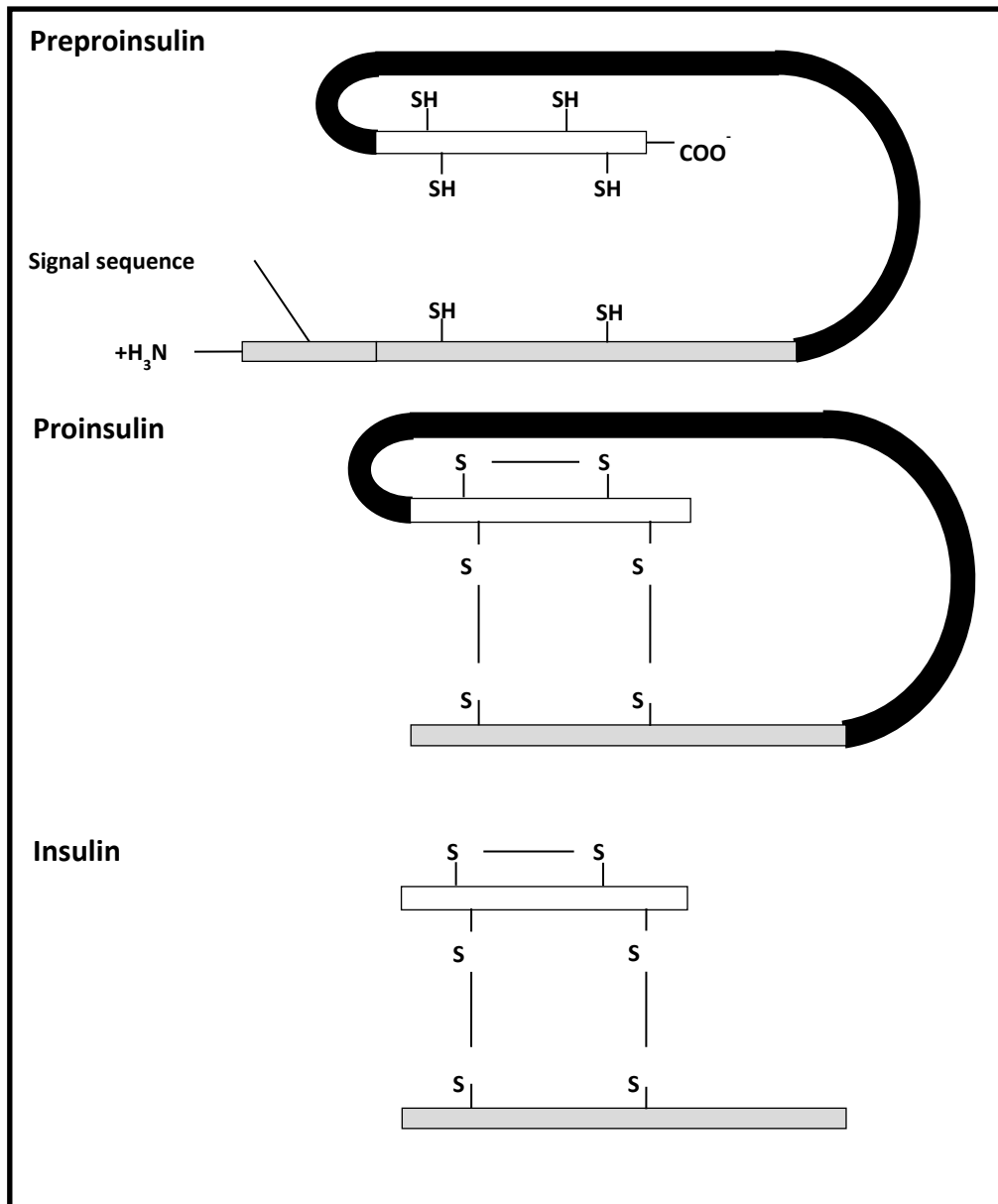
Figure-2-3 Production of insulin by proteolysis from preproinsulin. Proteolysis cleaves N-terminal signal sequence and c-peptide resulting in a two polypeptide chain structure. C-peptide (Black), A-chain (White), B-chain (Grey). Figure adapted from Joshi et al., (2007).

2.8.3   The insulin receptor

The insulin receptor is a receptor tyrosine kinase (RTK), which contains ligand-binding domains within the extracellular region, a trans membrane helix and a cytoplasmic region, which contains the tyrosine kinase domain that activates intracellular signalling pathways (*Figure* 2-4) (Lawrence, McKern, and Ward, 2007; Lemmon and Schlessinger, 2010). Exposure to insulin results in a conformational change in the insulin receptor, leading to trans-autophosphorlyation of the tyrosine kinase domain of the trans-membrane β subunit. *In vitro*, phosphorylation of the insulin receptor has been found to occur within a few seconds, phosphorylating upon three specific tyrosine residues enabling for sufficient kinase activity insulin of the insulin receptor (Tornqvist & Avruch, 1988; White & Kahn, 1989). The lucine rich domains (L1 and L2 respectively) are key binding sites in which insulin binds to the insulin receptor, as well as the fibronectin (FnIII), domain (De Meyts and Whittaker, 2002), and the two binding sites of insulin have been reported to interact with the insulin receptor (Menting et al., 2013). The high and low affinity of these binding sites can impact upon cooperatively between these respective proteins. Dose response curves have presented a bell shaped formation indicating a loss of dissociation at concentrations which exceed 100 nM (De Meyts and Whittaker, 2002). Subsequently, increasing concentrations ultimately leads to negative cooperatively[1] and further chronic exposure has been shown to lead to alterations in high and low affinity isoforms (Shanik et al., 2008), resulting in desensitisation of the insulin receptor proteins (Ueno et al., 2005).

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[1] **Negative cooperatively** is defined by De Meyts et al., (2002) as '*a property of a multi binding protein whereby the binding of one ligand molecule decreases the binding affinity of other ligand molecules to neighbouring binding sites.*'
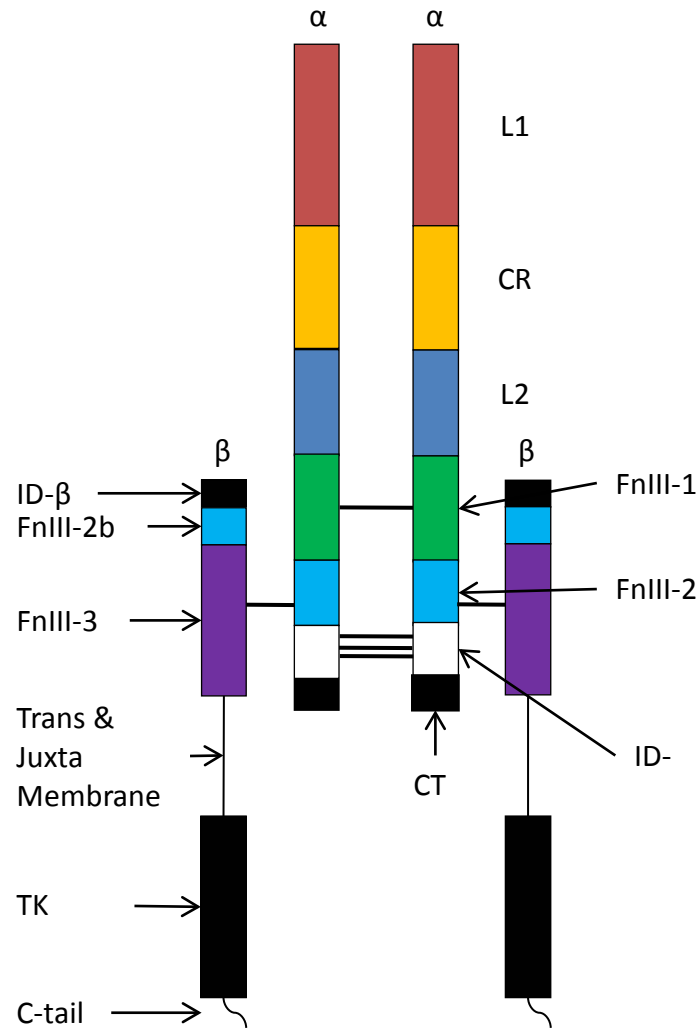
Figure 2-4 Structural domains of the insulin receptor homodimer. Leucine rich repeat domain (L1), Cysteine rich region (CR), Second leucine rich repeat domain (L2), Three fibrinectin type III domains (FnIII-1, FnIII-2, FnIII-3), Insert Domain (ID), C-terminus (CT), Tyrosine Kinase (TK). Black transverse lines represent disulphide bonds. Figure adapted from Lawrence et al., (2007).

2.8.4   Insulin Receptor Substrate 1 (IRS-1)

Tyrosine kinase phosphorylation of the insulin receptor residues results in recruitment of insulin receptor substrate 1 (IRS-1). IRS-1, a docking protein, contains several key features that initiate the recruitment of downstream insulin signalling proteins including a NH_2-terminal which aids protein-protein interactions (White, 1998). Furthermore, IRS-1 has several COOH-terminal tyrosine residues that create a *Sac* Homology (SH) -domain binding site (White and Kahn, 1994). The SH-domain is important in the recruitment of the p85 phosphoinositide 3-kinase (PI-3 kinase) subunit (Backer et al., 1992; Myers et al., 1992). Twenty-one tyrosine phosphorylation sites and thirty potential serine/threonine phosphorylation sites have been identified on IRS-1 (White & Kahn, 1994). Upon insulin stimulation, several tyrosine sites have been reported be phosphorylated (Mothe and Van Obberghen, 1996), of which Tyrosine 612 (Tyr[612]) and Tyrosine 632 (Tyr[632]) have found to be pivotal in the full activation of downstream PI-3 kinase activity and GLUT4 translocation (Esposito, Li, Cama, & Quon, 2001).

The IRS-1 protein is considered critical in effective insulin signalling (Taniguchi, Emanuelli, & Kahn, 2006), and insulin resistance has been implicated as a result of impaired phosphorylation of downstream signalling proteins. In humans, genetic studies have showed the IRS-1 gene to be variable in NIDDM (10-20%) compared with healthy individuals (5%) (Almind et al., 1993; Laakso, Malkki, Kekäläinen, Kuusisto, and Deeb, 1994). Furthermore, Animal models expressing null alleles of the IRS-1 gene, which reduces the expression of IRS-1 protein present approximately (~60%) reduction in total IRS-1 protein, attenuates IRS-1 phosphorylation and p85 PI-3 kinase associated activity (Brüning, Winnay, Cheatham, and Kahn, 1997). Yamauchi and colleagues have reported similar findings in knockout rodent models, which present approximately a 20% reduction in IRS-1 phosphorylation to that of wild-type mice (Yamauchi et al., 1996). Despite this reduction, IRS-1 null mice do not develop diabetes, but rather present an increased in insulin secretion resultant of beta cell hyperplasia suggesting the role of another protein which is implicated in the development of diabetes, potentially IRS-2 (Yamauchi et al., 1996).

The impairment in IRS-1 phosphorylation with relation to insulin resistance *in vivo* has been speculated to be due to an increase in serine phosphorylation (Hotamisligil et al., 1996). Exposure to pro-inflammatory cytokines such as tumour necrosis factor – alpha (TNF-α), activates protein kinases such as inhibitor κB Kinase (IKK) (de Alvaro, Teruel, Hernandez, & Lorenzo, 2004), and c-Jun-N-Terminal Kinase (JNK), (Lee, Giraud, Davis, & White, 2003),

which increase serine phosphorylation. *In vitro* research in skeletal muscle cells has shown serine phosphorylation to impair IRS-1 associated phosphorylation of PI-3 kinase in skeletal muscle cells as a result of pro-inflammatory cytokine exposure (del Aguila et al., 2011). TNF-α activates the stress protein kinase, c-Jun N-terminal kinases (JNK), which result in serine phosphorylation of IRS-1 and impairs insulin stimulated glucose uptake in healthy humans (Plomgaard et al., 2005). In addition, skeletal muscle cells from diabetic subjects have shown to express a fourfold increase in TNF-α compared to insulin sensitive subjects (Saghizadeh, Ong, Garvey, Henry, & Kern, 1996). Furthermore, *in vitro* experiments using the myogenic C2C12 cell line have found TNF-α to impair IRS-1 associated PI-3 kinase activity to the p85 subunit, and reduction in insulin stimulated glucose uptake (Del Aguila, Claffey, & Kirwan, 1999), demonstrating the strong effect of TNF- α upon impaired IRS-1 phosphorylation.

2.9   Phosphatidylinositol 3-Kinase (PI 3-Kinase): A regulator of insulin signalling.

Phosphatidylinositol 3-Kinase (PI 3-Kinase) is a hetero-dimer, composed of a p110 catalytic subunit (110 kDa) and p85 regulatory subunits (85 kDa). PI 3-Kinase is implicated in various signalling pathways, many of which are beyond the scope of this review, however, the implications upon insulin signalling are crucial in maintaining normal cell metabolism (Shepherd, Withers, & Siddle, 1998; Shepherd, 2005).

The initiation of tyrosine-phosphorylated substrates is mediated by Src Homology (SH2) domains, which are critical for cytoplasmic protein binding with auto phosphorylated receptor tyrosine kinases (Pawson and Gish, 1992). All PI-3 kinase isoforms contain two SH2 domains as well as inter SH2-domain to enable adaptor and catalytic unit interaction and recruitment of the complex to the membrane (Shepherd, 2005). Activation of PI-3 kinase through tyrosine phosphorylation by IRS-1 results in binding of the p85 subunit SH2 domain with the tyrosine phosphorylation sites (Myers et al., 1992). Insulin has been found to be a potent activator of PI-3 kinase (Navé, Haigh, Hayward, Siddle, and Shepherd, 1996) and cell permeable inhibitors of PI-Kinase activation (Wortmannin and LY294002), abate PI-3 kinase activity and subsequently insulin stimulated glucose uptake (Cusi and Maezono, 2000).

The recruitment of PI3-kinase as a result of IRS-1 activation is dependent upon phosphorylation of multiple tyrosine sites (Sun, Crimmins, Myers, Miralpeix, & White, 1993).

In addition, IRS-1 contains a number of serine phosphorylation sites, however, due to the low serine activity of p110 and p85 units, IRS-1 serine phosphorylation sites leads to impaired IRS-1, and PI-3 kinase associated activity (Cusi and Maezono, 2000). Serine phosphorylation of IRS-1 has been shown impair PI3-Kinase associated activity in adipocytes (Hotamisligil et al., 1996) and cultured skeletal muscle cells (Bouzakri et al., 2003). Additionally, *in vivo* research subjected humans to a 3 hour hyperinsulineamic euglycemic clamp to investigate skeletal muscle insulin signalling. Kim and colleagues showed there to be a progressive reduction in IRS-1 associated p85 subunit PI-3 kinase activity in type 2 diabetics (2.4 fold), compared with lean controls (6.1 fold) and non-diabetic obese subjects (3.7 fold), when (Kim, Nikoulina, Ciaraldi, Henry, & Kahn, 1999).

PI-3 kinase activity leads to the instigation of a number of phosphorylated inositol phospholipids (Shepherd, Navé, & O'Rahilly, 1996). Insulin has specifically been shown to acutely increase phosphatidylinositol-3,4,5-triphosphate (PIP_3) and phosphatidylinositol-3,4,5-biphosphate (PIP_2), a by-product of PIP_3 breakdown (Ruderman, Kapeller, White, and Cantley, 1990). Ruderman and colleges showed maximal activity of phosphorylated inositol phospholipids within a minute of exposure to insulin which was subsequently maintained for up to 60 minutes (Ruderman et al., 1990). Furthermore, PIP_3 has been shown to be an important mediator of downstream signalling. Pre-treatment with PI-3 kinase specific inhibitors have shown to attenuate PIP_3 accumulation and subsequent activation of downstream signalling. The increase in PIP_3 is important in enabling co-localisation between protein kinase b (Akt) and 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Kandel and Hay, 1999).

2.10 Metabolic role of Akt

Since its identification over 20 years ago (Jones, Jakubowicz, Pitossi, Maurer, & Hemmings, 1991), Protein Kinase B (Akt/PKB), or Akt as it will be referred to for the purpose of this review, has instigated a large amount interest within the literature with regards to its role in cellular signalling (Brazil and Hemmings, 2001; Kandel and Hay, 1999; Schultze, Jensen, Hemmings, Tschopp, and Niessen, 2011). Akt is a multifunctional protein kinase, that is implicated in various cellular responses, such as growth and development (Yang et al., 2003), as well as glucose homeostasis (Kohn, Summers, Birnbaum, and Roth, 1996; Wang et al., 1999).

Akt contains an amino terminal plackstrin homology (PH) domain and is a downstream target of PI3-Kinase (Hanada, Feng, and Hemmings, 2004). The PH domain of Akt, interacts with the phosphorylation of serine/threonine kinase, PI3-Kinase, by conversion PIP_2, to (PIP_3) at the plasma membrane (Alessi et al., 1997). PIP_3 is required for maximal activity of Akt via 3-phosphoinositide-dependent protein kinase (PDK) phosphorylation at the catalytic domain (Thr^{308}) (Schultze et al., 2011). In addition to phosphorylation at Thr^{308}, which accounts for 10% of the basal activity, phosphorylation at the C-terminal domain (Ser^{473}) is critical for complete kinase activity (Alessi and Cohen, 1998; Alessi et al., 1996). Activation of Akt leads to phosphorylation of a number of downstream molecules, such as glycogen synthesis via glycogen synthase (Friedrichsen et al., 2013), protein synthesis via the mTOR signalling pathway (Proud and Denton, 1997), and AS160, a Rab-GTPase protein that is implicated in GLUT4 exocytosis for insulin stimulated glucose uptake (Sano, 2003).

The Akt family comprises of three isoforms (Akt1, Akt2 and, Akt3), which are expressed in numerous tissues (Yang et al., 2003). Both Akt1 and Akt2 isoforms are expressed in insulin sensitive tissues, such as liver and adipose tissue. Skeletal muscle expresses all three Akt isoforms (Brozinick, Roberts, & Dohm, 2003) and all of them have specific functions (Cleasby, Reinten, Cooney, James, and Kraegen, 2007). While Akt1 and Akt3 have been shown to be involved in normal growth and brain development respectively (Schultze et al., 2011), Akt2 has predominantly been found to be involved in glucose homeostasis (Cho et al., 2001; Garofalo et al., 2003).

Both Akt1 and Akt2 are important in the proliferation and differentiation of skeletal muscle cells (Rotwein and Wilson, 2009; Wilson and Rotwein, 2007). However, the regulation of skeletal muscle differentiation by Akt1 and Akt2 isoforms remains debatable. The literature is divided with respect to the roles that Akt1 and Akt2 play within skeletal muscle differentiation with some authors suggesting Akt1 (Rotwein and Wilson, 2009; Wilson and Rotwein, 2007), while others suggesting Akt2 (Heron-Milhavet et al., 2006; Heron-Milhavet, Mamaeva, Rochat, Lamb, & Fernandez, 2008) to be the main regulator of skeletal muscle differentiation. Akt2 contains promoter regions, which allow for myodesmin and myocyte enhance factor interactions, two important basic helix-loop-helix proteins implicated in skeletal muscle development (Kaneko et al., 2002). During differentiation the increase in Akt2 expression also increases expression of myogenin through a positive feedback loop (Kaneko et al., 2002). Silence of Akt2 in skeletal muscle cells impairs differentiation, morphological compared to control myotubes. In contrast, ablation of Akt1 completely prevented differentiation through the down regulation of transcriptional regulators of myogenesis without affecting cell viability or proliferation (Wilson and Rotwein, 2007). It is clear that both of these proteins play important roles in skeletal muscle development in some form and the expression of Akt is a critical factor in skeletal muscle differentiation and development (Gardner, Anguiano, and Rotwein, 2012).

The transfection of mutant PKB/Akt1 in L6 myotubes reduces insulin stimulated Akt1 phosphorylation and GLUT4 translocation (Wang et al., 1999). Additional work by Katome and colleagues has shown that RNA silencing of both Akt1, but more so the Akt2 isoform, is implicated in the reduction of insulin stimulated glucose uptake in 3T3- L1 adipocytes (Katome et al., 2003). These findings have been further supported in skeletal muscle, with Akt2 knockout mice developing impaired insulin action and simulating a diabetic like syndrome (Cho et al., 2001; Garofalo et al., 2003). The over-expression of Akt increases glucose uptake in both L6 muscle cells (Hajduch, Alessi, Hemmings, & Hundal, 1998), and rat muscle fibres (Cleasby et al., 2007). Cleasby and colleagues have shown that the over-expression of Akt2 isoform in rodent skeletal muscle significantly enhances insulin stimulated glucose uptake and glycogen synthesis activity by 36% and 59% respectively identifying Akt2 important in glucose metabolism (Cleasby et al., 2007).

In human skeletal muscle, Akt expression is similar between NIDDM and controls subjects (Brozinick et al., 2003; Krook, Roth, Jiang, & Zierath, 1998), however, under

hyperinsulineamic euglycemic conditions, Akt phosphorylation is severely impaired in insulin resistant and type 2 diabetic individuals (Krook et al., 1998). More recent work has identified the reduction in phosphorylation of Akt to be a consequent of a significant reduction in Ser^{473} phosphorylation in comparison to Thr^{308}, which does not differ at basal levels or following insulin stimulation in NIDDM patients (Cozzone et al., 2008). Further work by Cozzone and colleagues supported previous research presenting specific isoform impairments in phosphorylation of Akt2 in skeletal muscle of NIDDM patients.

2.10.1 The role of AS160 substrate

The protein TBC1 domain family member 4 (TBC1D4) or, Akt substrate at 160 kDa (AS160 (160 kDa)) as it is more commonly known, has only recently emerged as a key protein in insulin stimulated glucose uptake. Since the identification of AS160 (Kane et al., 2002; Sano et al., 2003), there has been a profound interest in the mechanistic role that AS160 plays both in insulin dependent and independent glucose uptake (Cartee & Wojtaszewski, 2007; Sakamoto & Holman, 2008).

Structurally, AS160 has been found to comprise of a COOH-terminal Rab-GAP (GTPase-activating protein) domain, as well as several, but not exclusively, Akt phospho-motifs (Miinea et al., 2005). Rab proteins are highly implicated in vesicle trafficking (Lee, Mishra, & Lambright, 2009), and they are vital in the exocytosis of glucose transporters (Moyers, Bilan, Reynet, & Kahn, 1996). Under basal conditions, Rab-GAP activity remains in an inactive (guanosine-5'-diphosphate (GDP-loaded)) form, inhibiting GLUT4 translocation (Sakamoto & Holman, 2008). When AS160 is phosphorylated, Rab-GAP activity is subsequently inhibited, leading to GTP loading, which enables for translocation of GLUT4 vesicles from the peri-nuclear region of the plasma membrane.

Several serine and threonine sites are phosphorylated on AS160 following insulin stimulation (Kane et al., 2002; Sano et al., 2003). Although multiple phosphorylation sites have been identified, the work by Kane and colleagues identified Ser^{588} and, Thr^{642} as the major insulin-stimulated phosphorylation sites, with later work identifying up to a further three sites to be implicated in insulin stimulated AS160 phosphorylation (Sano et al., 2003). The phosphorylation of these sites is imperative in GLUT4 trafficking (Mîinea et al., 2005). This has been shown through inhibition of the aforementioned phosphorylation sites insulin(Sano, 2003). Research by Sakamoto's laboratory has shown that knock in mutation of AS160

dramatically impairs glucose tolerance by 25% and 14% in male and female mice respectively; specifically identifying that mutation of the Thr^{649} AS160 residue to be implicated in the increase in intercellular GLUT4 content is (Mîinea et al., 2005). This has been shown through inhibition of the aforementioned phosphorylation sites (Chen, Wasserman, MacKintosh, & Sakamoto, 2011). Skeletal muscle has also been shown to express AS160 (Bruss, Arias, Lienhard, and Cartee, 2005; Kramer et al., 2006), and is phosphorylated by insulin in a dose dependent manner (Arias, Kim, & Cartee, 2004), via the upstream phosphorylation of Akt at ser^{473} (Bruss et al., 2005).

2.11 Skeletal muscle glucose transporters.

Glucose uptake is imperative for the maintenance of energy homeostasis (Pessin, Thurmond, Elmendorf, Coker, & Okada, 1999). Insulin stimulation glucose uptake results in rapid (~1 minute) entry of glucose into the cell (Daniel, Love, and Pratt, 1975). The intercellular concentration of glucose is small compared to extracellular concentrations, resulting in a significant concentration gradient. However, due to the hydrophilic nature of the glucose molecule its transport into the cell is mediated by a family of integral membrane proteins known as glucose transporters (Kahn, 1992). The control of glucose transporter exocytosis is regulated by proteins which initiate translocation to the plasma membrane, as well as regulate recycling following glucose uptake (Leto and Saltiel, 2012).

Two glucose transporters, glucose transporter 1 (GLUT1) and glucose transporter 4 (GLUT4) are the primary regulators of glucose transport and are highly concentrated in both skeletal muscle and adipose tissue (DeFronzo et al., 1983). GLUT1, located at the plasma membrane, is responsible for basal glucose turnover, and is a mediator of glucose transport in prenatal skeletal muscle (Gaster, Handberg, Beck-Nielsen, and Schroder, 2000). In contrast, under basal conditions, GLUT4 is sequestered within intracellular glucose storage vesicles (Gould and Holman, 1993), with less than 5% located at the cell surface (Slot et al., 1991). Insulin stimulation, results in a 40-fold increase in cell surface GLUT4 concentrations in brown adipocytes (Slot et al., 1991). Both GLUT1 and GLUT4 mRNA and protein have been reported to be present within tissues from both humans and rats (Flier, Mueckler, McCall, and Lodish, 1987), including skeletal muscle (Klip & Pâquet, 1990). In human skeletal muscle, GLUT4 expression is approximately 400-fold higher in comparison to GLUT1 (Stuart et al., 2006) and is proportionally higher within red, oxidative (7:1) compared to white glycolytic (4:1) skeletal muscle (Marette et al., 1992). In addition, the expression is highly correlated with glucose transport at both the transcriptional and protein levels (Kern et al.,

1990), and is also reflected in the surface density of GLUT4 upon insulin stimulation and the rate of glucose uptake.

Hormones such as insulin regulate transcriptional and translational turnover in insulin sensitive tissues (Flores-Riveros, McLenithan, Ezaki, and Lane, 1993). Prolonged exposure to insulin has been shown to decrease GLUT4 mRNA within a relatively short exposure period, subsequently leading to a reduction in cellular GLUT4 protein by approximately 50-80% (Flores-Riveros et al., 1993; Sargeant and Pâquet, 1993). Contrary to this effect, GLUT1 expression increases to steady state levels 6-fold greater than initial values, resulting in the increase in cellular protein concentration (Sargeant and Pâquet, 1993). The translational control of GLUT4 mRNA has been elucidated through transfection of GLUT1 and GLUT4 cDNA in adipocytes and skeletal muscle cells, and has shown insulin to mediate increases in GLUT1 expression through a PI3-Kinase/Akt/mTOR signalling pathway (Taha et al., 1999).In order to further investigate the role of glucose transporters within skeletal muscle, both skeletal muscle cells lines and human skeletal muscle cells have been used due to the expression of basal and insulin stimulated glucose transporters, as well as their ability to respond to insulin stimulated glucose uptake (Sarabia, Lam, Burdett, Leiter, and Klip, 1992) .

The use of skeletal muscle cells can provide a valid *in vitro* model for investigation of metabolic response at the cellular level (Berggren, Tanner, and Houmard, 2007). Human muscle derived cells (MDC) in culture respond to insulin in a dose and time dependent manner (Al-Khalili et al., 2003; Ciaraldi, Abrams, Nikoulina, Mudaliar, & Henry, 1995; Henry et al., 1995). Both GLUT1 and GLUT4 have been shown to be present in human skeletal muscle cells maintained in culture from both healthy and NIDDM subjects (Al-Khalili et al., 2003; Ciaraldi et al., 1995; Henry, Abrams, Nikoulina, & Ciaraldi, 1995). Furthermore, Al-khalili and colleagues observed differences between myoblast cell and differentiated myotube glucose transporter concentrations, which at the single cell level, express predominantly GLUT1 and upon differentiation express predominantly GLUT4 (Al-Khalili et al., 2003).

2.11.1 Glucose Transport Endo/Exocytosis

Under basal conditions approximately 5% on the total transporter pool is located at the cell surface (Klip & Pâquet, 1990), while GLUT4 vesicles are mainly sequenced within the intracellular space, distributed between endosomes, the trans Golgi network and specialised glucose storage vesicles (GSV) (Rea and James, 1997). The translocation of glucose

transporters from vesicle storage compartments within the cytoplasm to the sarcolemma and T-tubules is regulated by distinct signalling pathways (Jessen and Goodyear, 2005) (*Figure 2-5*). Insulin stimulation initiates an increase in cell surface concentrations of GLUT4 within 5 minutes of exposure (Kern et al., 1990). Exocytosis[2] of GLUT4 to the plasma membrane is regulated by the hydrolysis of Rab-GTPase[3] protein complexes, which regulate GLUT4 trafficking (Larance et al., 2005; Sano, 2003). Both Rab8 and Rab14 have been found to be present in skeletal muscle and as previously mentioned are targets of the Rab-GTPase protein AS160 (*Section 2.10.1*). The final process of GLUT4 exocytosis is the binding between transporter and the plasma membrane. Proteins known as SNARE (soluble *N*-ethymaleimide-sensitive factor attachment protein receptor) proteins are regulated by the Sec1/Munc18 protein family, which regulate membrane fusion events allowing for docking GSV to the plasma membrane and enable facilitated diffusion of glucose across the membrane (Leto and Saltiel, 2012). In human skeletal muscle, glucose ingestion increases GLUT4 translation to the membrane (Goodyear et al., 1996). In contrast, GLUT4 dynamics have been shown to be reduced in human adipose cells from obese and insulin resistant donors compared to healthy controls (Lizunov et al., 2013).

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[2] GSV exocytosis has been described as three processes. Firstly translocation to the cell membrane, targeting and, fusion to the plasma membrane (Leto and Saltiel, 2012).
[3] Guanine activated proteins are small proteins which remained within an inactive state (GDP). Hydrolysis of GDP results in an active form (GTP). Activation of AS160 leads GSV exocytosis.
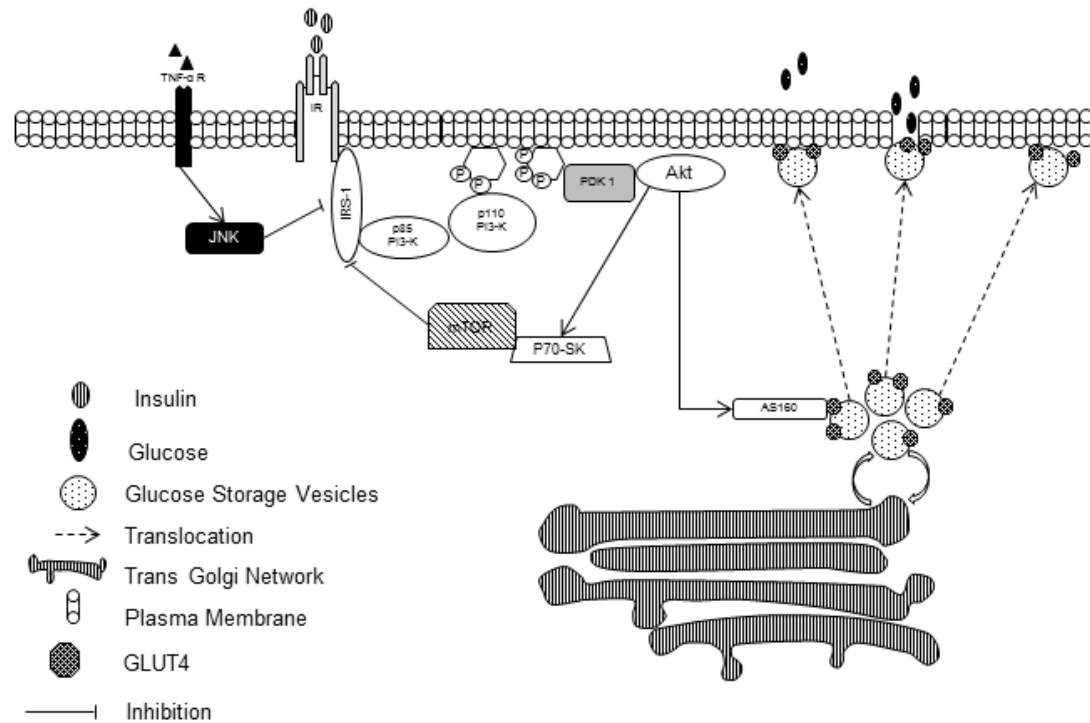
Figure 2-5 Insulin stimulated glucose uptake. Binding between the hormone, insulin and insulin receptor (IR) activates and autophosphorylates the insulin receptor substrate-1 (IRS-1). IRS-1 acts as a docking protein for the downstream subunit phosphatidylinositol 3-kinase (PI3-Kinase). Phosphorylation of PI3-Kinase leads to the recruitment of phosphate dependent kinase (PDK) which causes translocation and subsequent phosphorylation of Akt. Phosphorylation of Akt leads to downstream phosphorylation of the Akt substrate AS160 and release of the glucose storage vesicles to the plasma membrane to enable facilitated diffusion of glucose across the plasma membrane. Figure adapted from Leng et al., 2004 and Leto and Saltiel 2012.

2.12 Implication of insulin upon gene expression

In addition to its role in post-prandial glucose disposal, insulin is also implicated in positive and negative transcriptional regulation (O'Brien and Granner, 1996). The regulation of gene transcription by insulin is mediated through two basic mechanisms. Firstly, through the phosphorylation/dephophorlyation of plasma membrane bound signalling cascades and, more directly through intracellular receptors (O'Brien and Granner, 1996). Research in human skeletal muscle has reported several insulin responsive and non-responsive genes as well as transcriptional factors that are involved in vesicle trafficking, protein turnover and signal transduction. Research by Wu and colleagues reported 70 transcriptional regulated genes by insulin which include the vesicle trafficking regulator, glucose transporter 4 (GLUT4), Ras-related associated with diabetes (rRAD) and, peroxisome proliferative activated receptor γ (PGC-1α, a regulator of metabolism) (*Table 2-3*). The aforementioned transcriptional regulators could be considered what O'Brien and colleagues have previously described as candidate diabetic genes (*diabetogenes*), which have subsequently been found to be altered in skeletal muscle and adipose tissue of insulin resistance individuals (Bach et al., 2002; Ducluzeau et al., 2001).

2.8.3.1. *Glucose Transporter 4 (GLUT4)*

Expression levels of GLUT4 are highly regulated during embryonic development of rats (Santalucía et al., 1992). During rat foetal development, GLUT4 concentrations have been found to be low; however during the prenatal phase of development continuous expression of GLUT4 mRNA, which is proceeded by increases in posttranslational protein expression, results in GLUT4 being the predominant isoform for glucose transport within rat skeletal muscle (Santalucía et al., 1992). Both protein and transcriptional concentrations are highly present within mature skeletal muscle in humans (Stuart et al., 2006), and expression levels of GLUT4 are highly correlated with glucose transport (Kern et al., 1990). Several mechanisms regulate GLUT4 expression in skeletal muscle (Zorzano, Palacín, and Gumà, 2005). The use of transgenic animal models has identified the important transcriptional promoter regions which are responsible for increases in GLUT4 mRNA expression (Liu, Olson, Edgington, Moye-Rowley, and Pessin, 1994). Several authors have shown that insulin or pharmacological mediated stimulation significantly increases GLUT4 mRNA expression (Al-Khalili et al., 2005; Leick et al., 2010).

2.8.3.2. *Peroxisome proliferator-activated receptor coactivator – 1α (PGC-1α)*

Since its discovery within brown adipose tissue (Puigserver et al., 1998), Peroxisome proliferator-activated receptor (PPAR)-γ coactivator – 1α (PGC-1α), has received a large amount of interest within the literature due to its multi-faceted role in metabolism and physiological adaption (Lira, Benton, Yan, & Bonen, 2010; Olesen, Kiilerich, & Pilegaard, 2010). DNA microarray of skeletal muscle biopsies from NIDDM has reported approximately a 20% reduction in PGC-1α mRNA expression, and a similar concurrent reduction in oxidative phosphorylation genes (Mootha et al., 2003). The mechanistic role of PGC-1α has been investigated both *in vitro* research using stable cells lines (Michael et al., 2001), and in animal models (Leick et al., 2010). While the use of transgenic rodent models have shown some inconsistencies within the literature in relation to the effects of findings as to the effects of PGC-1α upon insulin sensitivity, research in the Zucker rat animal model have approximately a 50% reduction in PGC-1α expression in skeletal muscle (Jové et al., 2004; Sriwijitkamol et al., 2006). The use of cell culture models has identified PGC-1α as an activator of genes implicated in the, tricarboylic acid cycle, electron transport chain and mediation of GLUT4 mRNA expression in skeletal muscle (Koves et al., 2005; Michael et al., 2001). Koves and colleagues showed that PGC-1α overexpression also resulted in an increase in Peroxisome proliferator-activated receptor (PPAR) mediated genes, which regulated lipid metabolism (Koves et al., 2005). More recently, over expression of the PGC-1α related co-activator, a structurally similar protein to PGC-1α, has been shown to significantly increase mRNA expression of oxidative phosphorylation (OXPHOS) genes and proteins as well as GLUT4 mRNA and protein concentrations by as much as 350% and 14% respectively in C2C12 skeletal muscle cells (Philp et al., 2011; Philp, Perez-Schindler, Green, Hamilton, and Baar, 2010). These changes in concentrations at both the transcriptional and protein levels have unsurprisingly been found to increase glucose uptake (Philp et al., 2011).

These *in vivo* animal, and *in vitro* cell culture models support findings observed within skeletal muscle of pre-diabetic, insulin resistant and diabetic and humans (De Filippis et al., 2008). Recent work has shown that skeletal muscle cells from obese individuals increases lipid oxidation following over-expression of the PGC-1α gene, by as much as ~3.5 fold, similar to increases observed following exercise training (Consitt et al., 2010). The effect of exercise upon PGC-1α gene expression in skeletal muscle is clear within the literature based on the number of reviews in recent years (Handschin and Spiegelman, 2006; Olesen, Kiilerich, and Pilegaard, 2010). Exercise increases the expression of PGC-1α (Burgomaster

et al., 2008) and prolonged exercise training has been shown to transiently increase Increases in both PGC-1α mRNA and protein expression (Perry et al., 2010). Augmentation of PGC-1α expression by exercise has been attained through metabolic signalling via AMPK (Pilegaard, Saltin, and Neufer, 2003), as well as stretch activated protein kinase, p38 MAP Kinase (Koves et al., 2005). Furthermore, recent research by Barrès and colleagues have shown exercise intensity play an important role in the epigenetic regulation of the PGC-1 α promoter region and subsequently its transcriptional expression (Barrès et al., 2012). In addition to increases in PGC-1α expression, a number of whole body changes have been shown to be affected by exercise training including, aerobic capacity, lactate accumulation and, insulin sensitivity (Hood, Little, Tarnopolsky, Myslik, and Gibala, 2011; Perry, Heigenhauser, Bonen, and Spriet, 2008). Although the research is somewhat limited, De Fillipis and colleagues have shown that mRNA PGC-1α expression is blunted during the acute recovery phase of aerobic exercise in obese non-diabetic males compared to lean controls, however increases above base line expression (De Filippis et al., 2008). Furthermore, exercise intensity has been shown to be important in both transcriptional regulation and DNA methylation status (Barrès et al., 2012; Egan and Zierath, 2013). Recent research by Barrès and colleagues has identified beneficial effects of exercise upon DNA methylation of the PGC-1α promoter region (Barrès et al., 2012). These acute responses have been supported by Sriwijitkamol and colleagues who have shown PGC-1α to recover following seven weeks of aerobic training (Sriwijitkamol et al., 2007). Increases in both PGC-1α mRNA and protein expression are transient with exercise training (Perry et al., 2010). Augmentation of PGC-1α expression by exercise has been attained signalling via AMPK, as well as protein kinase, p38 MAP Kinase Furthermore, recent research by Barrès and colleagues have shown exercise intensity play an important role in the epigenetic regulation of the PGC-1 α promoter region and subsequently its transcriptional expression (Barrès et al., 2012). In addition to increases in PGC-1α expression, a number of whole body changes have been shown to be affected by exercise training including, aerobic capacity, lactate accumulation and, insulin sensitivity.

2.8.3.3. *Hexokinase II gene expression*

The phosphorylation of glucose to glucose-6-phosphate is catalysed by hexokinase enzymes. These enzymes have a high affinity for glucose and are regulated by the feedback loop of glucose-6-phosphate. The hexokinase isoforms, hexokinase I and hexokinase II (HKII) are expressed in skeletal muscle (Lawrence & Trayer, 1985), with the latter more abundant (~6-fold) compared to hexokinase I (Odoherty, Bracy, Osawa, Wasserman, & Granner, 1994). In

addition, the expression of HKII is fibre dependent with increased expression in slow compared fast twitch fibre skeletal muscle (Odoherty et al., 1994).

The expression of HKII has been shown to be critical, as homozygous deficient rodents die during prenatal phase, while heterozygous deficient rodents present with severe glucose intolerance (Heikkinen et al., 1999). In contrast, overexpression of HKII increases insulin dependent and independent glucose uptake (Chang et al., 1996; Halseth, Bracy, and Wasserman, 1999). Insulin stimulation increases HKII mRNA expression in animal cell lines and humans (Mandarino et al., 1995; Printz et al., 1993). Mandarino and colleagues showed that insulin stimulation increased HKII mRNA concentrations by approximately 3-fold over basal levels, however the subsequent transcriptional responses observed did not affect HKII protein expression following a 4-hour hyperinsulineamic euglycemic clamp in healthy males (Mandarino et al., 1995). In contrast, individuals with NIDDM express both significantly lower mRNA concentrations of HKII, by as much as 3-fold, compared to healthy control subjects, therefore suggested that HKII expression is a secondary impairment to metabolic abnormalities in subjects with NIDDM (Vestergaard et al., 1995).

2.8.3.4. *Ras-Associated diabetes*

Ras proteins are small GTP-ase activated proteins, which have been found to have multiple cellular functions, such as vesicle trafficking (Rudich and Klip, 2003). Ras Associated Diabetes (rRAD) is a member of the Ras superfamily and is increased upon insulin stimulation (~2-fold) in human skeletal muscle (Laville et al., 1996). rRAD has been termed based on the increases in gene expression in NIDDM patients, which has been speculated to be resultant of hyperinsulinemia observed in NIDDM populations insulin (Reynet & Kahn, 1993). Both *In vitro* and *in vivo* research has further implicated rRAD in the reduced glucose uptake in both adipocytes and skeletal muscle cells (Moyers, Bilan, Reynet, and Kahn, 1996).

Table 2.3 Summary of the genes affected by insulin signalling

Gene	Summary	References
Peroxisome proliferator-activated receptor (PPAR)-γ coactivator – 1α (PGC-1-α)	• Important mediator of mitochondrial biogenesis • Insulin stimulation increases PGC-1-α • PGC-1-α mRNA expression is reduced in NIDDM individuals • Overexpression of PGC-1-α increases insulin stimulated glucose uptake	Jove et al., (2004), Mootha et al., (2003), Michael et al., (2001)
Glucose Transporter 4 (GLUT4)	• Insulin stimulated glucose transporter which aids facilitated glucose uptake • Highly expressed in insulin sensitivity tissues. • No difference in GLUT4 mRNA expression between healthy and NIDDM individuals	Al-khalili et al., (2005), Zorzano et al., (2005)
Hexokinase II (HKII)	• Converting enzyme of glucose to glucose-6-phosphate during anaerobic glycolysis • Insulin stimulation increases HKII expression by approximately 3-fold • Impaired HKII mRNA expression in NIDDM	Vestergaard et al., (1995), Mandarino et al., (1995), Bouche et al., (2003)
rRAD	• rRAD involved in regulating vesicle trafficking • Increased mRNA expression in NIDDM • Increased rRAD expression reduces insulin stimulated glucose uptake	Laville et al., (1996), Reynet & Kahn (1993)

2.13 Summary

In summary, insulin stimulated glucose uptake in skeletal muscle is a major site of glucose disposal and the signalling cascade in which insulin initiates the translation of glucose transporters to the cell membrane for glucose uptake to occur is highly complex. The use of cell culture models has provided a platform for investigating the molecular basis of insulin stimulated glucose uptake. The use of tissue engineered skeletal muscle as a platform for investigating insulin signalling and glucose uptake is currently limited despite a rising interest in the use of these models for investigating skeletal muscle physiology. In order to further understand the potential role of these models for research in insulin signalling and glucose uptake, this thesis will firstly investigate if these models develop into mature skeletal muscle constructs which express the glucose transporters which are implicated in insulin stimulated glucose uptake. The development of three dimensional tissue engineering using biological hydrogels as extracellular matrices in which cells and grow and differentiating into a bio mimetic models which recapitulates *in vivo* tissue could provide an alternative model to investigate both the molecular basis of insulin signalling and glucose uptake.

3 General Methodology

3.1.   Cell Culture

3.1.1   Cell culture

All cell culture was performed in a class II microbiological flow hood under aseptic conditions. Cells were grown in a thermally controlled incubator (Thermo Scientific, Fisher-Scientific, Loughborough, UK) (37°C, 5% CO_2) using standard growth medium (GM) (Dulbecco's Modified Eagle's Medium (DMEM) (Fisher- Scientific, Loughborough, UK), 20% foetal bovine serum (FBS) (FBS Good, Dutscher Scientific, Germany), and 1% antibiotics (Penicillin/Streptomycin) (GIBCO/Invitrogen, Paisley, UK). Cells were grown in either T75 or T175 cell culture flasks (Fisher-Scientific, Loughborough, UK). The GM was changed every 2-3 days.

3.1.2   Cell passage

Once 80-90% confluence was attained, cells were counted and passaged. GM was removed and cells were washed 2X in PBS (Fisher-Scientific, Loughborough, UK). Trypsin-EDTA (TE) (Sigma-Aldrich, Dorset, UK) was used to detach cells from the flask surface. Detachment was confirmed by phase contrast microscopy. Double the volume of GM to TE was added to inhibit the action of the trypsin. The resultant solution was removed and placed into a test tube for cell counting.

Cells were counted using a haemocytometer (*Figure 3-1*) using the equation below. An aliquot of cell suspension was added to an Eppendorf tube with trypan blue (Sigma-Aldrich, Dorset, UK). A volume of 10µl solution was placed into each chamber of the haemocytometer and counted under a phase contrast microscope (Leica DM IL LED, Leica Microsystems (UK) Ltd, Milton Keynes, UK). Cell count was determined by the number of cells within a sixteen square quadrant, in four quadrants. The total number of cells was determined by the total number of counted cells, dived by the number of quadrants, times the cell dilution factor.

46
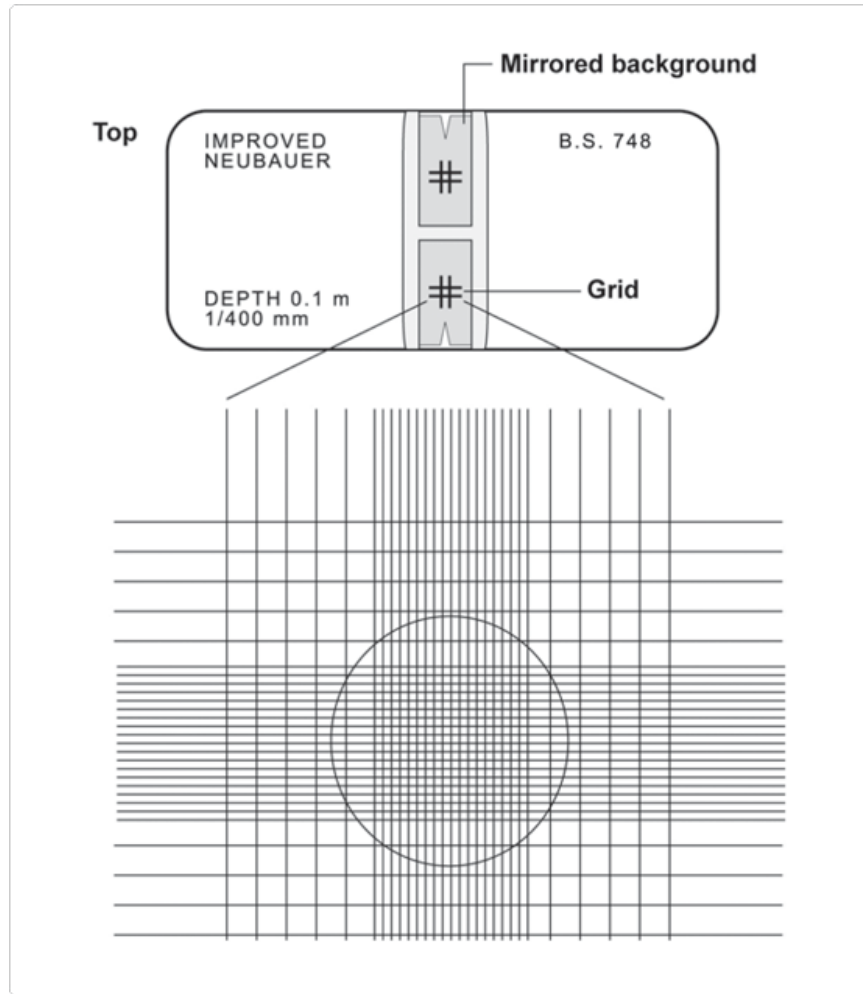
Figure 3-1 A representative diagram of a haemocytometer used for counting of skeletal muscle cells. Image from life technologies.
http://www.lifetechnologies.com/content/dam/LifeTech/migration/en/images/ics-organized/references/cell-culture-basics/data-diagram/560.par.32479.image.557.637.1.counting-cells-hemocytometer-gif.gif. Image downloaded on 14/01/2015.

$$Total\ cell\ count = \left(\frac{Total\ No.of\ cells}{No.of\ quadrants}\right) \times Df\ \times 1\ x\ 10^{-4}\ \times Cell\ suspension\ (mL)^4$$

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[4]Df: Dilution factor is the proportion of cells suspension to trypan blue for counting. A 1:1 or 1:10 dilution was used for cell counting in this thesis

3.1.3 Freezing and cryopreservation of cells

Excess cells were suspended in freezing media (90% GM with 10% DMSO) (DMSO, Fisher-Scientific, Loughborough, UK) at 1×10^{-6} in cryovials (Corning, UK). Vials were placed in a 'Mr frosty' (Fisher-Scientific, Loughborough, UK)[5] and stored at -80ºC overnight before being placed in liquid nitrogen for long term cryopreservation.

3.1.4 Resuscitation of cells

Vials of cryopreserved cells were thawed by placing in a water bath at 37ºC until the cell solution had completely thawed. Cells were placed in cell culture flasks and grown as described above (*Section 3.1.1).*

3.1.5 Monolayer experiments

All monolayer experiments were conducted in 6 well cell culture plates (Fisher-Scientific, Loughborough, UK), unless otherwise stated. Cells were grown and passaged as previously described (*Section 3.1.1 & 3.1.2*). Cells were seeded at 15,000 cells·cm^2 and grown in GM until 90% confluent before being changed to differentiation media (DM) (Dulbecco's Modified Eagle's Medium (DMEM) (Fisher- Scientific, Loughborough, UK), 2% horse serum (HS) (PAA, Somerset, UK), and 1% antibiotics (Penicillin/Streptomycin) (GIBCO/Invitrogen, Paisley, UK) for a period of 72 hours in order enable fusion of myoblasts into multinucleated myotubes. Chronic insulin exposure experiments differentiation media was supplemented with 100 nM of human recombinant insulin (Sigma-Aldrich, Dorset, UK). All differentiation media was sterile filtered prior to use.

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[5] Storage of vials in this container provides a -1°C per minute cooling rate for reliable and repeatable cryopreservation and recovery of cells.

3.2 Tissue engineering skeletal muscle constructs

The following Sections outline the methodologies used for the culture of two different skeletal muscle tissue engineering constructs. Both fibrin and collagen methods have previously been described within the literature from which these methods have been used with some slight modifications (Martin et al., 2013; Smith et al., 2012).

3.2.1 Development of fibrin based tissue engineered constructs

3.2.1.1. *Preparation of reagents*

All aliquots were thawed at 37°C unless otherwise stated. Fibrinogen was reconstituted in Hams F12 media (Sigma-Aldrich, Dorset, UK) at a concentration of 20 mg·mL and left to completely dissolve at room temperature. Prior to plating, fibrinogen was sterile filtered through a 0.45 µm syringe filter. Thrombin (Sigma-Aldrich, Dorset, UK) was dissolved in un-supplemented DMEM at a concentration of 200 U/mL. Aprotinin (Sigma-Aldrich, Dorset, UK) was dissolved in distilled water (dH$_2$O) at a concentration of 10 mg·mL and sterile filtered prior to use. Powdered aminocaproic acid (Sigma-Aldrich, Dorset, UK) was dissolved in un-supplemented DMEM at a concentration of 50 mg·mL.

3.2.1.2. *Preparation of sylgard plates*

Standard 35mm falcon petri dishes (VWR, Leicestershire, UK) coated with Sylgard (Sylgard 184 elastomer kit, Dow Chemical, USA) according to the manufacturer's instructions and allowed to cure for one week at room temperature.

Two 6mm long pieces of silk suture were placed 12mm apart in pre-treated plates and anchored into place with stainless steel minutien pins (*Figure 3-2*). Plates were sterilised under UV light for 60 minutes before being rinsed in 70% ethanol for 60 minutes.

3.2.1.3. *Tissue engineering of skeletal muscle in fibrin based gels*

Thrombin solution was made with growth media (DMEM, 20% FBS, 1% P/S), supplemented with 50 µl·mL of thrombin and, 4 µl·mL of aprotinin. Thrombin solution (500 µl·plate) was placed around both silk sutures before being evenly distributed evenly across the plate by gentle agitation. Fibrinogen was sterile filtered before 200 µl was placed around the plate. The solutions were mixed by gentle agitation before being allowed to stand at room temperature for 15 minutes. Plates were placed in the culture incubator for 1 hour. Cells were passaged (*Section 3.1.2*) as previously described and seeded at 10,000 cells·cm^2 in GM supplemented with 250 mg/mL of aminocaproic acid (AA). In order to ensure longevity of the three-dimensional constructs, growth, differentiation and maintenance media were

supplemented with aminocaproic acid unless otherwise stated. The media was changed every other day until confluence was attained. Upon confluence (80-90%) media was changed to DM for 2 days before being placed in maintenance media (DMEM, 7% FBS, P/S, AA) for the remainder of the experiment (10 days). The media was replaced every 1-2 days.
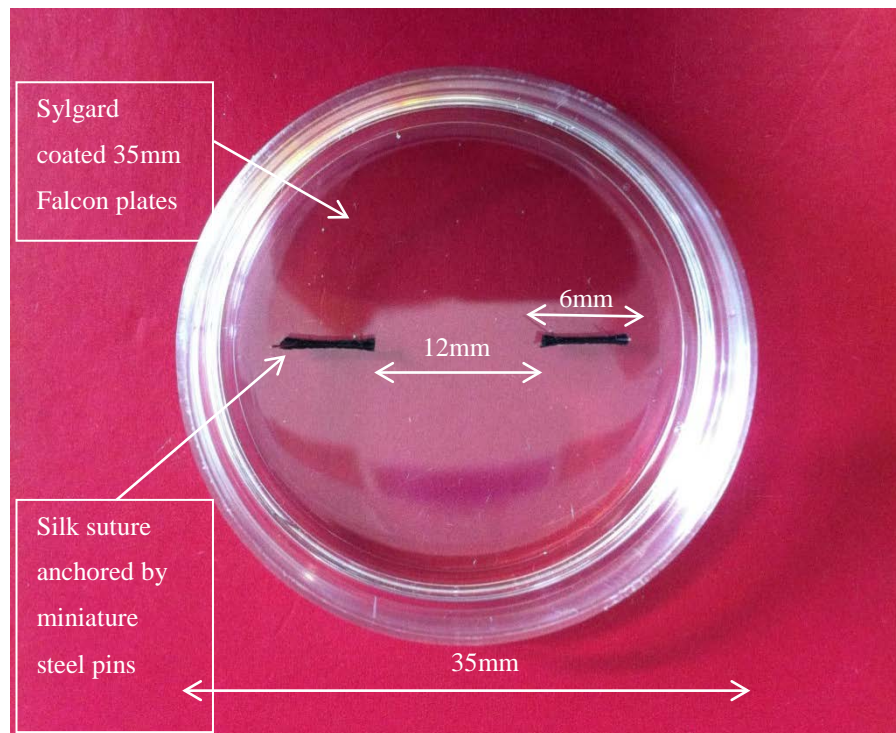
Figure 3-2 A prepared plate for use in fibrin based tissue engineered construct. Falcon plates were layered with sylgard and allowed to cure prior to fixation of silk suture by two steel miniature pins.

3.2.2 Collagen based tissue engineered constructs

The formation of collagen constructs using C2C12 skeletal muscle cells was based on previous literature (Player et al., 2014; Sharples et al., 2012), with some slight modifications.C2C12

3.2.3.1.   *Formation of flotation bars and anchor frames (A-Frames)*

Flotation bars were made by binding two 5x3 mm Sections of polyethylene plastic mesh (Darice Inc., Strongsville, Ohio, US) together using stainless steel dental wire (Scientific Wire Company, Great Dunmow, UK). The A-frames were constructed using 0.7mm thick stainless steel wire (Scientific Wire Company, Great Dunmow, UK) and fitted between the two bound pieces of plastic mesh (*Figure 3-3A*). Prior to use, floatation bars were washed with 70% ethanol and sterilised under UV light.

A-frames were hooked over the edge of an 8-well square reservoir cell culture plate (NUNC, Thermo-Scientific, Fisher-Scientific, Loughborough, UK) (well dimensions: L37.6mm x W27.9mm x D11.6mm) (*Figure 3-3B*). Sylgard and a-cellular collagen were used to create moulds within the plates to set the collagen gel between the A-frames.
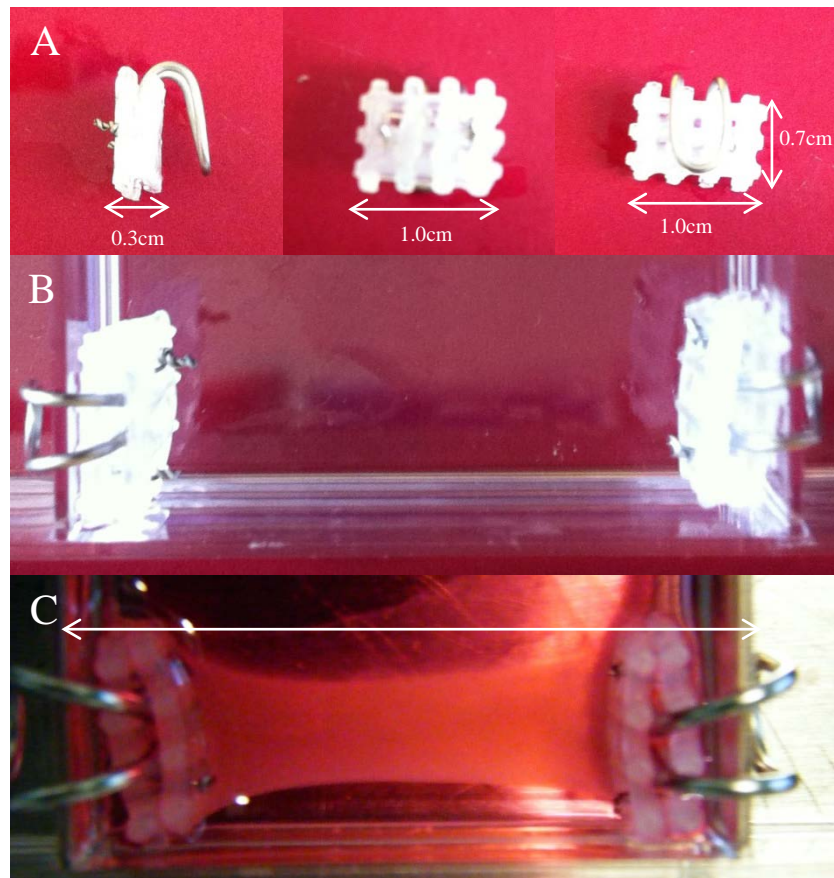
Figure 3-3 A prepared plate for use in collagen based tissue engineered construct. . A-frames (A) were constructed and placed within reservoir cell culture plates (B). Collagen gels (1mL) were set between A-Frames and cultured for 14 days (C). Diagram modified from Smith et al., (2012).

3.2.5.1. *Development of Collagen gels*

The preparation of collagen gel tissue engineered skeletal muscle constructs has been previously described in the literature (Cheema et al., 2003; Mudera et al., 2010; Sharples et al., 2012). In brief, cells were passaged and counted as previously described (*Section 3.1.2*). Cells at a density of 4×10^{-6} per mL were centrifuged at 2000 rpm for 5 minutes. A solution of type I rat tail collagen (2.6 g·mL^{-1}, first link, Birmingham, UK), and 10X minimal essential medium (MEM) (GIBCO/Invitrogen, Paisley, UK) was neutralised in a drop wise manner using 1M and 5M sodium hydroxide (NaOH) (Fisher-Scientific, Loughborough, UK) respectively. Cells were homogenised with neutralised collagen solution, before being set in pre-sterilised chamber slides between the two A-frames. The constructs were allowed to set for 10-15 minutes in an incubator (37°C, 5% CO_2) before being re-suspended in GM. The media was changed twice daily during the attachment phase before being changed to differentiation media (DMEM, 2% HS, 1% P/S), which was subsequently changed daily for the remainder of the culture period (10 days) (*Figure 3-3C*).

3.3. Transcriptional analysis

3.3.1 Principle

Transcriptional activity precedes translation of protein in response to external stimuli (Perry et al., 2007). Extraction of mRNA from lysates can be amplified by polymerase chain reaction (PCR) to investigate expression of specific target genes.

3.3.2 RNA extraction of monolayer lysates

RNA was extracted from samples using the TRIZOL method according to manufacturer's instructions (Sigma-Aldrich, Dorset, UK). Samples were lysed in TRI reagent and titrated several times to form a homogenous sample. Samples were transferred to RNase free Eppendorf tubes and stored at -80ºC for subsequent RNA purification. In order to separate RNA from DNA and protein, samples were thawed at room temperature for 5-10 minutes. Once a completely homogenous liquid, 0.2 mL of chloroform (Sigma-Aldrich, Dorset, UK), per 1 mL of TRI reagent used was added. Samples were homogenised for 15 seconds before being left to stand for 15 minutes at room temperature. The samples were centrifuged for 15 minutes at 12,000 g (4ºC) in order to separate liquid into RNA, DNA and, Protein phases respectively. The upper aqueous phase containing RNA was removed and placed into separate RNase free Eppendorf tubes with 0.5 mL 2-propanol (Sigma-Aldrich, Dorset, UK), per mL of TRI reagent used. Samples were left at room temperature for 10 minutes before being centrifuged at 12,000 g (4ºC). The supernatant was removed leaving the RNA pellet. The RNA pellet was washed in 1 mL of 75% ethanol and centrifuged at 7,500 g for 5 minutes. Excess supernatant was removed and samples were allowed to air dry for 10 minutes before being homogenised in RNAse free water (Sigma-Aldrich, Dorset, UK). Samples were stored at -20ºC for later PCR analysis. The concentrations (ng/µl) and purity of RNA samples were determined using UV spectroscopy (Nanodrop, Fisher Scientific, Loughborough, UK). Samples were deemed to be of sufficient RNA purity when they possessed an A_{260}/A_{280} ratio of ≥1.7. All samples were analysed in duplicate.

3.3.3 RNA extraction of tissue engineered constructs

Tissue engineered constructs were prepared for RNA extraction by washing in PBS and blot dried to remove excess PBS. Constructs were placed in RNAs free tubes containing Trizol. Prior to extraction, constructs were homogenised using a handheld homogeniser in order to breakdown the extracellular matrix. The homogeniser was washed in dH_2O, 70% ethanol, and dH_2O between each construct. RNA extraction was performed as described above (*Section 3.3.2*).

3.3.4 Primer design

Primers were synthesised by Sigma Aldrich in according with guidelines outlined below (*Table 3-1*). Primers were purchased from Sigma-Aldrich and were reconstituted to a concentration of 100 µM in Tris-EDTA buffer (Sigma-Aldrich, Dorset, UK) as recommended by the manufacturer. The primers and their respective sequences used for this thesis are outlined below (*Table 3-3*). Primer specificity was confirmed by BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and primer specificity with their respective target genes were confirmed by melt-curve analysis following the PCR run (*Section 10.1*), and by identification through agarose gel electrophoresis as described below (*Section 3.3.7*).

Table 3.1 Considerations and guidelines for primer design using quantitative-PCR.

Consideration	Guideline
Primer Length	18-24 base pairs
Amplification Length	≤ 200 base pairs long
% GC content	40-60%
Annealing Temperature (T_m)	60°C is the optimal annealing temperature for SYBR quantifast chemistries
General Consideration	Primers should span exon-exon boundaries

3.3.5 Conventional and Real-Time PCR (PCR$_{RT}$) analysis

Gene expression was analysed using One-step PCR (Stratagene Mx3005P, Aglient Technologies, Cheshire, UK). RNA samples were diluted in a concentration of 70 ng in 9.5 µl. PCR reactions were made to a total reaction volume of 20 µl. Master mix was made up 10 µl of Syber green fluorescent mix, and 0.2 µl RT mix (Qiagen Chemistries, Crawley, UK) and 0.15µl forward and reverse primers respectively. The specific primers sequences are outlined below (*Table 3-3*) and are described with each Chapter. A one-step thermal profile was used as outlined in the table below (*Table 3-2*). Primer specificity was assessed by running a dissociation/melt phase at the end of the profile or by agarose gel electrophoresis (*Section 3.3.7*). An example of C^t profile and dissociation/melt curves can be found in appendices A.

Table 3.2 Thermal profile setup for one-step PCR using Stratgene Mx3000P

Profile Stage	Temperature	Duration of cycling (min:sec)
Denaturation	50ºC	10:00
Initial Activation	95ºC	05:00
Two step-cycling (40 cycles)		
Denaturation	95ºC	00:10
Annealing and Extension	60ºC	00:30

3.3.6 qPCR data analysis

Gene expression data were analysed using the ΔΔC^t method as previously described (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). The principle involves the use of a normalisation gene, or reference gene to compare changes in expression between conditions. All gene expression data were compared to RNA polymerase II β (RPII-β) which has been reported as a suitable reference gene for when conducting qPCR analysis (Radonić et al., 2004), and has previously been used as a reference gene when conducting experiments with the C2C12 myoblast cell line (Sharples et al., 2011).

Table 3.3 primer sequences of mouse mRNA genes used for one-stop qPCR

Gene	Primer Sequence	Accession No:
RNA Polymerase II -Beta (RPIIβ)	F: 5'-GGTCAGAAGGGAACTTGTGGTAT	NM_153798
	R: 5'-GCATCATTAAATGGAGTAGCGTC	
Myogenin (MyoG)	F: CCAACTGAGATTGTCTGTC	NM_031189
	R: GGTGTTAGCCTTATGTGAAT	
Myocyte enhancer factor 2a (MEF2a)	F: TAGCGGAGACTCGGAATT	NM_001033713
	R: GCTGCCGTTGAAATTGTC	
Peroxisome Proliferator-Activated coactivator (PGC1-α)	F: 5'-AGACTATTGAGCGAACCT	NM_008904.2
	R: 5'-TATGAGGAGGAGTTGTGG	
Hexokinase II (HKII)	F: 5'- GGAGGAGGAGCAGTATGG	NM_013820
	R: 5'-TTCAGCCGTGTGAGGTAA	
GLUT4	F: 5'-CATCAGGATAAACAGCAGGG	NM_011480
	R: 5'-GGAGGCAGGGCTAGATTT	
GLUT1	F: CAGAGGCTTGCTTGTAGAG	NM_011400
	R: GTCACCTTCTTGCTGCTG	

3.3.7 Agarose Gel Electrophoresis

If a melt curve analysis was not conducted, PCR products were determined through agarose gel electrophoresis. Two percent agarose gels were prepared in 1X Tris Borate EDTA (pH 8.3) (TBE) and heated until a homogenous solution. SYBR safe staining solution (Thermo fisher, Fisher Scientific, Loughborough, UK) was added and gels were subsequently allowed to set for approximately 30 minutes in casting trays. PCR products were mixed with 6X DNA loading dye (Thermo fisher, Fisher Scientific, Loughborough, UK) and 10 µl of sample loaded into each well and 10 µl of DNA reference ladder (Thermo fisher, Fisher Scientific, Loughborough, UK) was loaded at the end of each gel. Gels were run at 110 volts for approximately 60 minutes before being visualised using a Chemi-Doc image analyser (Bio-Rad) and Lab View analysis software (Lab View Version 4.1, Bio-Rad) (*Figure 3-4*).
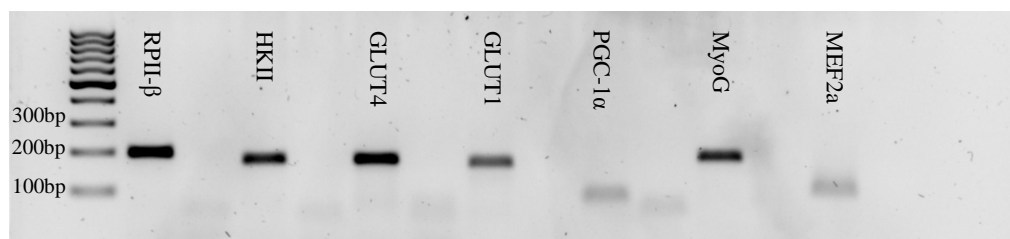
Figure 3-4 Agarose gel electrophoresis of PCR products alongside non-template controls (NTC). Ladder: 100 base pairs (bp).

3.4.   Immunohistochemical Analysis

3.4.1   Immunofluorescence Staining

Myoblast numbers and fusion efficiency were determined through immunocytochemical staining of samples. In brief, cells were washed in cold PBS before being incubated in a 1:1 solution of cold PBS and fixative (1:1 methanol/acetone) solution (Fisher-Scientific, Loughborough, UK). The solution was removed and replaced with 100% fixative for five minutes before being washed twice in PBS and stored for later analysis. Samples were blocked and permeabilised using 1X TRIS buffered saline solution (TBS), 5% goat serum (GS) and, 0.2% Triton X-100 for one hour. Coverslips were washed in TBS three times before being incubated for one hour at room temperature with a polyclonal, IgG, rabbit anti-mouse Desmin antibody (anti-desmin antibody, ab15200, Abcam, Cambridge, UK) diluted 1:200 in TBS (+2% GS and 0.2% Triton X-100). Samples were again washed in TBS before being incubated in a darkened chamber with a polyclonal goat anti-rabbit IgG antibody conjugated to TRTIC (, ab35181, Abcam, Cambridge, UK) diluted 1:200 in TBS (+2% GS and 0.2% Triton X-100). Nuclei were identified using a florescent DNA binding probe 4',6-diamidino-2-phenylindole, (DAPI) (Sigma-Aldrich, Dorset, UK), diluted 1:10,000 in purified water (dH_2O) for 15 minutes. Coverslips were washed in dH_2O before being mounted onto glass coverslips (Fisher-Scientific, Loughborough, UK) with 1µl of mounting medium (Fisher-Scientific, Loughborough, UK) and left to dry before being imaged by fluorescent microscopy.

3.4.2   Image Analysis

Images were obtained a fluorescent microscope (Leica DM 2500, fluorescent microscope, Leica DFC360 FX camera, Leica, Milton Keynes, UK). Images were obtained under X20 focus and analysed using image processing software (ImageJ, Version 1.46, http://rsbweb.nih.gov/ij/index.html). Cumulative frequency analysis was used to determine the number of images required from each slide. Images were analysed for the total number of myotubes per image, myotube width, and the number of nuclei formed into the myotubes.

3.5. Protein Extraction and Quantification

3.5.1 Principle

Exposure to various stimuli can result in the post-translational modification or re-localisation of cellular proteins. As a result the expression and phosphorylation of these proteins was investigated by the western blot technique. The technique of western blotting has been used for several decades in order to identify proteins of interest, which involves the separation of proteins by their molecular weight in kilo Daltons (kDa) through sodium-dodecyl-sulphate polyacrylamide gels (SDS-PAGE).

3.5.2 Protein extraction in monolayer culture

Cells were lysed in 200µl of protein lysis buffer protein lysis buffer (50mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 Mm EGTA, 50 mM NaF, 50 mM β-glycerolphosphate, 1 mM Na_{3}-VO_4, 1% Triton X-100 and 2 mM PMSF) or RIPA lysis buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0.). Cells were mechanically lifted by cell scraping from the well surface into a 1.5 mL Eppendorf and stored at -80°C ready for subsequent protein quantification. The specific lysis buffer are described in the respective Chapters and quantified using the protein assay as outline below (*Section 3.5.5*).

3.5.3 Cellular protein extraction from collagen gels

Following the experimental protocol, constructs were washed in cold PBS and blot dried before being snap frozen in liquid nitrogen. Samples were stored at -80°C until analysis. 1X laemmli buffer was added to samples, which were mixed by orbital rotation at 40 rpm for 60 minutes at 4°C in order to dissolve cellular material. 250 µl of 100% 2-propanol per 500 µl of laemmli lysis buffer was added and samples were centrifuged at 17,000 g for 10 minutes. The supernatant was discarded and the pellet was re-suspended in 300 µl of laemmli lysis buffer and allowed to rotate for a further 60 minutes at 40 rpm at 4°C. Samples were centrifuged for 5 minutes at 13,000 g after which, the supernatant was removed and placed in a fresh Eppendorf tube and quantified as described (*Section 3.5.5*).

3.5.4 Extraction of cellular protein from fibrin gels

Other laboratories have successfully extracted and blotted for protein using fibrin tissue engineered constructs (Khodabukus and Baar, 2009a). Therefore, protein extraction was conducted as previously described (Khodabukus and Baar, 2009a) with some slight modifications. Following 14 days in culture, constructs were washed in cold PBS before being removed from sutures, blot dried, snap frozen in liquid nitrogen, and stored at -80°C for later analysis. Samples were homogenised in lysis buffer. In order to ensure continuity, protein extracted using the RIPA lysis buffer (*Section 3.5.2*), or a sucrose lysis buffer described by Khodabukus and colleagues (Khodabukus and Baar, 2009b). Samples were mixed in the respective lysis buffer by orbital rotation for 1 hour at 4°C. Lysates were briefly homogenised and centrifuged down for 1 minute at 11,000 g. The supernatant was aspirated and placed in a separate Eppendorf tube and protein concentration quantified as previously described (*Section 3.5.5*). Protein concentrations were found to be greater using RIPA lysis buffer compared to sucrose lysis buffer (*Figure 3-5*) (t = 5.586, P<0.001). Subsequently, samples lysed in RIPA buffer when loaded onto 8% SDS-PAGE gels at serial dilutions of protein (0-20 µg protein) with laemmli loading buffer to identify the optimum loading concentration of protein (*Figure 3-6*).
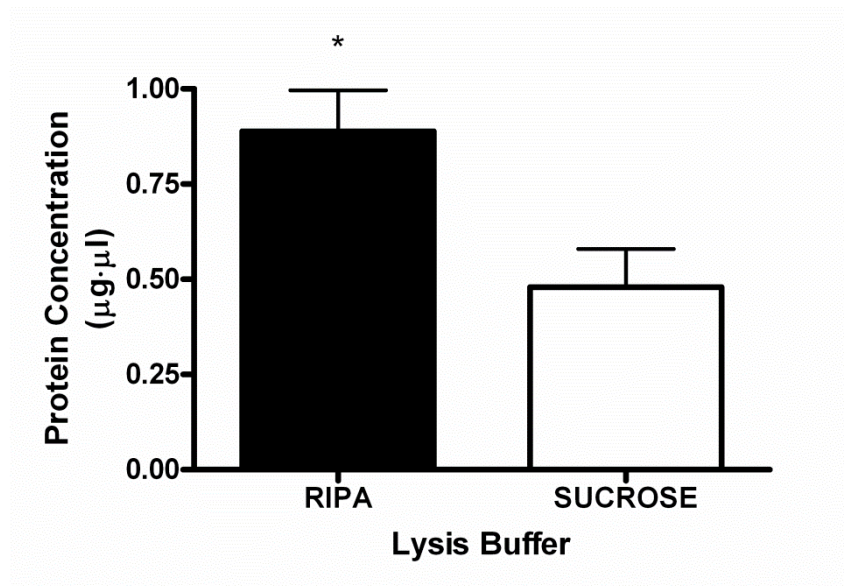
*Figure 3-5 Protein concentrations of 14 day old fibrin skeletal muscle constructs using RIPA or Sucrose lysis buffers. Data are mean ± SD from four constructs (N=4). * Significance P < 0.001.*

3.5.5   Protein quantification

Protein quantification was determined using a pierce 660 nm protein assay according to the manufacturer's instructions (Thermo Scientific, Fisher, Loughborough UK). Protein concentrations were determined against serially diluted BSA standards. Samples and standards (10µl) were analysed in triplicated in a 96 well plate with 150µl of assay reagent. Samples were shaken for 1 minute and incubated at 22°C for 5 minutes and measured at an absorbance of 660nm.

3.5.6   SDS-PAGE Silver Staining Protocol

3.5.6.1.   *Principle*

Silver staining provides a simple and cheap method of detecting proteins particularly at low concentrations (nano-gram range) making it a highly sensitive method (Chevallet, Luche, and Rabilloud, 2006). Because proteins bind to silver ions, silver-staining SDS-PAGE gels results in visualisation of protein separation through the gel (Chevallet et al., 2006). Protein extraction from tissue engineered constructs has presented some complications with regards to the concentrations of protein, particularly from fibrin gels (*Figure 3-7*), but also the clear separation of protein extracted from collagen gels, (due to the high concentration of extracellular matrix) has led to difficulties presenting clear western blots from these constructs (Player, 2013). Based on these factors, silver-staining was conducted with serial dilutions of protein from fibrin and collagen gels were separated by SDS-PAGE to establish a suitable concentration of protein to investigate what the optimal concentration protein could be used to ensure clear separation of proteins throughout the SDS-PAGE gel.

3.5.6.2.   *Silver staining protocol*

In brief, following electrophoresis, the gel was removed from the glass plates, and washed in ultrapure water before being incubated for 1 hour in fixative solution (40% ethanol, 10% acetone, 50% dH$_2$O). The gel was washed in ethanol and sensitising solution (30% Ethanol, 10% Sensitizer, 60% dH$_2$O) for 10 minutes. The gel was again washed in 30% ethanol for 10 minutes followed by further 10 minutes incubation in dH$_2$O. Staining solution (1% Stainer, 99% dH$_2$O) was applied for 15 minutes. Following aspiration of the staining solution the gel was briefly washed in dH$_2$O and incubated in developing solution for 5 minutes. The reaction of the developing solution was arrested by the addition of stop solution. The gel was rinsed in ultrapure water for 10 minutes before being exposed under white transilluminecant light (Chemi-Doc, Bio-Rad, UK).
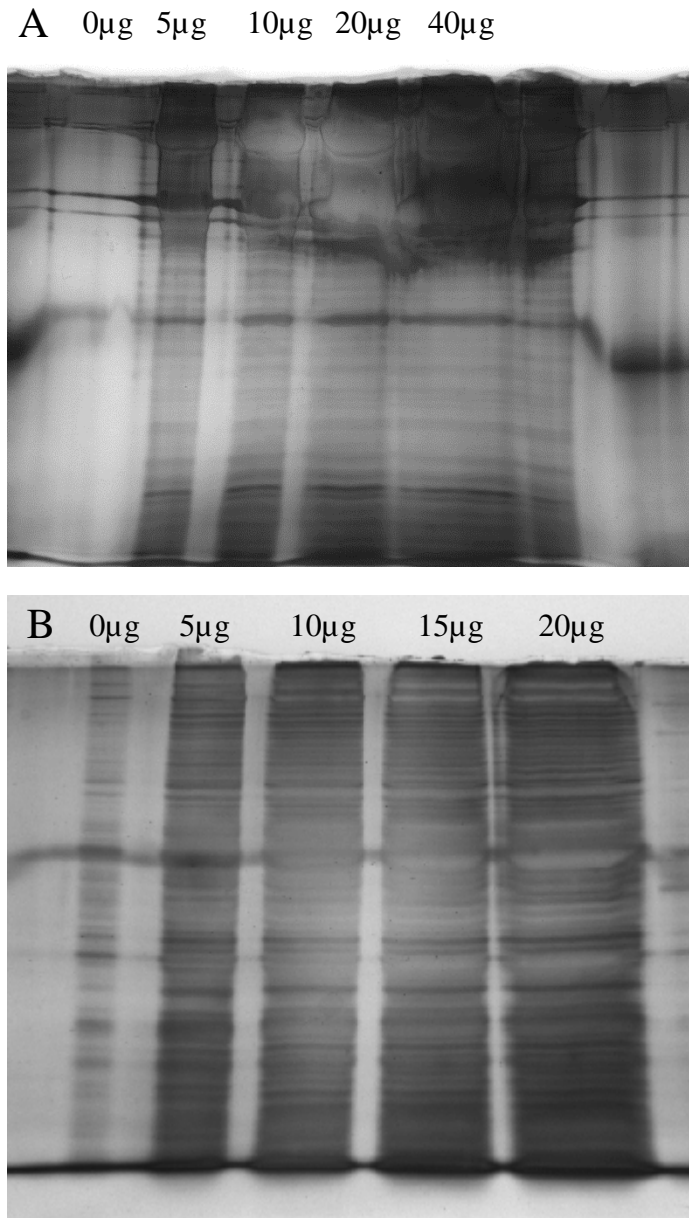
Figure 3-6 Silver stain with serial dilutions of cellular protein (μg). A: Collagen gels B; Fibrin Gels. Collagen gels (A) presented clearer protein separation at lower (5μg & 10μg) protein concentrations. Fibrin gels (B) showed clear separation at all concentrations of protein. Bands observed in figure B at 0μg are potentially a result of overspill within adjacent lanes. Lower concentrations of protein (5μg) were used for subsequent western blot analysis in both tissue engineering skeletal muscle models Image is of 1 experimental repeat.

3.5.7 Western Blotting

Cells lysates were mixed in Laemmli buffer (4 mL H_2O, 1 mL 1M TRIS HCl pH 6.8, 0.4 mL Glycerol, 0.4 mL 2-β-mercaptoethanol, 0.05% bromophenol blue), and boiled for 5 minutes at 95ºC. A volume equivalent to 20 µg of sample for monolayer experiments was loaded into SDS-polyacrylamide gels (SDS-PAGE) (4% stacking (6.05 mL H_2O, 1.3 mL 30% Acrylamide, 2.5 mL 1.5 M Tris HCl, pH 6.8, 0.2 mL 10% SDS, 0.1 mL ammonium persulfate (APS), 10 µl TEMED), and 12% resolving gels (6.7 mL H_2O, 8mL 30% Acrylamide, 5mL 1.5 M Tris HCl, pH 8.8, 0.1 mL 10% SDS, 0.05 mL APS, 10 µl TEMED). Samples were separated in Tris-glycine running buffer (1:10 10X Running buffer, 1 L dH_2O, 30.3 g Tris-Base, 144 g Glycine, 10 g SDS) using Bio-Rad mini proton electrophoresis kits (Bio-Rad laboratories, Hertfordshire, UK). Voltage was set at 80V through the stacking gel and 120V through the resolving gel, until the proteins had ran to the bottom of the gel.

Gels were wet transferred onto nitrocellulose membranes (Whatman Proton, Sigma-Aldrich, Dorset, UK), for 2 hours at a constant amplitude (0.35 Amps). Membranes were blocked (5% BSA/TBS) for 1h at 4ºC and rinsed three times in TBST (50 mL 5X TBS, 450 mL dH_2O, 50 µl Tween), before being incubated with primary antibody overnight at 4ºC. Details of the antibodies used and concentrations can be n in table 3.2. Following overnight primary antibody incubation, membranes underwent further 3 x 5 minute washes and were subsequently incubated for 1 hour with anti-rabbit horseradish peroxidase-conjugated secondary antibody in 5% Milk TBST (*Table 3-4*).

Following secondary antibody incubation membranes were washed as above and incubated in chemiluminescence reagent (ECL) (Supersignal, Thermo Fisher Scientific, Rockford, IIL, USA) for five minutes before being exposed using Bio-Rad Chemi-doc system (Bio-Rad Laboratories Ltd, Hertfordshire, UK). Band Densities were quantified using Quantity One image analysis software (Quality One 1-D analysis software version 4.6.8, Bio-Rad Laboratories Ltd, Hertfordshire, UK).

3.5.7.1. *Protein separation in tissue engineered skeletal muscle constructs*

For tissue engineered constructs, dose dependent concentrations of protein was separated by SDS-PAGE (8% resolving gels), which have previously be used within the literature to investigate protein expression with fibrin based skeletal muscle tissue engineered constructs (Khodabukus and Baar, 2012, 2014a). SDS-PAGE gels were silver stained using a commercially available kit according to the manufacturer's recommendations which is

outlined in Section 8expression3.5.6.2 (Invitrogen, Life-Technologies, CA, USA) or transferred onto nitrocellulose membranes as described above and blotted for the expression of total Akt which is one of the main target proteins in this thesis. From both silver staining and western blots, it was determined that 5 µg of protein was sufficient for protein separation as well as quantification of western blots in both types of construct (*Figure 3-7*).

Table 3.4 Primary and Secondary antibodies used for immunoblotting in this thesis.

Antibody	Company	Product No.	Isotype	Concentration	Molecular Weight (kDa)
Insulin Receptor Substrate 1 (IRS-1)	*Santa Cruz Biotech*	SC-559	Rabbit	1:1000	170-185
Phosphor IRS-1 (Try^{632})	*Santa Cruz Biotech*	SC-17196	Rabbit	1:1000	170-185
P85 PI-3 kinase	*Cell Signalling*	#4292	Rabbit	1:1000	85
Akt	*Cell Signalling*	#9272	Rabbit	1:1000	60
Phosphor Akt (Ser^{473})	*Cell Signalling*	#9271	Rabbit	1:1000	60
Phosphor Akt (Thr^{308})	*Cell Signalling*	#9275	Rabbit	1:1000	60
Glucose Transporter 4 (GLUT4)	*Santa Cruz Biotech*	SC-7938	Rabbit	1:1000	50-63
Beta-Actin (β-Actin)	*Cell Signalling*	#4967	Rabbit	1:1000	45
Secondary Antibody					
Anti-rabbit IgG – HRP	*Cell Signalling*	#7074	Rabbit	1:1000	-
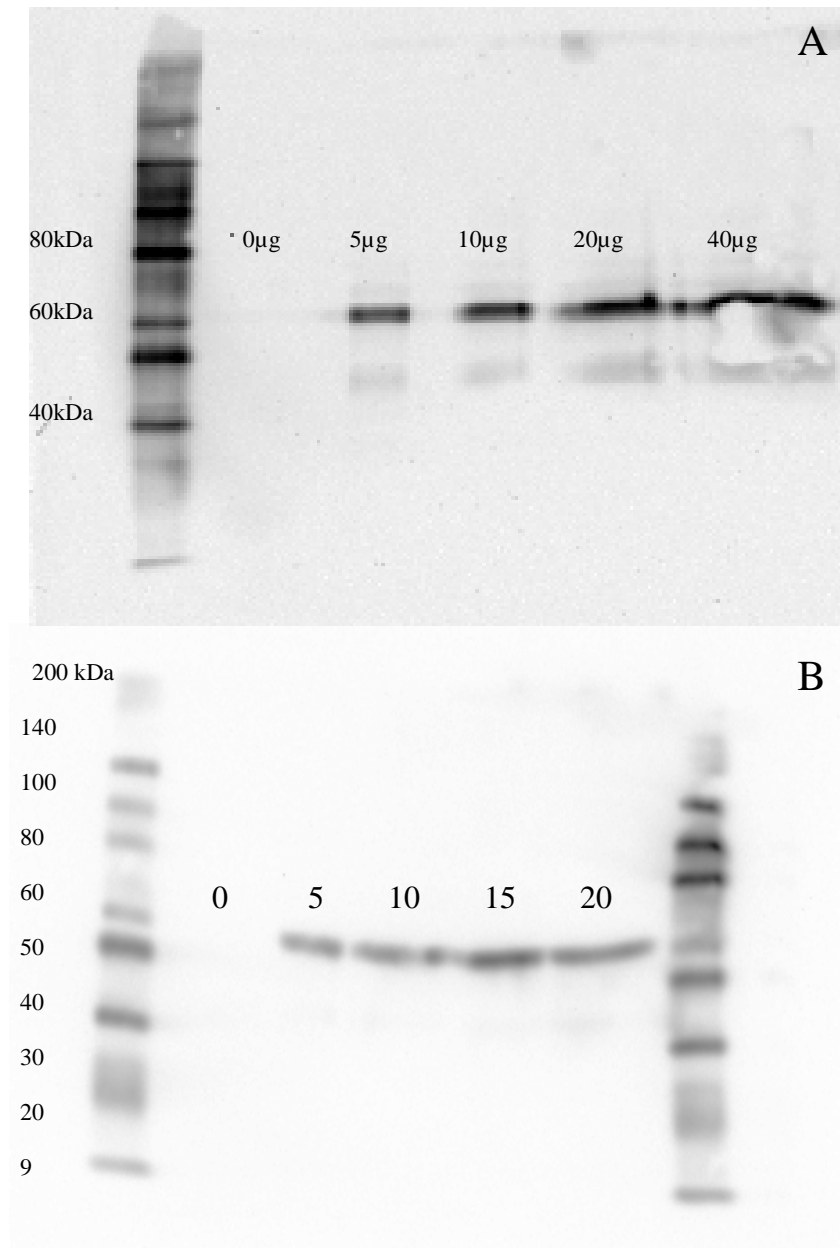
Figure 3-7 Immunoblot for total Akt with dose dependent protein concentrations (µg) from 14 day old A) collagen and B) fibrin gels.

3.5.8   Immunoprecipitation of Insulin Receptor Substrate-1 (IRS-1).

3.5.8.1.   *Principle*

The molecular weight of the IRS-1 protein has been determined to be 135kDa however, the high serine phosphorylation of IRS-1 results in migration to 185kDa when separated by SDS-PAGE (White, 1998). Initial attempts to quantify the IRS-1 protein from western blots were unsuccessful; previously research has employed immunoprecipitation of the IRS-1 protein in order to quantify its phosphorylation status by western blot (Ma et al., 2013).

3.5.8.2.   *Immunoprecipitation of IRS-1*

Samples were lysed in 500 µl of RIPA buffer (Sigma-Aldrich, Dorset, UK) and 10 µl/mL of protease/phosphatase inhibitor cocktail mix (Fisher-Scientific, Loughborough, UK) and rotated for 30 minutes at 4ºC before being frozen at -80ºC for later analysis.

Samples were sonicated at output 3 and 50% duty cycle for 10-30 pulses in order to completely homogenise the tissue within the lysis buffer. Samples were rotated for 60 minutes at 4ºC before being quantified using Pierce Protein Assay as previously described (*Section 3.5.5*).

1mg of total protein was diluted to 4 µg/µl in RIPA buffer and protease/phosphatase inhibitor cocktail mix. Samples were 'pre cleared' through adding 50 µl of protein A-conjugated agarose beads (Santa Cruz Biotechnology, CA, USA) and rotated for 60 minutes at 4ºC. Samples were centrifuged at 4000 rpm for 10 minutes at 4ºC. The supernatant was aspirated and incubated overnight with 5 µl of insulin receptor substrate (IRS-1) or phosphor IRS-1 (Tyr^{632}) antibody at 4ºC (Santa Cruz Biotechnology, CA, USA).

Following overnight incubation a further 50µl of protein agarose beads and samples were allowed to rotate for 60 minutes at 4ºC. Samples were washed three times by centrifugation at 2500 rpm for one minute, following which the supernatant was discarded and replaced with fresh RIPA + protease/phosphatase inhibitor buffer. Following aspiration of the wash buffer 7 µl of 3X reducing buffer (0.35 M Tris-HCl, 10% SDS, 36% Glycerol, 5% β-mercaptoethanol, 0.012% bromophenol blue and 0.3% Tween-20) was homogenised with the sample and boiled for 5 minutes at 95ºC before being loaded onto SDS polyacrylamide gels for immunoblotting (*Section 3.5.6*).

3.5.9 Migration front

A migration front was calculated (*Figure 3-8*) based on the distance travelled from the top of the gel to the point of the visual bands to confirm the visualised band was the protein of interest. The reference point was determined based upon the migration of the molecular weight ladder (New England Bio labs, Herts, UK).
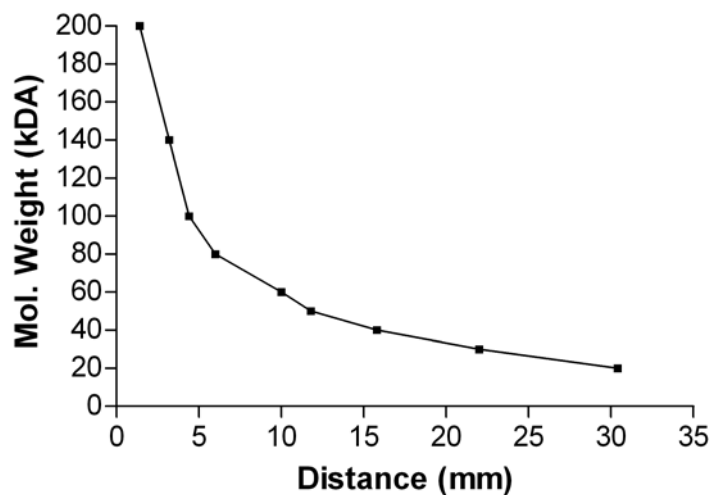
Figure 3-8 Migration front calculation based on distance of known molecular weight ladder.

3.5.10 Stripping and Re-probing of Nitrocellulose Membranes

Due to the limited concentration of proteins which were extracted from cultures, it was necessary to maintain nitrocellulose membranes to investigate other proteins of interest. Therefore a membrane stripping method was used to remove primary and secondary antibody from the membrane for subsequent analysis of different proteins of interest.

Following visualisation, nitrocellulose membranes were washed three times for 5 minutes in TBST. Membranes were agitated by rocking (60 rpm), twice for ten minutes in stripping buffer (0.15% Glycine, 0.01% SDS, and 10% Tween-20, pH 2.2) (*Figure 3-9*). Following the respective washes in stripping buffer, membranes were washed in TBST, before being blocked in BSA as previously described (*Section 3.5.6*) and incubated overnight at 4°C with appropriate primary antibody.
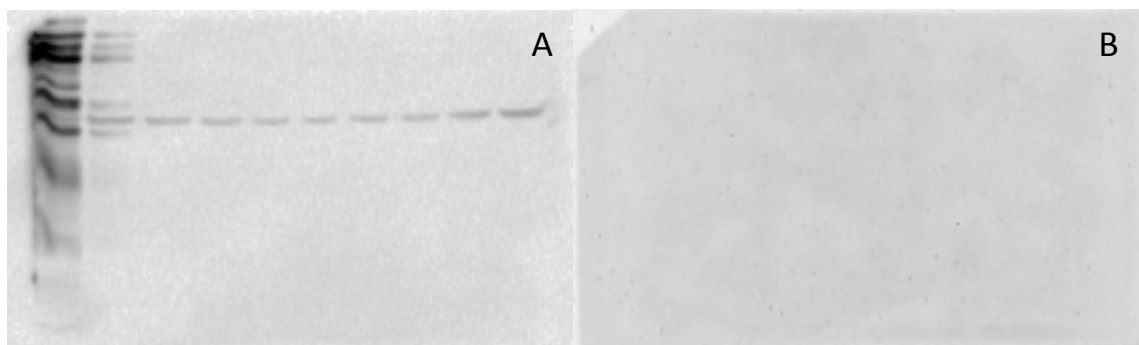
Figure 3-9 Exposed nitrocellulose membranes pre (A) and post (B) membrane stripping protocol

3.6.   Glucose Uptake Analysis

3.6.1   Measurement of ^{3}H-Deoxy-D-Glucose uptake in cell culture.

Following four hours serum starvation, experimental plates were washed twice with Krebs Ringer HEPES (KRH) buffer and incubated for 15 minutes in the presence or absence of 100 nM insulin. Glucose uptake was determined by the addition of ^{3}H-Deoxy-D-Glucose (0.1 mM, 0.5 µCi/mL; PerkinElmer Life and Analytical Science). After incubation, glucose uptake was stopped by the addition of 10 µM cytochalasin B (Sigma-Aldrich, Dorset, UK) in PBS. Cytochalasin B inhibits actin remodelling and subsequent vesicle trafficking of proteins from the cytoplasmic region to the plasma membrane (Jung & Rampal, 1977; Klip & Walker, 1983). This inhibitor is repeatedly used within the literature as an inhibitor in order to prevent GLUT4 mediated glucose uptake. The cells were washed twice in PBS and lysed in 0.2 M NaOH, PBS. Glucose uptake was measured by liquid scintillation counting (Packard-Bell). Cytochalasin B (10 µM) is added to scintillation fluid for the measurement of nonspecific background. Total protein was also quantified in concurrent 24 well plates using the protein assay described previously (*Section 3.5.5*).

3.6.2   Measurement of ^{3}H-Deoxy-D-Glucose uptake in tissue engineered constructs

Glucose uptake was measured as previously described (Baker et al., 2003), with some slight modifications. Following four hours serum starvation, experimental plates were washed twice with Krebs Ringer HEPES (KRH) buffer and either incubated for 30 minutes in the presence or absence of 100 nM insulin. Glucose uptake was determined by the addition of ^{3}H-Deoxy-D-Glucose (0.1 mM, 1 µCi/mL; PerkinElmer Life and Analytical Science). After incubation, the reaction was arrested by the addition of 10 µM cytochalasin B in PBS. Constructs were removed from the sutures, blotted and weighed. Individual constructs were dissolved in a 1:10 (wt./vol) dilution of 1 M potassium hydroxide (KOH) solution, placed in boiling water for 2 min, placed on ice for 5 min. Samples were neutralized by adding 1 M HCI in a 1:10 (wt./vol) dilution of construct wet weight. Samples were homogenised with 250 µl solution added to scintillation fluid. Uptake was analysed by scintillation counting as described above (*Section 3.6.1*).

3.6.3 Calculating ^{3}H-Deoxy-D-Glucose uptake in culture

The following calculations were used to determine the concentrations of ^{3}H-Deoxy-D-Glucose uptake in both monolayer cultures and tissue engineered constructs. In order to

Definitions and abbreviations of radioactive isotopes used for calculating glucose uptake

- **Curie (Ci)**: Is a unit of radioactivity, 1 Curie is defined as 3.7 X 10^{-10} disintegration per second (dps).

- **Disintegrations per minute (dpm)**: A unit of measurement which is directly obtained from the liquid scintillation counter.

- **Specific Activity (SA)**: the amount of radiolabelled mass within a sample (Ci·mmol)

The calculation of curies (Ci) was determined using based on the conversation between the disintegrations per minute (dpm) which were given as the standard output from the scintillation counter.

$1Ci = 3.7$ x 10^{-10} Bq $= 3.7$ x 10^{-10} disintegrations per second (dps) $= 2.22$ x 10^{-12} dpm

$$\text{Conversion of dpm to Ci} = \frac{dpm}{2.22 \times 10^{-12}}$$

Conversation of Ci to moles $= Concentration\ of\ glucose\ (mmol) \times 10^{-6} \times (1mmol \div SA)$

3.7. Statistical Analysis

Statistical analysis was conducted using SPSS statistical software package (IBM SPSS, Version 19, NY, USA). Specific statistical analysis is outline within the respective Chapters of this thesis.

4. Method Optimisation: Measurement of glucose uptake in C2C12 skeletal muscle cells using radioactive isotopes and fluorescent based methods.

Preface: The following brief Chapter outlines the investigation and optimisation of two methods for the quantification of cellular glucose uptake using radioactive isotope tracers and fluorescent based glucose assays.

4.1. Introduction

Glucose uptake is controlled by distinct mechanisms (Krook, Wallberg-Henriksson, and Zierath, 2004). As glucose is non-permeable to the plasma membrane, an increase in glucose concentrations results in an increase in insulin concentrations in order to initiate glucose transporter exocytosis and facilitate glucose uptake into the cell (Leto and Saltiel, 2012). The quantification of glucose uptake has provided a better understanding of the rate of glucose uptake and metabolism both *in vivo* and *in vitro* (Hansen, Gulve, and Holloszy, 1994; Sell, Jensen, and Eckel, 2012). Two methods have previously been used within the literature for the quantification of glucose uptake, using either fluorescence based glucose analogues (Manchester et al., 1990), and more conventionally, the use of radioactive isotopes for investigation of insulin sensitive tissues such as adipose and skeletal muscle (Sell et al., 2012; Tanti, Cormont, Grémeaux, and Le Marchand-Brustel, 2001). Therefore, the purpose of these optimisation experiments was to establish a method for investigating insulin stimulated glucose uptake in monolayer cell culture as well as tissue engineered skeletal muscle constructs.

4.1.1. Measurement of 2-deoxy-d-glucose uptake to investigate glucose metabolism

Investigation of glucose uptake has commonly used glucose analogues such as, 2-deoxy-d-glucose (2-DG) (Molecular Weight (MW) 164.16) in order to quantify cellular glucose uptake (Tanti et al., 2001). 2-DG is obtained up by solute membrane transporters however unlike glucose and other glucose analogues, 2-DG is metabolised by the hexokinase reaction to form a stable and impermeable derivative 2-deoxy-glucose-6-phosphate which is not metabolised further (Wick, Drury, and Morita, 1955). Early studies by Wick and colleagues suggested that the accumulation of 2-deoxy-glucose-6-phosphate inhibits the hexokinase reaction and subsequently results in a 'backing up' in 2-deoxy-glucose accumulation (Wick, Drury, Henry, and Chem, 1957), however the concentrations required before 2-DG becomes rate limiting are extremely high (Hansen et al., 1994), and are not reached within cell culture experiments (Sell et al., 2012; Tanti et al., 2001). As a result, the use of 2-DG in quantification of 2-DG uptake *in vitro* using fluorescent and radioisotope-based assays methods has been established and used extensively within the literature.

4.1.1.1.   Fluorescent based methods for measurement of 2-deoxy-d-glucose uptake

Although the use of radioisotopes are a sensitive and accurate method for measuring glucose uptake, which will be discussed within the proceeding Section (*Section 4.1.2*), the expensive nature, and specialist equipment and expertise that are required in order to perform radioactive experiments can be problematic. The development of enzymatic assays using fluorescent based methods with fluorescent D-Glucose analogue 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (2-NBDG) have been established since the early 1990's as an alternative method for investigating cellular glucose uptake (Manchester et al., 1990; Sasson, Oron, and Cerasi, 1993). It has been used to measure uptake in various tissues such as skeletal muscle, adipocytes and hepatocytes (Kanwal, Singh, Grover, and Banerjee, 2012; Zou, Wang, and Shen, 2005). One of the main advantages of the use of this assay over the more conventional radioisotopes assay is its use for quantification of glucose uptake in single cells, through the use of flow cytometry (Zou et al., 2005). However, Zou and colleagues have reported large variation in 2-NBDG uptake between cells (Zou et al., 2005), meaning that this method of quantification of glucose uptake in highly metabolic tissues such as skeletal muscle is yet to be fully validated. Alternatively, a 96 well plate based assay has been established for investigating more insulin sensitive tissues such as skeletal muscle and adipocytes (Saito et al., 2011; Yamamoto, Sato, Kawasaki, Murosaki, and Yamamoto, 2006). The use of this method has shown to be sensitive enough to quantify glucose uptake within the pico mole (pmol) range in adipocytes (Saito et al., 2011). Yet, the use of 2-NBDG with skeletal muscle cells is sparse within the literature, with the majority of the work being performed in the L6 rat myoblast cell line (Yamamoto et al., 2006; Zou et al., 2005). The use of this cell line has commonly been used within the literature to investigate insulin stimulated glucose uptake using radioactive isotopes and has been shown to respond highly to insulin (Philp et al., 2011). While alternative cells lines such as the C2C12 skeletal muscle cell line have been used for investigation of insulin signalling and glucose uptake using traditional radioactive isotopes (Nedachi et al., 2008; Nedachi and Kanzaki, 2006) there is currently no evidence for the use of a fluorescent based glucose uptake assay within this cell line. Therefore the purpose of this experiment was investigate the fluorescent cell based glucose uptake assay could be used to investigate insulin stimulated glucose uptake in C2C12 skeletal muscle cells. Principles of radioactive isotopes for *in vitro* metabolic research

Tracers are compounds, which are chemically and functionally identical to their natural counterparts, yet they provide a form of identification through radioactive labelling which enables detection (Wolfe and Chinkes, 2005). The use of radioactive isotopes for *in vitro* research has provided a useful tool for investigation of a number of cellular functions such as glucose metabolism within insulin sensitive tissue such as adipocytes and skeletal muscle (Sell et al., 2012; Tanti et al., 2001). Radioactive isotopes provide several advantages for use in biological research; Firstly, the isotope in question can be easily incorporated into the biological compound being quantified, in this case the use of tritium (^{3}H) (*Figure 4-1*), and secondly isotopes are required to have a suitable half-life. The half-life is the rate of decay to a non-radioactive state and in the case of tritium the half-life is approximately 12 years.

The quantification of isotopes within biological samples is measured through liquid scintillation counting (LSC) which provides a reproducible and sensitive method for quantification of aqueous biological samples. LSC detects radioactivity through light emission between the radioisotope and scintillation fluid. A reaction between these molecules leads to decay of the molecule to ground state leading to the production of light or 'photon' which is counted (*Figure 4-2*).

Radioactive isotopes using low beta emitting particles such as ^{3}H or ^{14}C are the two most common isotopes to be used to investigate glucose uptake and both of these radioactive isotopes have been utilised to investigate rates of glucose metabolism in skeletal muscle and adipocytes *in vivo* (Cooney, Astbury, Williams, and Caterson, 1987; Cooney, Caterson, and Newsholme, 1985; Hom and Goodner, 1984). In addition, the use of ^{3}H-2DG has frequently been used to investigate glucose metabolism in culture from primary skeletal muscle cells (Sell et al., 2008, 2012) as well as skeletal muscle cell lines (Ma et al., 2013; Philp et al., 2011) to investigate both mechanisms of insulin dependent (del Aguila et al., 2011; Kumar and Dey, 2003), and independent glucose uptake (Iwata et al., 2009; Lambernd et al., 2012).

HO

O ³H

OH

OH   OH

³H
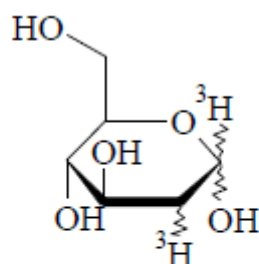
M.W. 164

$C_6H_{12}O_5$

Figure 4-1 Chemical structure of tritium labelled (³H) 2-deoxy-d-glucose used for investigation of glucose uptake in vitro. Image is obtained from Perkin Elmer certificate of analysis supplied with radioactive stock. Certificate of analysis for ³H-2DG used in this thesis can be found in appendices D.
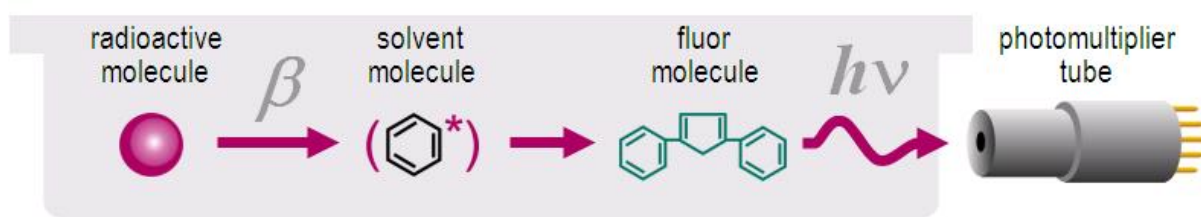
Figure 4-2 A simplistic schematic of the liquid scintillation process between a stable β emitting particle, such as tritium (³H), and scintillation fluid. A reaction between these two molecules results in the emission of light (photon) which is quantified. Accessed 25.7.14 from Perkin-Elmer. http://www.perkinelmer.co.uk/resources/technicalresources/applicationsupportknowledgebase/radio metric/liquid_scint.xhtmL

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4.2. Methods and Results

4.2.1. Non-labelled 2-DG glucose uptake assay

The measurement of glucose uptake was quantified according to the manufactures instructions using a commercially available cell based glucose uptake assay kit (Cayman Chemical, Ann Arbor, MI, USA). In brief, cells were seeded into a black sided, clear bottom, 96-well plate (Corning life sciences, Amsterdam, Netherlands). Cells were seeded at a density of 15,000 cells·cm^2 (this is consistent with all plating densities used through this thesis and requires 3000 cells per well) in 100µl of standard growth medium. The cells were grown and differentiated as outlined within the methods Section (*Section 3.1*). Following differentiation for a period of 96 hours, C2C12 myoblasts had differentiated into skeletal muscle myotubes (*Figure 4-3*). Myotubes were serum starved for 4 hours as previously described (Nedachi and Kanzaki, 2006) before being stimulated with insulin for 30 minutes. Cells were incubated in 100 µl of glucose free medium (Life Technologies, Paisley, UK) with 150µg/mL of 2-deoxy-2[(7-nitro-2, 1, 3-benzoxadiazol-4-yl) amino] – D-glucose) (2-NBDG) for 10 minutes. In parallel wells, cells were also incubated with the addition of 10µM of cytochalasin B with or without insulin (*Figure 4.4B*). Cytochalasin B inhibits insulin stimulated glucose uptake by impairing actin remodelling and GLUT4 translocation (Amira Klip et al., 2014), and was used as a negative control within these experiments. The supernatant was aspirated and 200 µl of cell based assay buffer was added to each well, the supernatant removed and replaced with 100 µl of fresh cell based assay buffer before being analysed on a fluorescent plate reader at a excitation/emission wavelength of 485/535 nm. However, despite the presence of the inhibitor the fluorescence emitted was similar between conditions (p = 0.99) (*Figure 4-4A*).
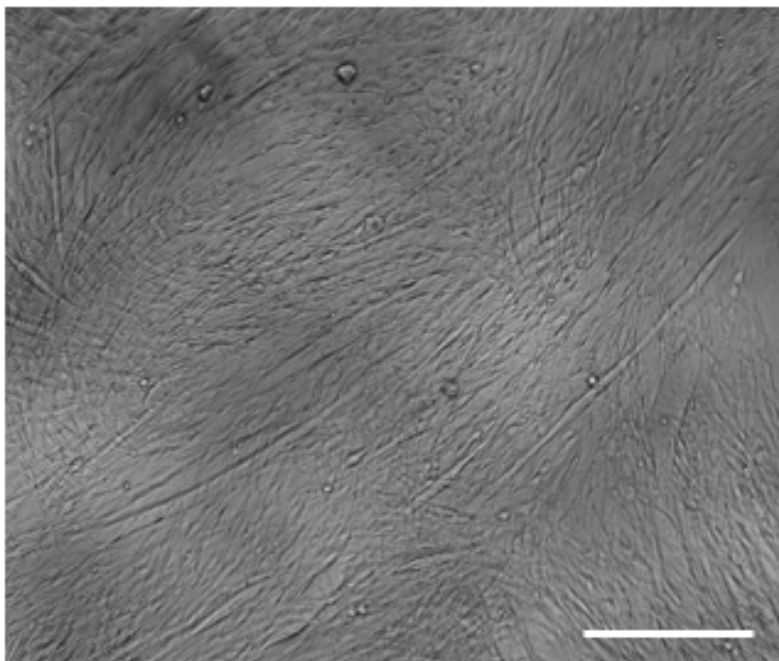
Figure 4-3 Light microscopy of differentiated C2C12 skeletal muscle cells in 96 well plates. Skeletal muscle cells were differentiated in black sided, clear bottom well plates for investigation of glucose uptake using the fluorescent based assay. Image was obtained at 10X magnification. Scale bar = 25μm

A

B
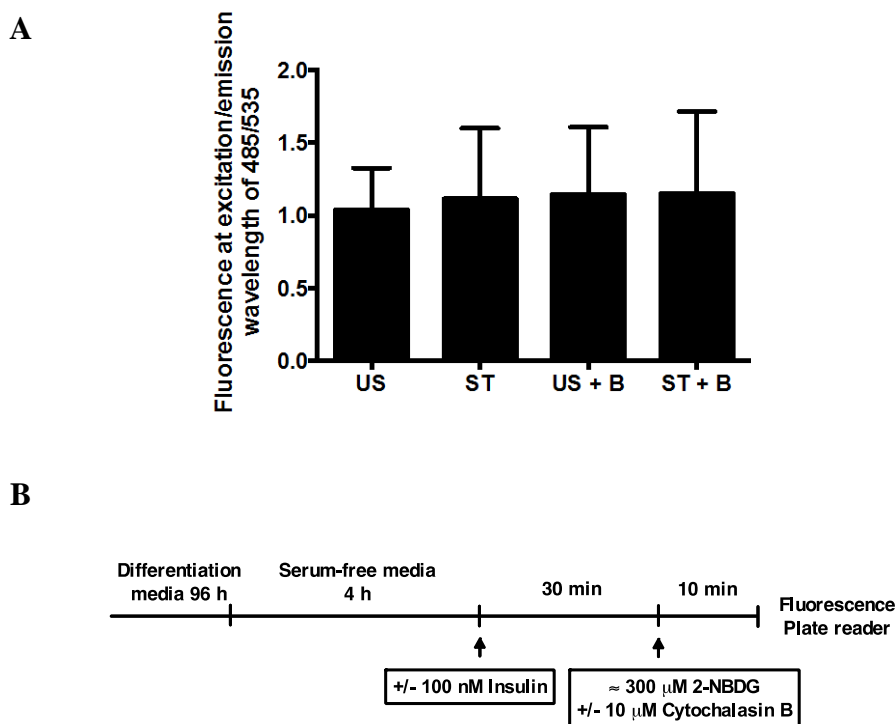
Figure 4-4 A, 2-NBDG uptake in C2C12 myotubes expressed as fluorescence in 4 different conditions, US: unstimulated, ST: stimulated with 100 nM insulin, US+B: US + blocker (cytochalasin B), ST+B: ST + blocker. A one-way ANOVA was non-significant (P = 0.99). Values are absolute value (mean ± SD) from 3 independent experiments performed in triplicate (n = 9). B, Experimental design.

4.2.2. Measurement of ^{3}H-Deoxy-D-Glucose uptake.

Following four hours serum starvation, experiment plates were washed twice in Krebs Ringer HEPES (KRH) buffer and either incubated for 15 minutes in the presence or absence of 100 nM insulin. Glucose uptake was determined by the addition of ^{3}H-2-deoxy-d-glucose (0.1 mM, 0.5 µCi/mL; PerkinElmer Life and Analytical Science). Wells were incubated with 10 µM cytochalasin B (Sigma-Aldrich, Dorset, UK) in PBS, in order to measure nonspecific uptake. The cells were washed two times in ice cold PBS, to remove any excess media. Cells were lysed in 0.2 M NaOH, PBS (Fisher Scientific, Loughborough, UK), with the whole cell lysate added to a scintillation vial containing scintillation cocktail (Gold Star scintillation fluid, Meriden biotechnologies, Surry, UK). Samples were briefly homogenised before being quantified by scintillation counting (Packard TRI-CARB 2750 TR/LL liquid scintillation counter). Total protein was also quantified in concurrent plates using 660 nM pierce protein assay as previously described (*Section 3.5.5*).

4.2.3. Optimisation experiments for ^{3}H-De-oxy-D-Glucose uptake.

Initial optimisation experiments were conducted in 24 well plates to investigate concentration and time dependent glucose uptake in skeletal muscle myotubes. C2C12 skeletal muscle cells were d at 15,000 cells·cm[2] in standard growth media as previously described. Upon confluence cells were changed to standard differentiation media for 72 hours following which skeletal muscle cells had fused into skeletal muscle myotubes as observed by phase contract microscopy (*Figure 4-5*).
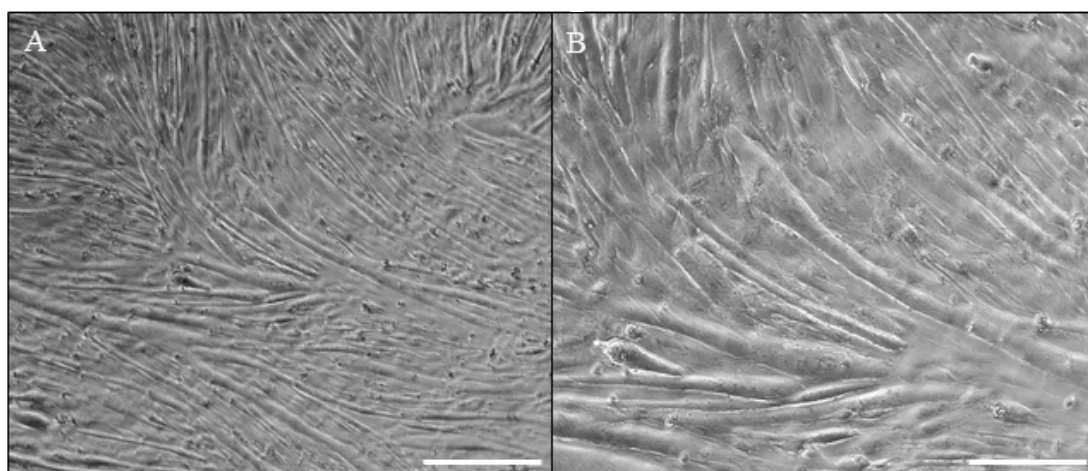
Figure 4-5 Representative phase contrast micrographs of C2C12 skeletal muscle myotubes following 72 hours of differentiation in 24 well cell culture plates for measurement of ^{3}H-De-oxy-D-Glucose uptake A; X20 focus; B; X40 focus. Scale Bar 50μm.

Glucose uptake was measured as described above (*Section 4.2.2*) following zero, five, ten, twenty and thirty minutes to [3]H-2DG glucose radioactive concentrations of glucose which had previously been described within the literature using C2C12 skeletal muscle cells (Nedachi and Kanzaki, 2006) (0.1 mM, 0.5 μCi/mL). Incubation times were determined based on previous experiments in monolayer which had incubated with insulin these periods of time (Kumar and Dey, 2003; Nedachi and Kanzaki, 2006).

Following the time response curve (*Figure 4-6*) it was established that thirty minutes was a potential time point that would provide a suitable assay window to investigate insulin stimulated glucose uptake in C2C12 skeletal muscle cells. It was therefore necessary to repeat the experiment at the same concentration but also investigate higher concentrations of radioactive glucose (0.1 mM, 1 μCi/mL) to investigate further improving the assay window.

The increase in concentration of ^{3}H-2DG would resulting increase the specific activity of the concentrations being used and potentially reduce the possibility of quenching within the samples. As tritium has a low emission of energy, even compared to ^{14}C, and the efficiency of scintillation counters themselves can be low (National Diagnostics, 2004), lower concentrations could result is production of photons below the detectable range and therefore would not register as a count. Therefore, skeletal muscle cells were grown and differentiated as previously described (*Section 3.1*) and glucose uptake measured and quantified as previously outlined (*Section 4.2.2*). Cells were incubated for 30 minutes before being lysed and measure by scintillation counting.
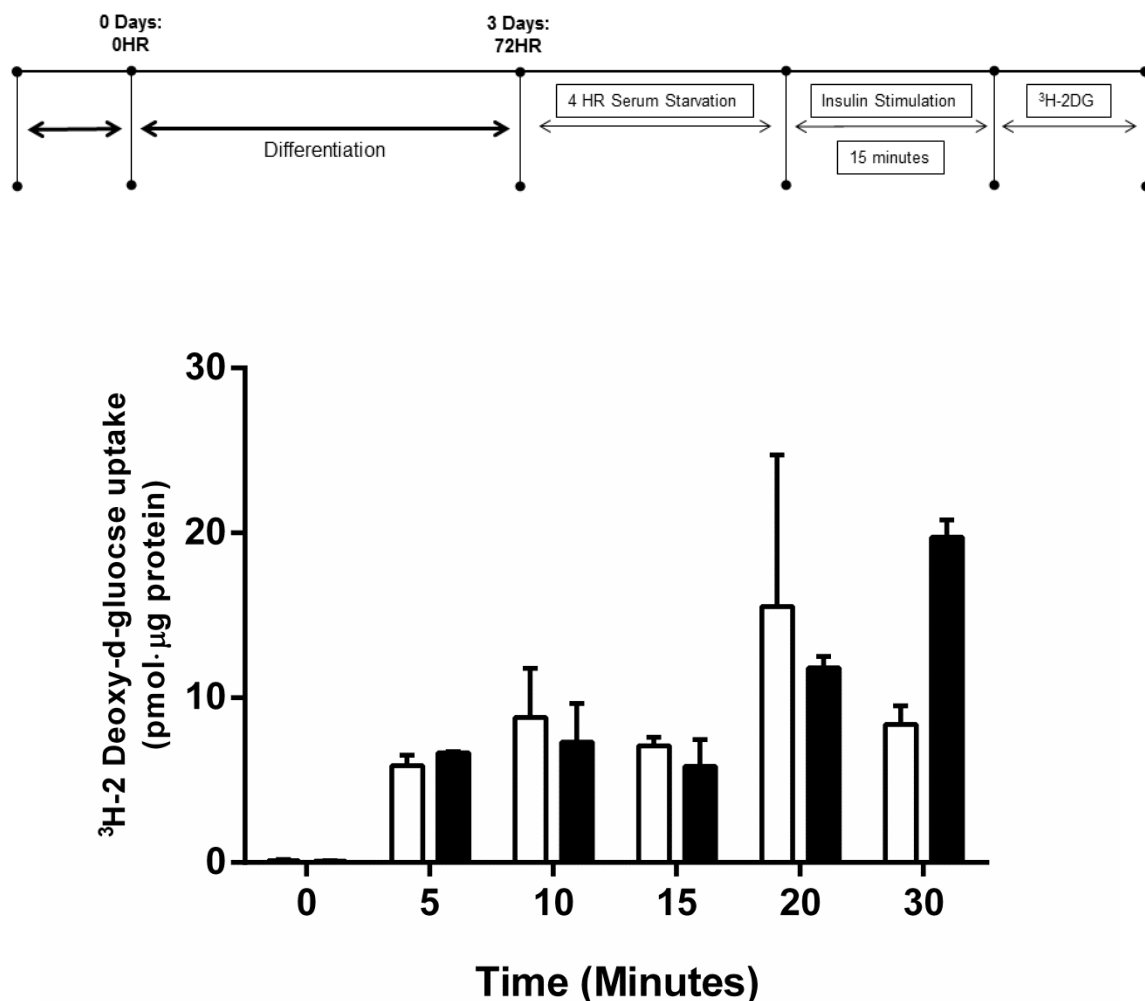
Figure 4-6 Time response of glucose uptake in C2C12 skeletal muscle myotubes incubated with ^{3}H-2-Deoxy-d-glucose (0.5μCi·mL). Samples are in duplicate (mean ± S.D) from one independent set of cells. Open bars represent unstimulated and Filled bars represent insulin stimulated (100 nM) cells.

After repeating the experiment with 0.5 µCi/mL ^{3}H-2DG there was a large variation within each condition which resulted in no significance between the two conditions by independent samples t-test t (6) -0.900 p =0.403 (*Figure 4-7*). The variation between conditions could be resultant of the limits of the scintillation counter and therefore normalisation of the data could potentially be skewed due to the detection limits.). However, increasing the concentration (1 µCi/mL) the variation between conditions was reduced and subsequently showed a significant difference between conditions t (2) -5.835 p = 0.028 (*Figure 4-8*). Therefore, based upon the findings of these optimisation experiments further experimental conditions in monolayer used a 30 minutes glucose incubation period using a radioactive concentration of 1 µCi/mL.
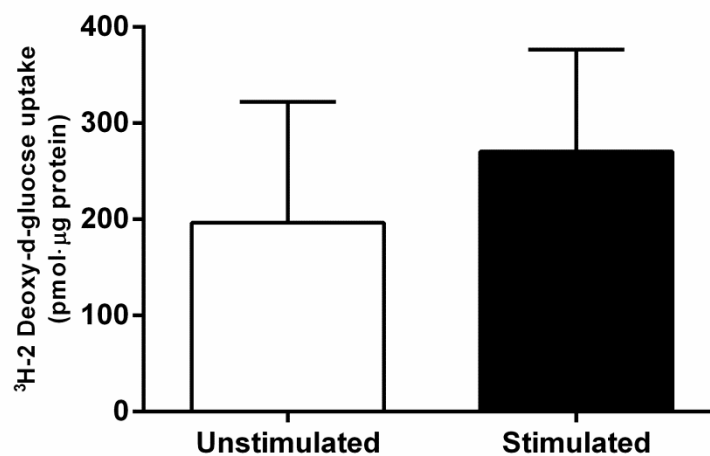
Figure 4-7 Glucose uptake in C2C12 skeletal muscle myotubes incubated for 30 minutes with ^{3}H-2-Deoxy-d-glucose (0.5 µCi·mL). Data is an accumulation of duplicate samples from two independent set of cells mean ± S.D. Open bars represent unstimulated and filled bars represent insulin stimulated (100 nM) cells for 15 minutes before 30 minutes incubation with ^{3}H-2DG.
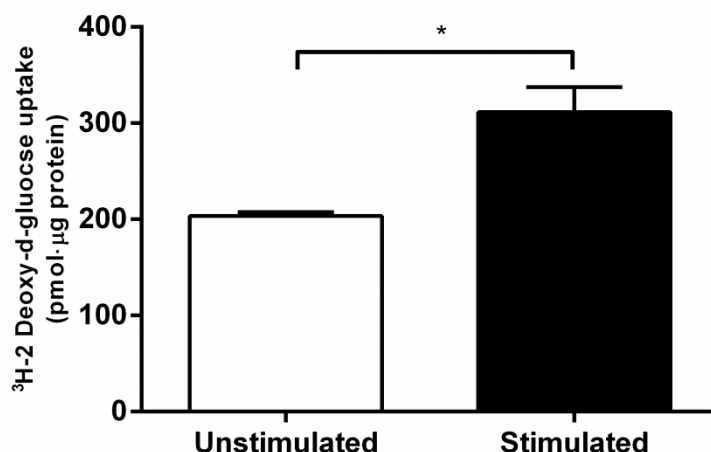
*Figure 4-8 Glucose uptake in C2C12 skeletal muscle myotubes incubated for 30 minutes with ^{3}H-2-Deoxy-d-glucose (1µCi·mL). Data is mean ± S.D from one independent set of cells. Open bars represent unstimulated and filled bars represent insulin stimulated (100 nM) cells for 15 minutes before 30 minutes incubation with ^{3}H-2DG. * Significantly different between conditions (p < 0.05).*

4.2.4. Optimisation of insulin stimulation and ^{3}H-Deoxy-D-Glucose uptake in tissue engineered constructs.

Following the findings in monolayer culture, it was important to establish glucose uptake in tissue engineered constructs. Investigation of glucose uptake in tissue engineering within the literature is limited, having only been established in constructs ded with primary rat cells (Baker et al., 2003). The use of C2C12 skeletal muscle cells have been well established within the literature for their use within tissue engineering (Khodabukus & Baar, 2009, 2014a; Player et al., 2014), Furthermore, the use of skeletal muscle cells have commonly been used within the literature to investigate insulin signalling and glucose uptake in 2D culture (del Aguila et al., 2011; Philp et al., 2010). Therefore based on these two elements of cell culture, these cells provide the ideal candidate for further investigating insulin stimulated glucose uptake in skeletal muscle tissue engineered constructs.

Using the collagen tissue engineered skeletal muscle constructs; C2C12 cells were grown and differentiated for a total of 14 days in culture as described within the methods Section (*Section 3.2.2).* In order to maintain consistency with the methods established in monolayer experiments (*Section 3.6.1*), constructs were stimulated with insulin for a period of 15 minutes before being incubated with ^{3}H-2DG (0.1 mM, 1.0 µCi/mL) for 30 minutes. Constructs were incubated in cytochalasin B (10 µM) for 5 minutes and washed twice with PBS, before being removed from the A-frames, blot dried and weighed. Despite following the

protocols established in monolayer there was no significant increase in insulin stimulated glucose uptake following 15 minutes insulin stimulation (*Figure 4-9*).
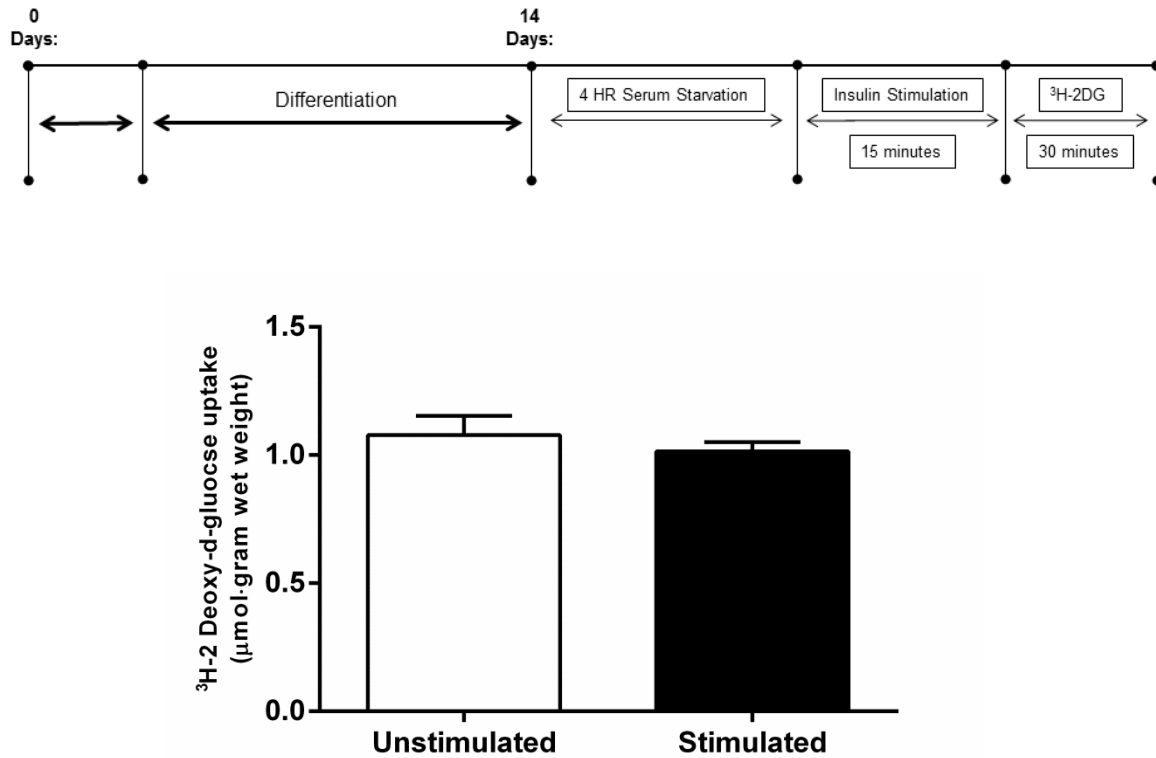
Figure 4-9 Glucose uptake C2C12 skeletal muscle in collagen skeletal muscle constructs. Myotubes incubated for 30 minutes with ^{3}H-2-Deoxy-d-glucose (1 μCi·mL). Data is mean ± S.D from two constructs per condition from one independent set of cells. Open bars represent unstimulated and filled bars represent insulin stimulated (100 nM) cells for 15 minutes before 30 minutes incubation with ^{3}H-2DG.

As a result of these findings it was important to establish if the lack of insulin stimulated glucose uptake was as result of the incubation time in insulin. Therefore, western blots were conducted for phosphorylation of Akt and key protein involved in insulin signalling. Collagen constructs were grown as previously described (*Section 3.2.4*). Prior to insulin stimulation constructs were washed twice and incubated twice for 30 minutes in KRH buffer as described in monolayer cultures (*Section 4.2.2*). Constructs were incubated in KRH

containing 100 nM of insulin at set time periods (*Figure 4-10*). KRH buffer was removed and constructs were washed in PBS, before being blot dried and snap frozen in liquid nitrogen. Protein from collagen constructs was extracted as outlined (*Section 3.5.3 & 3.5.4*) and separated by western blot (*Section 3.5.6*). Imaged bands for phosphorylation of Akt at serine 473 (Ser^{473}) and threonine 308 (Thr^{308}) were darker beyond 20 minutes and therefore insulin stimulation in subsequent experiments in tissue engineered constructs was increased to thirty minutes for investigation of insulin signalling as well as prior to incubation with ^{3}H-2DG (*Figure 4-10*).
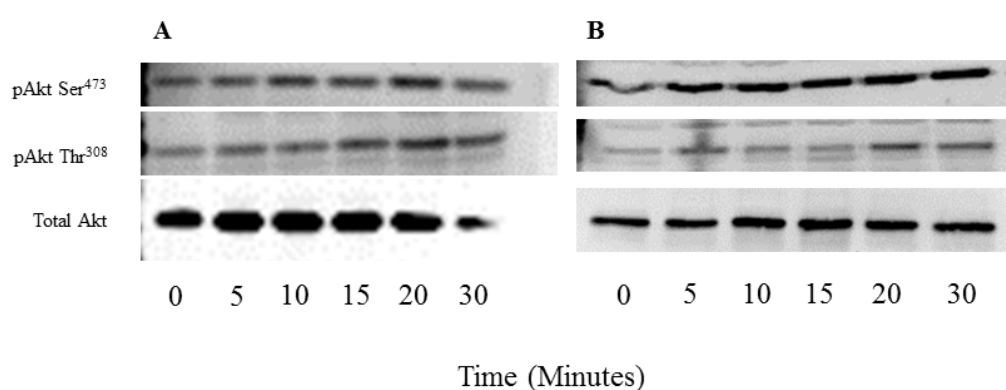
Figure 4-10 Immunoblots of time dependent response of phosphorylation of Akt (Ser^{437}) and Akt (Thr^{308}) (60 kDa) in response to insulin. A; Collagen gel skeletal muscle constructs B; Fibrin gel skeletal muscle constructs

Following the findings in collagen western blots were also conducted on Fibrin based constructs. Similarly to collagen tissue engineered constructs, insulin stimulation appeared to show phosphorylation to be at its greatest beyond 20 minutes insulin stimulation (*Figure 4-10B*) and therefore the same incubation periods were used to investigate glucose uptake and insulin signalling in these constructs. Constructs were stimulated for 30 minutes and incubated with ^{3}H-2DG for 30 minutes as described above. The data from these initial experiments was pooled into the control condition in fibrin experiments, which are outlined in this thesis (*Chapter 7*).

4.3. Discussion

The purpose of these experiments was to establish a method for investigation of cellular glucose uptake in C2C12 skeletal muscle cells. The use of the glucose analogue 2-deoxy-d-glucose is most suitable for investigation of glucose uptake within skeletal muscle due to the rapid flux across the membrane and the stability and impermeable nature of this analogue once phosphorylated to 2-deoxy-glucose-6-phosphate (Hansen et al., 1994).C2C12

The first optimisation experiments investigated the use of a 96 well plate fluorescent based assay which has previously been used to investigate glucose uptake in L6 skeletal muscle cells (Yamamoto et al., 2006). Compared to the work performed by Yamamoto and colleagues, the use of this assay did present evidence of insulin stimulated glucose uptake using the C2C12 skeletal muscle cell line. This particular cell line has been used for investigating skeletal muscle glucose uptake using radioactive isotopes (Nedachi and Kanzaki, 2006) and subsequently, the incubation periods used were based on the established work by Nedachi and colleagues. Although these time periods are effective in providing a suitable assay window between basal and insulin stimulated glucose uptake in a radioisotope based assay, the incubation period used within the fluorescent based assay using L6 myotubes was greater and therefore could potentially explain the differences in glucose uptake observed (Yamamoto et al., 2006).

C2C12 skeletal muscle cells present a high basal glucose turnover, and although insulin stimulated glucose uptake increases ^{3}H-2DG uptake by approximately 1.5 fold over basal levels (Nedachi and Kanzaki, 2006), it has been suggested that the effect of insulin stimulated glucose uptake are small compared to that of other skeletal muscle cell lines (Sarabia et al., 1990). Although insulin stimulated glucose uptake has been reported in L6 myotubes using this method (Yamamoto et al., 2006), further experiments would be required to determine if the fluorescent based methods is sensitive enough for detection of insulin stimulated glucose uptake within this particular cell line. A method for investigating this is with the use of transfected *myc*-tagged GLUT4 skeletal muscle cells lines, which have been used within the literature to investigate GLUT4 translation in skeletal muscle cells (Amira Klip et al., 2014; Nedachi and Kanzaki, 2006).

Due to the null findings observed with the fluorescent based assay, experiments were conducted using radioisotopes which have been well established and described within the literature for investigation of glucose uptake *in vitro* and have arguably termed the '*gold*

standard' in this field of research (Sell et al., 2012; Tanti et al., 2001). The use of 2-DG radioisotopes have been used within the literature to investigate cellular glucose uptake in both primary (Sell et al., 2008), and myogenic cell lines (Iwata et al., 2009; Nedachi and Kanzaki, 2006; Philp et al., 2010). Initial optimisation experiments were performed based on previous research by Nedachi and colleagues, whereby a time course was performed to identify the most suitable incubation time with ^{3}H-2DG. Contrary to previous findings, the greatest assay window to observed insulin stimulated glucose uptake was following 30 minutes incubation with ^{3}H-2DG. Yet, repeating the experiment shown there to be a large deviation within the mean between conditions (*Figure 4-7*). Although the variation between batches of cells could contribute to the variation observed, as outlined above (*Section 4.2.3*) the low efficiency of scintillation counters combined with the low specific activity that was used in these initial experiments would increase the risk of quenching and subsequently the counts per minute obtained from each sample. The radioactive concentrations of ^{3}H-2DG used for experiments using C2C12's has ranged (0.5 µCi·mL-1 µCi·mL) within the literature. The concentration of ^{3}H-2DG used in skeletal muscle constructs is higher than those used in 2D culture (1.5 µCi·mL) (Baker et al., 2003). Establishing a specific activity that would be detectable in both 2D and 3D systems, meant that for subsequent experiments, a specific activity of 1 µCi·mL would be used for the subsequent experimental Chapters (1 µCi·mL which has commonly been used within the literature to investigate glucose uptake within C2C12 skeletal muscle cells (Iwata et al., 2009; Philp et al., 2010). With this concentration, the difference in glucose uptake between insulin stimulated and unstimulated cells was discernible at 30 minutes and therefore these parameters were used in the subsequent chapters.

Following optimisation of insulin stimulated glucose uptake in monolayer cell culture, experiments were conducted in tissue engineered skeletal muscle constructs. Initial experiments conducted using collagen based skeletal muscle constructs followed the same methodology as those previously used in 2D culture. However, there was no increase in insulin stimulated glucose uptake following 15 minutes insulin stimulation and therefore, western blots were conducted for phosphorylation of Akt which is a key insulin signalling protein in skeletal muscle (Schultze et al., 2011). This optimisation experiment showed that phosphorylation occurred at all-time points. Twenty minutes of insulin stimulation is consistent with what has previously been reported by Baker and colleagues who incubated with insulin for 20 minutes in skeletal muscle tissue engineered constructs using primary rat skeletal muscle cells.

Experiments in fibrin skeletal muscle constructs, (*Chapter 7*), were incubated with insulin for 30 minutes prior to be exposed to ^{3}H-2DG. These constructs showed an insulin stimulated glucose uptake of approximately 2-fold over basal level. These findings are similar to the methods previously described by Baker and colleagues, using a comparable protocol. The research into the metabolic characteristics of tissue engineered skeletal muscle is limited to the paper by Baker and colleagues (Baker et al., 2003). The subsequent Chapters within this thesis will attempt to further elucidate these characteristics in two separate skeletal muscle tissue engineered constructs.

4.4. Chapter Summary

The purpose of this Chapter was to establish a method for the measurement of glucose uptake in C2C12 skeletal muscle cells, which could be used within the following Chapters of this thesis. Two methods were investigated using fluorescent 96 well plate and radioisotope assays respectively. Optimisation experiments using the fluorescent 96 well plate assay showed no effect of insulin stimulated glucose uptake and therefore, further experiments would be required to optimise this method with C2C12 skeletal muscle cells.

In contrast, the use of the radioisotope tracer assay with tritium labelled glucose showed through a time response experiment that 30 minutes of glucose exposure resulted in a significant assay window between unstimulated and stimulated C2C12's in 2D culture. Furthermore, it was established that higher a higher specific activity of ^{3}H-2DG was more suitable for investigating insulin stimulated glucose uptake in both monolayer cultures and subsequently tissue engineered skeletal muscle constructs. This increase in specific activity reduces the potential effect of quenching within samples; in addition to the inefficiency associated with scintillation counting. The findings in 2D culture correspond to those currently reported in the literature, and the concentrations used within these optimisation experiments are used in the subsequent Chapters of this thesis. In addition, these methods have shown that glucose uptake can be measured within skeletal muscle tissue engineered constructs using the myogenic C2C12 cell line and the response elicited within these constructs is similar to what has previously been shown within primary cell lines (Baker et al., 2003). Further experiments within this thesis will establish the effects of both acute and chronic insulin exposure in both monolayer and tissue engineered constructs upon insulin stimulated glucose uptake using radioactive isotopes.

5. A time course of transcriptional and translational expression of glucose transporters in C2C12 skeletal muscle cultures.

5.1. Introduction

A family of integral membrane proteins known as glucose transporters controls the regulation of glucose uptake. The importance of these proteins in glucose uptake has previously been discussed (*Section 2.11*). During foetal development, GLUT4 concentrations have been found to be low, while Glucose Transporter 1 (GLUT1) is the predominant mediator of glucose transport, however, during the perinatal phase of development continuous expression of GLUT4 mRNA, which is proceeded by increases in posttranscriptional protein expression, results in GLUT4 being the predominant isoform for glucose transport (Santalucia et al., 1992). Both protein and mRNA expression of these glucose transporters are present within mature skeletal muscle (Stuart et al., 2006), and are highly correlated with glucose transport (Kern et al., 1990).

GLUT4 mRNA expression is regulated by upstream promoter regions (Liu et al., 1992). One of these, Myocyte Enhancer Factor -2 (MEF-2), is an important developmental transcription factor. MEF-2 is implicated in the transcription of GLUT4 with a MEF-2 promoter region located upstream of the GLUT4 transcription site (Thai, Guruswamy, Cao, Pessin, and Olson, 1998). Four isoforms of MEF-2 have been identified. Of those four, MEF-2A has been shown to regulate GLUT4 expression in both rat (Mora & Pessin, 2000), and human skeletal muscle (Knight, Eyster, Griesel, & Olson, 2003). The importance of MEF2A has been shown in skeletal muscle cells lines (Liu, Olson, Edgington, Moyerowley, & Pessin, 1994), as well as transgenic rodent models, which have shown reduced MEF2A activity in skeletal muscle of diabetic animals to be correlated with reduced GLUT4 expression and insulin stimulated glucose uptake (Thai et al., 1998). What is more, the GLUT4 muscle specific enhancer has been found to be fibre type dependent and requires the integrity of the MEF-2 binding site in both oxidative and glycolytic muscle fibres (Moreno et al., 2003).

In the basal state, a high Concentrations of GLUT4 is located within the cytoplasmic region. The translocation of the GLUT4 vesicles to the cytoplasmic region to the plasma membrane as a result of insulin stimulation has been found to increase cell surface density within skeletal muscle from healthy individuals (Ryder et al., 2000). Furthermore, as previously discussed (*Section 2.11.1*), insulin or pharmacological mediated stimulation significantly increases transcriptional expression of GLUT4 both *in vitro* (Al-Khalili et al., 2005; Laville et al., 1996) and *in vivo* (Ryder et al., 2000). Although no differences in the basal expression of mRNA GLUT4 in skeletal muscle between NIDDM and control subjects has been reported, diabetic animal models have shown to have reduced binding between the MEF2A-MEF2D

heterodimer, which is responsible for regulation in the expression of the GLUT4 gene (Mora and Pessin, 2000).

While the developmental characteristics of glucose transporters within skeletal muscle have been elucidated in two dimensional cultures (Mitsumoto, Burdett, Grant, and Klip, 1991), and *in vivo* skeletal muscle (Jensen et al., 2008), less is understood about expression of glucose transporters, in particular their expression during myogenic development within skeletal muscle tissue engineered constructs. The work of Baker and colleagues has shown the expression of GLUT1 to be increased compared to GLUT4 in tissue engineered constructs using primary cell from rodents (Baker et al., 2003). Although these initial experiments have provided an insight into the expression of these proteins in tissue engineered skeletal muscle constructs (Baker et al., 2003), further work to characterise understanding of the expression of glucose transporters in these models is an area that remains a challenge within the field of tissue engineering and regenerative medicine (Cheng et al., 2014).

Due to the significance of GLUT4 expression in cellular glucose transport, it is important to understand the changes in mRNA expression of GLUT4 and GLUT1 during skeletal muscle differentiation in culture. In addition, myogenic regulatory factors such as myogenin, an important marker of skeletal muscle differentiation, and developmental genes such as MEF-2a, which are implicated in regulation of GLUT4 transcription, can provide further understanding of the development of skeletal muscle from single cells, to mature skeletal muscle fibres. Little is understood about the time course development of glucose transporter expression in tissue engineering particularly within a homogenous cell population from a murine skeletal muscle cell line. It is therefore important to understand the transcriptional and translation expression of glucose transporters within tissue engineered constructs in order to establish these models as a valid model for investigating insulin stimulated glucose uptake. Time course experiments were conducted to investigate the changes in expression of myogenic, development and glucose transporters during skeletal muscle myotube formation in monolayer culture and tissue engineered skeletal muscle constructs. It was hypothesised that with the formation of skeletal muscle myotube formation and increases in myogenic and developmental gene expression, there would be also an increase in expression of GLUT4, the insulin mediated glucose transporter expression

The objectives of this Chapter were to:

I. *Investigate the transcriptional response of glucose transporter mRNA expression in monolayer and tissue engineered constructs in order to establish if these culture models are suitable for investigating insulin stimulated glucose uptake.*

II. *To investigate glucose transporter protein expression of GLUT4 in cell culture.*

5.2. Methods

5.2.1. Experimental protocol in monolayer

C2C12 myoblasts were seeded in six well plates in standard growth media as previous described (*Section 3.1*). Upon confluence, cells were changed to differentiation media as previously described. Cells were lysed for RNA extraction (*Section 3.3.2*) at 0 days (D0), 3 days (D3) and day 6 (D6) time points (*Figure 5-1*)
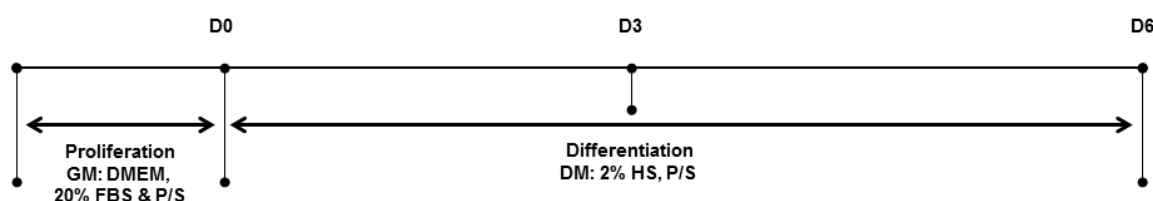
Figure 5-1 Experimental protocol schematic of the time course changes in GLUT4 expression in C2C12 myoblast cells.

5.2.2. Experimental protocol in collagen constructs

Collagen gels were constructed as described in the methods Section of this thesis (*Section 3.2.2*), and grown in standard growth media until for 4 days, before being changed to differentiation media (2% HS, 1%P/S, DMEM) for the remaining time in culture. Growth media was changed twice daily, while differentiation media was changed daily during the experiment. At each respective time point constructs were washed in PBS before being lysed in Tri-reagent for subsequent RNA extraction. Constructs were lysed at early (day 0 & 2) and late differentiation time points (day 8 & 10). Macroscopic images were obtained to measure gel width throughout differentiation and the formation of myotubes was observed by phase contract microscopy

5.2.3. Experimental protocol in Fibrin constructs

In addition, a similar time-course experiment was conducted in fibrin based tissue engineered constructs. The fibrin plates were developed and cultured as previously described (*Section 3.2.1.3*). Constructs were lysed at the change in constituents of the media to induce differentiation (day 2 to differentiation media) and when changed to maintenance media at day 4 (D4). Subsequent constructs were lysed every other day up until the constructs had been in culture for a total of 14 days. Constructs were lysed in tri-reagent for extraction of RNA for mRNA expression analysis (*Section 3.3.3*).
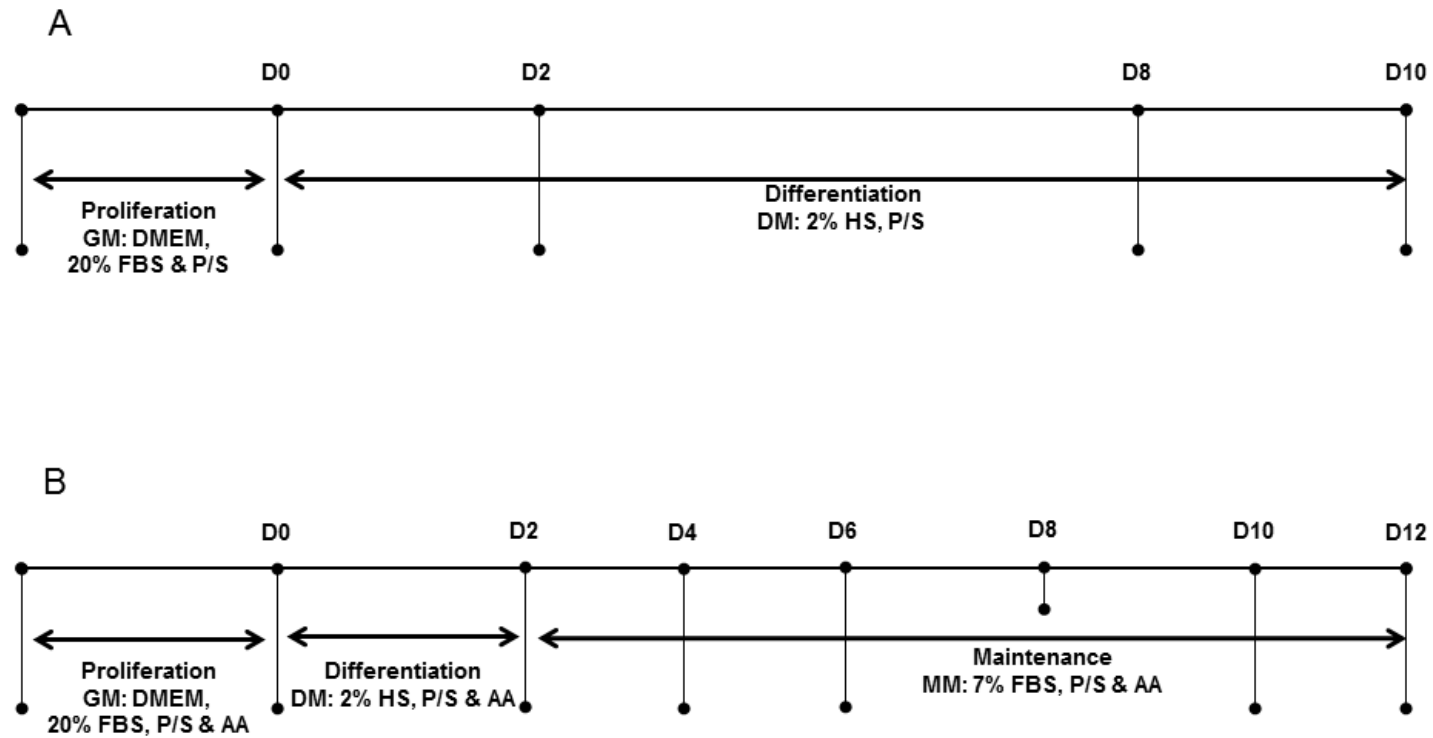
Figure 5-2 Time course experimental schematic of C2C12 skeletal muscle cells grown and differentiated in A, 1mL Collagen gels and B, Fibrin gels. Skeletal muscle constructs were grown in respective media and lysed at the time points on each timeline. Days (D).

5.2.4. RNA extraction and PCR

RNA was extracted using TRI-reagent according to manufactures instructions and quantified as previously described (*Section 3.3.2*). PCR was performed as previously described with respective primer sets for RPIIβ, GLUT4, GLUT1, MyoG, and MEF2α (*Table 3-3*). Changes in gene expression were calculated using $\Delta\Delta C_t$ method as previously outlined (*Section 3.3.6*).

5.2.5. Protein Extraction and Immunoblotting of GLUT4

Protein lysates were extracted from monolayer cultures with RIPA lysis buffer as previously described (*Section 3.5.2*) and quantified using piece 660nm protein assay (*Section 3.5.5*). Samples were mixed with laemmli buffer and loaded (10 μg) onto SDS-PAGE gels (8-12% resolving gels, 4% stacking gel). Western blots were performed as outlined in the methods section (*Section 3.5.6*), with some modifications. These modifications used due to the negative results obtained following the protocol outline in the methods section. The considerations and optimisation for investigating glucose transporter protein expression in cell culture models is outline in appendices C (*Page 217*). Membranes were blocked for one hour in 2% blotting milk (Bio-rad, Herts, UK) TBS, before being incubated overnight (4°C) with GLUT4 primary antibody (5% blotting milk, TBST). Membranes were washed and incubated with secondary antibody and exposed as described previously (*Section 3.5.6*).

5.2.6. Statistical Analysis

Statistical analysis was conducted using SPSS software (IBM SPSS statistics software, version 19.0.0, USA). Shapiro-Wilk was used to assess normality and Levine tests for homogeneity of variation. Log transformation was used if either normality or homogeneity assumptions were violated. Transformed data were analysed by One-way ANOVA to assess changes in mRNA expression with Bonferroni post-hoc differences used to identify differences between conditions when a significant F value was observed. An alpha level of $p < 0.05$ was used as the criterion for statistical significance. An independent samples t-test was conducted for differences in the expression of GLUT4 relative to GLUT1 mRNA expression at each time point. Values are mean ± standard deviation number; of replicates and independent experiments conducted are outlined in each figure legend.

5.3. Results

5.3.1. Glucose transporter expression in monolayer culture

During the course of myotubes differentiation, Myogenin, a marker of myogenic differentiation, was measured at zero, three and six day's differentiation. The mRNA levels of Myogenin were significantly different $F_{(2, 13)} = 726.03$ $p < 0.0001$ with both 72HR and 144HR expression levels significantly greater than at 0HR in differentiation media ($p < 0.0001$). There was also a significant difference between 3 and 6 days in differentiation media ($p < 0.005$) (*Figure 5-4A*). There was a significant effect on MEF2a mRNA expression $F_{(2, 22)} = 6.483$, $p = 0.06$ following 3 and 6 days in differentiation media (3 days $p = 0.016$; 6 days $p = 0.003$ vs. control). GLUT4 increased approximately 2-fold over the course of differentiation (*Figure 5-4B*), $F_{(2, 20)} = 4.592$, $p = 0.023$ with both differentiated skeletal muscle expressing greater levels of GLUT4 mRNA at 72 ($p = 0.011$) and 144 hours ($p = 0.037$) respectively compared to undifferentiated cells (*Figure 5-4C*). Despite this increase in GLUT4 during skeletal muscle differentiation there was no significant changes in GLUT1 mRNA levels $F_{(2, 22)} = 0.232$, $p = 0.795$ (*Figure 5-4D*).
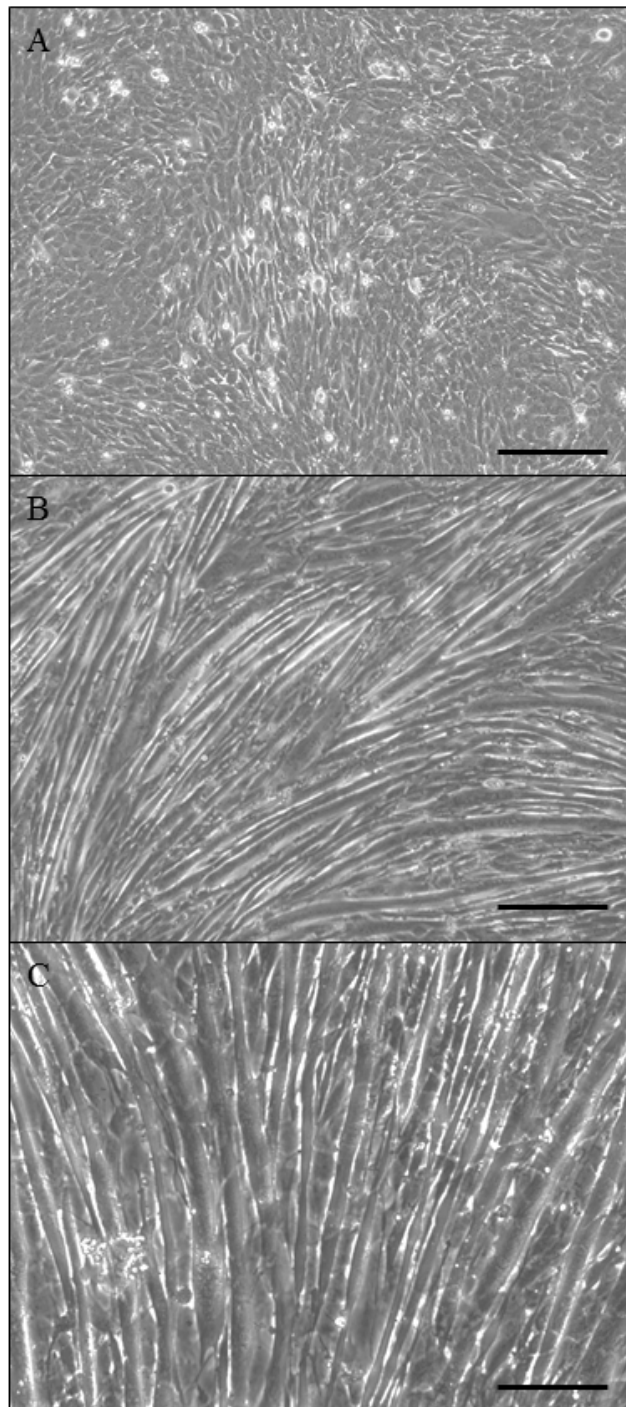
Figure 5-3 Representative phase contrast images of C2C12 skeletal muscle cells at A) 0 days DM B) 3 days DM and, C) 6 days DM in monolayer culture. Scale bar = 100μm of x20 magnification image.

Figure 5-4 Mean ± SD (N=3) of mRNA expression (ΔΔC$_t$) of glucose transporters, myogenic and, developmental genes, A; Myogenin (MyoG) and B; Myocyte Enhancer Factor 2a (MEF2a), C; Glucose transporter 4 (GLUT4), D; Glucose transporter 1(GLUT1) and in single cell (Day 0) and mature (Day 3-DM; Day 6-DM) C2C12 skeletal muscle myotubes in monolayer cell culture. * Significant difference vs. GM (p<0.05).

Table 5.1 Raw Ct Values (mean ± SD) genes of interest during skeletal muscle development of C2C12 skeletal muscle in monolayer culture.

Gene of Interest	D0	D3	D6
MyoG	23.69 ± 0.69	19.54 ± 0.72	20.32 ± 1.17
MEF2a	20.00 ± 0.71	21.13 ± 2.75	19.20 ± 0.74
GLUT4	25.81 ± 1.06	23.52 ± 2.42	25.04 ± 1.09
GLUT1	20.75 ± 1.10	22.47 ± 2.70	20.69 ± 0.56

5.3.2. GLUT4 protein expression in undifferentiated and differentiated C2C12 skeletal muscle

In order to investigate if the transcriptional changes in GLUT4 mRNA expression translated to an increase in protein concentration, western blots were conducted on samples at 0, 3 and 6 days of differentiation. (*Figure 5-5*). The data from this pilot experiment shows that GLUT4 protein abundance in increased at days 3 (9.1 fold) and 6 (20.3 fold) differentiation relative to day 0.
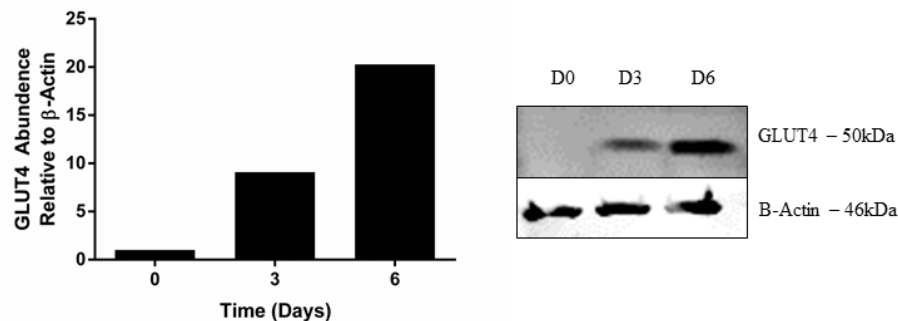
Figure 5-5 GLUT4 protein expression in C2C12 skeletal muscle myotubes differentiated at day 0, 3 and 6 days. Data is mean abundance from 1 independent experiment.

5.3.3. Collagen gel contraction and myotube formation during differentiation

In order to characterise the expression of glucose transporters in skeletal muscle tissue engineered constructs, time course experiments were conducted to investigate the transcriptional responses of these constructs. During differentiation, the formation of myotubes was observed by phase contrast microscopy (*Figure 5-6*). Furthermore, macroscopic images were obtained for the measurement of gel contraction during differentiation (*Figure 5-7A*). There was a reduction in gel width during differentiation which was significantly different between days 0 and 10 as identified by independent t-test t (26) = 2.181 p =0.38 (*Figure 5-7C*).
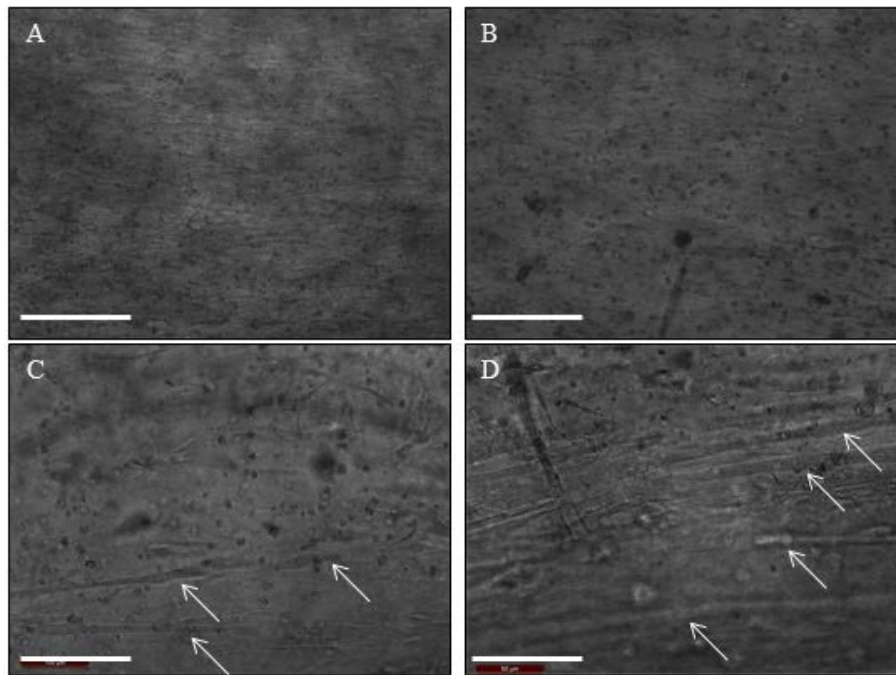
Figure 5-6 Phase contrast images of collagen based tissue engineered constructs culture for A) 0 days, B) 2 days, C) 8 days and D) 10 days in differentiation media Scale bar = 100µM of X20 magnification image. Arrows denote the formation of skeletal muscle myotubes.
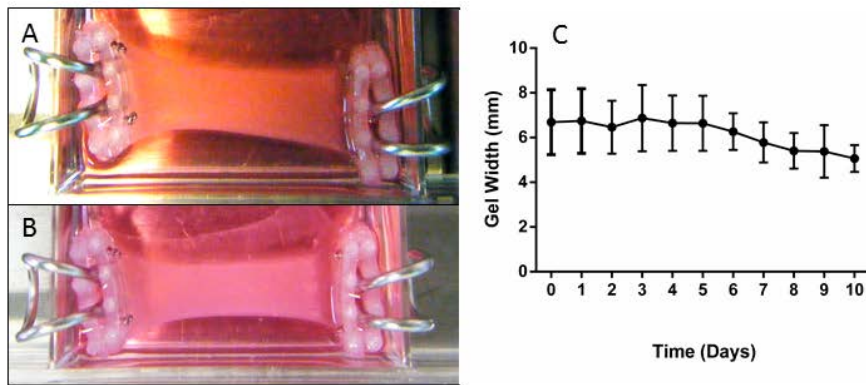
Figure 5-7 Contraction of collagen skeletal muscle gels during differentiation. Constructs were incubated in standard growth media before being switched to differentiation media at A; Day 0 for B; 10 days in culture C; Measurement of gel width (mm) of collagen gels. Data is mean ± SD.

5.3.4. Transcriptional responses of glucose transporters in collagen tissue engineered constructs

There was no significant differences in GLUT1 expression across time points, however it did approach significance F (3, 5.589) = 4.206 p = 0.069 (*See Figure 5-8A*). Interestingly, GLUT4 expression was shown to approach significance (F (3, 4.407) = 6.488 p = 0.055), with day 2 significantly greater than day 0 (p = 0.05), with expression returning to baseline at the later days in culture (*Figure 5-8B*). MEF2a expression significantly increased with time in culture F (3, 14) = 10.014 p = 0.002 increasing at day 2 (p = 0.01) and day 8 (p = 0.002) compared to day 0. Expression began to decline at day 10, approaching significance compared to day 0 (p = 0.055) (*Figure 5-8C*). Myogenin expression followed a similar trend F (3, 14) = 7.924 p = 0.004 with all days significantly greater than day 0 (p<0.05) (*Figure 5-8D*).

*Figure 5-8 Time-course transcriptional response of glucose transporter (GLUT1 & GLUT4), developmental (MEF2a) and myogenic (Myogenin) genes in collagen based tissue engineered constructs. A; GLUT 1, B; GLUT4, C; Myocyte Enhancer Factor 2a (MEF2a), D; Myogenin (MyoG) * Significant difference vs. Day 0 (p<0.05). Mean ± SD (N=4 constructs per time point).*

Table 5.2 Raw Ct Values (mean ± SD) genes of interest during skeletal muscle development of collagen C2C12 skeletal muscle tissue engineered constructs.

Gene of Interest	D0	D2	D8	D10
MyoG	26.19 ± 0.36	22.79 ± 0.43	23.07 ± 1.00	22.97 ± 0.92
MEF2a	21.67 ± 0.51	20.50 ± 0.27	20.63 ± 0.82	20.89 ± 0.85
GLUT4	29.28 ± 0.63	25.50 ± 1.34	27.94 ± 1.07	27.96 ± 0.61
GLUT1	20.24 ± 0.58	20.56 ± 0.40	21.25 ± 0.47	20.68 ± 0.54

5.3.5. Fibrin tissue engineered gel contraction during differentiation

Macroscopic images were obtained daily for measurement of gel width during differentiation and maintenance within fibrin skeletal muscle constructs. Gel width significantly reduced with time in culture F (6) = 32.437, p < 0.0001 with all times points significantly lower than day 0 in differentiation (*Figure 5-9*).
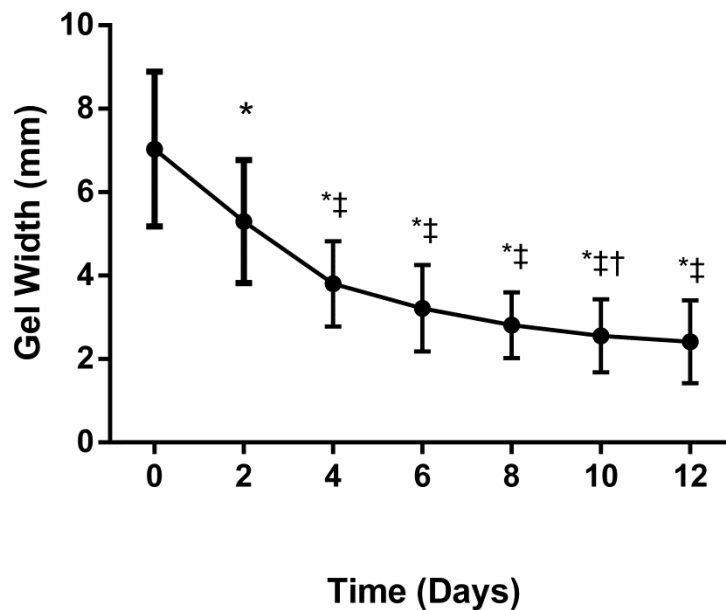
*Figure 5-9 Gel width (mm) measurements of fibrin based tissue engineered constructs during differentiation period. *Significantly different vs D0 (p<0.05), ‡ significantly different vs D2 (p<0.05), † significantly different vs D4 (p<0.05). Data is presented as mean ± SD (N=4 constructs per time point).*

5.3.6. Transcriptional responses of glucose transporters in fibrin based tissue engineered constructs

Despite an increase in myogenin expression at day 2 of differentiation and its maintenance for the duration in culture thereafter (*Figure 5-10B*), there was no significant difference between time-points F (6, 8.608) = 2.473 p = 0.111. Similarly, as expected there was no significant differences in GLUT1 expression throughout the culture period (*Figure 5-10C*). In contrast, MEF2a expression significantly increased at day two and remained significantly elevated compared to day 0 for the duration of time in differentiation F (6, 9.121) = 25.050 p < 0.001. GLUT4 expression steadily increased over time in differentiation F (6, 7.235) = 3.905 p = 0.047 with day 12 significantly different too day 0 (*Figure 5-11*).

*Figure 5-10 Time-course of transcriptional responses of glucose transporter (GLUT1), developmental (MEF2a) and myogenic (Myogenin) genes in fibrin based tissue engineered constructs. A; MEF2a, B; Myogenin, C; GLUT1. * Significant difference vs. Day 0 (p<0.05). Mean ± SD (N=4 constructs per time point).*

Figure 5-11 Time-course of transcriptional responses of glucose transporter GLUT4, gene in fibrin skeletal muscle tissue engineered constructs. Mean ± SD (N= 4 Constructs per time point) * significantly difference compared to day 0 (p<0.05).

Table 5.3 Raw Ct Values (mean ± SD) genes of interest during skeletal muscle development of fibrin C2C12 skeletal muscle tissue engineered constructs.

Gene of Interest	D0	D2	D4	D6	D8	D10	D12
MyoG	26.23 ± 1.33	23.71 ± 1.53	23.37 ± 1.60	23.33 ± 1.17	23.40 ± 0.93	23.04 ± 1.32	22.87 ± 0.98
MEF2a	21.10 ± 0.19	20.40 ± 0.68	20.18 ± 0.85	20.34 ± 0.59	20.42 ± 0.35	20.08 ± 0.53	20.28 ± 0.31
GLUT4	26.69 ± 1.85	26.59 ± 0.45	26.17 ± 1.04	26.05 ± 1.11	25.41 ± 0.89	25.54 ± 0.73	25.10 ± 0.77
GLUT1	21.21 ± 0.31	21.89 ± 0.80	21.90 ± 0.40	21.90 ± 0.50	21.75 ± 0.15	21.46 ± 0.27	21.15 ± 0.37

5.4. Discussion

Insulin stimulated glucose uptake in skeletal muscle is mediated by specific glucose transporters, mainly GLUT4. In addition, skeletal muscle both *in vivo* and *in vitro* has been shown to express GLUT1 and GLUT4 at both the transcriptional and translation levels (Liu et al., 1994; Mitsumoto et al., 1991; Santalucía et al., 1992). Since little research has been conducted investigating glucose transporters in tissue engineered skeletal muscle constructs, the experiments aimed to determine both the gene and protein expression of glucose transporters in the two tissue engineered skeletal muscle models. Because GLUT1 and GLUT4 is the predominant transporter of cellular glucose uptake in skeletal muscle (Gaster, Handberg, Beck-Nielsen, & Schroder, 2000), time-course analysis experiments were conducted in order to investigate the transcriptional changes of GLUT1 and GLUT4 during differentiation.

5.4.1. Glucose transporter expression in monolayer cell culture

During the process of differentiation, cells exit the cell cycle and fuse together to form multi-nucleated myotubes (Buckingham et al., 2006). The formation of these myotubes relies on an increases in gene expression of a number of myo-regulatory factors (Buckingham et al., 2003). Myogenin and myocyte enhancer factor 2a (MEF2a) are implicated in myogenesis and the regulation of GLUT4 mRNA expression. This is due to their location on the GLUT4 promoter region (Mora and Pessin, 2000). Exposure of cells to differentiation media, which resulted in the formation of multi-nuclear myotubes, unsurprisingly significantly increased Myogenin and MEF2a mRNA expression respectively (*Figure 5-3*). Despite the importance of MEF2a in GLUT4 expression, work by Jensen and colleagues using denervated skeletal muscle showed that MEF2a was not implicated in the down regulation of GLUT4 expression (Jensen et al., 2009), but rather the reduction in GLUT enhancer factor expression which further upstream of MEF2a on the GLUT4 transcription initiation site. These findings could potentially explain the increases in MEF2a that were observed with differentiation of skeletal muscle despite the small changes in GLUT4 expression which could be regulated by other promoter sites and signalling pathways (Jensen et al., 2009).

Although low expression levels of GLUT4 mRNA and protein have previously been reported in C2C12 skeletal muscle cells (Kotliar and Pilch, 1992), subsequent research has investigated glucose transporter expression within this cell line (del Aguila et al., 2011; Ma et al., 2013; Nedachi and Kanzaki, 2006). The use of both wild-type and myc-tagged GLUT4

transfected C2C12's (Nedachi and Kanzaki, 2006), has provided evidence that C21C12 skeletal muscle cells express, albeit low levels of GLUT4 mRNA and protein (Philp et al., 2010). The current findings showed that in response to differentiation these C2C12 skeletal muscle cells increase their mRNA expression of GLUT4. In addition, western blots of GLUT4 showed there to be an increase in GLUT4 abundance during skeletal muscle differentiation (*Figure 5-5*). This data initial data shows the potential for GLUT4 to be expressed in skeletal muscle myotubes. Following this, the expression of GLUT4 was investigated as a positive control in skeletal muscle lysate from a human biopsy; however identification of a clear band at 50kDa was not attainable and therefore unquantifiable despite clear visualisation of β-Actin, which was used as a normalisation protein (*Section 10.2*). Therefore further optimisation of for investigating the abundance of GLUT4 protein in skeletal muscle cultures and native tissue requires further optimisation and consideration (*Section 10.3*).

5.4.2. Glucose transporter expression in tissue engineered skeletal muscle

GLUT4 expression *in vitro* has been speculated to be of low abundance compared to the more embryonic GLUT1 transporter (Baker, Dennis, & Larkin, 2003). Within myoblasts, the expression of GLUT4 is relatively low compared to expression of GLUT1 (Santalucia et al., 1992). However, upon differentiation of myoblasts into multinucleated myotubes from both primary satellite cells as well as established cell lines, the expression of GLUT4 mRNA and proteins is increased (Liu et al., 1994). In both fibrin and collagen skeletal muscle constructs differentiation resulted in a significant increase in GLUT4 mRNA expression. However, further research is required in order to identify changes in the ratio of GLUT4: GLUT1 occurs during skeletal muscle development *in vitro*, particularly as denervation of skeletal muscle contributes to an elevation in GLUT1 expression. Denervation of *in vivo* skeletal muscle has shown to result in a reduction in GLUT4 expression within both oxidative and glycolytic skeletal muscle (Block, Menick, Robinson, & Buse, 1991; Handberg et al., 1996; Jones, Tapscott, Olson, Pessin, & Dohm, 1998). This results in greater basal glucose uptake and subsequently reduced insulin stimulated glucose uptake (Handberg et al., 1996). Although there was no observed increase in GLUT1 mRNA in these culture conditions, the fact that both the current tissue engineered skeletal muscle models and those developed previously (Baker et al., 2003), are a-neural, and could provide an alternative hypothesis for the expression profile observed in post-differentiated skeletal muscle cultures.

The high expression of GLUT1 in skeletal muscle is indicative of foetal development where it is expressed to a higher extent than GLUT4 (Gaster et al., 2000). Tissue engineered skeletal muscle models; in particular the fibrin based tissue engineered constructs, have been used to investigate electrical stimulation (Khodabukus et al., 2007), and more recently to investigate proteins involved in metabolism (Khodabukus and Baar, 2014a). Khodabukus and Baar have shown that growth of C2C12 fibrin tissue engineering constructs cultured in high glucose based medium express significantly higher GLUT4 protein levels compared to low glucose media. Similarly, the current findings concur with the continuous increase in GLUT4 mRNA expression during skeletal muscle development observed in the current experiments.

In addition, the use of collagen based tissue engineered constructs has been used more in detail to investigate skeletal muscle development (Cheema et al., 2003). The peak in GLUT4 mRNA expression in collagen gels during early skeletal muscle differentiation (day 2) is a similar expression profile to what has been observed previously for both IGF-1e and MGF mRNA (Cheema et al., 2003). The pattern of mRNA expression of both IGF-1e and MGF have been linked to force generation (Cheema et al., 2003), however the exact relevance in relation to the expression of GLUT4 remains to be elucidated. Additionally, although these constructs over time differentiate to form aligned skeletal muscle myotubes (*Figure 5-6*); further experiments are required to investigate the impact of the GLUT4 mRNA expression profile at the translational level, and ultimately its effect upon glucose uptake.

The skeletal muscle tissue engineered constructs developed within the literature have repeatedly been used to investigate their force production characteristics (Dennis & Dow, 2007; Dennis et al., 2001; Huang et al., 2005). However, in spite of the production of force in these constructs, and the advancements in the characterisation of *in vitro* models of skeletal muscle innervation (Larkin, Van der Meulen, Dennis, and Kennedy, 2006; Morimoto, Kato-Negishi, Onoe, and Takeuchi, 2013), they have remained anural. The implications of denervation upon GLUT4 expression has been identified (Handberg et al., 1996; Jensen et al., 2009; Jones et al., 1998). Several authors have reported a rapid reduction in GLUT4 mRNA and protein expression within skeletal muscle within less than a week of denervation within skeletal muscle from rodent models (Zhou, Vallega, Kandror, and Pilch, 2000). This reduction has been shown to result in an increase in GLUT1 expression both at the plasma membrane, but also within the cytosolic regions. Zhou and colleagues reported an increase GLUT1 protein concentration within intercellular fraction of skeletal muscle from rodents, which are separate from the GLUT4 storage vesicles (Zhou et al., 2000). This compensatory

mechanism leads to an increase in basal glucose uptake and GLUT1 mediated insulin stimulated glucose uptake within denervated skeletal muscle.

5.5. Conclusion and Summary

The purpose of this Chapter was to investigate the transcriptional and translation response of glucose transporters in cell culture and two tissue engineered skeletal muscle models using fibrin and collagen matrices. Time-course experiments using two-dimensional cultures and three-dimensional skeletal muscle tissue engineered constructs show that these systems express both GLUT1 and GLUT4 mRNA, the two main glucose transporters expressed in skeletal muscle. Furthermore, as skeletal muscle cells both in monolayer and skeletal muscle tissue engineered constructs differentiate to for multinucleated myotubes, all three models increase their expression of GLUT4, the major insulin stimulated glucose transporter expressed in mature skeletal muscle. As a result of these experiments, which have shown that the expression of the key insulin stimulated glucose transporter, GLUT4 increases its transcriptional expression during differentiation of tissue engineered constructs.

6. The effects of acute and chronic insulin exposure upon C2C12 skeletal muscle cells in monolayer cell culture

6.1. Introduction

Insulin stimulated glucose uptake in skeletal muscle accounts for the majority of postprandial glucose disposal (DeFronzo et al., 1983). Insulin signalling involves the phosphorylation of a number of key signalling proteins which initiate movement of glucose transporters to the cell membrane (Gaster et al., 2000). Although in principle the initiation of glucose uptake through insulin stimulation sounds simple, a number of proteins play critical roles in the successful activation of this signalling pathway, and impairments in activity of these proteins contributes to attenuated insulin signalling and subsequently, defective glucose homeostasis (Taniguchi et al., 2006).

The culture of skeletal muscle cells *in vitro* provides a system to understand cellular mechanisms independent of a systemic environment using cells from primary, or cell line sources. Primary cell culture from human muscle has presented an intrinsic defect in insulin signalling when grown *in vitro* (Gaster et al., 2002; Henry et al., 1995; Thompson et al., 1996). However, the use of primary human skeletal muscle cells in culture can have its limitations due to their reduced myogenic potential *in vitro* (Sell et al., 2008; Thompson et al., 1996). Therefore, stable cell lines such as C2C12 and L6 rat myoblast cells have been used as alternative candidates for investigating the mechanisms of skeletal muscle insulin signalling and glucose uptake (del Aguila et al., 2011; Kumar and Dey, 2003; Philp et al., 2010).

Although a number of proteins are involved in effective insulin signalling (*Figure 2-5*), a select group of proteins have been termed as '*critical nodes*' of insulin signalling (Taniguchi et al., 2006). Insulin receptor substrate one (IRS-1) is a tyrosine kinase docking protein, which is phosphorylated by the insulin receptor upon insulin stimulation (White, 1998). Several tyrosine residues have been identified which enable recruitment of downstream proteins such as p85 subunit of phosphatidylinositol 3-kinase (PI-3 kinase) (Shepherd, 2005). However, serine/threonine phosphorylation of IRS-1 has been found to impair IRS-1 signalling in adipocytes and skeletal muscle (Bouzakri et al., 2003). In addition, Akt, a 14-3-3 serine/threonine kinase is a downstream protein of p85 PI-3 kinase and is implicated in insulin stimulated glucose uptake (Brozinick & Birnbaum, 1998). Knockout models have implicated the importance of Akt in glucose metabolism which is severely impaired in Akt knockout rodents (Cleasby et al., 2007). Both Threonine 308 (Thr[308]) and Serine 473 (Ser[473]) are key phosphorylation sites (Cozzone et al., 2008). Phosphorylation of Ser[473] is required for full kinase activity and has been found to be impaired in skeletal muscle from both obese and diabetic subjects (Cozzone et al., 2008; Krook et al., 2000, 1998).

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In addition to the effects of insulin upon cellular signalling pathways, insulin is a major regulator of gene expression (O'Brien and Granner, 1996). The expression of metabolic genes such as hexokinase II, which catalyses glucose to glucose-6-phosphate (Printz et al., 1993), and peroxisome proliferator-activated receptor coactivator – 1α (PGC-1α), an important mediator of fat oxidation and mitochondrial biogenesis (Mootha et al., 2003), are activated upon acute insulin stimulation. Similarly to the translational response, the transcriptional mRNA expression of these genes as well as others involved in membrane trafficking, and lipid metabolism, have been found to differ between healthy and insulin resistant skeletal muscle (Bach et al., 2002; Ducluzeau et al., 2001).

Although the positive effects of insulin are well documented, chronic exposure to elevated insulin concentrations, which is often an associated symptom with hyperglycaemia, can have negative effects upon phosphorylation and activation of the insulin signalling cascade (Ueno et al., 2005). Previous research using C2C12 cells has found that hyperinsulinemia or exposure to inflammatory cytokines results in a reduction in insulin receptor signalling and glucose transport, leading to an insulin insensitive model of skeletal muscle (del Aguila et al., 2011; Kumar and Dey, 2003).

The exposure of differentiating skeletal muscle cells was a replication of the experiment and findings of Kumar and Dey (2003). Further experiments were conducted to investigate the effects of chronic insulin exposure upon post-mitotic skeletal muscle myotubes to determine if the findings observed previously in the literature and in the current experiments would translate in developed skeletal muscle myotubes. It was hypothesised that chronic exposure of skeletal muscle cells to insulin during and post differentiation would result in attenuated phosphorylation of proteins involved in insulin signalling and impaired mRNA expression of genes implicated in metabolism. The findings from these experiments would define the most appropriate method of exposing skeletal muscle cells to insulin and to investigate the effects of chronic insulin exposure upon skeletal muscle tissue engineered constructs. Therefore the purpose of these experiments was to:

1. *To identify the most appropriate method for inducing insulin insensitivity in C2C12 skeletal muscle cells which could be used for experiments in three dimensional skeletal muscle tissue engineered constructs.*

2. *Investigate the effects of exposure to a hyperinsulineamic environment upon differentiating and post-mitotic C2C12 murine myoblast mRNA expression, insulin signalling and glucose uptake.*

6.2. Methods

6.2.1. Experimental protocol

6.2.1.1. *Chronic insulin exposure upon differentiating skeletal muscle cells*

C2C12 myoblasts were seeded into six well plates and grown until confluent as previously described (*Section 3.1*). Cells were changed to either a control (CON) differentiation media (DMEM + 2% HS), or an insulin treated (IT) media (DMEM + 2% HS and 100 nM Insulin) (*Figure 6-1A*). The media was changed twice daily for three days, before being stimulated with insulin as outlined below (*Section 6.2.1.3*)

6.2.1.2. *Chronic insulin exposure upon post-mitotic skeletal muscle myotubes*

The purpose of this experiment was to investigate the effects of chronic insulin exposure upon insulin signalling, and glucose uptake in post-mitotic skeletal muscle myotubes. C2C12 murine myoblasts were cultured as previously described in standard growth media (*Section 3.1*). Upon confluence the media was changed to standard differentiation media as previously described (*Section 3.1.1*). Cells were allowed to differentiate for 72 hours (PRE) (*Figure 6-1B*). This duration was determined based on results in the previous Chapter which shown 72 hours in differentiation media to induce a significant increase in myogenic regulatory factor expression (*Chapter 5*). Skeletal muscle myotubes were exposed to either control media (CON) or insulin treated media (IT) as described above (*Section 6.2.1.1*). To assess insulin signalling of differentiated skeletal muscle myotubes, differentiation media was changed 1 hour prior to exposure to 100 nM of human recombinant insulin in Krebs ringer HEPES buffer or left unstimulated.

6.2.1.3. *Acute insulin stimulation of skeletal muscle in vitro*

The media was changed one hour prior to the start of the experiment, at which point cells were washed twice in Krebs Ringer HEPES (KRH) buffer (10mM HEPES pH7.4, 138mM NaCl, 4.7mM KCl, 1.25mM $CaCl_2$, 1.25mM MgSO, 5mM Glucose and 0.05% BSA). Samples were incubated twice for 30 minutes before being stimulated for 5 minutes with insulin (100 nM) or left un-stimulated. Cells were washed twice in PBS before being lysed within the respective lysis buffers.
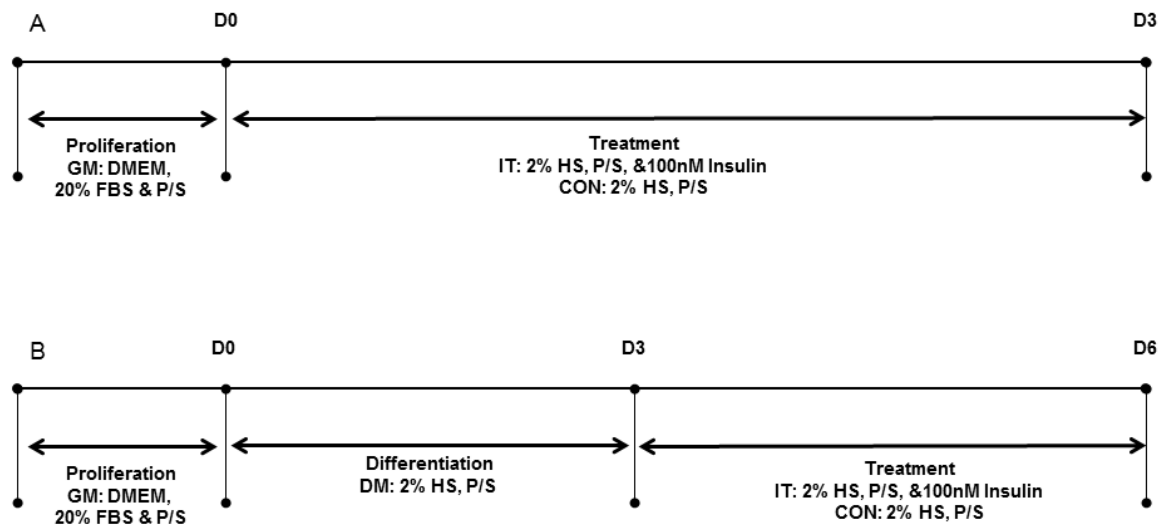
Figure 6-1 Experimental schematic for exposure of C2C12 skeletal muscle cells to insulin. A; Exposure to differentiating skeletal muscle cells. B; insulin exposure to post-mitotic skeletal muscle cells.

6.2.2. Immunostaining

Skeletal muscle cells were ded onto gelatine coated glass coverslips coated with 0.5% gelatin in PBS. Following completion of the experiment (*Section 6.2.1*), plates were fixed for immunocytochemistry as described previously (*Section 3.4*). Cells were imaged at20X magnification and analysed for the number of myotubes, myotube width, length and the number of nuclei per myotube for all conditions. Images were collected from three coverslips per well, three wells per conditions from two independent experiments.

6.2.3. RNA extraction and PCR analysis

RNA was extracted using TRI reagent lysis buffer and quantified as previously described (*Section 3.2*). Samples were diluted (*Section 3.2*) and analysed for a group of genes which have previously been identified to be attenuated in an insulin resistant state (Ducluzeau et al., 2001). The analysis of HKII, PGC-1-α, rRAD, GLUT4 and MyoG mRNA expression by qPCR as outlined (*Table 3-2*). Forward and reverse primer sequences are presented in the general methods Section of this thesis (*Table 3-3*).

6.2.4. Immunoblotting

Samples were lysed in protein lysis buffer (50mM HEPES, 150mM NaCl, 1.5 $MgCl_2$, 1Mm EGTA, 50mM NaF, 50mM β-glycerolphosphate, 1mM Na_3VO_4, 1% Triton X-100 and 2mM PMSF) as previously used in C2C12 myoblasts (Kumar & Dey, 2003). Samples were stored at -80ºC until later analysis. Protein concentrations were determined in triplicate using a protein assay as previously described (*Section 3.5.5*). Samples were separated by electrophoresis on 12% SDS-PAGE gels before being transferred onto nitrocellulose membranes for blotting with specific primary antibodies (*Table 3-4*) at a concentration of 1:1000 in TBST, 5% BSA. Following overnight incubation, membranes were washed and incubated in secondary antibody (1:1000 in TBST, 5% non-fat milk) for one hour before being exposed using ECL reagent as previously described (*Section 3.4*).

6.2.5. Immunoprecipitation

Samples were lysed in 500µl of Radio-Immunoprecipitation Assay (RIPA) buffer and 10µl/mL of protease/phosphatase inhibitor cocktail mix (Fisher-Scientific, Loughborough, UK) and rotated for 30 minutes at 4ºC before being frozen at -80ºC for later analysis. IRS-1 complex was isolated by immunoprecipitation as previously described (*Section 3.5.8.2*) before being boiled in 3X reducing buffer (0.35M Tris-HCl, 10% SDS, 36% Glycerol, 5% β-mercaptoethanol, 0.012% bromophenol blue and 0.3% Twwn-20) for 5 minutes at 100ºC. Samples were loaded and separated on 8% SDS polyacrylamide gels and analysed for the

expression of total and phosphorylated IRS-1 by western blot as previously outlined (*Section 3.5.8.2*).

6.2.6. ^{3}H-Deoxy-D-Glucose uptake

The measurement of glucose uptake was performed following insulin exposure to both differentiating and post-mitotic skeletal muscle cells (*Figure 6-2*), as described previously (*Section 3.6.1*). Concurrent 24 well plates were used to measure protein concentrations upon termination of the experiment (*Section 3.5.5*).
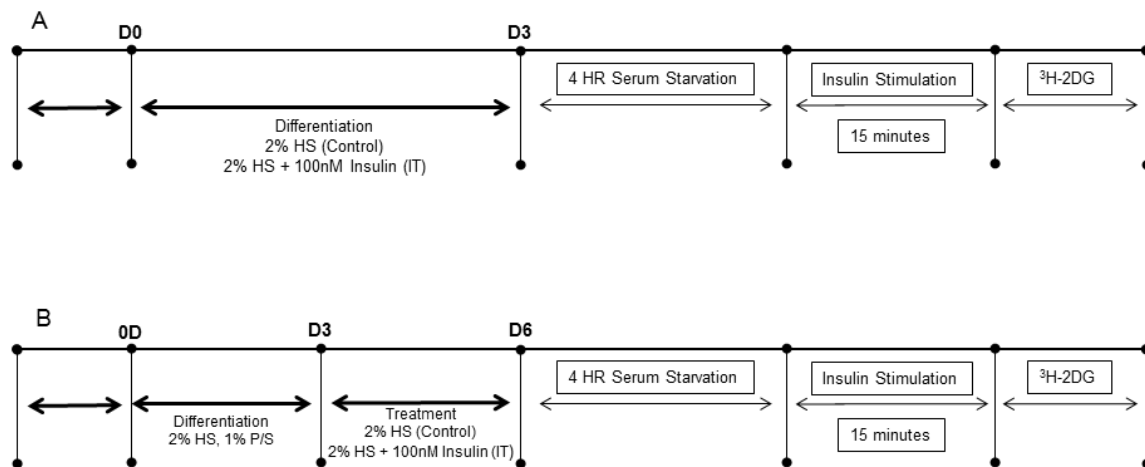
Figure 6-2 Protocol schematic for investigating insulin stimulated glucose uptake in A; differentiating and B; Post-mitotic skeletal muscle myotubes.

6.2.7. Statistical Analysis

Statistical analysis was conducted using SPSS statistical software (IBM SPSS, Version 19, NY, USA). A difference between groups was investigated by analysis of variance (ANOVA) with Bonferroni post-hoc comparisons to identify any differences between conditions. Data which violated the assumptions of homogeneity of variance and normal distribution was analysed by non-parametric tests. Immunofluorescence data was analysed by multiple analyses of variance (ANOVA) from two independent experiments conducted in triplicate wells containing three coverslips. Transcriptional and translational data is presented as mean ± SD and is an accumulation of three independent experiments (*n=3*). Differences in protein concentrations in differentiating skeletal muscle cells was analysed by independent samples t-test, which differences in myotube experiments analysed by ANOVA.

6.3. Results

The purpose of these experiments was to investigate the effects of chronic and acute insulin exposure upon differentiating skeletal muscle cells and post-mitotic skeletal muscle myotubes. Protein and gene expression data were obtained to investigate the molecular response and glucose uptake was investigated to establish the functional response of skeletal muscle cells as a result of insulin exposure. Therefore, this results Section will be divided into two Sections with the first Section presenting data relating to the effects observed in differentiating skeletal muscle cells and the second, the response of skeletal muscle myotubes to chronic and acute insulin exposure.

6.3.1. Chronic and acute insulin exposure upon differentiating skeletal muscle cells.

6.3.1.1. *Protein Concentration is increased following insulin exposure in differentiating skeletal muscle cells.*

C2C12 skeletal muscle cells were differentiated into skeletal muscle myotubes (*Figure 6-4*) whilst being exposed to either control or insulin supplemented media. Following 72 hours differentiation, protein assay was conducted to investigate if exposure to insulin increased protein concentration. Exposure to insulin resulted in a significant increase in protein concentration compared to control skeletal muscle cells t (22) = -2.651, p = 0.015 (*Figure 6-3*).
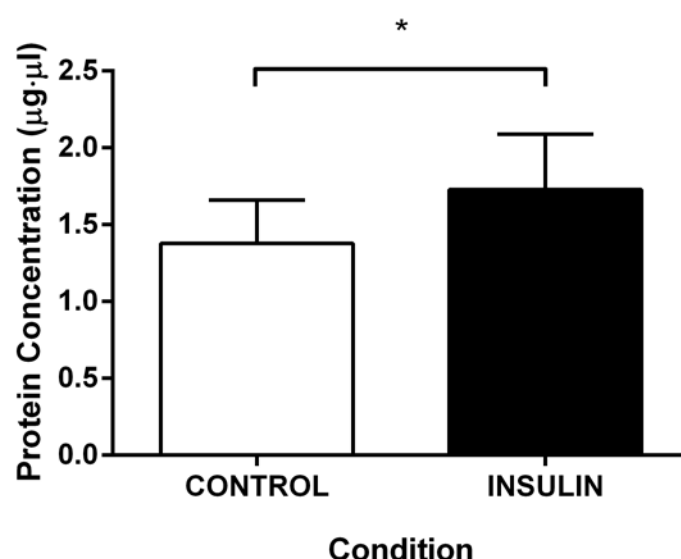
*Figure 6-3 Protein Concentration of differentiating C2C12 skeletal muscle cells exposed to control or insulin-supplemented media. Data is mean ± SD are triplicate samples from 2 independent experiments. * Significant difference between conditions (p<0.05).*

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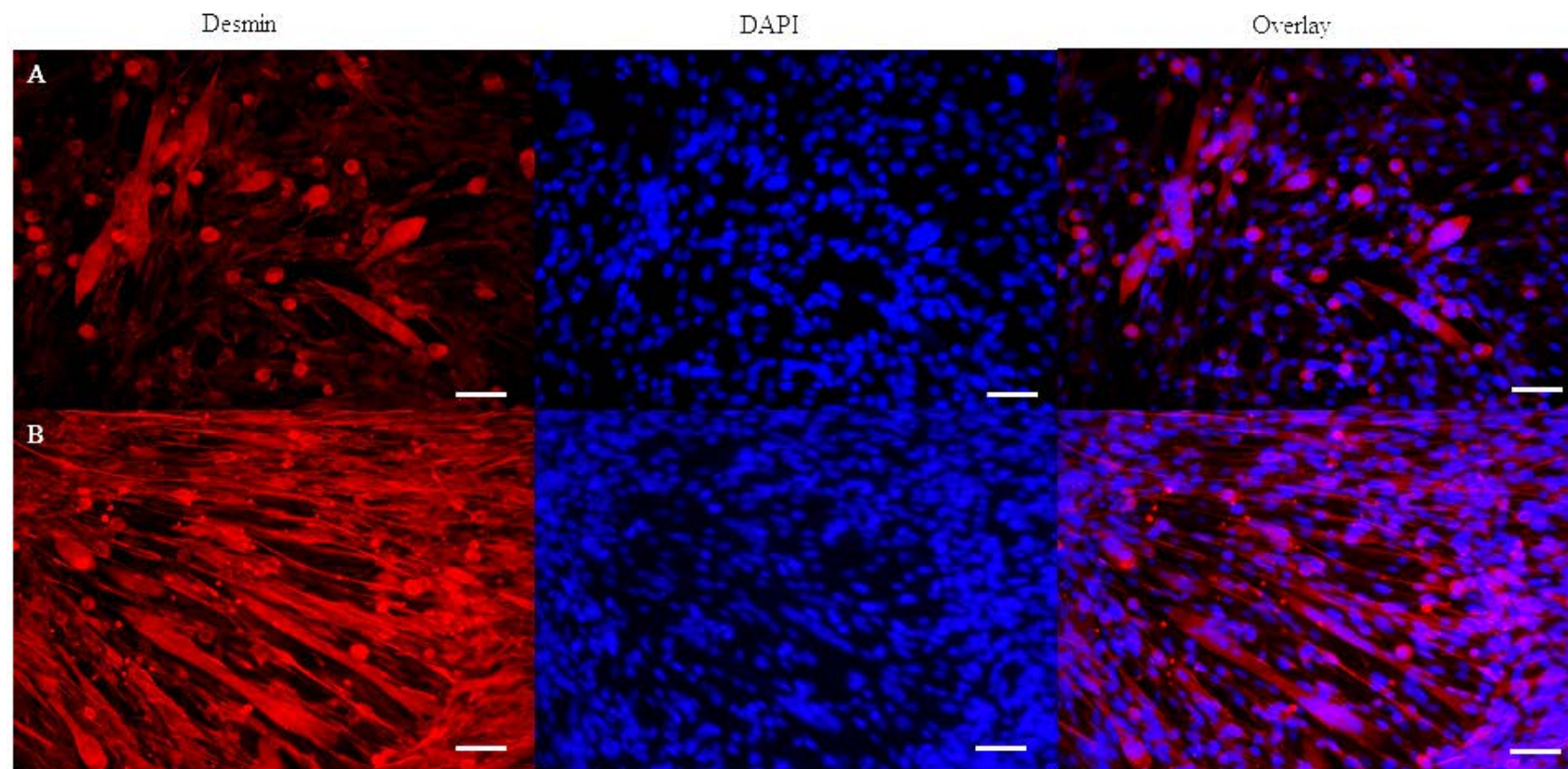
Figure 6-4 Representative Desmin staining (Red) C2C12 skeletal muscle cells counter stained with DAPI (Blue) during differentiation when exposed to A) control or B) insulin (100 nM) supplemented media. Scale bar = 100μm

6.3.1.1. *Protein expression in differentiating skeletal myoblast cells*

In order to assess the effect of hyperinsulinemia upon insulin signalling, we analysed three key proteins that are critical for insulin stimulated glucose uptake to occur. Phosphorylation of IRS-1 at tyrosine residue 632 indicated a significant difference between conditions ($F_{(2, 27)} = 5.061$, $p = 0.007$) (*Figure 6-5*). CON US was significantly greater compared to both IT treated conditions respectively ($p < 0.01$). Furthermore, CON ST was significantly greater than IT US ($p = 0.033$) and approached significance between IT ST condition ($p = 0.054$). The changes in phosphorylation came with no changes in total IRS-1 expression ($F_{(2, 27)} = 5.061$, $p = 0.007$) (*Figure 6-5C*).
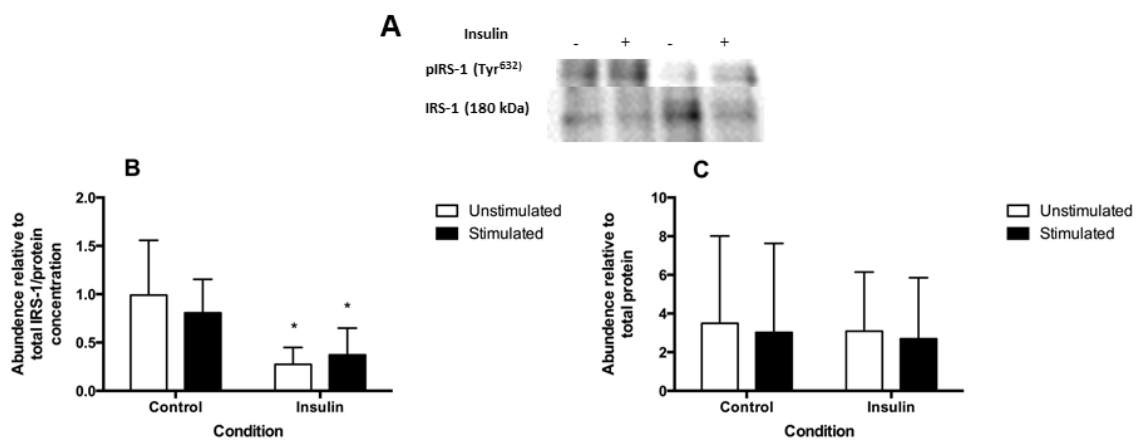
*Figure 6-5 Determination of phosphorylated and total Insulin Receptor Substrate -1 (IRS-1) expression in C2C12 myoblasts. C2C12's were exposed to either control media (CON) or insulin treatment media (IT) for 72 hours. Cells were either left un-stimulated (US) or re-stimulated (ST) with 100 nM insulin. * Significantly different vs. IT-US (P<0.05), # significantly different vs. IT-ST (P<0.05).*

6.3.1.1.   *P85 PI-3 kinase*

Although p85 protein content was elevated with insulin treated stimulated samples, one-way ANOVA revealed a violation of homogeneity of variances and was corrected for using Welch-F test for equity of means. Correction of the violated assumption showed a significant differences between conditions $F_{(3, 13)} = 8.08$ p= 0.003 with an increase within insulin stimulated condition compared to all other conditions ($p < 0.05$) (*Figure 6-6*).
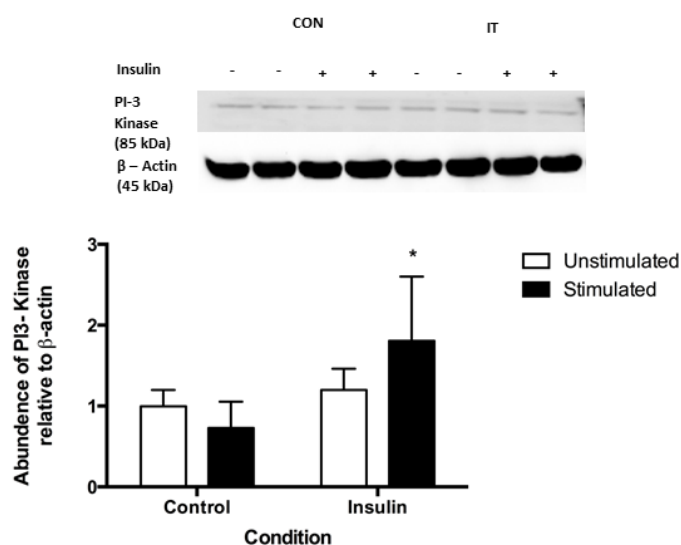
Figure 6-6 Protein abundance of p85 subunit Phosphatidylinositol 3-Kinase (PI 3-Kinase) in C2C12 myoblasts. C2C12's were exposed to either control media (CON) or insulin treatment media (IT) for 72 hours. Cells were either left un-stimulated (US) or re-stimulated (ST) with 100 nM insulin. * Significantly different vs. all other conditions (P<0.05).

6.3.1.2. *Total and Phosphorylation of Akt (Ser[473]).*

Akt is a key 14-3-3 domain protein which highly implicated within the insulin signalling cascade (Brozinick, Roberts, & Dohm, 2003), and has subsequently been found to have impaired phosphorylation activity upon its serine residue in type II diabetics (Krook et al., 1998). Although there was a small increase in total protein content within the insulin treated condition, unsurprisingly there were no significant experimental effects upon total Akt protein content between condition $F_{(3,28)} = 2.763$, $p = 0.61$. One-way ANOVA revealed a violation of homogeneity of variances and was corrected for using Welch-F test for equity of means. There was a significant experimental effect observed $F_{(3, 11)} = 3.33$ $p = 0.05$. Post-hoc analysis showed, unsurprisingly a significant increase in phosphorylation of Akt (Ser[473]) between un-stimulated and stimulated control samples ($p = 0.08$) and differences between control stimulated samples and insulin treated un-stimulated samples ($p = 0.03$). However, there was no significant difference between acute stimulation in chronically insulin treated cells ($p = 0.74$), or a differences between control unstimulated condition ($p = 0.37$) (*Figure 6-7*).
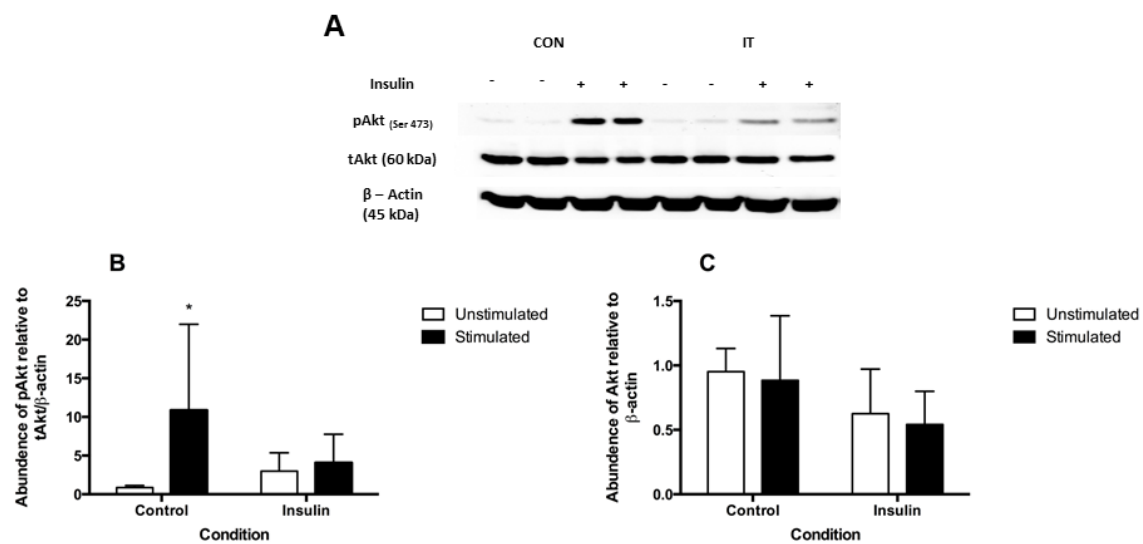
*Figure 6-7 Phosphorylated and total Akt protein abundance in C2C12 myoblasts. C2C12's were exposed to either control media (CON) or insulin treatment media (IT) for 72 hours. Cells were either left un-stimulated (US) or re-stimulated (ST) with 100 nM insulin. * Significantly different vs. all other conditions (P<0.05).*

6.3.1.1. *Gene expression following chronic insulin exposure in differentiating skeletal muscle cells*

Gene expression of PGC-1α mRNA expression was affected by both chronic $F_{(1,18)} = 10.633$, $p = 0.004$ and acute stimulation $F_{(1,18)} = 7.600$, $p = 0.013$ which increased in control cells ($p = 0.002$) but not in insulin cells ($p = 0.195$) (*Figure 6-8A*). HKII was significantly different between conditions $F_{(3,20)} = 8.209$, $p = 0.001$, with control stimulated samples showing greater mRNA expression compared to both insulin treated conditions ($p < 0.05$) (*Figure 6-8B*). There was a significant difference in GLUT4 mRNA expression between groups with unstimulated control samples showing a significantly higher expression compared to all other conditions $F_{(3,17)} = 9.988$, $p = 0.001$ (*Figure 6-8C*). Similarly, rRAD expression was significantly different between conditions $F_{(3,17)} = 9.988$, $p = 0.001$, with control stimulated samples significantly higher compared to all other conditions (*Figure 6-8D*). Myogenin mRNA expression showed there to be a significant difference between groups $F_{(3,6.183)} = 15.959$, $p = 0.003$, which acute insulin stimulation significantly in insulin treated samples to be lower compared to all other conditions ($p < 0.001$) (*Figure 6-9*).

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Figure 6-8 Gene expression of in C2C12 myoblast cells. C2C12's were exposed to either control media (CON) or insulin treatment media (IT) for 72 hours. Cells were either left un-stimulated (Open bars) or re-stimulated (Filled bars)., A; PGC-1α, B; Hexokinase II, C; Glucose Transporter 4 (GLUT4), D; Ras Associated Diabetes (rRAD). * Significantly different vs. all other condition (p<0.05). ‡ Significantly different vs. Insulin Unstimulated (p <0.05), † significantly different vs. Insulin Stimulated (p <0.05).

*Figure 6-9 Myogenin (MyoG) gene expression of in C2C12 myoblasts. C2C12's were exposed to either control media (CON) or insulin treatment media (IT) for 72 hours. Cells were either left un-stimulated (Open bars) or re-stimulated (Filled bars) * Significantly different vs. all other condition (p<0.05).*

Table 6.1 Raw Ct Values (mean ± SD) genes of interest during differentiation of skeletal muscle cells exposed to insulin.

Gene of Interest	Control - Unstimulated	Control - Stimulated	Insulin - Unstimulated	Insulin - Stimulated
HKII	23.43 ± 2.16	22.31 ± 2.18	21.91 ± 2.24	19.88 ± 1.15
GLUT4	27.89 ± 1.94	30.24 ± 2.83	27.17 ± 0.72	26.52 ± 1.01
PGC-1α	25.93 ± 1.31	27.13 ± 1.42	27.08 ± 1.83	25.27 ± 0.93
rRAD	25.64 ± 1.51	25.83 ± 1.40	24.83 ± 1.26	24.81 ± 1.82
MyoG	28.82 ± 3.20	29.38 ± 2.46	30.11 ± 2.69	28.00 ± 3.23

6.3.1.2. $^{3H\text{-}Deoxy\text{-}D\text{-}Glucose}$ *uptake*

Glucose uptake was measured as described in Section 3.7. As a result of chronic insulin exposure there was a significant difference in glucose uptake between conditions $F_{(1, 26)} = 5.251$ (p =0.030). As expected, there was a significant difference between acute stimulation $F_{(1, 26)} = 6.905$ (p = 0.014). Interaction effects $F_{(1, 26)} = 4.865$ (p =0.036) showed significant increases in glucose following insulin stimulation in the control skeletal muscle cells (p = 0.003) but interestingly a significant difference in unstimulated samples between conditions was observed (p = 0.005) (*Figure 6-10*).

Figure 6-10 ^{3}H-Deoxy-D-Glucose uptake in differentiating C2C12 skeletal muscle cells. Skeletal muscle cells were exposed to low serum media supplemented without (Control) or with (Insulin) the addition of insulin (100 nM). Data are Mean ± SD of four independent experiments (N=4).

6.3.2. Chronic and acute insulin exposure upon post-mitotic skeletal muscle myotubes
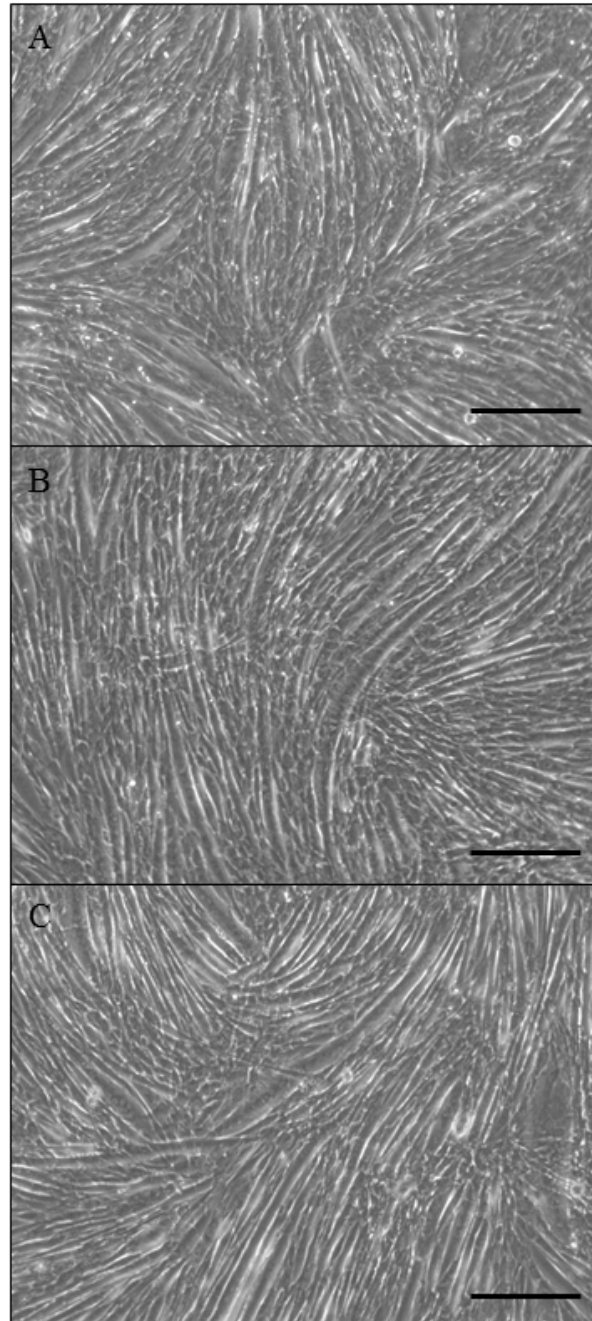
Figure 6-11 Phase contrast image of C2C12 skeletal muscle myotubes prior to treatment (A; PRE), following Control treatment (B; CON) or following Insulin treatment (C; INS). Scale bar = 100μm.

6.3.2.1. *Protein Expression in post-mitotic skeletal muscle myotubes*

Protein was extracted principally for investigation of specific proteins of interest in relation to insulin signalling. In addition to the role of insulin upon glucose metabolism, it also contributes to the regulation of protein synthesis (Proud, 2006). A significant difference was found between conditions in protein concentrations with post-hoc comparisons showing a significant difference $F_{(2, 50)} = 4.549$, $p = 0.016$ between myotubes exposed to insulin compared to initial differentiated myotubes (PRE-T vs. IT) ($p = 0.02$). Despite insulin showing a slight increase in protein concentrations, there was no significant difference in protein concentration between control and chronically incubated myotubes ($p = 0.064$) (*Figure 6-12*).
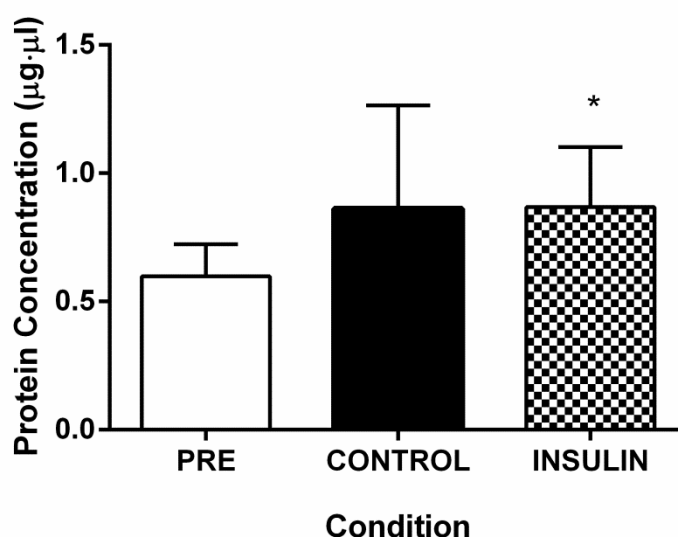
*Figure 6-12 Protein concentration (µg•µl) of protein lysates collected from C2C12 skeletal muscle myotubes incubated in control media (CON) or chronically exposed to insulin (100 nM) (IT) following differentiation (PRE). * Significantly difference vs. PRE (p <0.05)*

As investigated in skeletal muscle cells, phosphorylation of Akt (Ser^{473}) was used to determine the effects of insulin exposure upon the insulin signalling cascade. There were significant differences between means $F_{(2, 17)} = 5.632$, $p = 0.007$. Bonferroni corrections showed significant increase in Akt phosphorylation following acute insulin stimulation in post-mitotic skeletal muscle myotubes ($p = 0.034$). Acute stimulation in post-mitotic skeletal muscle myotubes was also significantly greater than control unstimulated ($p = 0.016$) and stimulated myotubes following chronic insulin stimulation ($p = 0.01$) (*Figure 6-13A*). As shown in previous experiments, there was no significant difference in total Akt expression $F_{(2, 27)} = 0.252$, $p = 0.935$ (*Figure 6-13B*). In addition, there was no significant difference in total p85 PI-kinase expression between conditions $F_{(2, 17)} = 2.081$, $p = 0.138$ (*Figure 6-14*).
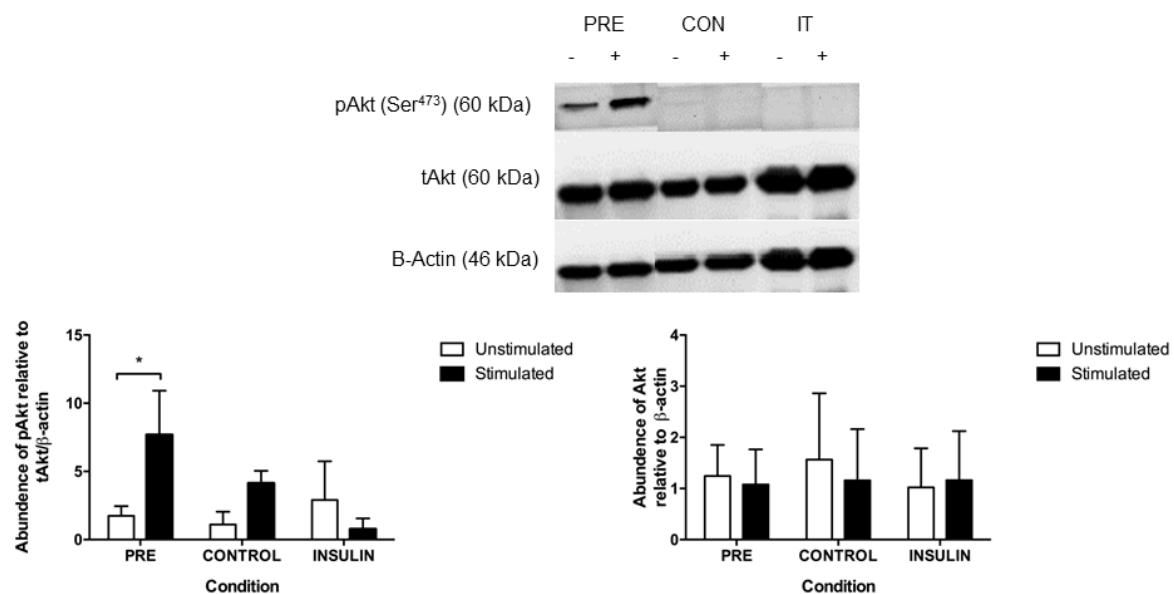
*Figure 6-13 Total and phosphorylated Akt protein expression in C2C12 skeletal muscle myotubes incubated in control media (CON) or chronically exposed to insulin (100 nM) (IT) following differentiation (PRE). * Significantly difference between PRE ST (p <0.05).*
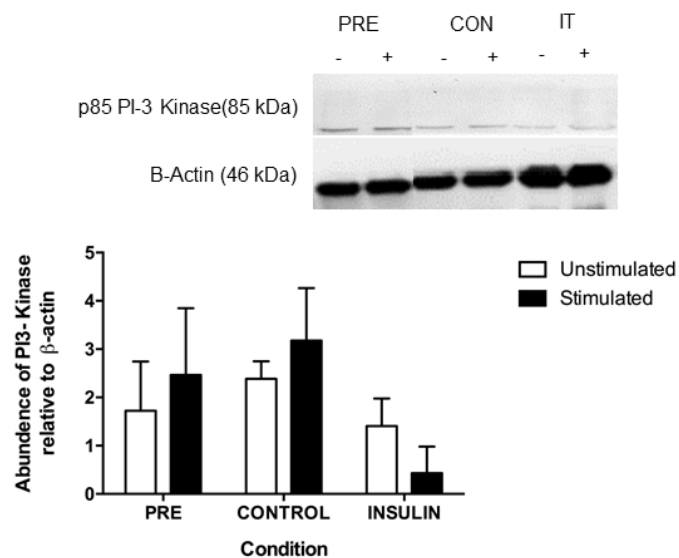
Figure 6-14 Total p85 PI-3 kinase protein expression in C2C12 skeletal muscle myotubes incubated in control media (CON) or chronically exposed to insulin (100 nM) (IT) following differentiation (PRE).

6.3.2.2. *Skeletal muscle myotubes image analysis*

Immunohistochemical analysis was conducted following exposure of skeletal muscle myotubes to insulin or control media. Morphological characteristics were determined following formation of skeletal muscle myotubes and following chronic exposure to insulin.

Myotubes length increased as a result of time in culture $F_{(2,219)} = 37.604$ $p < 0.001$ with myotubes significantly longer in both control and insulin exposed myotubes compared to pre-exposed myotubes ($p < 0.001$) (*Figure 6-15*). Differences in myotube length was observed between control and insulin exposed myotubes, however with no significantly different ($p = 0.081$). Furthermore, as expected with an increased in myotube length (PRE 224.20 ± 46.28 µm; CON 277.98 ± 64.68 µm; IT 299.16 ± 51.72 µm), there was a significant increase in the average number of nuclei per myotube (PRE 24.71 ± 6.60 µm; CON 26.21 ± 7.35 µm; IT 23.94 ± 6.30 µm) $F_{(2,217)} = 45.584$ $p < 0.001$ with both control and insulin myotubes significantly greater compared to pre-exposed myotubes ($p < 0.001$) (*Figure 6-15*).

Other morphological characteristics were analysed by non-parametric tests to investigate differences between groups and further multiple independent t-tests, with Bonferroni correction for multiple comparisons to identify post-hoc differences between conditions. The number of myotubes significantly increased in culture $t_{(2)} = 42.875$ $p < 0.0001$. Both control $t_{(2)} = 42.875$ $p = 0.001$ and insulin exposed cultures presented significantly more myotubes compared to pre-exposed (*Figure 6-15*). In addition, there was significantly more myotubes in control compared to insulin exposed myotubes $t_{(1)} = 10.041$ $p = 0.001$. There was no significant differences in myotube width between conditions $t_{(2)} = 3.411$ $p = 0.182$ (*Figure 6-15*).
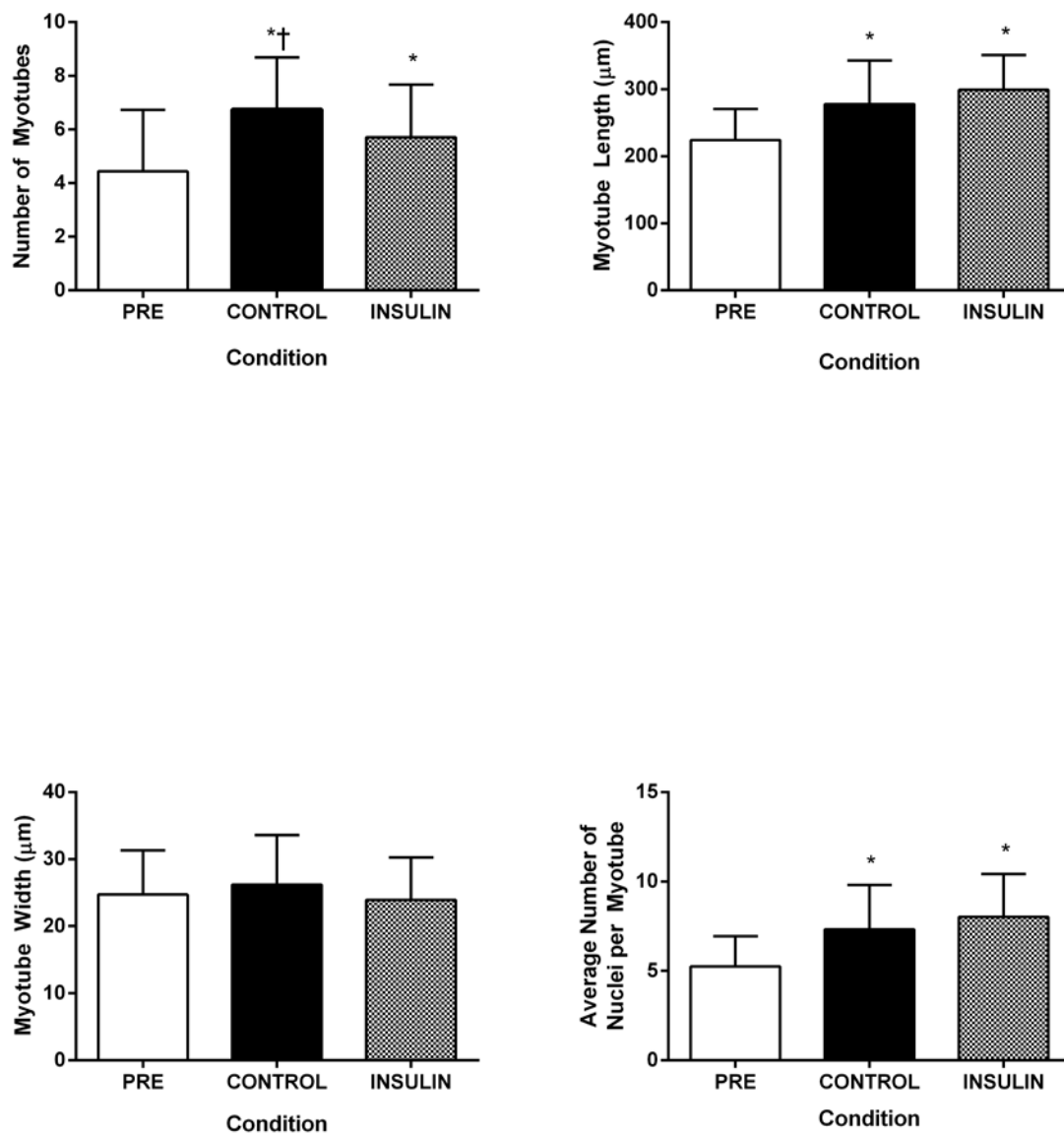
*Figure 6-15 Immunhistolchemical Analysis of C2C12 skeletal muscle myotubes. Data is mean ± SD from two independent experiments. * Significantly difference compared to PRE (p <0.01). † Significantly different compared to insulin (p <0.01).*
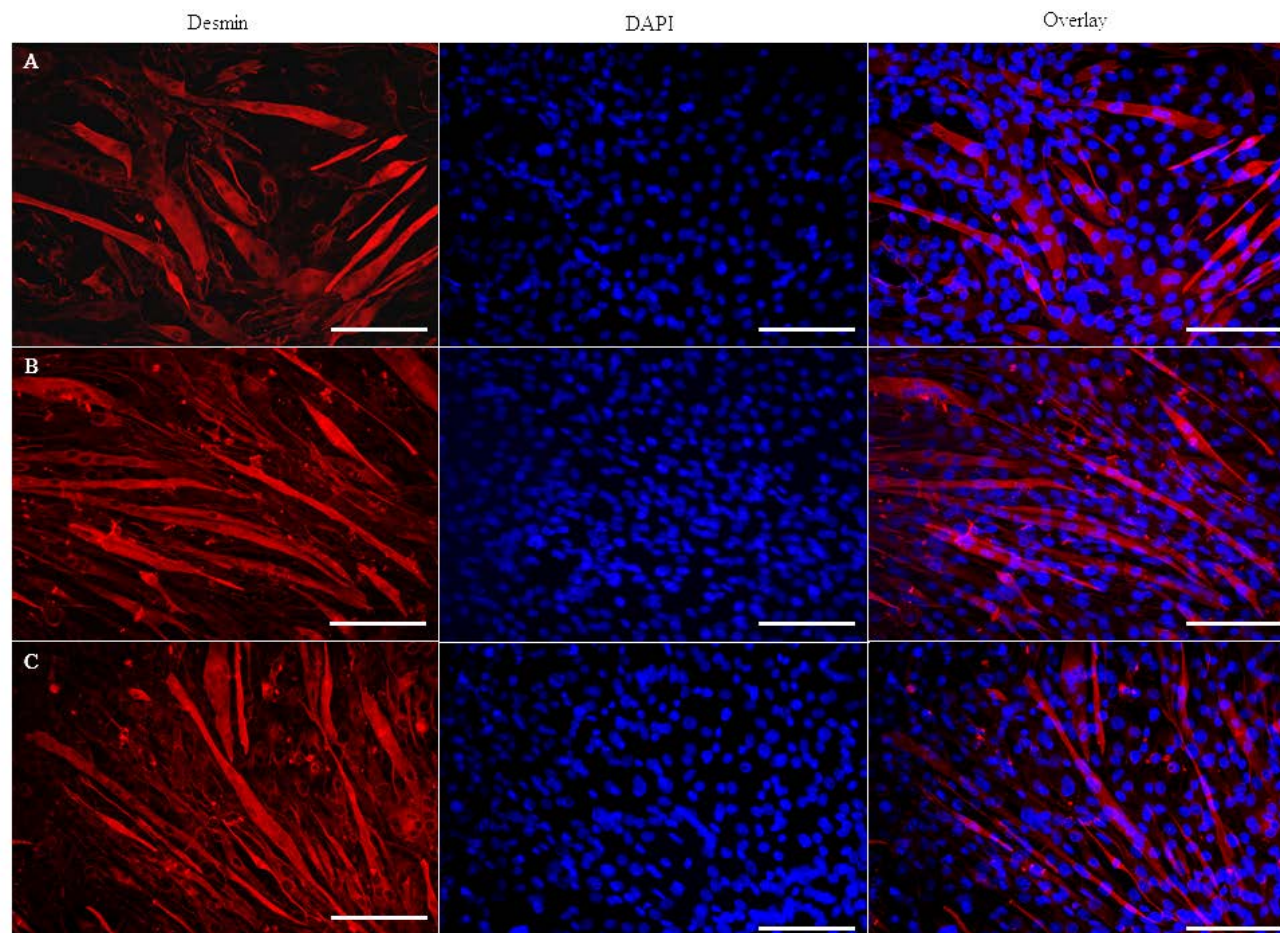
Figure 6-16 Representative Desmin staining (Red) C2C12 skeletal muscle myotubes counter stained with DAPI (Blue) following (A) 72 hours differentiation, and a further 72 hours incubation in either (B) control or (C) insulin (100 nM) supplemented media. Scale bar = 100μm.

6.3.2.1. *Gene expression in post-mitotic skeletal muscle myotubes*

Insulin has been reported to affect a number of transcriptional regulators in skeletal muscle (O'Brien and Granner, 1996). Therefore genes involved in myogenesis (Myogenin) and glucose metabolism (PGC-1, HKII and, GLUT4) were investigated. There was a main effect of GLUT4 expression between the same conditions (p = 0.043) (*Figure 6-17A*). In addition, there was a main effect between conditions (p=0.012) for HKII expression, with post-hoc comparisons showing a difference between pre-treated and insulin exposed myotubes (p=0.012) (*Figure 6-17B*). On the other hand, PGC-1α expression was not different between conditions, but was significantly affected by acute insulin stimulation (p = 0.011) (*Figure 6-17C*). Myogenin mRNA expression showed no main effects for condition $F_{(2, 38)} = 0.116$ p = 0.89 or stimulation $F_{(1, 38)} = 0.689$ p=0.41 however, there was a significant interaction affect $F_{(2, 38)} = 4.987$ p = 0.012). Acute insulin stimulation significantly increased within pre-treatment condition (p = 0.016). Although this was not observed in the control condition, surprisingly there was a trend for down regulation of expression upon stimulation within the insulin treated condition (p = 0.065), which was lower compared to stimulated pre-treatment samples (p = 0.041) (*Figure 6-17D*).

Figure 6-17 Gene expression analysis (ΔΔC^t) of C2C12 skeletal muscle myotubes incubated in control media (CON) or chronically exposed to insulin (100 nM) (IT) following differentiation (PRE). A; GLUT4, B; HKII, C; PGC-1α, D; MyoG. # Significantly different between unstimulated (open bars) vs. stimulated (closed bars). **Significant main effect between unstimulated vs. stimulated. § Significant main effect between PRE-T vs. IT. Data is Mean ± SD of three independent experiments (N=3).

Table 6.2 Raw Ct Values (mean ± SD) genes of interest during skeletal muscle myotubes exposed to insulin.

Gene of Interest	PRE TREATMENT		CONTROL TREATMENT		INSULIN TREATMENT	
	Unstimulated	*Stimulated*	*Unstimulated*	*Stimulated*	*Unstimulated*	*Stimulated*
HKII	20.30 ± 1.97	20.06 ± 0.55	19.96 ± 0.37	19.85 ± 0.72	20.80 ± 0.66	20.01 ± 0.72
GLUT4	25.31 ± 0.41	25.44 ± 0.49	25.66 ± 0.44	25.14 ± 0.69	25.98 ± 0.62	25.25 ± 0.64
PGC-1α	23.72 ± 0.74	23.50 ± 1.36	24.02 ± 0.48	22.37 ± 1.17	23.54 ± 0.87	21.87 ± 1.67
MyoG	23.65 ± 1.52	22.23 ± 0.99	22.53 ± 0.64	21.80 ± 0.83	22.63 ± 0.61	22.15 ± 0.74

6.3.2.2. ^{3}H-2-deoxyglucose uptake in post-mitotic skeletal muscle myotubes

Glucose uptake was measured in skeletal muscle myotubes following acute and chronic insulin exposure. Non-parametric tests were conducted to investigate differences between conditions however no significant differences were observed ($p > 0.05$) (*Figure 6-18*).

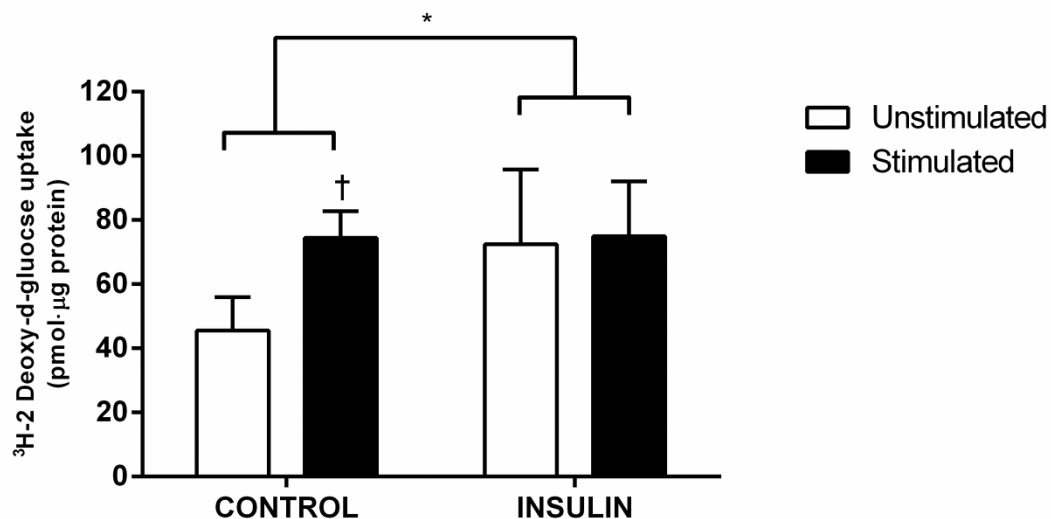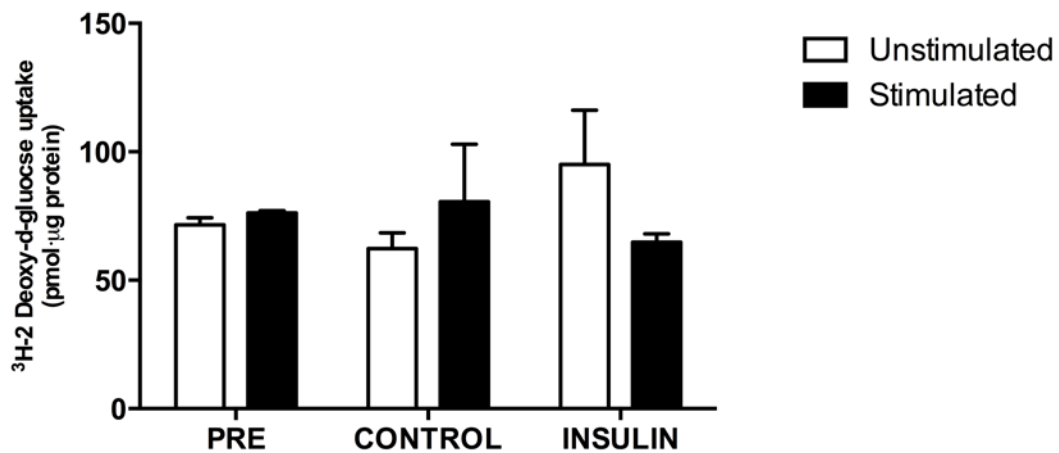*Figure 6-18 ^{3}H-De-oxy-D-Glucose uptake in C2C12 skeletal muscle myotubes. Skeletal muscle myotubes were exposed to low serum media supplemented without (Control) or with (Insulin) the addition of insulin (100 nM). Open bars represent unstimulated filled bars represented acute 30 minutes insulin incubation. Data are Mean ± SD of three independent experiments (N=3).*

6.4. Discussion

The purpose of these experiments was to investigate the effect of insulin exposure upon skeletal muscle cells during and post differentiation. The experiments conducted in monolayer culture aimed to inform subsequent experiments as to the most appropriate method of for developing a model of insulin insensitive skeletal muscle through exposure to hyperinsulinemia. Hyperinsulinemia is a symptom of non-insulin dependent diabetes mellitus (NIDDM) (Gaster et al., 2002), and has been shown to affect protein signalling within insulin sensitive tissues (Ueno et al., 2005). A method of investigating these effects upon skeletal muscle, a major endocrine organ, has been through the use of cell culture models (del Aguila et al., 2011; Kumar and Dey, 2003).

PI-3 kinase contains a catalytic (p110) and regulatory (p85) subunit which are activated upon phosphorylation of the upstream IRS-1 tyrosine kinase (Peter R Shepherd et al., 1998). In normal conditions a pool of p85 monomers exist in balance with p85-p110 heterodimer. Subsequently, both monomer and heterodimer compete for the same binding sites therefore any imbalance between the two could potentially result in alterations in PI-3 kinase activity (Draznin, 2006). The current finding shows that within insulin treated samples, upon acute insulin stimulation, there was a significant increase in protein concentrations of p85 PI-3 kinase. Similar findings have previously been reported in L6 skeletal muscle cells when exposed to dexamethasone (Giorgino, Pedrini, Matera, and Smith, 1997), as well as in human skeletal muscle from insulin resistant populations (Bandyopadhyay, Yu, Ofrecio, and Olefsky, 2005). Therefore it could be speculated that exposure to a hyperinsulineamic environment during differentiation of skeletal muscle cells results in an alteration in the p85 monomer: p85-p110 heterodimer ratio resulting in a negative PI-3 kinase activity and therefore an impairment of effective phosphorylation of downstream insulin signalling proteins. If this is true, one would expect impairment in the serine/threonine kinase, Akt, and indeed this was the case in these experiments.

Exposure of skeletal muscle cells to insulin during differentiation resulted in impaired acute insulin stimulated phosphorylation of Akt at Ser[473], as observed in human primary skeletal muscle cells (Cozzone et al., 2008). Chronic insulin exposure has been shown to lead to degradation of IRS-1 leading to attenuated downstream signalling (Haruta et al., 2000), in the case of previous research and the current findings, attenuated Akt phosphorylation (Haruta et

al., 2000; Tremblay et al., 2001). In contrast to differentiating skeletal muscle cells, chronic insulin exposure did not appear to have any effect upon phosphorylation of Akt in post-mitotic myotubes. The protein Akt, is involved in a number of cellular functions, plays a key role in metabolism in insulin sensitive tissues (Brozinick & Birnbaum, 1998; Brozinick, Roberts, & Dohm, 2003). Phosphorylation of Akt at Serine 473 (Ser[473]), a key residue for ensuring full kinase activity, has been found to be impaired in both skeletal muscle cells (Cozzone et al., 2008) and muscle tissue from diabetic individuals (Krook et al., 1998).

Metabolic diseases have been shown to alter transcriptional expression compared to healthy individuals (Bach et al., 2002; Ducluzeau et al., 2001). Although previous research has shown a large number of genes have been identified to be affected by insulin, a group which have previously been categorised as '*diabetogenes*' were measured to investigate their response to chronic insulin exposure during both differentiation, and in post-mitotic skeletal muscle myotubes. Hexokinase II (HKII), which is important in catalysing glucose to form glucose-6-phosphate, and mRNA expression has previously been shown to be increased upon insulin stimulation (Mandarino et al., 1995). In differentiating myoblasts, an increased HKII expression was found in control cells as a result of acute insulin treatment, however this increase was not evident when cells were chronically pre-treated with insulin. These findings concur with those reported in human skeletal muscle which has found impairment in mRNA expression of HKII upon insulin stimulation in both obese and diabetic subjects (Pendergrass et al., 1998). Skeletal muscle myotubes showed a different mRNA expression pattern with a reduction in mRNA expression when exposed to insulin, however were not affected by acute insulin stimulation.

Peroxisome proliferator-activated receptor coactivator – 1α (PGC-1α), is an important mediator of mitochondrial biogenesis and has consequently been shown to be down regulated in insulin resistant subjects (Mootha et al., 2003). Chronic insulin treatment resulted in a reduced mRNA expression of PGC-1α upon acute stimulation, which is similar to recent findings in C2C12 myotubes (Philp et al., 2010). Philp and colleagues showed that chronic exposure to pyruvate resulted in a significant reduction in PGC-1α and subsequently reduction in glucose oxidation within these cells, developing a '*diabetic-like phenotype*'. Therefore, based on the current findings, under a hyperinsulineamic environment, a symptom of type II diabetes mellitus (Cha et al., 2005), and in concordance with the findings of Philp and colleagues, it could be suggested that the impairment in PGC-1α observed in the current models replicates that observed in diabetic human skeletal muscle (Cha et al., 2005).

Glucose transporters are integral proteins for glucose uptake. Glucose transporter 4 (GLUT4) is highly concentrated at the protein and transcriptional levels (Stuart et al., 2006) and is predominantly responsible for insulin stimulated glucose uptake in skeletal muscle (Ueyama, Yaworsky, Wang, Ebina, and Klip, 1999). Insulin stimulation increases GLUT4 mRNA in human and rodent skeletal muscle (Laville et al., 1996), primary human muscle cells (Al-Khalili et al., 2003; Sarabia et al., 1992) as well as cell lines (Mitsumoto et al., 1991). Acute insulin stimulation increases GLUT4 mRNA expression in differentiating skeletal muscle cells however did not change in skeletal muscle myotubes. Furthermore, the effects of chronic insulin exposure showed to reduce GLUT4 mRNA expression. These findings support previous work conducted in adipocyte cells which, similar to skeletal muscle cells, has shown acute insulin stimulation to induce an increase in GLUT4 mRNA expression, with chronic stimulation with insulin abating an increase in gene expression (Kozka, Clark, and Holman, 1991). It has been proposed that the reduction in GLUT4 mRNA expression following chronic insulin exposure is due to the change in glucose uptake through alternative glucose transporters (Kozka et al., 1991). Furthermore, the reduced expression has been shown to reduce protein expression and subsequently insulin stimulated glucose transport via the GLUT4 protein (Tordjman, Leingang, James, and Mueckler, 1989). Although GLUT4 or GLUT1 protein concentration was not measured in these experiments, the impaired insulin stimulated glucose uptake following chronic insulin exposure, which has previously been reported in both adipocytes (Kozka et al., 1991; Tordjman et al., 1989) and skeletal muscle cells (Kumar and Dey, 2003), could suggest there to be a change in specific glucose transporter mediated uptake.

Glucose uptake in skeletal muscle is mediated through insulin dependent and independent mechanisms (Antonescu, Foti, Sauvonnet, and Klip, 2009). Subsequent insulin stimulated glucose uptake is important for post-prandial glucose disposal with an impairment in the insulin signalling cascade resulting in attenuation of this response (R A DeFronzo et al., 1983). Glucose uptake was therefore measured using a radioactive isotope tracer glucose analogue, ^{3}H-Deoxy-D-Glucose uptake (^{3}H-2DG) used widely within the literature the investigate cellular glucose uptake *in vitro* and considered the 'gold standard' for such experiments. From the experiments in differentiating C2C12 skeletal muscle cells, within control cells, acute insulin stimulated was shown to increase glucose uptake by approximately 1.5 fold which supports the increases previously observed within the literature using this cell line (Nedachi and Kanzaki, 2006; Philp et al., 2010). However, in comparison

to the work of Kumar and Dey with which this experimental protocol was based upon, there was a significant increase in glucose uptake within chronically exposed cells to insulin at the basal level which was maintained following acute insulin stimulation. In contrast to Kumar and Dey (2003), the result here resemble those previously reported in L6 skeletal muscle cells by Huang and colleagues (Huang et al., 2002). Importantly, they observed a reduction in GLUT4 exocytosis, without changes in GLUT4 expression following sustained insulin exposure (Huang et al., 2002). A potential cause of this increase in glucose uptake is mostly likely due to increases in basal glucose transporters namely GLUT1, which is responsible for basal glucose turnover (Klip & Pâquet, 1990), has been shown to be increased following high insulin exposure at the protein level within L6 muscle cells (Huang et al., 2002), and in the C2C12 skeletal muscle cell line (Nedachi and Kanzaki, 2006).

6.5. Conclusion

In this Chapter, C2C12 skeletal muscle cells were grown in standard culture conditions to investigate the effects of acute and chronic insulin exposure upon differentiating and post-mitotic skeletal muscle. Skeletal muscle cells exposed to insulin during differentiation showed a reduction in cell signalling and mRNA expression in response to acute insulin exposure. The reduction in sensitivity at the molecular level in differentiating skeletal muscle cells translates to an increase in basal glucose uptake, ablating insulin stimulated glucose uptake. In contrast, despite a molecular response to acute insulin exposure in post-mitotic skeletal muscle myotubes, myotubes grown for longer periods and in the presence of insulin were not acutely affected by insulin at the translational level. In addition, the effect of insulin exposure upon skeletal muscle myotubes had minimal effect upon insulin stimulated glucose uptake. These findings would suggest that insulin has a greater effect upon differentiating skeletal muscle cells compared to post-mitotic skeletal muscle myotubes.

7 Insulin signalling and glucose uptake in skeletal muscle tissue engineering: The effect of acute and chronic insulin exposure.

7.1 Introduction

Skeletal muscle is comprised of highly organised multinucleated myotubes surrounded by an extracellular matrix (Gillies and Lieber, 2011), mainly in the form of collagen (Kjaer, 2004). In order to further understand the complexities of skeletal muscle physiology, the use of human subjects, *in vivo* animal models (Joost, Al-Hasani, and Schürmann, 2012), as well as *in vitro* cell culture models have sought to disseminate individual factors which potentially contribute to skeletal muscle pathophysiology (Abdul-Ghani and Defronzo, 2010). As previously discussed (*Section 2.4*), cell culture provides an isolated environment in which single variables can be manipulated whilst in a controlled environment. Furthermore, the use of cell lines such as L6 and C2C12 skeletal muscle cell lines, provide a consistent population of cells, which are easy and readily available to grow and investigate various aspects of skeletal muscle physiology, including insulin signalling and glucose uptake (del Aguila et al., 2011; Kumar and Dey, 2003).

7.1.1 *In Vitro* skeletal muscle tissue engineering for investigating physiological applications

Emerging research in the field of tissue engineering and the development of three dimensional (3D) skeletal muscle tissue engineered constructs, have advanced within the literature as an *in vitro* model to investigate physiological and clinical applications (Baar, 2005; Stern-Straeter, Riedel, Bran, Hörmann, and Goessler, 2007). The development of 3D models have utilised various methods of ding skeletal muscle cells within biological hydrogels (Hinds et al., 2011) in order to mimic the extracellular *milieu* in which skeletal muscle resides *in vivo*. The ding of skeletal muscle cells between fixed anchor points results in ECM remodelling as the cells self-organise to form uni-axial multinucleated myotubes (Hinds et al., 2011; Khodabukus and Baar, 2009a). Previous literature has used 3D tissue engineered skeletal muscle models to investigate skeletal muscle development and maturation (Cheema et al., 2005, 2003; Mudera et al., 2010), force generation (Robert G Dennis and Dow, 2007; Robert G Dennis and Kosnik, 2000; Robert G Dennis et al., 2001), as well as the molecular responses to mechanical load (D J Player et al., 2014; Sharples et al., 2012). As a result, three dimensional tissue engineered skeletal muscle models have provided an alternative culture system, which bridges the gap between *in vitro*, *ex vivo* and, *in vivo* models (Baar, 2005).

The field of skeletal muscle tissue engineering has advanced the field of regenerative medicine (Juhas et al., 2014). In addition, the use of these models for drug screening has

postulated these models as a method of reducing, refining and replacing the number of animals used in research (Vandenburgh, 2010). Furthermore, the use of 3D tissue engineered models has the potential provide an insight into various phenomena relating to skeletal muscle physiology and function (Khodabukus et al., 2007). Recent research has shown the metabolic phenotype of tissue engineered skeletal muscle models can be manipulated by glucose and serum exposure during development (Khodabukus and Baar, 2014a, 2014b). Despite these recent findings, the use of tissue engineered constructs to investigate insulin signalling and glucose uptake is very limited (Baker et al., 2003), despite a number of characterised models using various ECMs to investigate physiological responses of skeletal muscle (Martin et al., 2013; Player et al., 2014; Powell et al., 2002).

As previously discussed cell lines such as C2C12 and L6 skeletal muscle cells have commonly been used to investigate the insulin signalling and glucose uptake (Nedachi and Kanzaki, 2006; Walker et al., 1990). Unlike L6 cells, the use of C2C12 skeletal muscle cells have been well characterised in models of skeletal muscle tissue engineering (Khodabukus et al., 2009; Player et al., 2014), and while there metabolic phenotype has recently been eluded to (Khodabukus and Baar, 2014a, 2014b), the effects of insulin stimulation upon skeletal muscle tissue engineered constructs is yet to be elucidated. Establishment of a 3D tissue engineered skeletal muscle model which is responsive to insulin and can be manipulated by this hormone would enable further understanding of the effects of a 3D environment upon the insulin signalling pathway. In addition, development of a culture model which recapitulates both the morphological and physiological aspects of native skeletal muscle tissue, and could have the potential to be used for a number of applications (Cheng et al., 2014). It was hypothesised that similar to the findings observed in monolayer culture (*Chapter 6*), exposure to insulin during differentiation of skeletal muscle tissue engineered constructs would impair insulin signalling and therefore, the objectives of this Chapter were to:

1. *Investigate the transcriptional and translational responses to acute and chronic insulin exposure in two 3D based skeletal muscle constructs.*
2. *To investigate if the effect of chronic insulin exposure can recapitulate the altered signalling and transcriptional responses observed in monolayer culture.*
3. *To investigate insulin stimulated glucose uptake in 3D tissue engineered constructs.*

7.2 Methodology

7.2.1 Cell Culture

C2C12 skeletal muscle myoblasts were culture in GM as outlined (*Section 3.1.1*). Cells were passaged and cell counted (*Section 3.1.2*), before being seeded onto fibrin or into collagen based hydrogels.

7.2.2 Preparation of fibrin constructs

Fibrin gels were prepared and developed as previously described (*Section 3.2.1*). Constructs were allowed to proliferate in GM (20% FBS, 1% P/S) supplemented with aminocaproic acid (AA) until confluence was attained, before being switched to DM (2% HS, 1% P/S) supplemented with AA for two days. Thereafter, constructs were grown in maintenance media (7% FBS, 1% P/S, AA) with or without the addition of 100 nM insulin (*Figure 7-1A*). Media was changed daily with constructs maintained in culture for a total of 14 days. At day 14, macroscopic images were obtained to measure gel width and images for observation of myotube formation were obtained by phase contrast microscopy.

7.2.3 Preparation of collagen constructs

Collagen tissue engineered constructs were prepared and cast as previously described (*Section 3.2.2*). Constructs were maintained in standard growth media for 4 days with media changed twice daily. Thereafter, constructs were changed to standard DM supplemented without or with insulin (100 nM) for the remainder of the culture period, with media changed daily (*Figure 7-1B*). Constructs were maintained in culture for a total of 14 days. Upon completion of the days in culture, macroscopic images were obtained to measure gel width and images for observation of myotube formation were obtained by phase contrast microscopy.
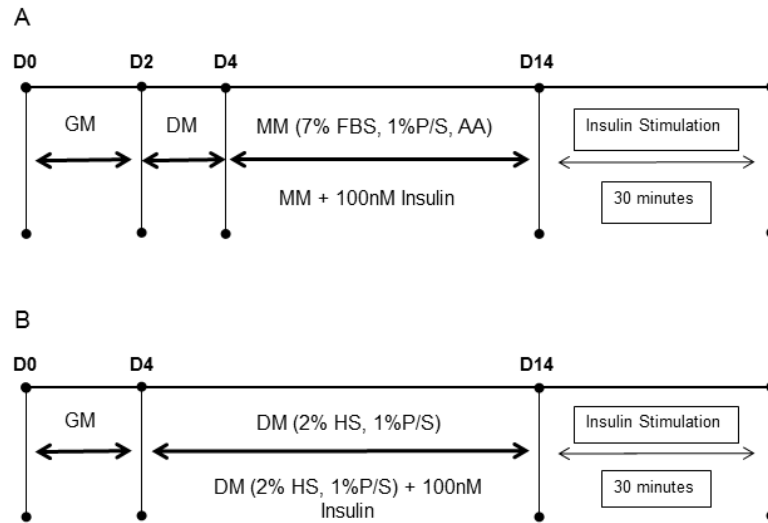
Figure 7-1 Protocol schematic of acute and chronic insulin exposure in A; Fibrin and B; Collagen skeletal muscle tissue engineered constructs.

7.2.4. Optimisation of acute insulin stimulation in skeletal muscle tissue engineered constructs.

Initial experiments were conducted to establish the responses to acute insulin stimulation. Acute stimulation was performed following 14 days total in culture. This culture period was based on the previous Chapter which investigated the transcriptional response of glucose transporters in fibrin and collagen skeletal muscle constructs (*Chapter 5*). On day 14 in culture, constructs were acutely exposed to insulin as previously described with some slight modifications (*Section 6.2.1.3*). In brief, the media was changed 1 hour prior to being washed twice in KRH buffer. Constructs were incubated twice for 30 minutes in KRH buffer before being exposed to insulin (100 nM) in a time dependent manner (0-30 minutes). Constructs were washed in PBS and blot dried before being snap frozen in liquid nitrogen before being quantified for protein as described above. Following these experiments it was decided that both fibrin and collagen constructs would be incubated in the presence of absence of insulin (100 nM) for 30 minutes and obtained for protein and gene expression analysis.

7.2.5 Construct lysis for RNA extraction and qPCR

Both fibrin (N = 4 constructs per condition) and collagen constructs (N = 3 constructs per condition) were lysed in TRI-reagent and immediately frozen at -20°C. Constructs were homogenised using an electronic stick homogeniser (IKA T10, Fisher-Scientific) in order to break down the ECM prior to RNA extraction which was performed as previously described (*Section 3.3.3*). As in the previous Chapter of this thesis (*Chapter 6*) qPCR analysis was

performed for the mRNA expression of HKII, GLUT4, PGC-1α and RPIIβ. Sequences for these primers are outline within the methods Section (*Table 3-3*) and PCR was conducted using the Stratagene MX3005p as described previously (*Section 3.3.5*). Analysis of mRNA expression was performed using the $\Delta\Delta C^t$ method.

7.2.6 Construct lysis for protein extraction

Following the experimental protocol (*Figure 7-1*), constructs (N = 4 fibrin constructs per condition and N = 3 collagen constructs per condition) were washed in cold PBS and blot dried before being snap frozen in liquid nitrogen. Samples were stored and -80°C for later analysis.

Fibrin constructs were homogenised by a 2 minute cycle using a tissue lyser in RIPA lysis buffer as previously described (*Section 3.5.4*). Samples were allowed to rotate at 40rpm for 60 minutes at 4°C in order to further dissolve cellular material. Samples were centrifuged at 13,000g for 10 minutes and the supernatant transferred to a fresh 1.5mL Eppendorf tube. Protein concentration was determined as previously described (*Section 3.5.5*).

Collagen constructs were homogenised by a 2 minute cycle using a tissue lyser (Qiagen Tisssuelyser, Qiagen, UK) in laemmli lysis buffer as previously used within this thesis (*Section 3.5.3*). Samples were allowed to rotate at 40 rpm for 60 minutes at 4°C in order to further dissolve cellular material. 100% 2-propanol (250 µl per 500 µl of lysis buffer) was added and samples were centrifuged at 17,000g for 10 minutes. The supernatant was discarded and the pellet was re-suspended in 300 µl of laemmli lysis buffer and allowed to rotate for a further 60 minutes at 40 rpm at 4°C. Samples were centrifuged for 5 minutes at 13,000g after which, the supernatant was removed and placed in a fresh 1.5 mL Eppendorf tube. Protein concentration was quantified as previously described (*Section 3.5.5*).

7.2.7 SDS-PAGE and Western Blots

Based on previous optimisation experiments (*Section 3.5.6*), 5 µg of protein was loaded onto 7.5% SDS-PAGE gels. Gels were run by electrophoresis for western blot as previously described (*Section 3.5.7*).

7.2.8 Measurement of ^{3}H-Deoxy-D-Glucose uptakes in fibrin skeletal muscle tissue engineered constructs.

Glucose uptake was measured as previously described (*Section 3.6.2*). In brief, following [3]H-2DG exposure, constructs were removed from the sutures, blotted and weighed. Individual constructs were dissolved in a 1:10 (wt/vol) dilution of 1M potassium hydroxide (KOH)

solution, placed in boiling water for 2 min, placed on ice for 5 minutes. After this, samples were neutralized by adding 1M HCI in a 1:10 (wt/vol) dilution of construct wet weight, and 20 µl of each sample was placed in a scintillation vial and counted on a scintillation counter.

The calculation of ^{3}H-2DG in these constructs was determined as previously described (*Section 3.6.3*). The amount of ^{3}H-2DG uptake was made relative to the wet weight of each construct.

7.2.9   Statistical analysis

Statistical analysis was conducted using SPSS statistical software (IBM SPSS, Version 19, NY, USA) by analysis of covariance (ANOVA) with Bonferroni post-hoc comparisons to identify any differences between conditions and stimulation. Comparisons between conditions for gel width and protein concentrations were conducted using independent t-test. Non-parametric kruskal-wallis test was conducted on data which violated homogeneity of variance and/or normal distribution. All data is presented as mean ± standard deviation. Significance was set at an alpha level of $p < 0.05$. Data from fibrin constructs is presented as an accumulation of 4 constructs per condition from two independent sets of cells. Data from collagen constructs is an accumulation of three constructs per condition (*n=3*) with cells ranging from passage 4-10.

7.3 Results

In this Chapter, two models of skeletal muscle tissue engineering were used to investigate their molecular and functional responses to chronic and acute insulin exposure during maturation. Previously in monolayer culture (*Chapter 6*), chronic insulin exposure to skeletal muscle cells during differentiation resulted in the greatest changes in both molecular and physiological response. Therefore, both skeletal muscle constructs were exposed to chronic insulin media for the same duration (10 days) during differentiation.

7.3.1 Macroscopic and microscopy observations in tissue engineered constructs

7.3.1.1. *Fibrin tissue engineered constructs*

In the previous Chapter markers of myogenic differentiation and development were found to remain stable during incubation in maintenance media (*Chapter 5*). Insulin has been shown to affect proliferation and differentiation of cell in culture (Vandenburgh, Karlisch, Shansky, & Feldstein, 1991) and therefore, incubation with insulin began at this time point. Macroscopic (*Figure 7-2A*) and microscopic images (*Figure* 7-3) were obtained following exposure to control (7% FBS, 1% P/S) or insulin (7% FBS 1% P/S, 100 nM Insulin) maintenance media, which did not affect final gel width t (30) = 1.071 p = 0.293 (*Figure 7-2B*).
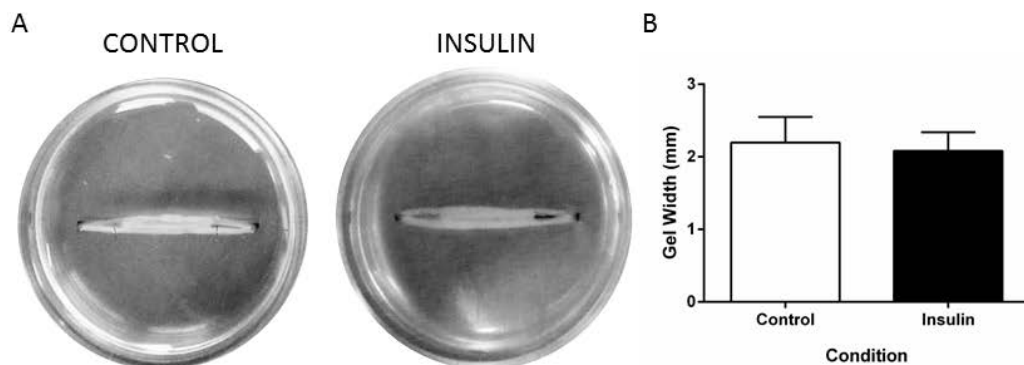
Figure 7-2 A; Macroscopic images of fibrin gel skeletal muscle constructs following either control or chronic insulin exposure. B; Gel width measurement of fibrin gels incubated in the presence of absence of insulin. Data is mean ± SD from 16 constructs per condition.
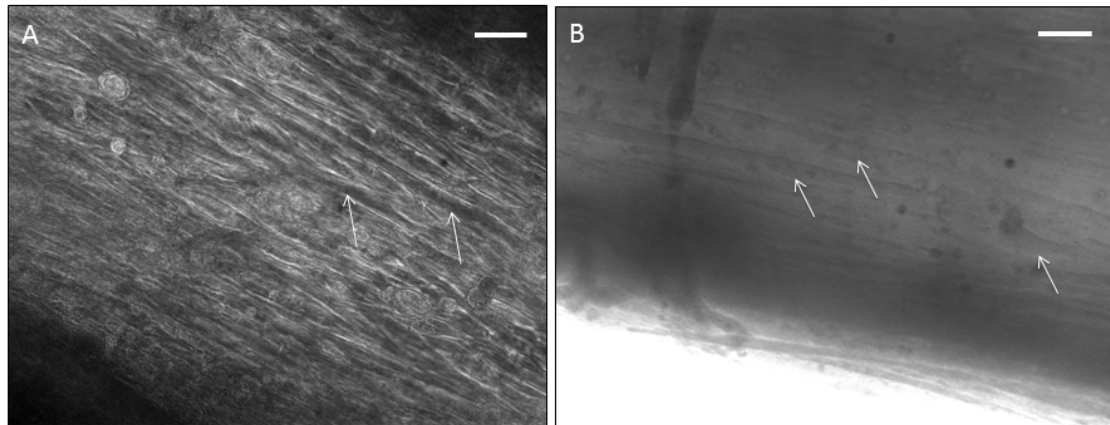
Figure 7-3 Phase contrast images of fibrin gel skeletal muscle constructs following incubation in either A; control or B; insulin supplemented media. Arrow denotes myotubes in fibrin gels. Scale bar = 100μm.

7.3.1.2. *Collagen tissue engineered constructs*

Following a 4 day growth period, collagen skeletal muscle tissue engineered constructs were incubated, in differentiation media supplemented without or with insulin (100 nM). Macroscopic images were obtained to analyse gel width at the end of the experimental period and compared by independent t-test (*Figure 7-4A*). Construct width was measured and subsequently showed a significant difference between conditions t (14) = 5.883 p < 0.001 (*Figure 7-4B*). The development of myotubes was observed by phase contrast microscopy (*Figure 7-4C*), which showed clear formation skeletal muscle myotubes in both control and insulin treated constructs.
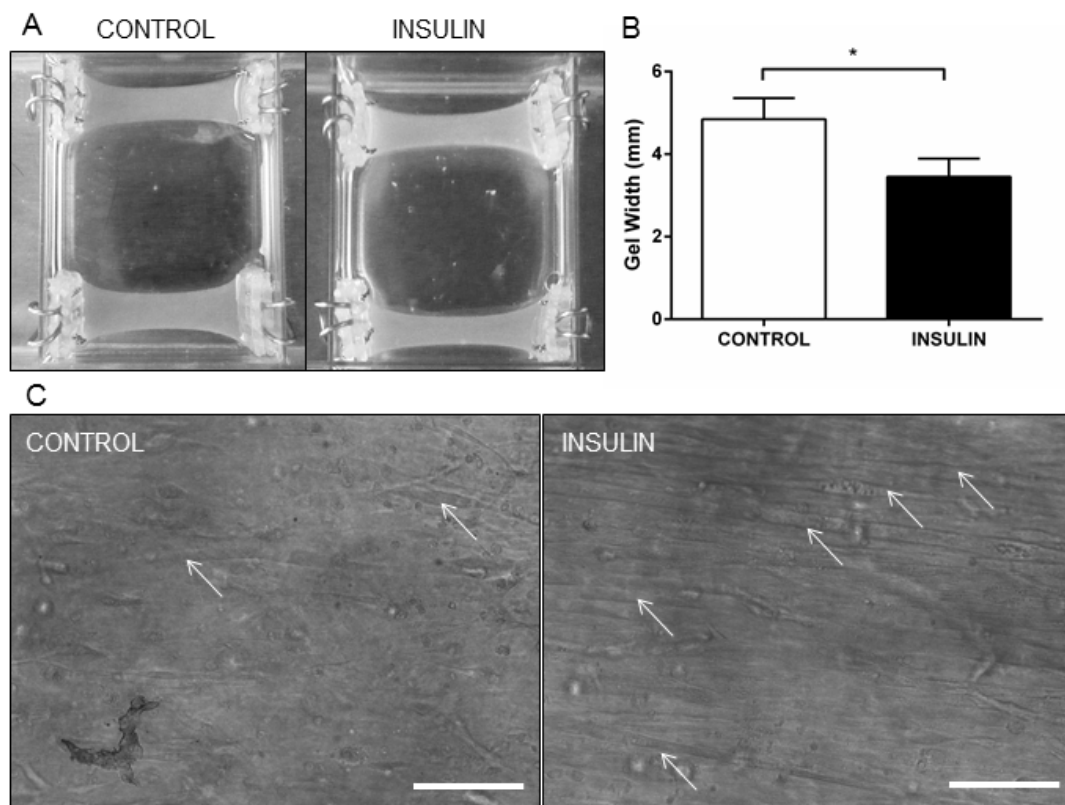
*Figure 7-4 Macro and microscopic images of collagen gel skeletal muscle constructs following 10 days incubation in either control or insulin supplemented media. A; Macroscopic images of 1 mL skeletal muscle collagen gels. B; Gel width measurement of collagen gels incubated in the presence or absence of insulin (100 nM) for 10 days. Data is means ± SD from 6 constructs per condition. Phase contrast images of skeletal muscle myotubes in collagen gels following incubation in control of insulin supplemented media. Arrows denote the presence of myotubes. *Significant difference between conditions p <0.05. Scale bar = 100µm.*

7.3.2   Chronic insulin exposure upon mRNA expression in tissue engineered constructs.

7.3.2.1.   *Fibrin Skeletal Muscle Constructs*

As previously conducted in monolayer cultures, the mRNA expression of HKII, PGC-1α, MyoG (key transcription factors involved in metabolism and myogenic development) were investigated in fibrin based constructs following incubation with or without insulin. GLUT4 mRNA expression was significantly affected by acute insulin stimulation $F_{(1, 10)} = 7.269$, $p = 0.012$, with mRNA expression increasing following acute stimulation in constructs which were chronically treated with insulin ($p = 0.004$), yet no significant difference between control constructs was observed (*Figure 7-5A*). Myogenin expression showed no differences between conditions $F_{(1, 11)} = 3.168$, $p = 0.103$, or as a result of acute insulin stimulation $F_{(1, 11)} = 1.286$, $p = 0.281$ (*Figure 7-7*). Surprisingly, PGC-1α expression was not effected by acute insulin stimulation $F_{(1, 11)} = 2.775$, $p = 0.124$, or by chronic exposure to insulin $F_{(1, 11)} = 0.078$, $p = 0.786$. HKII mRNA expression data was found to have violated assumptions of the ANOVA by significant Levine statistics for homogeneity of variance, and shaprio-wilk statistics for normal distribution. Therefore, non-parametric test were conducted using kruskal-wallis test, which showed no significant differences between groups in for HKII mRNA expression $H_{(3)} = 0.800$, $p = 0.874$ (*Figure 7-6*).

*Figure 7-5 mRNA expression (ΔΔC^t) in fibrin skeletal muscle constructs following insulin exposure. Constructs were exposed to control media or high insulin media for a period of 10 days in culture before being unstimulated (open bars) or stimulated (filled bars) with 100 nM insulin for 30 minutes A) GLUT4, B) PGC-1α. * Significantly different vs. unstimulated condition (p <0.05). Data is mean ± SD of 4 constructs per condition derived from two independent experiments.*

Figure 7-6 Hexokinase II (HKII) mRNA expression (ΔΔC^t) in fibrin skeletal muscle constructs following chronic insulin exposure. Constructs were exposed to control media or high insulin media for a period of 10 days in culture before being unstimulated (open bars) or stimulated (filled bars) with 100 nM insulin for 30 minutes. Data is mean ± SD of 4 (N=4) constructs per condition derived from two independent experiments.

Figure 7-7 Myogenin (MyoG) mRNA expression ($\Delta\Delta C^t$) in fibrin skeletal muscle constructs following chronic insulin exposure. Constructs were exposed to control media or high insulin media for a period of 10 days in culture before being unstimulated (open bars) or stimulated (filled bars) with 100 nM insulin for 30 minutes. Data is mean ± SD of 4 (N=4) constructs per condition derived from two independent experiments.

Gene of Interest	Control - Unstimulated	Control - Stimulated	Insulin - Unstimulated	Insulin - Stimulated
HKII	22.18 ± 0.38	21.86 ± 0.27	22.03 ± 1.07	22.12 ± 0.89
GLUT4	26.57 ± 0.40	25.87 ± 0.33	26.67 ± 0.74	25.79 ± 0.28
PGC-1α	26.50 ± 0.42	25.91 ± 0.25	25.91 ± 1.01	25.92 ± 0.46
MyoG	23.34 ± 1.21	22.61 ± 0.35	23.29 ± 1.57	23.31 ± 0.60

7.3.2.2. *Collagen Skeletal Muscle Constructs*

Within native tissue, skeletal muscle fibres are surrounded by the extracellular matrix which is predominantly in the form of collagen (Kjaer, 2004). The ECM plays an important role in the function of metabolic tissues, and, in the case of skeletal muscle has been shown to be implicated in insulin stimulated glucose uptake (Berria et al., 2006). While the use of collagen as a biological hydrogel in 3D tissue engineering is common within the literature (Cheema et al., 2003; Player et al., 2014; Sharples et al., 2012), currently no work has investigated the role of insulin exposure upon collagen tissue engineered skeletal muscle constructs.

Hexokinase II (HKII) is an important protein regulated the early stages of glucose metabolism (Bouché et al., 2004) and is responsive to insulin at the mRNA level (Vestergaard et al., 1995). Although, there was no significant effect of acute 30 minute stimulation upon HKII mRNA expression $F_{(1, 12)} = 0.059$, $p = 0.814$, chronic insulin exposure significantly increased HKII expression compared to control constructs $F_{(1, 12)} = 7.952$, $p = 0.025$ (*Figure 7-8A*). Similarly, the mRNA expression of the insulin stimulated glucose transporter, GLUT4, was not affected by acute stimulation $F_{(1, 12)} = 0.089$, $p = 0.762$, however, in contrast to an increase in HKII expression, chronic insulin treatment significantly lowered the expression of GLUT4 $F_{(1, 12)} = 8.448$, $p = 0.023$ (*Figure 7-8B*). The expression of myogenin mRNA was not significantly different in between conditions $F_{(1, 7)} = 0.955$, $p = 0.361$, however its expression levels were significantly affected by acute stimulation $F_{(1, 7)} = 11.270$, $p = 0.012$ in control samples (*Figure 7-9*). A one-way ANOVA was conducted to compare means between conditions for changes in PGC-1α expression. There was no significant differences in PGC-1α expression between conditions $F_{(3)} = 0.202$, $p = 0.892$ (*Figure 7-10*).

Figure 7-8 mRNA expression ($\Delta\Delta C^t$) in collagen skeletal muscle constructs following chronic insulin exposure. Constructs were exposed to control media or high insulin media for a period of 10 days in culture before being exposed to an acute insulin challenge A) HKII, B) GLUT4. Open bars represent unstimulated constructs and filled bars represent stimulated (100 nM insulin) constructs. * Significantly different between conditions (p <0.05). Data is mean ± SD of 3(N=3) constructs per condition.

Figure 7-9 Myogenin mRNA expression ($\Delta\Delta C^t$) in collagen skeletal muscle constructs following chronic insulin exposure. Open bars represent unstimulated constructs and filled bars represent stimulated (100 nM insulin) constructs. * Significantly difference between control unstimulated constructs. Data is mean ± SD of 3 constructs per condition.

Figure 7-10 PGC-1α mRNA expression (ΔΔC^t) in collagen skeletal muscle constructs following chronic insulin exposure. Open bars represent unstimulated constructs and filled bars represent stimulated (100 nM insulin) constructs. Data is mean ± SD of 3 constructs per condition.

Gene of Interest	Control - Unstimulated	Control - Stimulated	Insulin - Unstimulated	Insulin - Stimulated
HKII	23.01 ± 0.73	23.49 ± 0.47	21.91 ± 0.55	21.94 ± 0.36
GLUT4	26.59 ± 0.64	27.26 ± 0.80	26.64 ± 0.60	26.54 ± 0.70
PGC-1α	25.53 ± 0.62	25.63 ± 0.54	25.08 ± 0.39	24.87 ± 0.43
MyoG	21.88 ± 0.55	22.69 ± 0.64	21.40 ± 0.82	21.77 ± 0.61

7.3.3 Chronic insulin exposure increases protein concentrations in tissue engineered constructs

In addition to quantifying loading volumes for subsequent immunoblotting of insulin signalling proteins, protein concentrations as previously described (*Section 3.5.5*) to determine potential effects of chronic insulin exposure upon construct protein concentrations. Independent samples t-test was conducted between control and insulin exposed samples which showed insulin to significantly increase protein concentrations compared to control constructs in both fibrin t (14) = -2.323 p =0.036 (*Figure 7-11A*), and collagen tissue engineered constructs t (10) = -2.318 p =0.043 (*Figure 7-11B*).
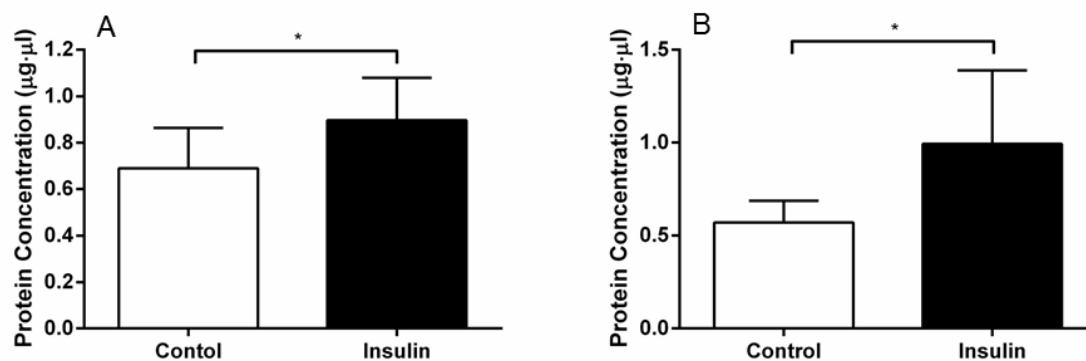
*Figure 7-11 Protein concentrations (µg·µl) from A) fibrin and B) collagen skeletal muscle tissue engineered constructs exposed to control (Open) or chronic insulin (Filled) media. * Significantly different vs. control condition (p <0.05). Data is mean ± SD of 8 constructs per condition in fibrin and 6 constructs per condition in collagen from two independent experiments.*

7.3.4 Insulin signalling protein response in skeletal muscle tissue engineered constructs

Exposure of insulin to skeletal muscle cells results in a cascade of events which enables facilitated glucose uptake into the cell. Several proteins are involved in the process of insulin stimulated glucose uptake of which a select group have been characterised as a 'critical node' of insulin signalling (Taniguchi et al., 2006). One of these proteins, Akt plays an important role within the insulin signalling cascade and within previous Chapters has been shown to increase as a result of insulin stimulation

7.3.4.1. *Fibrin skeletal muscle constructs*

The expression of the p85 subunit, PI-3 kinase was not affected by acute $F_{(1, 7)} = 0.19$, $p = 0.894$ or chronic insulin stimulation $F_{(1, 7)} = 0.020$, $p = 0.892$ (*Figure 7-13*). Similarly, despite a small increase in phosphorylation of Akt at serine 473 (Ser^{473}), there was no significant effect of acute insulin stimulation $F_{(1, 4)} = 0.549$, $p = 0.500$, $r = 0.121$ or chronic insulin exposure $F_{(1, 4)} = 4.136$, $p = 0.112$, $r = 0.501$ upon Ser^{473} phosphorylation (*Figure 7-12A*). Furthermore, there was no significant effect upon total Akt protein expression as a result of acute $F_{(1, 8)} = 0.514$, $p = 0.494$, or chronic insulin exposure $F_{(1, 8)} = 0.105$, $p = 0.754$ (*Figure 7-12B*).
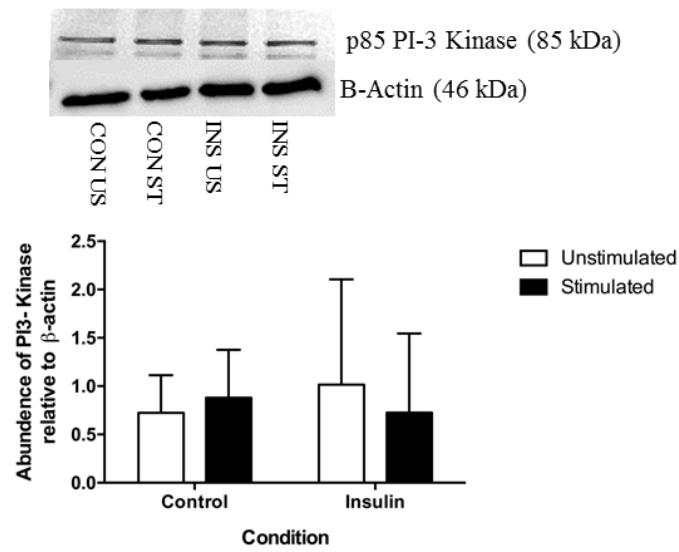
Figure 7-12 Western blots analysis of p85 PI-3 kinase following acute and chronic insulin stimulation. Constructs were either left Unstimulated (open bars) or stimulated (filled bars) with 100 nM insulin for 30 minutes. Representative images of western blots for p85 PI-3 kinase and B-actin. Data is mean ± SD of 3 constructs per condition.
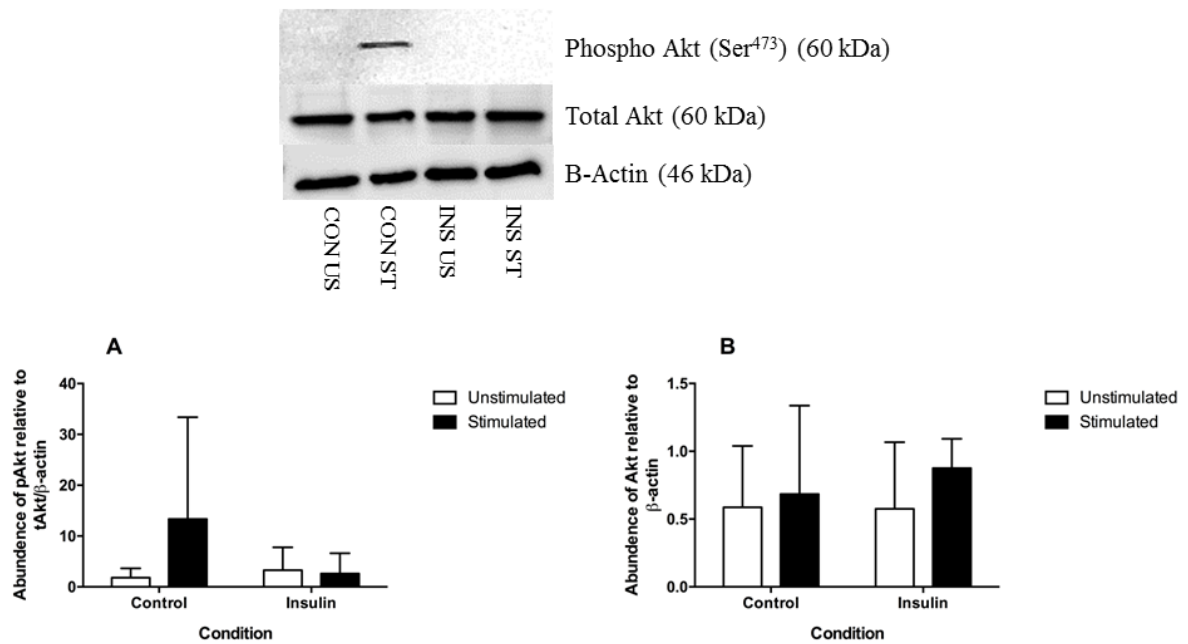
Figure 7-13 Western blots analysis of total Akt and phosphorylation of Akt (Ser^473) following acute and chronic insulin stimulation. Constructs were either left Unstimulated (open bars) or stimulated (filled bars) with 100 nM insulin for 30 minutes. A; phosphor Akt Ser^473 B; total Akt, Data is mean ± SD of pAkt N=2 constructs per condition. Data is mean ± SD of tAkt N=3 constructs per condition.

7.3.4.2. *Collagen skeletal muscle constructs*

Protein expression of the p85 subunit of PI-3 kinase was also not affected by acute insulin stimulation $F_{(1, 8)} = 0.0339$, $p = 0.577$, however there was a trend toward significance an increase in expression within chronically exposed constructs $F_{(1, 8)} = 4.957$, $p = 0.057$, $r = 0.380$ (*Figure 7-15*).Optimisation of insulin stimulation within collagen tissue engineered constructs showed an increase in phosphorylation of Akt as a result of acute insulin stimulation (*Figure 7-14A*). Although there was a tendency for an increase in Akt Ser[473], phosphorylation (*Figure 7-14B*), there was no significant effect of acute stimulation $F_{(1, 7)} = 2.248$, $p = 0.177$, $r = 0.243$ or effect of chronic insulin exposure $F_{(1, 7)} = 2.2015$, $p = 0.199$, $r = 0.224$. Furthermore, there was no effect of acute stimulation $F_{(1, 7)} = 0.052$, $p = 0.792$ or chronic exposure $F_{(1, 7)} = 0.052$, $p = 0.826$ upon total Akt expression (*Figure 7-14C*).
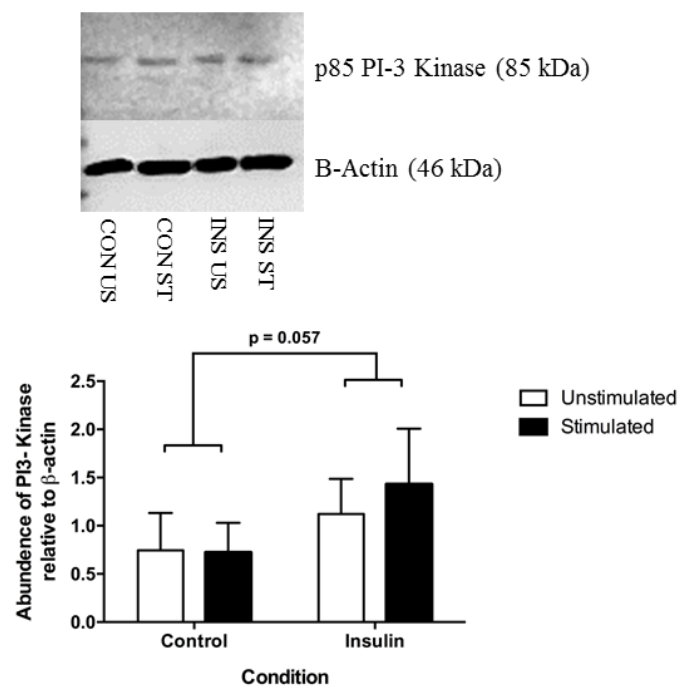
Figure 7-14 Western blots analysis of p85 PI-3 kinase following acute and chronic insulin stimulation. Representative images of western blots for p85 PI-3 kinase and B-actin. Data is mean ± SD of 3 constructs per condition.
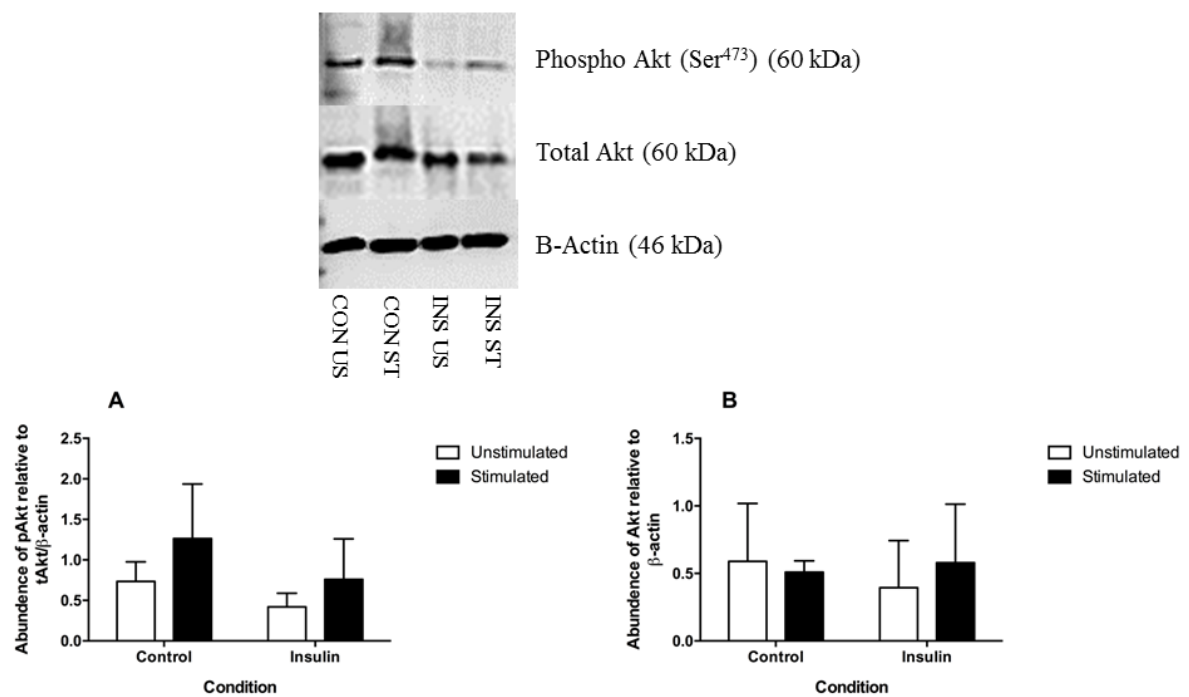
Figure 7-15 Western blots analysis of total Akt and phosphorylation of Akt (Ser^{473}) following acute and chronic insulin stimulation in collagen gel skeletal muscle constructs. A; phospho Akt Ser^{473} graph B; total Akt graph. . Data is mean ± SD of 3 constructs per condition.

7.3.5   Glucose uptake of fibrin skeletal muscle constructs

As outlined within the previous Chapters of this thesis (*Chapter 4*) the measurement of glucose uptake through the use of radioactive tracers, in particular tritium labelled (^{3}H) glucose analogue 2-D-oxy-d-glucose (^{3}H-2DG) was used to investigate changes in insulin stimulated glucose uptake in fibrin tissue engineered skeletal muscle constructs. Despite a mean increase in ^{3}H-2DG uptake within control fibrin based skeletal muscle constructs following 30 minutes insulin stimulation, there was no significant difference in insulin stimulated glucose uptake within these constructs t (10) = -1.831 p =0.09 (*Figure 7-16*). However, the differences between groups showed there to be a large effect of insulin stimulation upon glucose uptake in these constructs (r = 0.50).
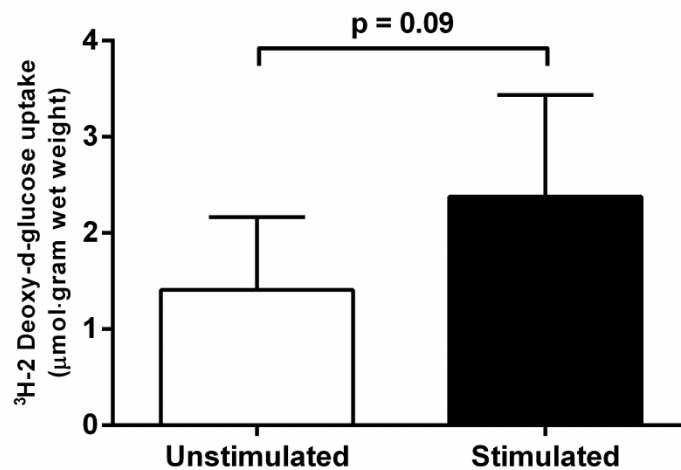
Figure 7-16 Insulin stimulated glucose uptake (^{3}H-2DG) in fibrin based skeletal muscle constructs. Constructs were incubated for 30 minutes in the presence or absence of insulin followed by 30 minutes ^{3}H-2DG incubation. Data is mean ± SD from 6 constructs per condition.

7.4 Discussion

Skeletal muscle is responsible for the majority of insulin stimulated glucose uptake (DeFronzo, Tobin, & Andres, 1979), however impaired glucose clearance within skeletal muscle is a major symptom of metabolic disease and often precedes the onset of metabolic diseases such as diabetes *mellitus* (Abdul-Ghani and Defronzo, 2010). While the use of monolayer cell culture continues to be utilised to investigate the complexities of insulin signalling and glucose uptake within skeletal muscle (Rudich and Klip, 2003; Sell et al., 2012), little research has investigated the response of skeletal muscle to insulin in three-dimensional culture at the molecular level (Baker et al., 2003). Therefore, the objective of this Chapter was to investigate insulin signalling and glucose uptake within three-dimensional skeletal muscle tissue engineered models.

7.4.1 Protein concentration differences in tissue engineered constructs following chronic insulin exposure

Although protein concentrations were primarily used to quantify the loading volume for subsequent immunoblotting, in both fibrin and collagen constructs, chronic insulin exposure increased protein concentration. These findings support previous research in skeletal muscle tissue engineered constructs which have shown large increases in protein concentration following incubation with insulin (Vandenburgh, Karlisch, Shansky, & Feldstein, 1991). A number of protein-protein interactions, particularly within the proximal insulin-signalling cascade affect a number of signalling pathways (Virkamäki, Ueki, and Kahn, 1999). Although some of these signalling pathways have known mechanisms, some, such as those which affect structural proteins (integrin's) and are implicated in the function of metabolic tissues (Huang et al., 2012), have been shown to increase IRS-1 phosphorylation independent of glucose transport (Bruning et al., 1998). While the mechanisms by which the increase in protein concentrations occur within these constructs as a result of chronic exposure to insulin requires further investigation, previous literature has shown constituently activation of Akt result in skeletal muscle hypertrophy (Bodine et al., 2001; Rommel et al., 2001). Due to the role of insulin upon phosphorylation of this protein and the various cellular functions Akt is implicated in (Schultz et al., 2000), it is clear that insulin doses exert a hypertrophic effect upon skeletal muscle in both fibrin and collagen tissue engineered constructs.

7.4.2   Insulin signalling response in skeletal muscle tissue engineered constructs

Although the role of insulin and glucose stimulation within three-dimensional culture has focused predominantly around β-cell secretion, stimulation and survival (Weber et al., 2008), currently no research has investigated skeletal muscle insulin signalling and glucose uptake in this environment. Optimisation of acute insulin stimulation on these constructs showed that 30 minutes stimulation within insulin (100 nM) would increase phosphorylation of Akt Ser[473] (*Section 3.6.2*).

7.4.2.1.   *Insulin signalling in fibrin skeletal muscle tissue engineered constructs*

The use of fibrin skeletal muscle tissue engineering constructs has provided an *in vitro* model for investigation of skeletal muscle force production (Huang et al., 2005; Khodabukus & Baar, 2009). However more recent research has used these models to investigate changes skeletal muscle phenotype *in vitro* (Khodabukus and Baar, 2014a). These current experiments are advancement on the current work in these models, in which the effects of hormonal exposure used to manipulate the molecular responses in these constructs. Although there was not a significant effect of acute insulin stimulation upon phosphorylation of Akt, the observed increase in serine phosphorylation and effect size observed suggest a level of biological effect as a result of acute stimulation in these constructs. The incubation period with insulin is similar to what has previously been shown to induce Akt phosphorylation of Akt in *ex vivo* skeletal muscle (Saini et al., 2014). Insulin stimulation *in vitro* has shown to range from five minutes, as conducted in the previous Chapter (*Chapter 6*), up to 30 minutes plus in skeletal muscle cells (Al-Khalili et al., 2003; Sell et al., 2008). The use of tissue engineered skeletal muscle aims to bridge the gap between monolayer, 2D cultures, *ex vivo* and *in vivo* animal models and therefore further time points would give further insight into the phosphorylation Akt as well as other proteins involved in the insulin signalling pathway. Previous research using C2C12's in fibrin skeletal muscle constructs has shown there to be high variability between clones of constructs and therefore the large variation in insulin signalling response is mostly like resultant of the batch to batch variation in their physiological and functional responses that have been associated with these constructs (Khodabukus et al., 2009).

7.4.2.2.   *Insulin signalling in collagen skeletal muscle tissue engineered constructs*

The use of collagen (collagen type I), has frequently been used as a representative matrix that present morphological and functional characteristics of the skeletal muscle ECM *in vitro*

(Cheema et al., 2003; Player et al., 2014; Sharples et al., 2012). The ECM plays an important role in metabolic tissues (Berria et al., 2006), and the ding of cells within a collagen matrix can improve insulin and glucose response (Weber et al., 2008). Although these constructs did respond to acute insulin exposure, the increase in phosphorylation of Akt was not significantly different within control conditions. However unlike findings observed in fibrin, there was a small but not significant increase in phosphorylation of Akt. With the exception of time in differentiation media and the ECM, these experiments involved exposure of insulin within the same differentiation media as experiments performed in monolayer culture. It is unsurprising that the results observed between the two culture systems are similar. Although further work is required in order to establish if insulin stimulated glucose uptake within these constructs is affected by the extracellular matrix, such variables are important to consider in further characterisation of this culture model for investigating insulin stimulated glucose uptake.

In addition to the phosphorylation of Akt, like in monolayer, the p85 subunit of PI-3 kinases was investigated following acute and chronic insulin exposure. PI-3 kinase is an important mediator of insulin signalling (Shepard, 1999) and is activated by associated activity with the IRS-1, ultimately leading to recruitment and phosphorylation of Akt downstream (Shepherd et al., 1998). Chronic insulin exposure to collagen skeletal muscle constructs showed there to be a trend toward significance ($p=0.057$) in expression levels of p85 PI-3 kinase. Expression of p85 has been shown to be important in recruitment and facilitation of proximal insulin signalling and subsequently is the predominant isoform which is recruited upon insulin stimulation (Shepherd et al., 1998). However, recruitment of p85-p110 heterodimers for effective insulin signalling can be effected by overexpression of p85 monomers. Research in skeletal muscle from rodents has shown that increases in p85 monomers result in impaired insulin signalling (Bandyopadhyay et al., 2005; Ueki et al., 2000) and the these findings which were replicated in monolayer culture (*Chapter 6*), could explain the alterations in insulin signalling as a result of chronic insulin exposure in culture and potentially show that the signalling responses to insulin are translatable between monolayer cultures and collagen tissue engineered skeletal muscle constructs.

7.4.3 Transcriptional response to insulin in skeletal muscle tissue engineered constructs

In addition to the primary role of glucose uptake, insulin contributes to both positive and negative transcriptional regulation (O'Brien and Granner, 1996). Although insulin has been shown to regulate are large number of genes *in vivo* (Rome et al., 2003), a small number have been investigated in their role of glucose metabolism (Ducluzeau et al., 2001).

7.4.3.1. *Transcriptional responses to insulin in fibrin tissue engineered skeletal muscle constructs*

Understanding the transcriptional responses of fibrin skeletal muscle constructs was a novel insight into the effects of insulin upon these models. Although a number of factors including serum origin and type have been found to effect a number proteins involved in glucose metabolism of fibrin C2C12 fibrin skeletal muscle constructs (Khodabukus and Baar, 2014a, 2014b), the current work is the first to investigate mRNA expression using this cell line. The insulin stimulated glucose transporter, GLUT4, is an important candidate at both the transcription and protein levels for understanding insulin stimulated glucose uptake. In fibrin skeletal muscle constructs, acute insulin significantly increased GLUT4 mRNA expression following chronic insulin exposure. Acute stimulation with insulin has been shown in both cell culture (Yu et al., 2001) and *ex vivo* skeletal muscle (Moraes et al., 2014).

As discussed in previous Chapters (*Section 2.8.3*), metabolic genes, which have been shown to be responsive to insulin such as, PGC-1α, and HKII and subsequently altered in monolayer cultures, showed no changes in expression following chronic or acute insulin stimulation. is an important regulator of metabolism in skeletal muscle (Olesen et al., 2010). As well as its role in the regulation of GLUT4 expression within skeletal muscle cells (Michael et al., 2001). These findings are surprising due to those previously observed in monolayer cultures (*Chapter 6*) as well as those recently reported by Khodabukus (Khodabukus and Baar, 2014a, 2014b). Indeed, the only previous work which has reported transcriptional changes in these constructs, investigated the development and maturation of human skeletal muscle cells in this system (Martin et al., 2013). Neither acute nor chronic insulin exposure resulted in changes in mRNA expression of myogenin in fibrin skeletal muscle constructs (*Figure 7-7*). Previous experiments (*Chapter 5*), which showed that these constructs maintain a stable myogenic expression in maintenance media during skeletal muscle development, also show that this gene is not affected by exposure to insulin during the same time frame. This would

therefore suggest that the increase in protein concentration observed in these constructs (*Figure 7-11*) is not resultant of further myogenin-regulated differentiation of non-differentiated skeletal muscle cells.

7.4.3.2. *Transcriptional responses to insulin in collagen tissue engineered skeletal muscle constructs*

However in the collagen constructs, chronic insulin treatment resulted in a mean reduction in the expression of GLUT4. Both *in vivo* and *in vitro* research has shown there to be differential effects upon GLUT4 expression in response to insulin depending upon incubation periods and insulin concentrations observed (Moraes et al., 2014). As reported in the previous Chapter (*Chapter 5*), GLUT4 mRNA expression showed a significant increase during early skeletal muscle development (Day 2 of differentiation) and a subsequent return to basal levels thereafter. It could be speculated that the reduction in expression following insulin treatment could accelerate this transcriptional response in these constructs, Alternatively, insulin resistance and diabetes has been shown to reduce both protein and mRNA expression of GLUT4 in skeletal muscle (Camps et al., 1992). The exact cause of this response is not currently know and further research is required to investigate if the changes in mRNA expression following chronic insulin exposure result in a translational response to GLUT4 protein expression either in a positive or negative fashion. Particularly as the mRNA expression of HKII, was shown to be increased following chronic insulin treatment. Acute insulin stimulation has been shown to increase HKII mRNA in response to insulin *in vivo* (Pendergrass et al., 1998) and *in vitro* (Printz et al., 1993). However, this is shown to be attenuated in insulin resistant human who present hyperglycaemia and hyperinsulinemia symptoms (Vestergaard et al., 1995), and diabetic animal models (Burcelin et al., 1993). However, collagen constructs which were exposed to chronic insulin had an increase in HKII mRNA expression. The increase in expression of HKII as a result of exposure to a hyperinsulineamic environment could suggest that this environment is not responsible for the impaired HKII expression observed *in vivo*, but rather other systemic factors which are associated with metabolic diseases (Vestergaard et al., 1995). In contrast, the response n with in these constructs are potentially more indicative of the transcriptional responses associated with acute physiological hyperinsulinemia which increases, but no exclusively, skeletal muscle HKII mRNA expression (Coletta et al., 2008).

A surprising finding in collagen skeletal muscle constructs was the attenuated myogenin expression following acute stimulation (*Figure 7-9*). Both monolayer and fibrin skeletal muscle constructs showed either no change or an increase in MyoG expression following acute stimulation. Previous research using IGF-1 to induce skeletal muscle differentiation has shown opposing effects upon myogenin mRNA expression during skeletal muscle differentiation (Adi et al., 2000). Due to the similarities in structure and function of insulin and IGF-1 (Werner, Weinstein, and Bentov, 2008), further explanation to the effects of insulin upon the regulation of skeletal muscle myogenisis in collagen skeletal muscle tissue engineered constructs is required.

7.4.4   Insulin stimulated glucose uptake in fibrin skeletal muscle constructs

In order to investigate the function response of these constructs to insulin stimulation, glucose uptake was assessed by ^{3}H-2DG as performed within previous Chapters of this thesis (*Chapter 4*). Currently, only one paper has previously investigated the effect of insulin stimulated glucose uptake in tissue engineered constructs (Baker et al., 2003). Although there was a large effect ($r = 0.50$), there was no significant difference between unstimulated and insulin stimulated glucose uptake. Skeletal muscle tissue engineered constructs have been shown to present with a high level of basal uptake and although the basal level observed in the current experiments is not as high as that previously reported in primary cell constructs (Baker et al., 2003). Nonetheless, C2C12 themselves have a high basal glucose turnover in monolayer cultures (Nedachi and Kanzaki, 2006) and these characteristics are also present when these cells are grown in a three-dimensional environment. The elevated basal turnover is indicative of early skeletal muscle development during which the expression of the basal glucose transporter GLUT1, is the predominant mediator of glucose turnover (Santalucía et al., 1992). Therefore, although these constructs respond to insulin, similar to primary skeletal muscle cells in three-dimensional constructs C2C12 skeletal muscle in fibrin tissue engineered constructs.

7.5   Conclusions

In this Chapter, two types of skeletal muscle tissue engineered construct were investigated for their transcriptional and molecular signalling response to acute and chronic insulin exposure. In fibrin constructs, chronic insulin exposure has little effect upon construct width despite small but significant increases in protein concentration. In addition, while protein phosphorylation increased, but not significantly, there was little effect upon the transcriptional response to insulin within these constructs. In contrast, collagen skeletal

muscle constructs showed a response to insulin through significant differences in gel width and protein concentration, and although protein phosphorylation increased but was not significant, these constructs showed to be effected by insulin exposure at the transcriptional level through significant changes in HKII and GLUT4 expression.

8. Discussion

8.1 General Discussion

Using the well-established C2C12 skeletal muscle cell line, a series of experiments were conducted in conventional monolayer culture and in three-dimensional tissue engineered constructs, to investigate the molecular response to insulin in these culture models. By exposing these models to insulin for acute and chronic periods, both 2D and 3D models were investigated for their molecular and functional responses to acute and chronic insulin exposure. In particular, the effect of insulin upon three-dimensional skeletal muscle constructs would provide the first insight into the potential use of skeletal muscle tissue engineering constructs as a novel *in vitro* model to investigate skeletal muscle insulin signalling and glucose uptake.

Research investigating insulin signalling and glucose metabolism continues to be a major priority due to the increasing global prevalence of metabolic diseases, such as diabetes *mellitus* (Zimmet, Alberti, and Shaw, 2001). A combination of *in vivo* human trials (Krook et al., 2000), animal (Joost et al., 2012), and cell culture models (Sarabia et al., 1990; Sell et al., 2012), are used to understand the physiological and patho-physiological factors associated with insulin signalling and glucose uptake. The use of cell culture models can be a useful model to better investigate the intricacies of insulin stimulated glucose uptake (Rudich and Klip, 2003). While the use of different cell types, primary or cell lines, have been used to study insulin signalling in skeletal muscle, the use of different models of cell culture, such as three dimensional skeletal muscle tissue engineered constructs, have not been used before.

Techniques such as tissue engineering of skeletal muscle are enabling advancements in regenerative medicine (Juhas et al., 2014), but are also providing a platform to investigate skeletal muscle physiology and function (Cheng et al., 2014; Khodabukus et al., 2007), due to the ability of these models to recapitulate skeletal muscle tissue *in vitro* (Tibbitt and Anseth, 2009). However, the use of these models to investigate glucose uptake and in particular insulin signalling has not be investigated extensively despite these culture models systems being identified as a key area for investigation within the field (Cheng et al., 2014). Development of tissue engineered skeletal muscle models which respond to insulin at the molecular and functional levels with regards to glucose uptake, would be an advancement upon the current literature which has investigated this area (Baker et al., 2003).

8.2 Key findings

During development, glucose transporters 1 and 4 which are responsible for basal and insulin stimulated glucose uptake respectively, alter their transcriptional expression as skeletal muscle matures to a more insulin responsive tissue where GLUT4 becomes the predominantly expressed glucose transporter (Santalucía et al., 1992). In Chapter 4, both two-dimensional cultures and the two three-dimensional skeletal muscle tissue engineered constructs, differentiation and development of multinucleated myotubes resulted in an increase in the mRNA expression of developmental genes as well as the expression of GLUT4, the insulin stimulated glucose transporter. The increase in GLUT4 mRNA expression in two and three-dimensional cultures provides evidence that all of these models have the potential to express this protein and could be a valid model for investigating insulin stimulated glucose uptake.

Due to the complexity and numerous symptoms that are associated with insulin resistance and diabetes *mellitus* (Abdul-Ghani and Defronzo, 2010), various methods have been used to recapitulate the insulin resistance phenotype of skeletal muscle *in vitro*. For example, the use of cytokines (del Aguila et al., 2011), conditioned media (Eckardt et al., 2008; Sell et al., 2008), and the exposure to a hyperinsulineamic environment (Kumar and Dey, 2003) have all shown to attenuate the insulin signalling pathway and subsequently insulin stimulated glucose uptake. By replicating previously published methodologies (Kumar and Dey, 2003), chronic insulin exposure impaired phosphorylation of key insulin signalling proteins but also increased basal glucose uptake at the expense of insulin stimulated glucose uptake in differentiating skeletal muscle cells. However, these effects were not replicated in skeletal muscle myotubes, suggestive that the greatest effect of insulin exposure upon insulin sensitivity and glucose uptake occurs during regeneration of skeletal muscle.

Providing cells with a three-dimensional extracellular matrix in which to proliferate and differentiate results in cell behaviour which better replicates that observed *in vivo* (Tibbitt and Anseth, 2009). Skeletal muscle tissue engineering has advanced significantly within the past decade and exhibits great utility as a potential model for investigating skeletal muscle physiology and adaption (Baar, 2005; Cheng et al., 2014; Khodabukus et al., 2007). The final Chapter of this thesis aimed to investigate the response of fibrin and collagen skeletal muscle constructs to acute and chronic insulin exposure as well as the effect if any upon insulin stimulated glucose uptake. In both fibrin and collagen based models acute insulin exposure increased phosphorylation of insulin signalling proteins. Although these findings were not

statistically significant, the effect of insulin exposure to these constructs showed a large effect size, potentially a large biological effect. In addition, insulin stimulated glucose uptake was not significantly different from un-stimulated constructs; nonetheless there was a large experimental effect as a result of acute insulin treatment upon glucose uptake. These findings are the first to investigate the effect of insulin stimulated glucose uptake upon fibrin based skeletal muscle tissue engineered constructs, and the first to use an established cell culture line. Although there are a number of factors which require further investigation for the use of these constructs to investigate the physiological responses to insulin, the findings from this Chapter demonstrate a progression in the use of these models for research into insulin signalling and glucose uptake in skeletal muscle from what has previously been published.

As reported in previous research, the most noticeable difference between two-dimensional and three dimensional skeletal muscle tissue engineering constructs is the uniaxial formation of skeletal muscle myotubes within 3D skeletal muscle constructs compared to the randomised formation of myotubes observed in 2D culture (Cheng et al., 2014; Hinds et al., 2011). In addition, both 2D culture and 3D tissue engineered constructs followed a similar transcriptional pattern of myogenisis and development. However, differences in the expression pattern of GLUT4 mRNA during development differed during development between fibrin and collagen constructs. Further research is required in order to determine if the increase in mRNA expression in both of these 3D models results in a translational response. Furthermore, another difference between these culture models is the measurement of insulin stimulated glucose uptake. Although the use of radioactive isotope labelled glucose remains the most sensitive method for measurement of glucose uptake in both 2D (Sell et al., 2012), and 3D skeletal muscle cultures (Baker et al., 2003), the high basal glucose uptake of 3D cultures could have potential implications upon the assay window observed as a result of insulin stimulation. Therefore, in order to further characterise insulin stimulated glucose uptake in 3D skeletal muscle tissue engineering constructs, time and dose responses experiments provide further insight into this phenomena.

Previous literature in the field of skeletal muscle tissue engineering has used larger collagen constructs to investigate skeletal muscle development and molecular physiology (Cheema et al., 2005; Cheema, Yang, Mudera, Goldspink, & Brown, 2003; Player et al., 2014). The use of a scaled down 1mL collagen construct, which developed to form mature myotubes, is a further refinement on previously used models and as a result are a more economic model for investigating transcriptional, translation and function responses. Additionally, the extraction

of cellular protein from these constructs is a novel technique which has been further optimised from previous work in collagen tissue engineered constructs (Player, 2013). As a result, the optimisation of these methods for extraction of cellular protein and visualisation of quantifiable western blots is a significant advancement with respect to investigating cellular signalling pathways in these models.

Table 8.1 Key findings from experimental chapters in this thesis.

Thesis Chapter	Summary
Chapter 4 – *Measurement of glucose uptake in C2C12 skeletal muscle cells using radioactive isotopes and fluorescent based methods.*	- Using a 96 well non-radiolabelled based assay did not provide a sufficient assay window to measure insulin stimulated glucose uptake in skeletal muscle myotubes. - Optimised specific activity of ^{3}H-2DG (1µCi) for investigating insulin stimulated glucose uptake in both monolayer and tissue engineered skeletal muscle constructs. - Incubation of 15 minutes with ^{3}H-2DG was chosen as an appropriate time in the monolayer culture. - Incubation with insulin and ^{3}H-2DG was increased to 30 minutes in tissue engineered constructs.
Chapter 5 – *A time course of transcriptional and translational expression of glucose transporters in C2C12 skeletal muscle cultures.*	- Differentiation of skeletal muscle cells in monolayer, fibrin and collagen tissue engineered skeletal muscle constructs resulted in an increase mRNA expression of the myogenin. - The developmental gene MEF2a, which is an important transcription factor which regulates GLUT4 expression, was increase during differentiation of skeletal muscle cells in monolayer and tissue engineered skeletal muscle constructs. - GLUT4 expression increased with time in differentiation in both monolayer and fibrin skeletal muscle tissue engineered constructs. - GLUT1 mRNA expression was unchanged throughout differentiation in both monolayer and tissue engineered cultures.
Chapter 6 – *The effects of acute and chronic insulin exposure upon C2C12 skeletal muscle cells in monolayer cell culture.*	- Chronic exposure to insulin impaired phosphorylation of insulin signalling proteins in differentiating skeletal muscle cells. - The mRNA expression of metabolic genes was impaired following acute stimulation in differentiating skeletal muscle cells chronically exposed insulin. - Basal glucose uptake in differentiating skeletal muscle cells was increased following chronic insulin exposure. This increase hindered any observation of insulin stimulated glucose uptake in these cultures. - Acute insulin stimulation did not change mRNA expression of skeletal muscle myotubes. - Chronic insulin exposure increased GLUT4 mRNA expression while reducing HKII mRNA expression. - Chronic insulin exposure in skeletal muscle myotubes did not alter insulin stimulated glucose uptake.

Thesis Chapter	Summary
Chapter 7 – *Insulin signalling and glucose uptake in skeletal muscle tissue engineering: The effect of acute and chronic insulin exposure.*	- Acute insulin stimulation showed a trend toward an increase in phosphorylation of Akt (Ser^{473}) in both fibrin and collagen skeletal muscle tissue engineered constructs. - Chronic insulin exposure increased hexokinase II mRNA expression while reducing GLUT4 mRNA expression in collagen skeletal muscle tissue engineered constructs. - Chronic insulin stimulation had no effect upon mRNA expression of metabolic genes in fibrin skeletal muscle tissue engineered constructs. - Incubation time was increased to 30 minutes insulin stimulation and 30 minutes ^{3}H-2DG in tissue engineered skeletal muscle constructs. Insulin stimulated glucose uptake approached significance in fibrin skeletal muscle constructs ($p = 0.09$).

8.3 Future Directions

The development of the methods within this thesis for investigating insulin signalling and glucose uptake in 2D and 3D cultures has the potential to be translated into primary cells across species.

The use of human skeletal muscle cells from donors have been used to investigate insulin signalling and insulin stimulated glucose uptake of skeletal muscle *in vitro* (Aas et al., 2013). The use of this cell type enables for further elucidation of the specific response of the donor to insulin within a controlled *milieu* independent of extracellular factors (Aas et al., 2013). A number of researchers have used human skeletal muscle cells to further understand glucose metabolism in healthy and diseased populations (Al-Khalili et al., 2005; Al-Khalili, Krämer, Wretenberg, and Krook, 2004; Gaster et al., 2002; Henry et al., 1995) (*Table 2-1*). While the use of human skeletal muscle cells from diseased populations differentiate to form skeletal muscle myotubes, (Al-Khalili et al., 2004; Thompson et al., 1996), differences in expression of myogenic genes have shown to differ between insulin resistant and healthy individuals. Similar responses have been reported in healthy human skeletal muscle cells and when exposed differentiated in adipocyte conditioned medium (Sell et al., 2008). None of the aforementioned studies have investigated the use of skeletal muscle cells from diseased populations, in particular insulin resistant or diabetic skeletal muscle cells in 3D tissue engineering. Although recent research using skeletal muscle cells within 3D tissue engineered constructs has established the optimum conditions to culture human skeletal muscle cells from healthy donors in fibrin skeletal muscle constructs (Martin et al., 2013), further research would be required in order to understand if the findings for optimal tissue engineering of healthy human skeletal muscle can be translated to diseased skeletal muscle cells. Indeed, utilisation of 3D human skeletal muscle models for investigation of insulin signalling and glucose uptake would potentially provide further insight into the role of a 3D environment upon development of glucose transporter expression and the response of these cells to insulin, as well as provide a more valid cell culture model for investigating human skeletal muscle metabolism.

In addition to the role of the 3D environment upon glucose transporter expression, the role of innervation upon skeletal muscle glucose transporter expression is important in GLUT4 expression and insulin stimulate glucose uptake mediated through this protein (Jensen et al., 2009; Jones et al., 1998). Within the literature the use of skeletal muscle cells from human

donors (Ciaraldi et al., 1995) and rodent cell lines (Sarabia et al., 1990) have reported increases in basal glucose uptake, which is likely attributed to the increased GLUT1:GLUT4 ratio reported in this cell culture (Nedachi and Kanzaki, 2006; Sarabia et al., 1992), compared to *ex vivo* skeletal muscle (Baker et al., 2003). Subsequently, a number of researchers have attempted to develop and characterise an *in vitro* model of a neuromuscular junction (Larkin et al., 2006; Morimoto et al., 2013). While such models are used to investigate contraction and force generation of 3D tissue engineered models, innervation of skeletal muscle in culture could enable maturation and development of glucose transporters and thus, a mature skeletal muscle model with which to investigate insulin stimulated glucose uptake. Previous literature has established the use of electrical stimulation as a method of innervation, particularly in fibrin skeletal muscle constructs using C2C12 and primary cell sources (Khodabukus and Baar, 2009a; Powell et al., 2002). However, the effect of upon insulin signalling and glucose metabolism using these methods could not only affect the development of these skeletal muscle models, but also a more valid models for investigating insulin independent glucose uptake in skeletal muscle.

Since a number of authors have successfully developed tissue engineered skeletal muscle models to investigate force production (Dennis & Kosnik, 2000; Khodabukus & Baar, 2009; Mudera et al., 2010), a natural line of research is to investigate the effects of electrical field stimulation, in order to mimic a controlled action potential initiation, upon insulin independent signalling pathways and contraction mediated glucose uptake (Richter and Hargreaves, 2013). While previous authors have elucidated to the effects of cyclic stretch (Iwata et al., 2009) and electrical stimulation (Nedachi et al., 2008) upon insulin independent signalling and glucose uptake in C2C12 skeletal muscle cells, currently no research has investigated these parameters in a 3D skeletal muscle tissue engineering model. Recent research by Player and colleagues has elucidated to the effects of mechanical strain upon lactate production in collagen 3D models using the C2C12 cell line (Player et al., 2014) however the effects of mechanical or electrical stimulation upon glucose uptake and insulin independent signalling pathways in three-dimensional tissue engineered constructs are yet to be investigated.

8.4 Conclusions

The work described in this thesis provides a basis for investigating insulin signalling and glucose uptake in two-dimensional and three-dimensional skeletal muscle cultures. These models respond to exposure to insulin and are affected by prolonged incubation with this hormone. In monolayer culture, skeletal muscle cells exposed to insulin during differentiation presented with attenuated phosphorylation of Akt, which is implicated in insulin signalling as well as attenuation of mRNA expression of genes implicated in metabolic function upon acute insulin stimulation. In contrast, post-mitotic skeletal muscle myotubes presented with small responses to acute insulin stimulation, however, were affected by chronic insulin exposure particularly at the mRNA level. In tissue engineered skeletal muscle constructs, both fibrin and collagen skeletal muscle tissue engineered constructs show a tendency to respond to insulin through the phosphorylation of insulin signalling proteins such as Akt. In contrast, these constructs differ in their mRNA expression of metabolic genes following acute and chronic insulin exposure. Further research is required to understand the difference between the tissue-engineered skeletal muscle models and their response to insulin both acutely and chronically. These findings show that skeletal muscle tissue engineered constructs are a model which could be used to further elucidate the mechanisms of insulin signalling and glucose uptake.

9   References

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10 Appendices

10.1 Appendices A

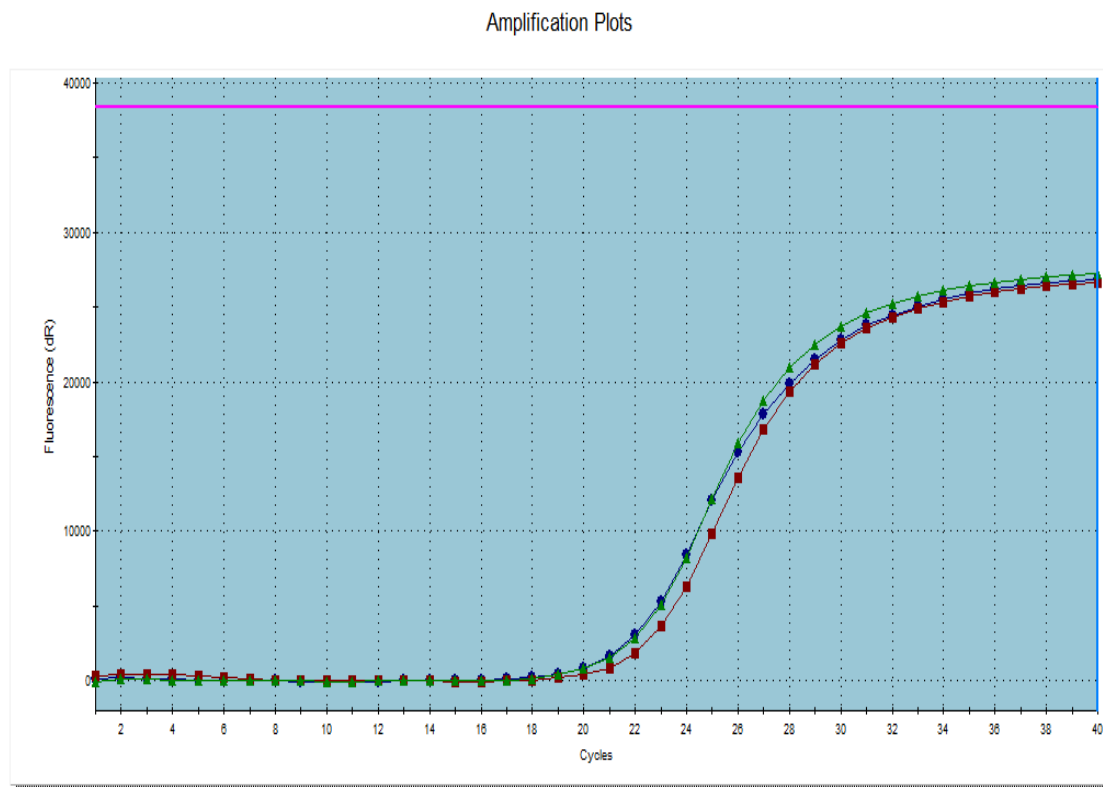### 10.1.1 Amplification plot from qPCR

Figure 10-1 Amplification plot of RNA-Polymerase II beta (RP-IIβ) mRNA from qPCR using Stratagene Mx3005p PCR machine.

10.1.2 Dissociation/Melt Curve Profile

In order to identify primer specificity, a dissociation melt curve was run following amplification.
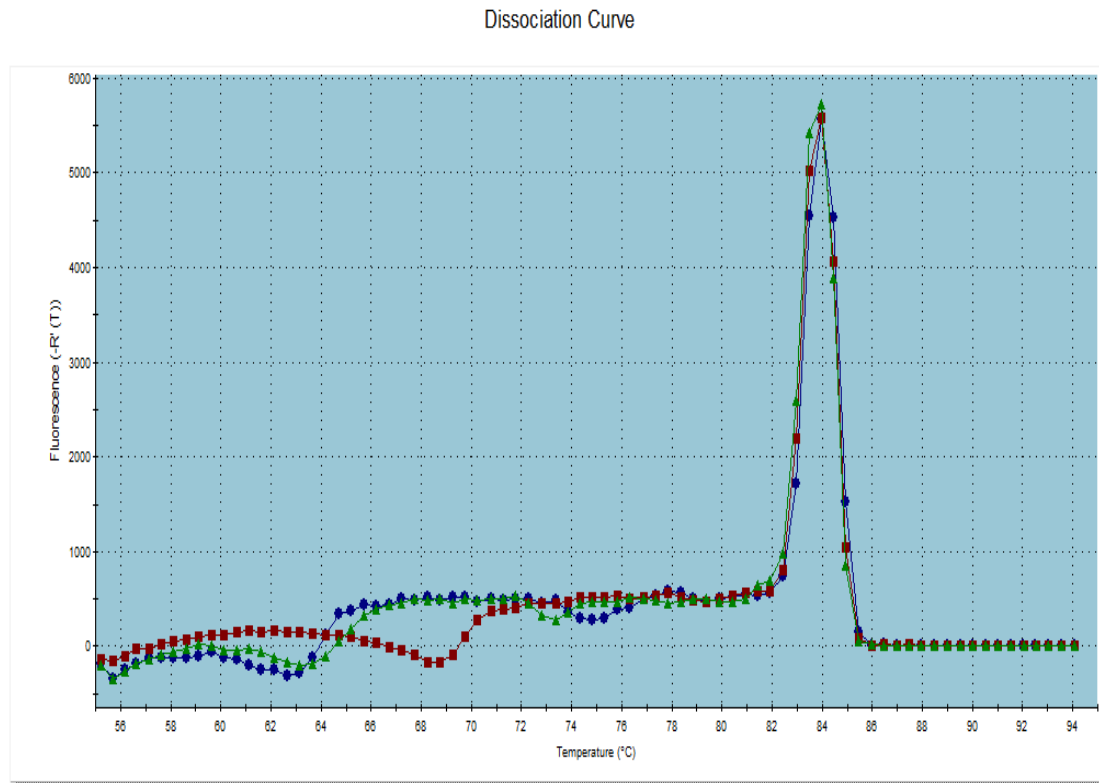

Figure 10-2 Dissociation/melt curve analysis of RNA-Polymerase II beta (RP-IIβ) mRNA from qPCR using Stratagene Mx3005p PCR machine.

10.2 Appendices B

Can skeletal muscle grown *in vitro* be used to investigate insulin resistance?

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INTRODUCTION: Hyperinsulinemia is a potent cause of insulin resistance (1), and thus impaired glucose uptake, which in turn can result in type II diabetes. Since skeletal muscle accounts for the majority of glucose uptake in the body (2), investigation in to the underlying mechanisms involved in the development of insulin resistance in this tissue is warranted. *In vitro* experimentation is an ideal methodology to help understand the development of insulin resistance on a cellular and molecular basis, and has already led to a degree of understanding of the aetiology of the disease (3,4). The purpose of the present experiments was to establish culture parameters that would drive insulin resistance in cultured skeletal muscle cells. These parameters would then form the basis of a system to develop "diabetic" tissue engineered skeletal muscle.

METHODS:
C_2C_{12} myoblast were grown until confluent, before being incubated with either a control (CON) differentiation media (DMEM + 2% HS), or an insulin treated (IT) media (DMEM + 2% HS and 100nM Insulin). The media was changed every 12 hours for 72 hours as previously described (3). Cells were then re-stimulated (ST) with insulin (100nM) or left un-stimulated (US). The subunit p85 of phosphoinositide 3-kinase (p85 PI-3 Kinase) and Akt, two proteins implicated in insulin signalling were measured by immunoblotting. Furthermore, mRNA expression of Hexokinase II and Peroxisome Proliferator-Activated Receptor (PPAR)-coactivator (PGC)-1α, two important genes involved in cellular metabolism were investigated by qRT-PCR. Statistical analysis was conducted using a one-way ANOVA or equivalent non-parametric test. The data is presented as mean ± SEM and were an accumulation of three independent experiments.

RESULTS:
There were no changes in total p85 PI-3 Kinase expression ($p > 0.05$). There was an increase in phosphorylation of Akt at residue serine 473 (Ser^{473}) in CON-ST vs. CON-US ($p < 0.01$), however this effect was blunted in the insulin treated condition ($p > 0.05$). There was a significant main effect for treatment on mRNA expression of Hexokinase II (HKII) ($p < 0.01$),

with significant differences between CON-US and CON-ST conditions ($p < 0.01$), however, there were no differences between CON and IT groups. PGC-1α expression was significantly lower ($p < 0.01$) following IT-ST compared to CON-US and CON-ST, respectively.

DISCUSSION & CONCLUSIONS:
Exposure of skeletal muscle cells to a hyperinsulineamic environment reduces phosphorylation of Akt, a key regulator in insulin signalling. In addition, mRNA expression of genes implicated in both mitochondrial biogenesis and glucose metabolisms were also impaired. This cell culture model replicates a selection of the protein and transcriptional features of an *in vivo* diabetic skeletal muscle. In future experiments we hope to recreate these findings when skeletal muscle cells are cultured in a three-dimensional scaffold, in order to create a truly physiological model of insulin resistant skeletal muscle.

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Tissue engineering skeletal muscle to investigate glucose uptake.

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INTRODUCTION:

In vivo, skeletal muscle contains, multi-nucleated aligned myotubes surrounded by an extracellular matrix [1], and functionally, skeletal muscle is responsible for a large proportion of post-prandial glucose uptake [2]. The development of skeletal muscle tissue engineered constructs which recapitulate both morphological and physiological characteristics *in vitro* provides the potential to investigate metabolic diseases, such as skeletal muscle insulin resistance.

METHODS:

C2C12 skeletal muscle myoblast cells were seeded at 1×10^5 cells in fibrin based constructs previously described [1]. Constructs were grown in growth medium supplemented with 250µg aminocaproic acid (AA) (DMEM, 20% FBS, 1% P/S). Upon confluence, media was changed to differentiation media (DMEM, 2% HS, 1% P/S, AA), for two days. Constructs remained in maintenance media for the duration of the experiment (DMEM, 7% FBS, 1% P/S, AA). Constructs were then analysed by qPCR for mRNA of GLUT1, GLUT4. Protein was obtained and separated by western blot to measure concentrations of protein kinase B (Akt) and GLUT4. Glucose uptake was quantified through scintillation counting using $[H^3]$-2-Deoxy-D-Glucose.

RESULTS:

Time duration in differentiation and maintenance media resulted in contraction of fibrin based constructs ($P < 0.0001$, ANOVA). At 14 days total in culture constructs had developed multi nucleated myotubes (*figure 1*) and express total Akt protein, a key mediator within the insulin signalling cascade (*figure 2*).
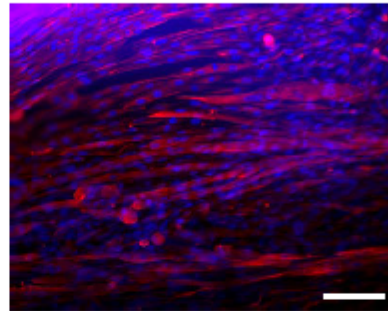

Figure 1: Immunofluorescence of C2C12 cells in fibrin gels at day 14 in culture. Myodesmin (Red) counterstained with DAPI (Blue). Scale Bar = 100µM (N = 1 construct).
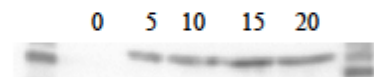

Figure 2: Immunoblot for total Akt with dose dependent protein concentrations (µg) from 14 day old fibrin gels.

DISCUSSION & CONCLUSIONS:

These findings present the characteristics of a skeletal muscle tissue engineered constructs which express fundamental genes and proteins required for insulin stimulated glucose uptake.

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Chronic insulin exposure results in transcriptional alternations in metabolic and myogenic genes in *in vitro* skeletal muscle.

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Introduction

Skeletal muscle is a major site of insulin stimulated glucose disposal. Furthermore, insulin regulates the transcription of a number of metabolically important genes, which are subsequently impaired within insulin resistant individuals'. Cell culture models using pure myogenic cells can provide a controlled environment to investigate the effects of insulin exposure upon transcriptional changes. Therefore, the purpose of these experiments was to investigate the effects of chronic insulin exposure upon pre- and post-mitotic skeletal muscle *in vitro*.

Methods

C_2C_{12} myoblast were grown until confluent, and either differentiated into myotubes before being incubated with either a control media (DMEM + 2% Horse serumserum (HS)), or insulin supplemented media (DMEM + 2% HS and 100nM Insulin), or were differentiated in the presence of the above medias. The media was changed twice daily for 72 hours as previously described (3). RNA was extracted to analyse mRNA expression of Hexokinase II (HKII), GLUT4, Peroxisome proliferator-activated receptor coactivator (PGC)-1α, and Myogenin (*MyoG*) by qRT-PCR.

Results

There was no effect of chronic insulin exposure upon GLUT4 mRNA expression in C_2C_{12} myotubes ($P>0.05$), although it was reduced in insulin exposed differentiating myoblasts ($P=0.051$). In addition HKII expression was impaired in differentiating myoblasts exposed to insulin ($P<0.05$). PGC-1α expression was not effected by condition in myotubes ($P>0.05$) however was different between conditions in myoblasts ($P<0.05$). MyoG expression was reduced in myoblasts treated with insulin ($P<0.05$), but not within myotubes ($P>0.05$).

Conclusions

Chronic insulin exposure negatively impacts upon key metabolic genes in regenerating of skeletal muscle, whilst it appears not to affect these same genes in differentiated skeletal muscle. These findings support *in vivo* research, which suggests a mechanistic defect within peripheral tissue of insulin resistant individuals at the transcriptional level.

10.3 Appendices C

10.3.1 Optimization of glucose transporter expression in skeletal muscle cell culture

Based on the findings of glucose transporter mRNA expression (*Chapter 5*), further analysis was conducted on protein lysates from monolayer cultures and skeletal muscle tissue engineered constructs. The objective was to investigate if the increases in mRNA expression observed translated to an increase in protein of GLUT1 and GLUT4 respectively in monolayer and tissue engineered skeletal muscle constructs.

We firstly attempted to investigate GLUT4 protein expression by western blot as performed for other proteins in this thesis (*Section 3.5.7*). Despite this, the expression of GLUT4 protein was not visible by the conventional method used to analyse the expression of the other proteins in this thesis. A potential cause of this could be due to the reducing method of the samples prior to loading.

The addition of sample buffer and subsequent boiling of protein lysate's denatures the out membrane of the cell. The denaturing of the lysate would therefore destroy any proteins located at the plasma membrane. A high concentration of the GLUT4 protein pool is located at the plasma membrane following insulin stimulation (Rudich and Klip, 2003) and GLUT1 is mainly concentrated at the membrane. As a result samples were not boiled prior to loading and analysed for the abundance of GLUT4 protein. As shown in both monolayer (*Figure 5-5*) and fibrin constructs (*Figure 10-3*), GLUT4 protein was expressed.

Despite higher expression of GLUT1 mRNA in skeletal muscle cultures, analysis of this protein could not be optimised despite initial attempts through western blot.

The findings from this initial optimisation for investigating glucose transporter expression; however further optimisation is required for investigating the expression of GLUT4 in tissue-engineered constructs. This is a result of the multiple bands presented upon ECL exposure. This is potentially due to factors such as inadequate blocking prior to primary antibody exposure, potentially resulting in non-specific proteins binding. .

10.3.2 Considerations for investigating glucose transporter protein expression in cell culture models.

Previous work has outlined a number of important considerations should be taken into account when analysing protein expression using antibody based techniques such as western

blot (Murphy & Lamb 2013). Although analysis of GLUT4 expression is has previously been investigated in skeletal muscle cell culture research (Philp et al. 2010; Wang et al. 1999) future research using skeletal muscle tissue engineered constructs should consider the following factors in order to further optimise the measurement of glucose transporter expression in this culture system.

Typically, the concentration of protein loaded for the purpose of western blot analysis can be small. As previously outlined the expression of GLUT4 is lower in vitro and therefore by loading a greater concentration of protein. Alternatively, immunoprecipitation of the GLUT4 protein would enable for investigation of GLUT4 abundance in these cultures. As previously discussed in the literature review (*Section 2.11.2*), the majority of the GLUT4 pool is sequenced within the cytoplasm of the cell. Upon insulin stimulation glucose storage vesicles, containing GLUT4 protein, translocate to the plasma membrane in order to facilitate glucose uptake. In order to investigate the distribution of GLUT4, subcellular fractionation is a crude method of disseminating the effects of insulin stimulation upon the location of GLUT4 as a result of both insulin dependent and independent stimulation.

10.3.3 GLUT4 Protein expression in fibrin skeletal muscle tissue engineered constructs

In Chapter 5, the mRNA and protein response of glucose transporters in both monolayer and tissue engineered skeletal muscle constructs were investigated (*Chapter5*). Data from monolayer cultures is presented within the respective Chapter (*Section 5.3.1*).*See section* Following a similar the same protocol as that in monolayer with the exception of loading concentration (10µg), protein from 0 days and 14 day old fibrin skeletal muscle constructs was loaded and separated on 7.5% SDS-PAGE gels. In addition human skeletal muscle lysate was used as a positive control as a comparison to tissue engineered constructs. Proteins were transferred onto nitrocellulose membranes. Membranes for GLUT4 were block in non-fat dry milk (2% milk in TBS) with membranes for β-Actin blocked in BSA (2%, TBS). Antibodies were made up in 5% milk TBST and 5% BSA, TBST for GLUT4 and β-Actin at a 1:1000 concentration. Membranes were washed in TBST and incubating overnight (4°C) in their respective antibodies. Membranes were washed and incubated in secondary antibody. A detailed description of the western blot protocol is outline within the methods section (*Section 3.5.7*).

Western blot for GLUT4 were exposed for 2 hours however presented multiple bands above the molecular weight of GLUT4 (50 kDa) (*Figure 10-3A*).*See figure* In addition, the positive

control did not present a clean band and therefore quantification of this protein despite clear identification of bands for β-Actin, which was used as a reference protein (*Figure 10-3B*).
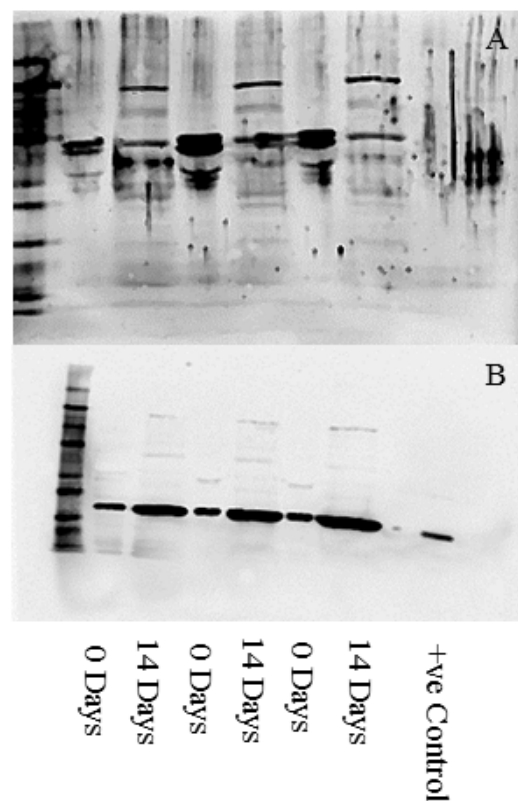

Figure 10-3 Immunoblot from 0 and 14 day old fibrin gels for A; GLUT4 and B: B-Actin. Protein lysate (10µg) were separated on 7.5% SDS-PAGE gels as outlined in the methods section.

10.4 Appendices D

Caution: For Laboratory Use. A product for research purposes only.

DEOXY-D-GLUCOSE, 2-[1,2-^{3}H (N)]-

Product Number: NET549

LOT SPECIFIC INFORMATION

Lot Number:	1754428
Specific Activity:	16.2 Ci/mmol
	599.4 GBq/mmol
Production Date:	17 May 2013

M.W. 164
$C_6H_{12}O_5$
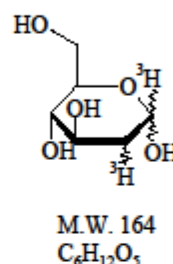
PACKAGING: 1.0 mCi/ml (37 MBq/ml) in ethanol : water (9:1).

STABILITY AND STORAGE RECOMMENDATIONS: When deoxy-D-glucose, 2-[1,2-^{3}H(N)]- is stored at -20°C in its original solvent and at its original concentration, the rate of decomposition is initially 1% for 12 months from date of purification. Stability is nonlinear and not correlated to isotope half-life. Lot to lot variation may occur.

SPECIFIC ACTIVITY RANGE: 20-50 Ci/mmol (740 -1850 GBq/mmol)

RADIOCHEMICAL PURITY: This product was initially found to be greater than 97% when determined by the following methods. The rate of decomposition can accelerate. It is advisable to check prior to use:

High pressure liquid chromatography on Aminex HPX-87C column using the following mobile phase:
water at 85°C.

Paper chromatography on cellulose using the following solvent systems:
n-butanol : ethanol : water, (50:32:18).

QUALITY CONTROL: The radiochemical purity of deoxy-D-glucose, 2-[1,2-^{3}H (N)]- is checked at appropriate intervals using the first listed chromatography method.

HAZARD INFORMATION: WARNING: This product contains a chemical known to the state of California to cause cancer.