



**Cytokine-induced skeletal muscle atrophy:  
protective effect of resveratrol**

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By

**Miss Rachel McCormick**

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## ABSTRACT

Sarcopenia, the loss of muscle mass and function as we age, affects all individuals from approximately the 4<sup>th</sup> decade of life and results in a poor quality of life. The mechanisms responsible for sarcopenia are unclear and it is likely that it is a multifactorial disease. However, it is hypothesised that an increase in systemic and/or muscle pro-inflammatory cytokine levels play a major role. Interferon induced protein 10 (IP10) is a chemokine that has been shown to be increased in serum as we age. Polyphenols are extracts from plants and have been shown to have anti-inflammatory effects with benefits in multiple diseases. Therefore, the main aim of this thesis was to establish an *in vitro* model to study the effect of increased levels of IP10 on muscle atrophy and inflammation and to examine whether resveratrol treatment was able to protect against IP10 induced effects on skeletal muscle atrophy or inflammation.

Primary myoblasts were isolated from rat muscles and treated with 0.1 $\mu$ M, 1 $\mu$ M and 10 $\mu$ M of resveratrol to identify a functional concentration. Treatment of cells with 1 $\mu$ M of resveratrol increased myoblast growth, increased myotube diameter and decreased production of hydrogen peroxide of the cell media. Furthermore, treatment of myoblasts with 1 $\mu$ M resveratrol led to increases in the protein content of known resveratrol targets; MnSOD and catalase and caused transient increases in Sirt1 protein content. Treatment of cells with 1 $\mu$ M resveratrol also increased MnSOD and catalase content of myotubes and resulted in a decrease in the content of Sirt1, coupled with an increase in the acetylation status of proteins compared with untreated myotubes. As these data suggested that 1 $\mu$ M of resveratrol was functional in myotubes, this concentration was used to pre-treat myotubes before IP10 treatment. Treatment of myotubes with IP10 at levels found in older people (200pg/ml) led to decreases in myotube diameter and increases in the atrophy marker, Atrogin1. Pre-treatment of myotubes with resveratrol provided protection against both of these effects. Treatment of myotubes with a concentration of IP10 found in the young (150pg/ml) had no effect on markers of atrophy. Both concentrations of IP10 were found to have similar effects on cytokine release by myotubes and resveratrol treatment had only marginal effects on this.

The second aim of this study was to identify an effective concentration of resveratrol *in vivo* and examine the effect of this on skeletal muscle force generation in adult and old mice. Treatment of mice with 125mg resveratrol/kg/day resulted in an increase in MnSOD and Sirt1 contents of skeletal muscle from young mice but had no effect on skeletal muscle force generation by EDL muscles of either adult or old mice.

Overall data suggest that the increase in IP10 levels seen in ageing contributes to sarcopenia; however it is unlikely this acts through changes to the cytokine profile of muscle. Resveratrol prevented some atrophic effects induced by IP10, suggesting that, the mechanism through which resveratrol may protect against sarcopenia may be through the prevention of increases in atrophic pathways.

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## DEDICATION

*To my Grandad, Peter Thomas Hewson*

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## ABBREVIATIONS

4E-BP1	4E-Binding Protein 1
ADP	Adenosine Di-phosphate
ADP	Adenosine Diphosphate
AGE	Advanced Glycation End-product
Akt	Protein Kinase B
ALS	Amyotrophic lateral sclerosis
AMPK	5' AMP-activated protein kinase
APS	Ammonium Pear-Sulfate
ARE	Antioxidant Response Element
ATP	Adenosine Tri-phosphate
BCA	Bicinchoninic acid
Bcl2	B-cell lymphoma 2
BSA	Bovine Serum Albumin
Ca <sup>2+</sup>	Calcium
cAMP	Cyclic adenosine monophosphate
CRP	C-reactive protein
CuZnSOD/SOD1	Copper Zinc Superoxide Dismutase
CXCL10	C-X-C motif chemokine 10
CXCR3	C-X-C motif chemokine 3
DH <sub>2</sub> O	Distilled Water
DNA	Deoxyribonucleic Acid
DPBS	Distilled Phosphate Buffered Saline
ECL	Enhanced Chemi-Luminescence
EDL	Extensor Digitorum Longus
EDTA	Ethylenediaminetetraacetic acid
eIF-4E	Eukaryotic Initiation Factor 4E
eIF-4G	Eukaryotic Initiation Factor 4G
EPO	Erythropoietin
ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated kinase
ERR $\alpha$	Estrogen-related receptor alpha
ETC	Electron Transport Chain
FGF	Fibroblast Growth Factor
FOXO	Forkhead Box
FOXO3	Forkhead Box O3
g	Grams
GRO-KC	growth-regulated oncogene
GSK-3 $\beta$	Glycogen Synthase 3 $\beta$
H	Hour

H <sub>2</sub> O	Water
H <sub>2</sub> O <sub>2</sub>	Hydrogen Peroxide
HBSS	Hepes Buffered Saline Solution
HRP	Horseradish Peroxidase
IFN- $\gamma$	Interferon gamma
IGF1	Insulin-like growth factor 1
IGF-1	Insulin growth factor 1
IL-1	Interleukin-1
IL-12p	Interleukin-12p
IL-13	Interleukin-13
IL-1 $\beta$	Interleukin-1 $\beta$
IL-5	Interleukin-5
IL-6	Interleukin 6
IL-7	Interleukin-7
IL-8	Interleukin 8
IMAT	Intermuscular Adipose Tissue
IP10	Interferon Gamma Induced Protein 10
kg	Kilograms
KO	Knock out
LDH	Lactate dehydrogenase
MAFbx	Muscle Atrophy F-Box
MCP1	Monocyte Chemoattractant Protein 1
M-CSF	Macrophage Colony-Stimulating Factor
mdx	Muscle Dystrophy
mg	Miligram
MHC	Myosin Heavy Chain
Mip1 $\alpha$	Macrophage inflammatory protein 1-alpha
Mip3 $\alpha$	Macrophage inflammatory protein 3-alpha
MnSOD/SOD2	Manganese Superoxide Dismutase
MRF	Muscle Regulatory Factor
MRF's	Myogenic Regulatory Factors
mRNA	Messenger RNA
mtDNA	Mitochondrial Deoxyribonucleic Acid
mTOR	Mechanistic Target of Rapamycin
Murf1	Muscle RING-finger protein-1
Myf-5	Myogenic Factor 5
MyoD	Myogenic Differentiation 1
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide
NAM	Nicotinamide
NF $\kappa$ B	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
nm	Nano Metre
NRF1	Nuclear Respiratory Factor 1
NRF2	Nuclear Respiratory Factor 2

O <sub>2</sub> <sup>•-</sup>	Superoxide Anion
P13K	Phosphoinositide 3-Kinase
p70s6k	P70-S6 Kinase 1
PBS	Phosphate buffered solution
PC3	Prostate Cancer Cell line 3
PCR	Polymerase Chain Reaction
PGC-1 $\alpha$	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SAMP1	Senescence Accelerated Prone 1
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
ShRNA	small hairpin RNA
Sirt1	Sirtuin 1
SOD	Superoxide Dismutase
TEMED	Tetramethylethylenediamine
TNF- $\alpha$	Tumour Necrosis Factor Alpha
TRIM32	Tripartite motif-containing protein 32
UPS	Ubiquitin Proteasomal System
US	United States
UV	Ultraviolet
VEGF	Vascular endothelium growth factor
WHO	World Health Organisation
$\mu$ M	Micrometre

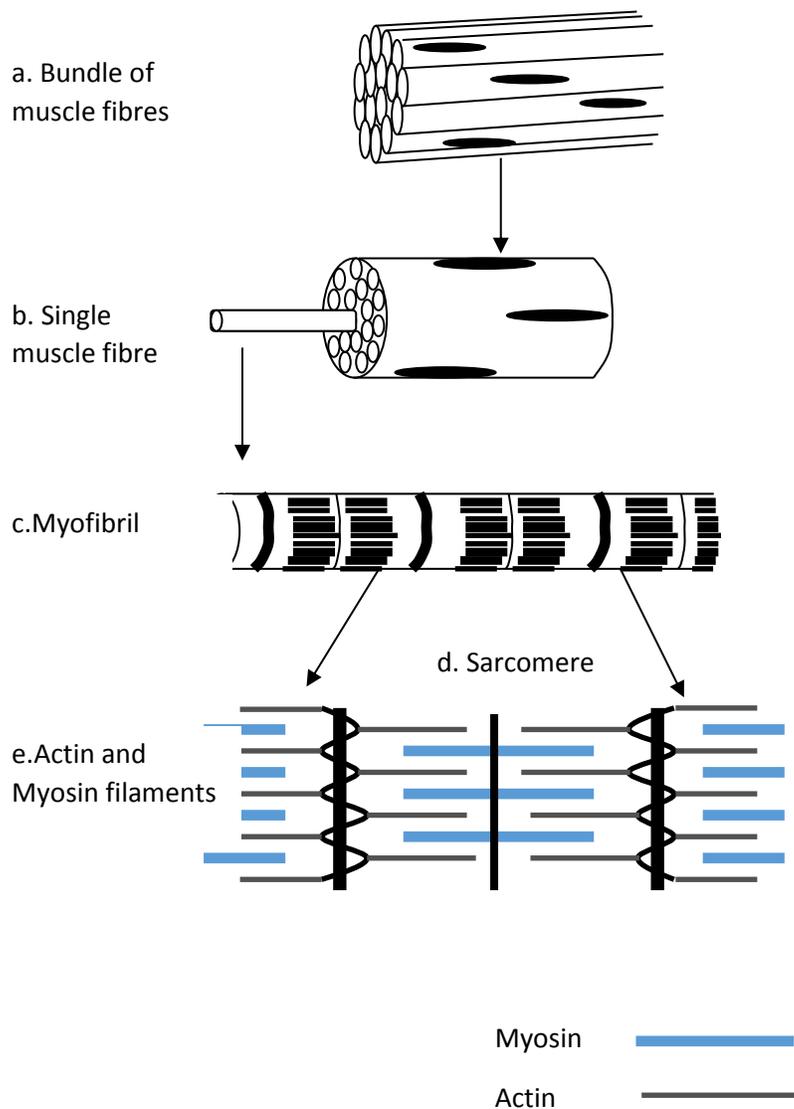
# **1. GENERAL INTRODUCTION**

## **1.1. Skeletal Muscle Structure and Function**

Skeletal muscle accounts for approximately 40% of total body weight and is characterised by multi peripheral nuclei and striations. The striations seen in skeletal muscle are due to the arrangement of the sub cellular contractile components; actin and myosin. Skeletal muscle is comprised primarily of contractile materials but also contains nerves, blood vessels and connective tissue – all of which contribute to the function of skeletal muscle. There are 640 skeletal muscles in the body that are attached to the bones via tendons throughout the body and have important roles including locomotion, stabilising body positions and producing heat.

### **1.1.1. Skeletal muscle structure**

Mature skeletal muscle consists of numerous multi nucleated muscle fibres (Figure 1.1). Each of these muscle fibres is composed of bundles of myofibrils that can run the entire length of the fibre (Figure 1.1). The number of parallel myofibres determines the force generating ability of the fibre. The number of these myofibres in a muscle is determined at birth and little change occurs during life span (Alnaqeeb et al., 1986; Klein et al., 2003). However, the cross-sectional area of a muscle fibre is very dynamic and is able to change throughout life in response to numerous stimuli such as exercise (Maughan et al., 1984), unloading (Berg et al., 1991) and ageing (Lexell et al., 1988; Frontera et al., 2000). Myofibrils consist of sarcomeres (Figure 1.1) which contain thick and thin protein filaments named myosin and actin that are arranged in a longitudinal repeated banding pattern (Figure 1.1).

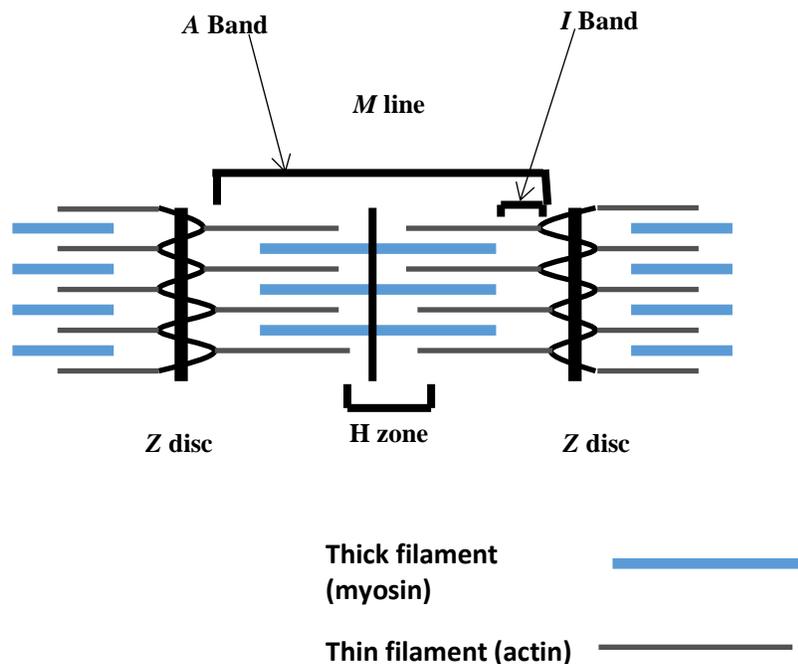


**Figure 1.1 Structure of skeletal muscle (Adapted from Vasilaki *et al* 2003).**

**1.1.2. Skeletal muscle molecular structure**

A single sarcomere is composed of repetitions of dark A bands and light I bands, it is this orientation that causes the striations that are characteristic of skeletal muscle. The light I bands contain the thin filaments, actin, the H zone contains solely

myosin and the dark A band is where myosin and actin overlap. The A band is separated by a dark line known as the M line and it is this distance between two Z discs that is known as a sarcomere. Skeletal muscle contraction involves the sarcomere, ATP, troponin and tropomyosin (Bailey, 1948; Ebashi, 1963; Hartshorne et al., 1967).



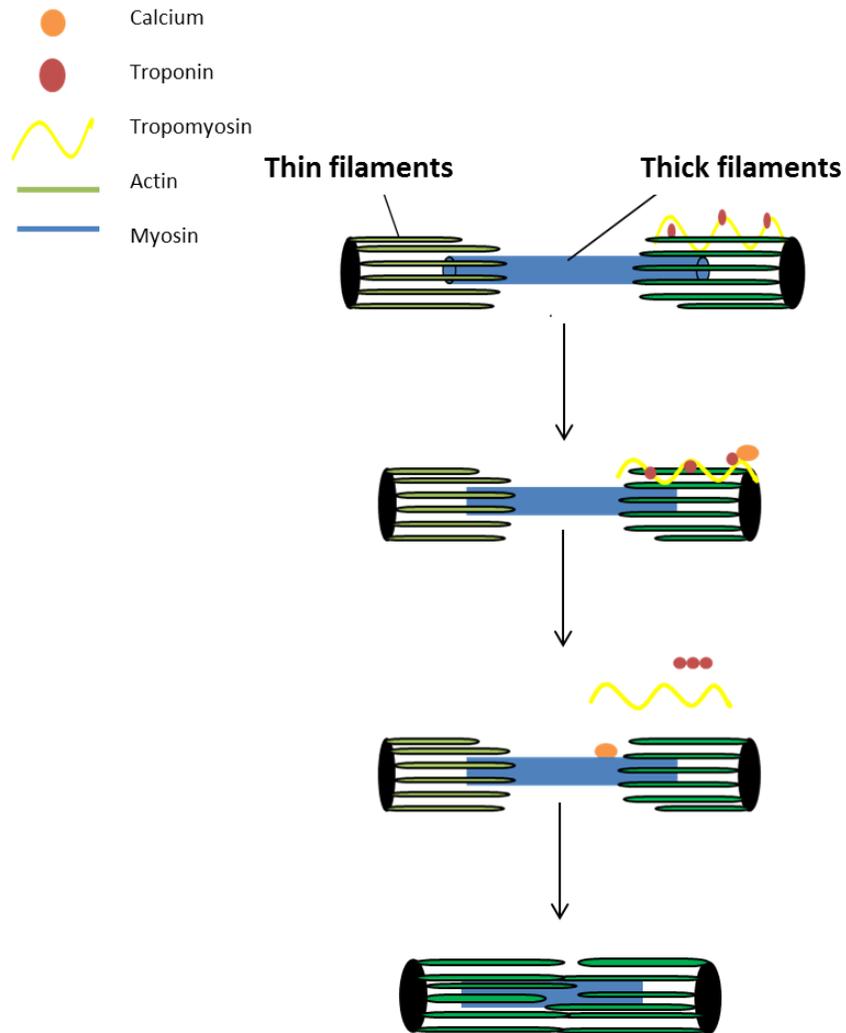
**Figure 1.2 Molecular structure of the sarcomere. The sarcomere is made of thick and thin filaments known as myosin and actin and the orientation gives skeletal muscle its striated appearance (Adapted from Vasilaki et al, 2003).**

### 1.1.3. Sliding filament theory of muscle contraction

The basic mechanism of skeletal muscle contraction is known as the sliding filament theory and was first proposed by Huxley in 1954 (Huxley et al., 1954) and confirmed in 1957 (Huxley, 1957). The sliding filament theory describes the action of

actin binding to and overlapping with myosin in the H Zone with the Z discs making contact with the A band. The ability of the sarcomere to act in such a way is due to presence and organisation of the actin and myosin filaments in the sarcomere.

At rest, actin and myosin are not cross-linked due to the presence of the troponin-tropomyosin complex. Tropomyosin occupies the groove in the actin filament which contains the myosin binding region (Parry et al., 1973). Skeletal muscle contraction occurs when an action potential which has exceeded the threshold arrives at the neuromuscular junction of a skeletal muscle fibre and ATP is available. The arrival of an action potential causes the release of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum (Ashley et al., 1968). The  $\text{Ca}^{2+}$  will then bind to troponin, causing a change in formation that will initiate the movement of tropomyosin away from the myosin binding site (Lehman et al., 1994). This allows the myosin head to form a cross-bridge with the actin filaments. If ATP is available, ATP will bind to the head of myosin and will be hydrolysed into ADP and inorganic phosphate, releasing the energy needed to activate the myosin head and for the actin to move across the myosin. This hydrolysis of ATP strengthens the cross-link between actin and myosin, and this ATPase activity of myosin dictates the speed of the contraction (Bárány, 1967). The myosin head then undergoes a conformational change that propels the actin approximately 10nm along the sarcomere towards the H Zone; this is known as a power stroke (Figure 1.3). Following the power stroke, an ATP molecule will again bind to the myosin head and release actin from the myosin head ready to repeat the cycle.



**Figure 1.3 Sliding Filament Theory.** The action of skeletal muscle contraction is known as the sliding filament theory. Following activation by an action potential,  $\text{Ca}^{2+}$  will bind to troponin causing tropomyosin to rearrange and expose the myosin binding site of the actin filaments.

#### 1.1.4. Skeletal muscle fibre types

Skeletal muscle is a very versatile organ with a range of different requirements for different tasks. To meet these demands there are two types of skeletal muscle fibres; type I and type II. The skeletal muscle fibres are identified by the presence of different isoforms of myosin heavy chain (MHC) and they are generally classified into 3

groups; MHC I, MHC IIa and MHC IIx which correspond to different isoforms of myosin ATPases; I, IIa and IIx (Brooke et al., 1970).

Type I fibres are known as slow oxidative fibres and primarily use oxidative phosphorylation of triglycerides as a source of energy (MacIntosh et al., 2006). These fibres produce ample amounts of ATP and have a high resistance to fatigue and are therefore capable of producing low level contractions for a long period of time under aerobic conditions. Characteristics of type I muscle fibres include high capillary density, mitochondria and myoglobin content (Ingjer, 1979; Jansson et al., 1983; Dueya et al., 1997) and type I fibres are commonly a red colour. Type I fibres have lower maximum shortening velocity, maximum power output, optimal velocity, optimal force, specific tension and isometric tension/cross-sectional area than type II fibres (Bottinelli et al., 1996). Type I fibres are innervated by slow motor neurons.

Type IIB (also known as Type IIx) fibres are known as fast-twitch glycolytic fibres and are generally whiter in colour. In contrast to type I fibres, type IIB fibres have low levels of mitochondria, myoglobin and capillaries (Ingjer, 1979) and rely mostly on glycolysis of ATP and creatine phosphate for energy. Type IIB fibres are used for short anaerobic exercises that require rapid high forces and quickly become fatigued. Type IIB fibres are innervated by fast fatigue motor neurons.

Type IIA (also known as Type IIA) fibres are known as fast-twitch oxidative fibres and are usually classed as intermediate fibres. These fibres contain moderate levels of mitochondria, capillaries and myoglobin (Ingjer, 1979; Jansson et al., 1983)

and use both glycolysis and oxidative phosphorylation of glycogen and creatine phosphate as sources of energy and are fairly resistant to fatigue. These fibres are used during long term anaerobic exercises. Type IIA fibres are innervated by fatigue resistant motor neurons.

**Table 1.1. Characteristics of skeletal muscle fibre types.**

	Type I	Type IIA	Type IIX
<b>Energy source</b>	Oxidative phosphorylation	Glycolysis and Oxidative Phosphorylation	Glycolysis
<b>Mitochondria Content</b>	High	Moderate	Low
<b>Capillary Content</b>	High	Moderate	Low
<b>Fatigue Resistance</b>	High	Moderate	Low
<b>Colour</b>	Red	White	White
<b>Innervated by</b>	Slow motor neurons	Fatigue resistant motor neurons	Fast fatigued motor neurons

In humans, most muscles are classified as type I or II however the majority of muscles contain both type I and type IIA/B fibres (Edgerton et al., 1975; Soukup et al., 2002). Furthermore, the muscle fibre type proportion can change following certain activities such as exercise (Simoneau et al., 1985), primarily this occurs if fibres are lost, although fibre type per se can only change if changes in innervation occur.

## **1.2. Plasticity of Skeletal Muscle**

### **1.2.1. Satellite cells**

It has been known since the 1800s that skeletal muscle has the ability to regenerate following injury. The process of regeneration is similar to the development of muscle, and depends on satellite cells. Satellite cells were first identified in 1960's (Katz, 1961; Mauro, 1961) when it was postulated that these cells were involved in

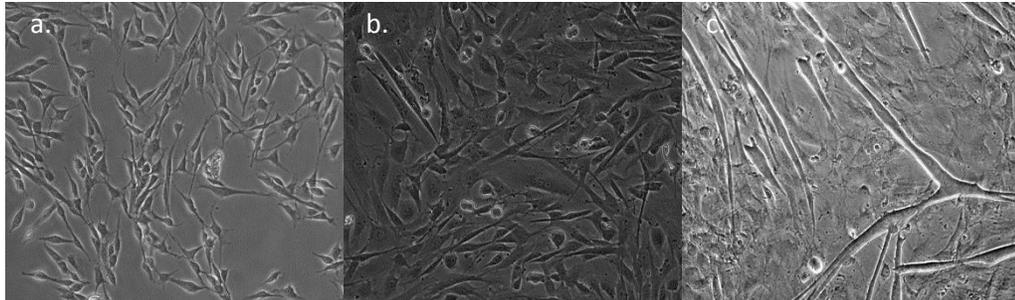
muscle regeneration, but it was not until later on in that decade that this was confirmed (Shafiq et al., 1965; Reznik, 1969).

Satellite cells are usually mitotically quiescent until they are required (Schultz et al., 1978). These cells reside in between the basal lamina and the sarcolemma (skeletal muscle cell cytoplasm) (Mauro, 1961) and become mitotically activated following signals from damaged muscle, or other signals such as inflammation (Li, 2003) or exercise (Crameri et al., 2004). Activated satellite cells migrate to the site of injury (Reznik, 1969), where they fuse to existing myofibrils to repair damage or fuse with each other to form new myofibrils. Satellite cells also replenish the reserve population of satellite cells (Zammit et al., 2004). Approximately 80% of activated satellite cells will be used for repair and 20% will replenish the satellite cell population (Schultz, 1996). Despite satellite cells being well accepted as major contributors to repair and regeneration, their involvement in hypertrophy is debated; some studies have shown satellite cells are involved in hypertrophy (Schultz et al., 1978; Crameri et al., 2004) whereas others have found there was no effect on hypertrophy with the prior ablation of satellite cells (McCarthy et al., 2011).

### **1.2.2. Culture of primary skeletal muscle cells**

Satellite cells can be isolated from muscles of humans and animals to allow cell culture studies on skeletal muscle (Simoneau et al., 1985; Larkin et al., 2006). Once activated, satellite cells will enter myogenic development were they become single nucleated muscle cells known as myoblasts (Figure 1.4). Following proliferation,

myoblasts can be induced to fuse with other myoblasts to become multinucleated myotubes (Figure 1.4) (Capers, 1960).



**Figure 1.4 Myogenesis: Representative bright field images of primary rat (a) myoblasts, (b) immature myotubes and (c) mature myotubes in cell culture.**

### **1.2.3. Skeletal muscle atrophy**

Skeletal muscle atrophy is characterised by a decline in protein content and fibre diameter. Both lead to the decline in force production. Skeletal muscle atrophy can be caused by numerous situations such as disuse, injury and denervation and is common in the ageing population.

The correct balance between protein synthesis and protein degradation is essential for the maintenance of muscle protein homeostasis. Although different models of atrophy (disuse, cachexia etc.) can act through different mechanisms, they all lead to the same common end result of fibre and muscle atrophy. The two main systems involved in protein degradation are the ubiquitin-proteasomal system (UPS) and the autophagy-lysosome system.

### **1.2.3.1. Ubiquitination of proteins**

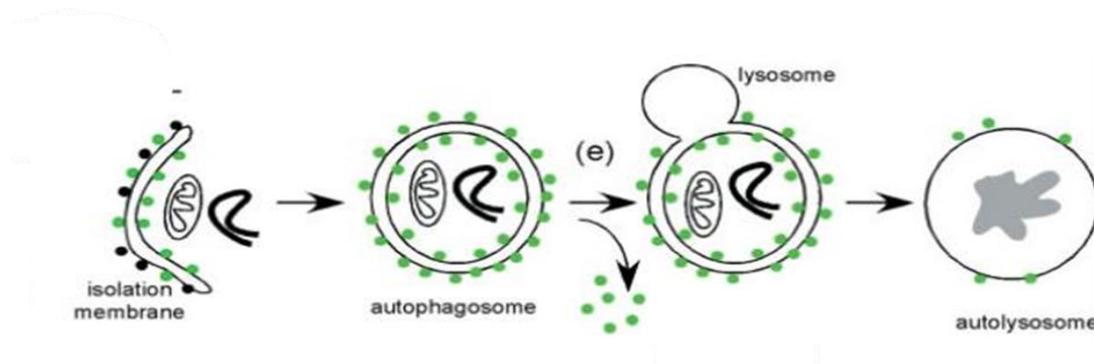
Ubiquitination is a post-translational modification that labels proteins for degradation. Ubiquitins are small highly conserved molecules that are activated by ubiquitin activating enzymes (E1) (Sharp et al.). Following activation of the ubiquitin, the ubiquitin molecule is transferred to an ubiquitin-conjugating enzyme (E2). Finally the ubiquitin ligases (E3) bind to E2 and the protein substrate to catalyse the movement of the ubiquitin from the E2 to the protein to be degraded. This cycle repeats so the protein will be polyubiquitinated, and labelled for proteasomal degradation (Hershko et al., 1983).

The UPS is used for the remodelling of sarcomere proteins in response to muscle use (Murton et al., 2008) and it is well established that, during atrophy, the UPS is upregulated. The involvement of the UPS in skeletal muscle atrophy was shown in elegant studies by Goldberg's (Gomes et al., 2001) and Glass's groups (Bodine et al., 2001b), who identified genes whose expression had changed in numerous models of atrophy. These genes were termed "atrogenes". Two of the most upregulated genes during atrophy were muscle specific ubiquitin ligases Atrogin1 and Murf1 (Section 1.4.4.2). The role of Atrogin1 and Murf1 in atrophy have been shown in genetically modified mice, with deletion of Murf1 and Atrogin1 leading to an atrophy resistant mouse (Murton et al., 2008). Furthermore, Atrogin1 specifically targets the key muscle transcription factor MyoD (Tintignac et al., 2005; Lagirand-Cantaloube et al., 2009) and the protein synthesis activator eIF3f (Csibi et al., 2009) for degradation. Murf1 has also been shown to target structural muscle proteins such as troponin (Kedar et al., 2004)

and myosin heavy and light chain (Clarke et al., 2007). There is a role for other E3 ligases in skeletal muscle atrophy such as Tripartite motif-containing protein 32 (TRIM32) which causes degradation of structural muscle proteins (Cohen et al., 2012). These data demonstrate that the UPS leads to protein degradation and inhibition of synthesis of important muscle proteins, signifying the importance of the UPS in muscle atrophy.

#### **1.2.3.2. Autophagy**

Autophagy is the process of “self-eating” and is crucial for the turnover of cell components, both in normal circumstances as well as in cellular stress such as starvation (Pfeifer et al., 1983). A portion of the cell cytoplasm, including organelles, is engulfed by a phagophore (a double membrane that engulfs the cytoplasmic components during autophagy) and leads to the formation of an autophagosome. Subsequently, the outer membrane of the autophagosome fuses with the endosome (a membrane bound compartment derived from the golgi body) this is followed by the fusion with the lysosome leading to the degradation of the internal components (Arstila et al., 1968) (Figure 1.5).



**Figure 1.5 Autophagy is a self-degradative process that involves the formation of a phagophore which engulfs cytoplasmic compartments to form an autophagosome. The autophagosome will fuse with an endosome and a lysosome which will lead to degradation (Glick *et al.*, 2010).**

Autophagy has been shown to play a role in skeletal muscle atrophy of genetically modified animals, such as mice with the transgenic deletion of CuZnSOD. This deletion results in muscle atrophy this was, at least partially, caused through upregulation of autophagy (Dobrowolny *et al.*, 2008). FoxO3 induced atrophy was also shown to occur through autophagy (Mammucari *et al.*, 2007).

The role of autophagy in skeletal muscle atrophy is further characterised by autophagy of the mitochondria (mitophagy). In many models of muscle atrophy, the mitochondria are dramatically remodelled. One of the systems activated during this remodelling is fission (the breakdown of the mitochondrial reticulum into multiple mitochondria) (Hoppins *et al.*, 2007). Inhibition of fission leads to the protection against denervation-induced atrophy and the upregulation of fission leads to atrophy in mice (Romanello *et al.*, 2010).

One of the pathways regulating the UPS and autophagy is the IGF1-Akt pathway (Section 1.2.4) which can inhibit Atrogin1, Murf1 and other genes required for

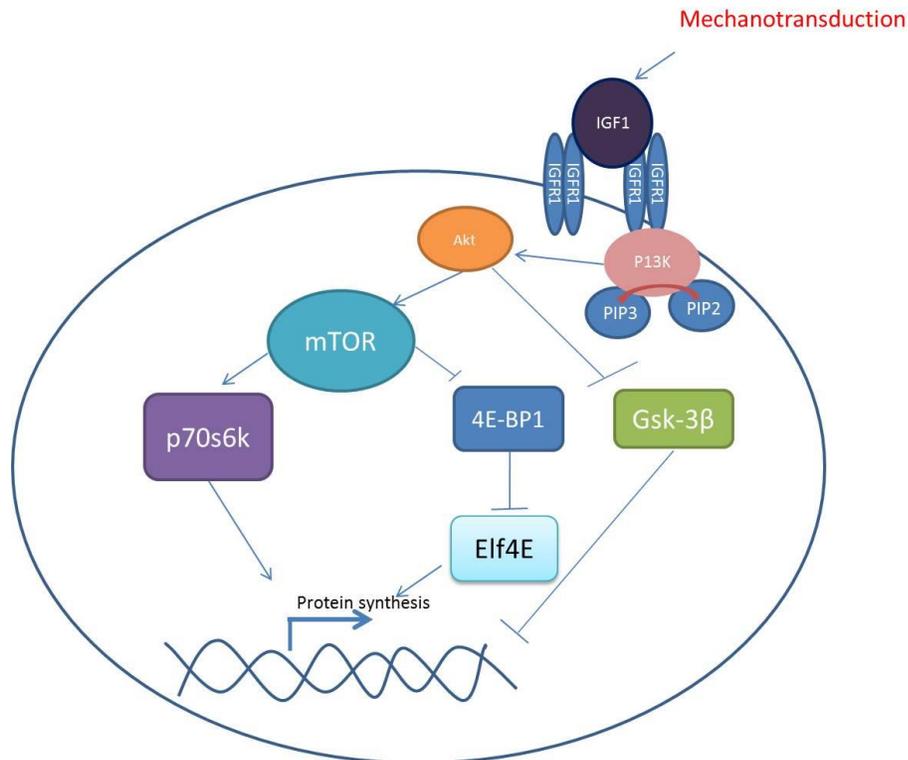
autophagy (Stitt et al., 2004). An increase in inflammation, and in particular an increase in the master inflammation regulator, NF- $\kappa$ B (Section 1.4.8) leads to an increase in Murf1 expression (Wu et al., 2014), whereas the genetic down-regulation of NF- $\kappa$ B results in a reduction in denervation-induced atrophy (Cai et al., 2004). TNF- $\alpha$  has also been shown to regulate Atrogin1 and Murf1 (Wang *et al.*, 2014). Myostatin is a well-known negative regulator of skeletal muscle mass and deletion of myostatin genes in young and old mice results in hypertrophy (Latres et al., 2015). Furthermore, myostatin is able to inhibit the IGF-Akt pathway, as Akt can inhibit Atrogin1 and Murf1; an increase in myostatin results in the indirect upregulation of Atrogin1 and Murf1 by preventing their inhibition by Akt. This myostatin induced upregulation of Atrogin1 and Murf1 has been shown to induce autophagy in C2C12 cells (Wang et al., 2015a).

#### **1.2.4. Skeletal muscle hypertrophy**

Skeletal muscle hypertrophy is an increase in muscle mass due to an increase in cross-sectional area of muscle fibres and is usually achieved when the muscle is continuously exposed to work that is beyond its current capacity, e.g. resistance training (Donnelly et al., 1993; Abe et al., 2003). For hypertrophy to occur, protein synthesis must exceed protein degradation. The main pathway involved in protein synthesis and muscle hypertrophy is the IGF1-PI3K-Akt-mTOR pathway (Bodine et al., 2001a; Lai et al., 2004). Insulin growth factor 1 (IGF1) is a small protein similar to insulin. A major role for IGF-1 in muscle hypertrophy has been demonstrated extensively, whereby inhibition of IGF-1 reduces proliferation and fusion of myoblasts *in vitro* and *in vivo* (Mavalli et al., 2010) and overexpression of IGF-1 in skeletal muscle

of mice results in hypertrophy (Musaro et al., 2001). Evidence that the PI3K-Akt pathway acts downstream of IGF-1 is found in studies where activation of P13K and Akt pathway led to hypertrophy (Lai et al., 2004) and IGF-1 dependent protein synthesis has been shown to require P13K (Mendez *et al.*, 1996; Welsh *et al.*, 1997a).

Hypertrophy occurs when IGF-1 binds to the IGF-1 receptor (IGFR-1). This causes activation of P13K resulting in the conversion of phosphatidylinositol (3, 4)-bisphosphate (PIP<sub>2</sub>) lipids to phosphatidylinositol (3, 4, 5)-trisphosphate (PIP<sub>3</sub>). Akt binds to PIP<sub>3</sub>, which results in phosphorylation of Akt by PDK1. Akt then phosphorylates and activates mammalian target of rapamycin (mTOR), which further phosphorylates and activates p70s6k leading to increased protein synthesis. mTOR also phosphorylates eukaryotic translation binding factor 4E- binding protein 1 (4E-BP1). Phosphorylation of 4E-BP1 results in release from the protein synthesis inhibitory complex with eLF-4E (Eukaryotic initiation factor 4E), allowing the binding of eLF-4E to eLF4G (Eukaryotic initiation factor 4G) thus promoting protein translation initiation (Bodine et al., 2001a). PI3K/Akt has also been shown to increase protein synthesis through the phosphorylation and inhibition of glycogen synthase 3 $\beta$  (GSK-3 $\beta$ ) (Welsh *et al.*, 1997b) (Figure 1.6).



**Figure 1.6 Mechanism of hypertrophy. P13K phosphorylates and activates Akt. Akt activates mTOR and mTOR phosphorylates p70s6k resulting in increased protein synthesis. mTOR also inhibits the translation suppressors' 4E-BP1 and GSK-3β leading to further increases in protein synthesis. One of the common activators of this pathway is mechanotransduction.**

### 1.3. Reactive oxygen species (ROS) and ROS defences

Reactive oxygen species (ROS) are extremely reactive molecules that are derived from molecular oxygen. ROS are thought to be predominantly produced by the mitochondria as a product of the electron transport chain (Chen et al., 2003b). The main source of ROS is thought to be the mitochondria; however recent studies have identified other sources of ROS (Sakellariou et al., 2013). ROS have vital roles in cell signalling; however in excess they can also cause damage to macromolecules (Powers et al., 2010). ROS production is relatively high in skeletal muscle, as skeletal muscle has high oxygen consumption and it is well known that ROS, in particular superoxide, are

produced during skeletal muscle contraction (Reid et al., 1992; Sakellariou et al., 2013). To contend with the production of ROS skeletal muscle cells have an antioxidant defence system which converts ROS into none or less damaging products to prevent any deleterious effects. The antioxidant defence system includes antioxidant defence enzymes such as the superoxide dismutases (SODs) and catalase.

### 1.3.1. Superoxide dismutase

Superoxide dismutase was first discovered by McCord and Fridovich (McCord et al., 1969) and is responsible for the dismutation of superoxide radical into hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and oxygen through the following reaction:

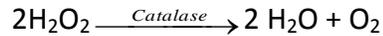


Mammalian skeletal muscle contains two prominent types of SOD; copper zinc and superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD). MnSOD is also known as SOD2 and is located in the mitochondria (Weisiger et al., 1973) with a manganese ion attached to its active site whereas CuZn is also known as SOD1 and contains copper and zinc. CuZnSOD is localised to the inter mitochondrial space and in the cytoplasm (Crapo et al., 1992). Mammalian skeletal muscle also expresses a third superoxide; extracellular SOD (Folz et al., 1994), which scavenges extracellular produced  $\text{O}_2^{\bullet-}$  however much less is known about its function.

### 1.3.2. Catalase

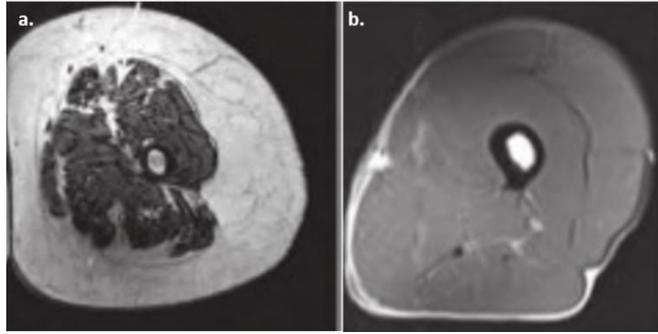
The dismutation of the superoxide anion produces hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). Although  $\text{H}_2\text{O}_2$  is more reactive than  $\text{O}_2^{\bullet-}$  it is not as damaging, but it is still capable of

causing cell damage to skeletal muscle cells. Therefore, the role of catalase is to further protect the cell from damage by converting H<sub>2</sub>O<sub>2</sub> into oxygen and water (Ji, 1993).



#### **1.4. Skeletal muscle ageing**

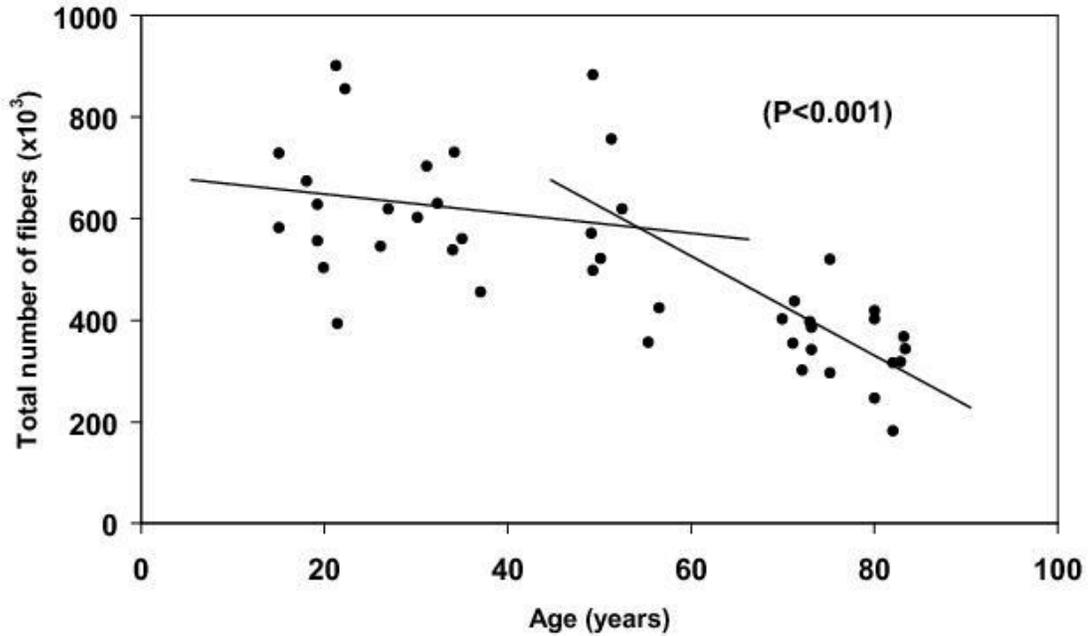
Sarcopenia is the loss of muscle mass and function as we age (Rosenberg, 1989) and has been shown to occur in human and animal models (Lushaj et al., 2008; Sousa-Victor et al., 2014). In humans, sarcopenia will affect individuals from approximately the 4<sup>th</sup> decade of life (Lexell et al., 1988), with a decrease of 30-50% in skeletal muscle mass and function by the time individuals reach approximately 80 years of age (Akima et al., 2001). However this percentage loss varies depending on personal characteristics (Gallagher et al., 1997) as well as the use of different criteria for defining sarcopenia (Coin et al., 2013). Sedentary people are most affected by sarcopenia and this is worsened by unloading of muscle in inactive old subjects (Bamman et al., 1998; Breen et al., 2013). Lifelong exercise is associated with modest improvements in muscle mass in the quadriceps of mice (McMahon et al., 2014) and lifelong triathlon training is able to preserve muscle mass in the mid-thigh of humans (Wroblewski et al., 2011) (Figure 1.7). However, the benefits of exercise on muscle function are controversial since lifelong exercise has not been capable of preventing loss of strength, since master athletes still undergo a loss in strength, power and endurance with age (Grassi et al., 1991; Kayani et al., 2008).



**Figure 1.7 MRI of a cross sectional area of a (a) sedentary 74 year old man and (b) a lifelong triathlon 70 year old man (Wroblewski et al., 2011).**

The ageing population is growing substantially with demographic analysis predicting that the world population of over 60's will reach 2 billion by 2050, nearly tripling from the beginning of the millennium, resulting in the over 80's being the fastest growing sub-population in the developed world (WHO, 2014). Although there have been improvements in lifespan, the same advances have not been made in health-span, meaning that the extra years of living are under poor health. Sarcopenia is a major contributor to frailty in the older population resulting in further immobility with a loss of independence, as well as increasing the risk of other chronic diseases and morbidity (Coin et al., 2013). Thus sarcopenia has major socio-economic costs – in 2000 the US spent 1.5% of their national budget (\$18.5 billion) on sarcopenia (Janssen et al., 2004). This means just a 10% reduction in sarcopenia prevalence would result in a saving of \$1.1 billion per year in U.S healthcare costs, signifying the importance of finding a treatment or preventative therapy for sarcopenia. The mechanisms that cause sarcopenia are not completely understood and it is likely that it is a multi-factorial disease with a network of interacting dysfunctional systems. There are several

proposed processes; decrease in protein synthesis (Welle et al., 1993), infiltration of fat and connective tissue into skeletal muscle (Brack et al., 2007; Addison et al., 2014), dysregulation of proteasomal degradation pathways (Chondrogiannia et al., 2000; Cuervo et al., 2000), mitochondrial dysfunction (Short et al., 2005; Sakellariou et al., 2013), reduced number of satellite cells, increased ROS production (Broome et al., 2006; Palomero et al., 2013) and increased inflammation (Fagiolo et al., 1993). These dysfunctions are proposed to lead to a decrease in fibre number, decreased muscle cross sectional area and poor regeneration seen in old humans (Figure 1.8) (Lexell et al., 1988). Type II fibres have been shown to be more susceptible than type I fibres to sarcopenia (Larsson et al., 1978; Lexell et al., 1988; Nilwik et al., 2013). There is also evidence for increases in the ratio of type I to type II fibres (Larsson et al., 1978; Holloszy et al., 1995; Andersen, 2003; Lee et al., 2006). However there are some conflicting results with other studies providing evidence of no difference in the percentage of type I and type II fibres with age (Lexell et al., 1988). The technique used to identify fibre type is mainly for identification of myosins, therefore, an explanation for these differences could be differential expression of the different myosins along the length of the fibre since there is evidence of an increase in the number of mixed fibres in old humans (Andersen, 2003). Mixed fibre types have been shown in chronically stimulated tibialis anterior of the rabbit (Staron et al., 1987). Therefore specific fibre typing would depend on analysis of the whole fibre for full fibre typing. Nevertheless, total numbers of muscle fibres are lost in older individuals (Figure 1.8).



**Figure 1.8 Relationship between the total number of fibres in the vastus lateralis muscles and age in males (Faulkner et al., 2007).**

#### **1.4.1. Changes in satellite cells during sarcopenia**

Satellite cells (Section 1.1.4) are stem cells present in muscle and are necessary for skeletal muscle to regenerate following injury (Shafiq et al., 1965). During ageing, the number of satellite cells have been shown to decline (Day et al., 2010; Chakkalakal et al., 2012) in a fibre type specific manner; in humans there was a decrease in satellite cells in type II fibres, with no differences seen in type I fibres (Verdijk et al., 2014). Furthermore, a decline in the satellite cell content of EDL muscles (primarily type II fibre) of mice is seen at 1 year of age, whereas a decline was not seen in the satellite cell content of the soleus muscle (predominately type I fibre) until mice were 2.5 years old (Shefer et al., 2006). Remarkably this study also showed the presence of whole myofibres with no satellite cells present in 2.5 year old mice (Shefer

et al., 2006) . However, there are also studies that show no change in satellite cell number in the soleus of rats (Brooks et al., 2009) and a study by Carlson et al (2001) showed that satellite cell number was increased in muscles of old rats suffering from hind-limb neuropathy (Carlson et al., 2001). These studies used different species as well as different muscles and suggest that changes to satellite cell number may be specific to the muscle and species used. Furthermore, the proliferation of satellite cells from mice is not grossly affected during ageing (Ballak et al., 2015), however the initial proliferation rate of isolated satellite cells from old mice in culture has been shown to be lower than in cells isolated from younger mice (Shefer et al., 2006).

Satellite cells self-renew the quiescent pool of satellite cells (Zammit et al., 2004). During ageing the ability of satellite cells to self-renew is reduced (Shefer et al., 2006) due to the loss of inactivation and increase in proliferation (Chakkalakal et al., 2012) which can lead to apoptosis or senescence (Sousa-Victor et al., 2014). This loss in the ability to self-populate the reserve population of satellite cells may contribute to sarcopenia and this is associated with poor regeneration of muscle of aged animals (Carlson et al., 2001).

The role of this modified behaviour of satellite cells as a cause of sarcopenia is unclear, as depletion of satellite cells had no effect on the cross-sectional area seen in the muscle of old mice (Fry et al., 2015) or on muscle growth after unloading (Jackson et al., 2012). Parabiosis studies showed that muscle of old mice can regenerate successfully when placed in a young host (Carlson et al., 1989; Conboy et al., 2005). Furthermore, cell culture studies have shown that satellite cells isolated from old mice

can fully differentiate into mature myotubes (Shefer et al., 2006) and when supplemented with fibroblast growth factor (FGF) showed there was no difference in the ability of satellite cells to proliferate ex vivo compared with those from young mice. These data suggest that the change in the environment during ageing causes the dysfunction in satellite cells rather than loss of function within satellite cells per se (Shefer et al., 2006). However, the ablation of satellite cells results in increased fibrosis suggesting satellite cell function may play a role in preventing fibrosis. Further studies have also shown that satellite cells are essential for regeneration following damage in muscle since ablation of these cells had profound effects on the ability of muscles to successfully regenerate (Fry et al., 2015).

#### **1.4.2. Changes in protein synthesis during sarcopenia**

A balance between protein synthesis and degradation is vital to maintain muscle mass and the relevant gains or losses in protein synthesis and degradation rates are required for hypertrophy (Section 1.2.4) and atrophy (Section 1.2.3). Much research has focused on the basal protein synthesis rates in the muscles in the old. These studies have shown contradicting results with some studies showing a decreased rate of overall protein synthesis in the muscle of old compared with muscle of adult humans, (Hasten et al., 2000) and decreased rates of synthesis of individual muscle proteins such as myosin heavy chain proteins (Balagopal et al., 1997). Other studies showed no difference in protein synthesis in muscles of old humans compared with muscles of adults (Volpi et al., 2001; Wall et al., 2015). Thus there is a lack of evidence for differences in basal protein synthesis between young and old people. Therefore

research has focused on studying protein synthesis in the post-prandial state to identify whether older subjects can utilise protein as efficiently as younger people. These studies have shown that older subjects have a blunted protein synthesis response to nutrients (Cuthbertson et al., 2005; Wall et al., 2015) and to exercise (Fry et al., 2011); this is known as anabolic resistance. Data by Kooperman et al (2009) demonstrated that there was no difference in either digestion or absorption of proteins between the old and young people so it was proposed that anabolic resistance is likely to be due to an increase in the amount of protein required to reach a 'threshold' for protein synthesis to occur (Koopman et al., 2009). This is further evidenced by studies that show blunted mTOR activation following protein intake in the muscle of older people (Cuthbertson et al., 2005). Additional studies have shown that this anabolic resistance could be overcome by giving >40g of protein to older men (Moore et al., 2015). This has led to researchers to suggest that the recommended quantity and daily intake of protein for older individuals to be increased from 0.8g/kg per day to up to 1-1.5g/kg (Morley et al., 2010). However it should be noted that a 90g dose of protein did not cause an increase in protein synthesis any more than a 30g dose suggesting just an increase in overall protein content is not sufficient to overcome anabolic resistance. Furthermore higher doses of protein intake (3g protein x kg fat-free mass (FFM)(-1) x day(-1)) have been linked with a decrease in the glomerular filtration rate in older people, suggesting that high levels of protein may have damaging effects on the kidney (Walrand et al., 2008) and thus prescribing increased intake of protein in old people is controversial. Increases in protein synthesis have also

been seen if the protein supplement contains a high percentage of leucine (Katsanos et al., 2006) or essential amino acids (Paddon-Jones et al., 2004). Increased protein synthesis was also achieved by inhibiting co-ingestion of carbohydrates and protein (Katsanos et al., 2006). This increase in the amount and quality of protein needed to activate protein synthesis in older people may be the reason that some studies have found no indication of anabolic resistance in the old (Koopman et al., 2009).

#### **1.4.3. Infiltration of fat and fibrosis during sarcopenia**

Fibrosis is the accumulation of extracellular matrix (Alnaqeeb et al., 1984; Goldspink et al., 1994) and it is well established that during ageing fibrosis and the infiltration of adipose tissue (Evans et al., 1995; Song et al., 2004) occurs in skeletal muscle. This decrease in the quality of skeletal muscle is thought to contribute to the age-related impairment in force generation, particularly in lateral transfer of force throughout the muscle fibres (Ramaswamy et al., 2011).

The accumulation of extracellular matrix, particularly collagen, seems to be the result of incomplete repair of muscle following damage (Serrano et al., 2010). Skeletal muscle regeneration following injury depends upon a series of well-coordinated events involving numerous cell types that modify the microenvironment of the damaged muscle. Infiltration of inflammatory cells and remodelling of the extracellular matrix is essential for normal muscle regeneration to preserve muscle architecture. During ageing, this remodelling becomes dysregulated possibly due to changes in the inflammatory response to damage (Mann et al., 2011). Dysfunction in remodelling is coupled with a switch in myogenic progenitor cells from a myogenic to

fibrotic fate (Shefer et al., 2006; Brack et al., 2007) or an adipogenic fate (Vettor et al., 2009; Pisani et al., 2010) suggesting satellite cells as a possible source of intramuscular fat deposition. This change in cell fate is possibly due to changes in the Wnt signalling pathway which has been shown to be involved in the myogenic fate of satellite cells and increased Wnt signalling has been shown to occur in ageing (Vertino et al., 2005; Brack et al., 2007). Alternatively changes in the inflammatory response such as those seen during ageing may also play a role in determining cell fate (Wang et al., 2015b).

Increases in collagen deposition leads to an increase in advanced glycation end (AGE) products in skeletal muscle in humans (Haus et al., 2007). AGE is the non-enzymatic crosslinking of collagen; this crosslinking increases the strength of the collagen leading to stiffness within the muscle. Goldspink et al (2004) showed no difference in the transcription levels of collagen in the muscle of old mice (Goldspink et al., 1994). Given that total collagen levels are elevated in muscle of old mice these data suggest that there may be a reduction in collagen degradation likely due to the increased crosslinking, making the collagen somewhat resistant to degradation by collagenase.

#### **1.4.4. Changes in protein degradation during sarcopenia**

The appropriate quality control of protein is vital for the correct functioning of the cell. The two most common mechanisms responsible for this are autophagy and the proteasomal degradation pathway. These two pathways have been shown to be dysregulated in ageing and therefore are hypothesised to contribute to the loss of muscle mass with age.

#### **1.4.4.1. Autophagy during sarcopenia**

There is evidence that autophagy (Section 1.2.3.2) is dysregulated in the muscle of old rodents (Russ et al., 2012; Joseph et al., 2013b; Russ et al., 2015a) and studies in drosophila show that there is an accumulation of protein aggregates that lead to impaired muscle function (Demontis et al., 2010) providing evidence for autophagic dysregulation in the development of sarcopenia.

Impairment of mitophagy (autophagy of the mitochondria) is detrimental to muscle homeostasis, and leads to the accumulation of damaged and dysfunctional mitochondria (Grumati et al., 2010). Dysfunctional mitophagy has been shown to occur in the muscle of old men (Gouspillou et al., 2014) and women (Drummond et al., 2014) and is therefore hypothesised to play a role in the mitochondrial dysfunction that is thought to be one of the major contributors to sarcopenia (Section 1.4.7).

#### **1.4.4.2. Proteasomal degradation during sarcopenia**

The role of the ubiquitin-proteasome system (UPS) (Section 1.2.3.1) is to regulate protein degradation and maintain protein homeostasis. Proteins are labelled with ubiquitin molecules for degradation and are passed to the proteasome where they are degraded.

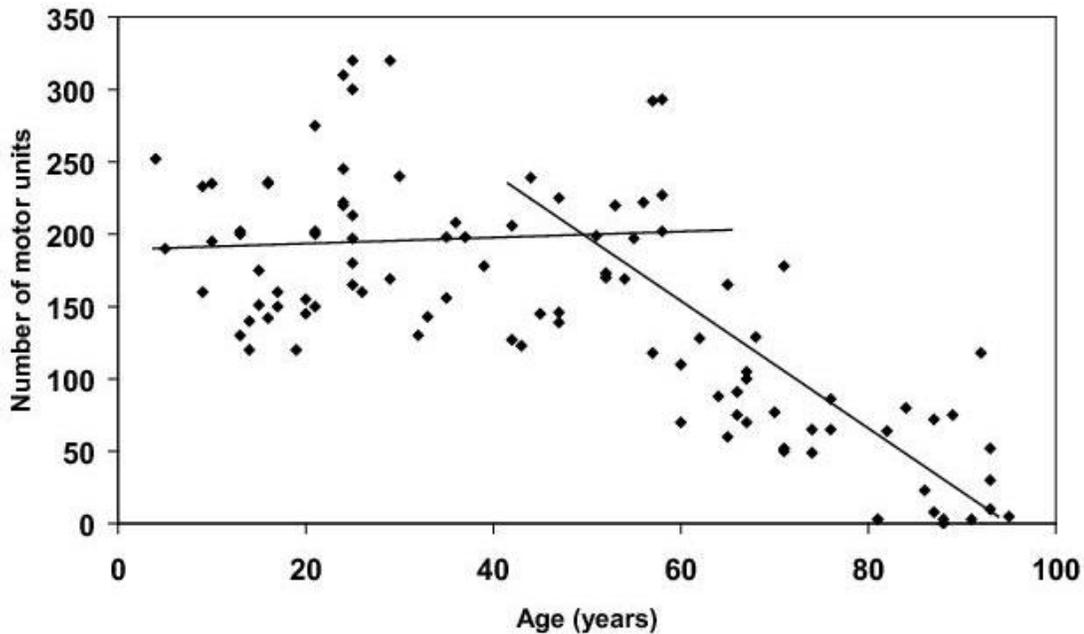
There are numerous ligases that are able to carry out protein degradation; however Atrogin1 and Murf1 are muscle specific ligases that have been shown to play a role in numerous models of atrophy (Bodine et al., 2001b). Despite the evidence for a role of the UPS in muscle atrophy, the role of the UPS in muscle ageing is controversial.

Some studies show upregulation of both muscle specific ligases Atrogin1 and Murf1 in the muscle of old rats (Clavel et al., 2006), whilst others show no difference between age groups (Gaugler et al., 2011) or the upregulation of only one of the atrogenes (Altun et al., 2010). Others have shown that Atrogin1 and Murf1 are down regulated in the muscle of old rats (Edström et al., 2006). Although these results from rodent studies show contradicting results, it is generally thought that these two atrogenes and the UPS play a role in sarcopenia.

#### **1.4.5. Changes to the neuromuscular system during sarcopenia**

During ageing there is a decrease in motor unit number in rodents (Ling et al., 2009) and humans (Figure 1.9) (Piasecki et al., 2015) and this has been shown to occur in various muscles (McComas et al., 1993). A decrease in the number of motor axons innervating fibres has been observed in rodents (Ansved et al., 1990) and humans (Tomlinson et al., 1977). Denervation leads to the sprouting of axons of existing functional nerves to innervate fibres in close proximity. This is known as motor unit remodelling and is evidenced by an increase in reinnervation in old mice (Holloszy et al., 1995). Reinnervation is speculated to cause some of the age-related fibre type switching that occurs (Larsson et al., 1978; Andersen, 2003; Lee et al., 2006), as slow motor neurons may be more adapted to reinnervation which leads to an age-related loss in fast motor neurons (Kadhiresan et al., 1996). If reinnervation does not occur it is likely that the muscle fibre will eventually undergo cell death (Borisov et al., 2000; Borisov et al., 2001). Research has provided evidence that neuromuscular remodelling

is a pre-requisite for muscle atrophy (Deschenes et al., 2010), however this is still poorly understood.



**Figure 1.9** The relationship between the number of motor units in the extensor digitorum brevis muscles and the age of men between 5 and 88 years of age. The number of motor units remained constant from 5 years to 50 years of age, but then decreased linearly with an apparent zero intercept at approximately ninety-five years of age (Faulkner et al., 2007)

#### **1.4.6. Increases in reactive oxygen species and alterations in antioxidant defence systems during sarcopenia**

Reactive oxygen species are extremely reactive molecules and have important roles in metabolism and cell signalling (Thannickal et al., 2000), particularly during muscle contraction (Reid et al., 1993). Though ROS have important functions in cells, when in excess, ROS can be damaging to the macromolecules of the cells such as lipids, proteins and DNA, leading to cell death, if not eliminated by the antioxidant defence system (Section 1.3.1).

The role of ROS in ageing was first hypothesised by Harman in the 1950s who proposed the 'free radical theory of ageing'. This theory states that ageing occurs due to the accumulation of damage to the cellular macromolecules by ROS over time (Harman, 1956). This is particularly relevant to skeletal muscle, as ROS are produced at a high rate in skeletal muscle due to the large amount of oxygen consumption and this increase in ROS is proposed to be one of the master regulators of sarcopenia. There is evidence that ROS are increased in the satellite cells of older subjects (Minet et al., 2012) which may contribute to the loss of regeneration potential in muscles of older animals and humans. One possible mechanism for this is a decrease in myogenic regulatory factors (Ardite et al., 2004). ROS are also increased at basal levels in mouse muscle (Palomero et al., 2013) and this is hypothesised to be due to mitochondria dysfunction (Section 1.4.7). This increase in ROS in older skeletal muscle has been shown to be detrimental to skeletal muscle as is reflected by increases in markers of oxidative damage such as an increase in protein carbonyl and malonaldehyde (Broome et al., 2006) and oxidation of lipids, DNAs and proteins in the muscles of old mice (Mecocci et al., 1999). This modified redox status has also been shown to be detrimental for processes such as calcium transport (Fulle et al., 2003) and increased degradation of important proteins such as myogenin. These changes result in impaired autophagy (Scherz-Shouval et al., 2007) and differentiation of muscle cells (Ardite et al., 2004). These impairments are possibly due to increased inflammation as well as an increase in proteasomal degradation (Li et al., 2003). ROS are also thought to play a role in the chronic inflammation state seen in old humans and animals (Section 1.4.8).

ROS are eliminated by the antioxidant defence system (Section 1.3). During ageing, it has been shown there is a constituent upregulation of the antioxidant defence system activity in skeletal muscle (Vasilaki et al., 2006; Palomero et al., 2013; Sullivan-Gunn et al., 2013). Following a stress such as muscle contraction, there is no further increase in antioxidant defence enzyme activities in the muscle of old humans and animals (Vasilaki et al., 2006; Ryan et al., 2008) leaving the cells exposed to oxidative damage.

The contribution that ROS play in the ageing process remains unclear. Overexpression of CuZnSOD extends the lifespan in *Drosophila melanogaster* (Sun et al., 1999). However the deletion of the SOD antioxidant enzymes did not affect the lifespan of *C.Elegans* (Honda et al., 2008) whereas the lifespan of the *C.Elegans* with deleted MnSOD was increased (Van Raamsdonk et al., 2009). Furthermore, the oral intake of juglone, a ROS generator that comes from the plants and leaves from the Juglandaceae family, increased the lifespan of *C.Elegans* (Ji et al., 2012). Overexpression of CuZnSOD in muscle led to increased oxidant production and resulted in atrophy (Rando et al., 1998). The deletion of CuZnSOD resulted in a decrease in modified ROS generation, inability of muscle to adapt and decreased muscle force (Muller et al., 2006; Vasilaki et al., 2010; Larkin et al., 2011; Sakellariou et al., 2014b) suggesting the correct balance of ROS as an important modulator of sarcopenia.

#### **1.4.7. Mitochondria dysfunction during sarcopenia**

Mitochondria are essential for providing the ATP required for muscle contraction and are also central to the redox regulation and quality control of the cell

and therefore essential for the viability of muscle cells. Given this essential role of mitochondria in skeletal muscle maintenance and survival, alterations in mitochondria are considered one of the primary contributors driving the sarcopenic process.

Due to the high levels of oxidative phosphorylation in skeletal muscle, mitochondria are proposed to be responsible for the majority of ROS produced. The role of the mitochondria in sarcopenia was proposed by Miquel in the mitochondrial free radical theory of ageing (Miquel et al., 1980). This stated that mitochondrial dysfunction in ageing occurs from the increase in ROS and blunted antioxidant defences, these damaging effects change the redox status of the cell which in turn leads to mutations in the mitochondrial DNA (mtDNA) leading to the production of dysfunctional components of the electron transport chain (ETC). Impairment of the ETC leads to compromised oxidative phosphorylation which causes a further rise in ROS, causing a vicious circle which will exacerbate the ageing phenotype (Miquel et al., 1980).

This hypothesis was confirmed in skeletal muscle by studies showing that during ageing there was an increase in ROS, mtDNA deletions and mitochondrial dysfunction which were associated with skeletal muscle atrophy in non-human primates (Lee et al., 1998a) rodents (Wanagat et al., 2001) and humans (Bua et al., 2006). Interestingly, these observations were not seen in the phenotypical normal regions of the muscle fibres. Furthermore mice which contain error prone mtDNA polymerase accumulate high levels of mtDNA mutations and show severe muscle atrophy due to increased apoptosis (Kujoth et al., 2005). Further studies show that

mtDNA mutations lead to increased ROS production (Logan et al., 2014), and overexpression of antioxidants have been shown to protect against some of the oxidative and nucleic acid damage as well as changes to mitochondrial respiration and ATP production in skeletal muscle (Lee et al., 2010) which prevent age related mitochondrial dysfunction. Furthermore, the overexpression of PGC1- $\alpha$ , the master mitochondrial regulator, led to improved mitochondrial function and protection against sarcopenia in mice (Wenz et al., 2009). These data suggests that both ROS and mitochondrial dysfunction are likely to contribute to sarcopenia. However, more recently the theory of mitochondrial free radical theory of ageing has become debatable as non-mitochondrial sources of ROS generation have been identified (Sakellariou et al., 2013; Sakellariou et al., 2014a; Jackson et al., 2016).

Mitochondria in the muscle of sarcopenic individuals also show increased fusion and decreased fission (Yoon et al., 2006) and impairment of mitochondrial autophagic (Gouspillou et al., 2014) and proteasomal machinery (Marzetti et al., 2008). The release of damaged mitochondrial components into the extracellular matrix correlates with increases in pro-inflammatory cytokines in the plasma of elderly humans (Pinti et al., 2014). Mitochondria undergo complex morphological changes during age which are also likely to affect their function (Leduc-Gaudet et al., 2015) and thus give further evidence of a role for dysfunctional mitochondria in sarcopenia.

#### **1.4.8. Increased inflammation during sarcopenia**

The inflammatory response is the secretion of pro-inflammatory mediators in response to the appropriate stimuli such as toxins, bacteria and damage. Primarily,

neutrophils migrate to the site of injury/infection (Zhang et al., 2004); during later stages the infiltration of other inflammatory cells such as leukocytes, lymphocytes and eosinophils contributes to the breakdown and removal of any damaged or foreign tissue. The inflammatory response is then resolved by the production of anti-inflammatory cytokines such as IL4 and IL13 which can then initiate repair to the site of damage (Marie et al., 1996). The migration of the immune cells to the site of damage is regulated by the production of growth factors, chemokines and cytokines. Therefore the inflammatory response prevents damage, infection, restores homeostasis and initiates repair.

The acute pro-inflammatory state is vital for the protection of cells but too much for too long can be detrimental; for example chronic low grade inflammation has been associated with ageing and has been implicated in numerous diseases such as diabetes (Duncan et al., 2003), cardiovascular disease (Lagrand et al., 1999) and neurodegenerative disease (Frischer et al., 2009).

Immunosenescence is the decline in the function of the immune system with age (Lazuardi et al., 2005; Gruver et al., 2007). Immunosenescence has been implicated in the cause of chronic low state of inflammation that occurs with age (Khodr et al., 2001). This constant state of inflammation is due to the systemic decrease in anti-inflammatory cytokines and increases in the pro-inflammatory cytokines, TNF- $\alpha$ , IL6 and C-reactive protein (CRP) (Alley et al., 2007; Giovannini et al., 2011). Such changes are correlated with an increased risk of mortality and morbidity although ageing without co-morbidities is still shown to be associated with low grade

inflammation (Krabbe et al., 2004). The increase in ROS that occurs with age (Section 1.4.6) is thought to be one of the major causes of this chronic low grade inflammation, due to the activation of redox sensitive inflammatory markers such as the transcription factor NF- $\kappa$ B.

NF- $\kappa$ B is a transcription factor that is expressed in skeletal muscle (Bar-Shai et al., 2005) and has been shown to increase with age in skeletal muscle (Vasilaki et al., 2006). In muscles of adult mice, non-damaging isometric contractions result in activation of NF- $\kappa$ B and this is related to an increase in transcription of cytoprotective proteins (Vasilaki et al., 2006). In contrast to this, muscle of old mice and humans has constitutively active NF- $\kappa$ B and no there is no further activation following contractions (Vasilaki et al., 2006; Buford et al., 2009). This increase in NF- $\kappa$ B has been associated with increases in muscle content of pro-inflammatory cytokines and skeletal muscle atrophy (Cai et al., 2004; Hunter et al., 2004) and the inability of muscles to regenerate in old mice (Broome et al., 2006).

Low level chronic inflammation coupled with immunosenescence that occurs in ageing has been termed 'inflamm-ageing' (Franceschi et al., 2000). Inflamm-ageing has been associated with numerous age-related diseases (Chung et al., 2009) such as obesity-related diabetes (Xu et al., 2003), Alzheimer's disease (Leea et al., 2008) and has been implicated as a major contributor to sarcopenia (Schaap et al., 2006; Schaap et al., 2009).

Serum levels of TNF- $\alpha$ , IL6 and C-reactive protein (CRP) are all increased in ageing and have been proposed to be important mediators of sarcopenia as changes

are correlated with a decrease in muscle mass (Pedersen et al., 2003; Aleman et al., 2011), performance (Thalacker-Mercer et al., 2010), function (Bautmans et al., 2011), strength (Tiainen et al., 2010; Norman et al., 2014) and fitness (Levinger et al., 2010). It should be noted however that TNF- $\alpha$ , IL6 and CRP have all been shown to have beneficial effects in skeletal muscle growth; IL6 and TNF- $\alpha$  at low levels has been shown to cause satellite cell proliferation and differentiation (Li, 2003; Kurosaka et al., 2013), therefore it is likely that the effect of systemic inflammation on muscle mass and function during ageing may only occur when it surpasses a certain threshold and/or persists for an extended period (Degens, 2010).

The increase in inflammation leads to a further increase in ROS production by skeletal muscle (Li et al., 1998). As well as an increase in skeletal muscle cell apoptosis (Phillips et al., 2005), inflammation has also been shown to play a role in the anabolic resistance that leads to a lack of increase in protein synthesis following protein ingestion that is correlated to Akt and IGF-1 levels (Del Aguila et al., 2000; Mercier et al., 2002). High levels of inflammation have been associated with catabolism in the skeletal muscle (Li et al., 1998) .

#### **1.4.8.1. The role of IP10 during sarcopenia**

The majority of studies examining a role of cytokines in muscle atrophy have focused on the effect of IL6, TNF- $\alpha$  and CRP (Pedersen et al., 2003; Phillips et al., 2005; Cartier et al., 2009). However, recent research into interferon-gamma induced protein 10 (IP10) has shown the importance of this chemokine in both systemic ageing and sarcopenia. IP10 is also known as CXCL10 and is a small chemokine. IP10 binds to the

CXCR3 receptor and is induced by IFN- $\gamma$  (Luster *et al.*, 1985) and other cytokines. Following induction, IP10 acts as a chemo-attractant for CXCR3 positive cells such as activated T-lymphocytes. This is evidenced in IP10 deficient mice that have an impaired contact hypersensitivity response and a reduction in inflammatory cell infiltrates (Dufour *et al.*, 2002). Furthermore IP10 levels are increased in humans and mice infected with viruses (Haeberle *et al.*, 2001) and bacteria (Azzurri *et al.*, 2005).

Increases in IP10 is associated with numerous diseases such as Behcet's syndrome (Ambrose *et al.*, 2015), hepatitis C (Reiberger *et al.*, 2008) and has been shown to have both beneficial (Kobayashi *et al.*, 2015) and negative effects in cancer. IP10 has also been implicated in inflammatory diseases (Medoff *et al.*, 2002; Grebel *et al.*, 2013) as well as increasing apoptosis in virus infected (Klein *et al.*, 2004) and healthy cells (Vinet *et al.*, 2010). Furthermore, IP10 has a prominent role in numerous autoimmune diseases (Antonelli *et al.*, 2014).

Unpublished work from our laboratory and other studies have shown an increase in IP10 in the plasma (Chen *et al.*, 2003a) and other tissues (Duan *et al.*, 2008) during ageing. Most importantly, a study that took into consideration other co-morbidities showed that IP10 was still significantly upregulated in the plasma of older people, when many cytokines that had previously been associated with ageing were not elevated (Miles *et al.*, 2008). These data suggest that IP10 is correlated directly with ageing whereas changes in other cytokines are potentially due to the presence of aged-related diseases. However, it should be noted that contrary to the above data

Banerjee et al (2011) found a decrease in the IP10 content in the plasma of older men compared with younger men (Banerjee et al., 2011).

In skeletal muscle, IP10 has been shown to be upregulated in unloading induced atrophic muscle, likely through activation of NF- $\kappa$ B (Wang et al., 2013). IP10 and its receptor are upregulated in the serum of humans suffering from myositis (Limongi, 2015) and *in vitro* work on human skeletal muscle cells show that an increase in IP10 is likely to be one of the first stages of self-promoted muscular inflammation in myositis (Crescioli et al., 2012). Furthermore, therapeutics to decrease IP10 have decreased inflammation in a mouse model of myositis (Kim et al., 2014).

Despite the evidence for changes in levels of IP10 during ageing and muscular diseases, only a sparse amount of work has been undertaken on the role of IP10 in sarcopenia. As it is now evident in numerous diseases, it is possible that IP10 could be used as a therapeutic target for sarcopenia.

### **1.5. Current therapeutics for sarcopenia**

Sarcopenia is a massive economic burden with billions of dollars being spent on direct and indirect effects of the condition every year (WHO, 2014). Current treatments for sarcopenia include both pharmacological and lifestyle interventions with evidence showing that loss of muscle function is at least partially reversible, particularly if interventions are started at the early stages of the onset of sarcopenia.

### **1.5.1. Physical exercise**

The importance of physical activity in sarcopenia has been shown in studies where people who are less physically active have a higher chance of developing a decrease in skeletal muscle mass and strength and thus sarcopenia (Lee et al., 2007). It is generally thought that exercise is beneficial and may slow down the progression of sarcopenia as well as the detrimental effects of unloading and bed rest on muscle loss in adult and old individuals. There are various different exercise programmes that are recommended for the older population in an attempt to combat sarcopenia.

#### **1.5.1.1. Resistance training**

There is mutual agreement that resistance exercise is effective at improving muscle function and the overall quality of life of older people. Resistance training is the requirement to generate force to move or resist weight such as weight lifting/push ups/leg press. Numerous studies have shown beneficial effects of resistance training in the function of skeletal muscle of the old; increasing muscle mass and strength (Fiatarone et al., 1994; Maltais et al., 2015) as well as cross-sectional area of myofibres (Fiatarone et al., 1994; Leenders et al., 2013) and motility (Fiatarone et al., 1994). Interestingly, Raue et al (2012) carried out analysis of the skeletal muscle transcriptome in young and old humans and the results suggested that the gene response following resistance exercise was most pronounced in type II rich fibres (Raue et al., 2012).

Studies have used a variety of different intensities and training programmes but there is data to suggest that just one bout of resistance exercise training per week

is sufficient to gain the same benefits as training up to three times a week (Taaffe et al., 1999). Although higher intensity resistance training leads to bigger muscle size than at a lower intensity, lower intensity is still capable of instigating increases in muscle mass (Bemben et al., 2000; Beneka et al., 2005; Csapo et al., 2015). However, it is worth noting that some studies have shown no correlation between physical activity and muscle mass (Mitchell et al., 2003) and only a higher level of physical activity and not 'leisure-time' activity are able to prevent or delay some sarcopenic effects (Raguso et al., 2006).

Improvements in muscle function following resistance training are thought to be due to an improved neuromuscular system (Taaffe et al., 1999), increased protein synthesis and attenuation of anabolic resistance (Schulte et al., 2001) and an increase in the satellite cell content of type II fibres (Verdijk et al., 2009a; Leenders et al., 2013).

#### **1.5.1.2. Aerobic training**

Aerobic training stimulates the heart and blood flow and provides cardiovascular conditioning. Examples of aerobic exercises include running, cycling and swimming. Although, the majority of the beneficial effects of aerobic exercise are cardiovascular, aerobic exercise has shown to increase the cross-sectional area of muscle fibres and can result in hypertrophy of old human muscles (Schwartz et al., 1991; Konopka et al., 2013). However the effects of aerobic exercise are not as well established as resistance exercise and it is likely that the hypertrophic effects of aerobic exercise depend on the frequency, intensity and length of exercise.

The effects of aerobic exercise on skeletal muscle are primarily through increases in mitochondrial proteins such as cytochrome C in the electron transport chain, and, the master of mitochondria biogenesis PGC-1 $\alpha$  (Short et al., 2003; Konopka et al., 2013). Increases in mitochondrial biogenesis result in improved mitochondrial function, metabolic control and respiratory capacity (Coggan et al., 1992; Short et al., 2003) consequently increasing the endurance of the individual. Furthermore, long term aerobic exercise programmes have shown an ability to reduce ROS production by muscle in old people (Ghosh et al., 2011). Aerobic exercise has also been shown to decrease anabolic resistance (Section 1.4.2) through the upregulation of protein synthesis through the Akt/mTOR pathway (Fujita et al., 2007), as well decreasing inflammation (Kohut et al., 2006).

#### **1.5.1.3. Other forms of physical activity**

Other forms of physical activity include power training. Power declines at a rate of 3-4% per year in older people and this is detrimental for everyday activities such as climbing stairs. To improve power, fast shortening resistance training is implemented. Improvements in skeletal muscle power (Fielding et al., 2002; Henwood et al., 2005; Reid et al., 2008) and in the ability to carry out every day activities in older people have been seen following power exercises (Henwood et al., 2005). These improvements are thought to be due to changes in the neuromuscular junction that allow a greater motor unit recruitment and firing rate of fast twitch fibres (Fielding et al., 2002; Reid et al., 2008). Some studies have shown power training to be more effective than slow velocity resistance training (Reid et al., 2008).

Many suggested physical activities may be too intense for older adults to maintain over a prolonged time. To combat this, less impact exercises such as whole-body vibrations and whole body electromyostimulation have been developed. These techniques use impulses that cause involuntary contractions of the muscles to preferentially recruit the fast twitch fibres that are most affected by ageing and this approach has been shown to increase maximum isometric strength and muscle mass (Kemmler et al., 2010; Kemmler et al., 2014) and grip strength (Stengel et al., 2015) in older women. These low impact exercises may be more suited for subjects who are unable to perform intense exercise programmes during sarcopenia.

Despite the evidence for some benefits of different types of exercise on muscle mass, even lifelong exercise is not the complete answer as a treatment or preventative for sarcopenia as lifelong exercise does not prevent sarcopenia (Kayani et al., 2008).

### **1.5.2. Nutrition**

Protein and other nutrients are vital for the protein synthesis required for muscle growth and maintenance. The effects that changes in protein synthesis and degradation have on sarcopenia have been discussed previously (Section 1.4.2). It is likely that nutritional intake may play a role in sarcopenia and altering nutritional intake may be able to relieve some symptoms of sarcopenia.

#### **1.5.2.1. Increase in protein intake**

Anabolic resistance is the inability of nutritional protein to be translated into protein synthesis effectively by muscle and this inability to increase protein synthesis

contributes to sarcopenia. In addition to anabolic resistance, around 30-40% of women over 50 and 20-40% of men over 50 do not reach the recommended daily intake of protein and it has been shown a low protein diet can be detrimental to muscle mass (Oumi et al., 2000). Therefore, a considerable number of studies examining interventions against sarcopenia have focused on increasing protein intake.

Studies using whole protein supplementation have shown that increasing the amount of overall protein intake can overcome the anabolic resistance in older people leading to an increase in protein synthesis, muscle mass and decrease proteolysis in rodents (Mosoni et al., 2014) and humans (Norton et al., 2015; Verreijen et al., 2015). Protein supplementation was also shown to increase SOD content (Shahar et al., 2013) in the blood of humans and glutathione concentrations in mice (Pouget et al., 2015), suggesting that increased protein supplementation may indirectly protect against oxidative damage. Furthermore a correlation between higher protein intake and muscle mass was found in women over 65 (Genaro et al., 2015) and it has been shown that protein intake of mothers during the pregnancy and lactation can affect the autophagy mechanism in the muscle of the offspring, demonstrating the importance of early life protein intake on muscle mass retention throughout life (Wanga et al., 2015).

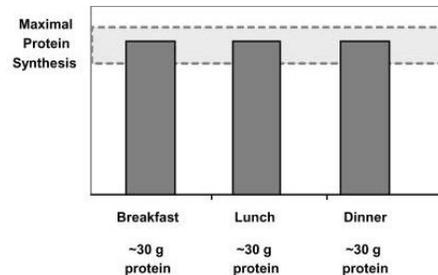
The essential amino acid profile, digestibility and bioavailability of ingested protein are important elements of the anabolic potential of protein. This was demonstrated in studies where anabolic resistance was overcome by increasing the percentage of leucine contained in the ingested protein rather than the total amount of protein (Volpi et al., 2003). The mechanism through which leucine achieves this

improvement in anabolic resistance in the old is likely to be through the upregulation of the Akt/mTOR pathway as well as decreasing proteolysis and autophagy (Volpi et al., 2003). Increases in Akt/mTOR pathway and decreases in proteolysis have also been shown *in vitro* (Sato et al., 2014) and *in vivo* which led to an increase in muscle mass when used as a single supplement of leucine or in combination with other nutrients (Sato et al., 2013b; Sato et al., 2015). Leucine supplementation leads to improved muscle regeneration in old rats through a decrease in inflammation and an increase in satellite cell proliferation resulting in an increase in the cross-sectional area of regenerated fibres compared with control animals (Pereira et al., 2015).

In contrast, meta-analysis of protein supplementation (Xu et al., 2014) showed no difference between the effect of protein supplementation and that of placebo groups on muscle mass, protein synthesis and muscle strength in older men (Dirks et al., 2014) or women (Zhu et al., 2015). Furthermore, Russ et al (2015) showed that protein supplementation attenuated muscle degradation through decreasing Murf1 expression, this did not translate to any functional benefits to muscles of old rats (Russ et al., 2015b).

One of the explanations of these negative studies may be the times at which intake of protein occurred. Symons et al (2007) showed that a 90g of protein meal in humans does not offer any more protein synthesis than a 30g meal in humans (Symons et al., 2007), suggesting that ingestion of more than 30g of protein in one meal is an energetically inefficient means of protein synthesis and that protein intake should be

spread out throughout the day to optimise muscle protein synthesis as shown below (Figure 1.10).



**Figure 1.10 Optimal distribution of protein intake throughout the day to maximise protein synthesis (Paddon-Jones et al., 2009)**

Benefits have been seen in muscle following protein supplementation however it is important to note the potential detrimental effects of a high protein diet. The most cited harmful effect of a high protein diet is the effect on renal function. Although it has been shown that high protein diets are relatively non-toxic to healthy kidneys (Manninen, 2004), it is possible that where other conditions are evident there may be detrimental effects on the kidney. High protein diets led to low glomerular filter rates in older humans (Walrand et al., 2008) and in mice where a high protein diet led to hypertrophy and increases in the size of the kidney (Hammond et al., 1998). A high protein diet can also be detrimental to the musculoskeletal system as high protein diets have shown negative calcium balance that could lead to osteoporosis in men (Allen et al., 1979). Other side effects of a high protein diet include weakness, nausea, diarrhoea problems and even death. Despite this, it is generally believed that 25-30g of high quality protein in a single meal is adequate to stimulate skeletal muscle protein synthesis in the old and that this may be beneficial as a treatment against sarcopenia.

### **1.5.3. Protein supplementation paired with exercise**

There is considerable evidence for beneficial effects of exercise and protein intake together on sarcopenia; therefore there are several studies that have combined the two as a potential therapeutic option for sarcopenia. These studies have shown that combined together, protein and exercise can increase muscle strength and mass in the old (Tieland et al., 2012; Shahar et al., 2013; Maltais et al., 2015; Palop et al., 2015), as well as increasing IGF1 and decreasing serum levels of IL6.

The ability of protein supplementation to increase muscle mass and strength further than with exercise alone is debateable. Some research groups suggest that protein supplementation will only enhance exercise-induced muscular improvements if there is an existing protein deficiency (Verdijk et al., 2009b). Thus there is increasing evidence that an increase in protein intake may be beneficial for attenuating sarcopenia where protein intake is sub optimal. This is particularly relevant in those who do not already reach the recommended daily intake of protein, where the amount and distribution of protein throughout the day alongside an effective exercise plan may play an essential part in whether the supplement will be effective.

### **1.5.4. Calorie restriction**

Calorie restriction is thought to be one of the most effective interventions of attenuating ageing, long term 30% reduction in calorie intake changed the transcriptional profile of an older individual similar to that of a younger subject, increased the production of antioxidants and decreased inflammation (Mercken et al., 2013). Restriction of the number of calories eaten has been proved to be life-extending

in numerous species (Weindruch et al., 1986; Lakowski et al., 1989; Jiang et al., 2000) as well as reducing all-cause mortality in rhesus monkeys (Colman et al., 2014).

The benefits of calorie restriction have been extended into sarcopenia. In rats, a 6 week 20% reduction in calorie intake led to an attenuation of age-related loss of muscle mass and function in the soleus and gastrocnemius muscles through an upregulation of PGC-1 $\alpha$  (Joseph et al., 2013a). Calorie restriction also preserved fibre number and type and protected mitochondrial DNA from deletion (Lee et al., 1998b). In rats, calorie restriction decreased apoptosis and protected from oxidative stress (Dirks et al., 2004) as well as a decrease in the overall oxidation (Hepple et al., 2008) in skeletal muscle. These data suggest that calorie restriction prevents sarcopenia potentially through an inhibition of apoptosis and enhancement of the mitochondrial function and this has been shown to occur through the upregulation of the NAD-deacetylase Sirt1 (Cohen et al., 2004). Sarcopenia was also attenuated by calorie restriction in the rhesus monkey (Colman et al., 2008).

## **1.6. Polyphenols**

Polyphenols are secondary metabolites of plants, where their role is predominantly protection from UV radiation and pathogens (Manach et al., 2004). Polyphenols are found in various foods and drinks such as fruits, vegetables, cereals and teas (Manach et al., 2004). There are thousands of molecules with a polyphenolic structure (aromatic rings with several hydroxyl groups) and depending on their structure, these polyphenolic molecules are further classified into flavanoids, phenolic acids, lignans and stilbenes (Bravo, 1998). The majority of polyphenols cannot be

absorbed in their native form and are therefore metabolised by intestinal enzymes or colonic microflora and further modified to allow entry into the blood and tissues, therefore the state of the polyphenols that are present in food are often very different to the ones that reach the blood and tissues (Scalbert et al., 2002).

Recent research in health and disease has focused on polyphenols as therapeutics for many diseases due to their apparent antioxidant (Young et al., 1999; Sies et al., 2005) and anti-inflammatory (Scoditti et al., 2012) effects, with fruit and vegetable intake associated with decreased IL6, CRP, TNF- $\alpha$  levels in the blood and reduced oxidative stress in adolescents (Holt et al., 2009) and adults (Folchetti et al., 2014). Therefore, polyphenols are thought to be beneficial in the fight against numerous diseases such as cardiovascular diseases for example, pomegranate juice reduced LDL oxidation in humans and mice (Aviram et al., 2000) and in mice this led to a decrease in the size of atherosclerotic lesion (Aviram et al., 2000). Black tea was found to lower blood pressure (Hodgson et al., 2012). Polyphenol intake has also shown to reduce the number of tumours in different types of cancer; green tea and quercetin both prevented proliferation of breast cancer (Sartippour et al., 2001; Deng et al., 2013) *in vitro* and curcumin inhibited prostate cancer in humans (Dhillon et al., 2008). Although numerous polyphenols have been shown to have anti-carcinogenic effects, the mechanisms of action are thought to differ. For example, curcumin decreased NF- $\kappa$ B activity in pancreatic cancer in humans (Dhillon et al., 2008), whereas, *in vitro*, a treatment of Du145 prostate carcinoma cells with black tea inhibited proliferation of the cancerous cells by inhibiting IGF-1 signalling (Klein et al., 2002) and

quercetin inhibited breast cancer cell expansion by retaining cells in the G0/G1 phase of cell proliferation (Deng et al., 2013). This and many other *in vitro* and *in vivo* studies provide a strong rationale in support of the use of dietary polyphenols as a human cancer chemoprevention. Polyphenols have been shown to be beneficial in diabetes, with quercetin (Rizvi et al., 2009) and tea catechins (Rizvi et al., 2005) reducing oxidation in blood cells isolated from diabetic patients and ferulic acid has been shown to lower blood glucose and increase plasma insulin levels leading to decreased cholesterol levels in diabetic mice (Jung et al., 2007).

Polyphenols have also been shown to have protective effects in neurodegeneration with a 10 year period of flavonoid supplementation negatively correlating to cognitive decline (Letenneur et al., 2007). A Mediterranean diet, which contains high amount of polyphenols, also correlates with a lower risk of Alzheimer's disease and Alzheimer's disease mortality (Scarmeas et al., 2006).

The anti-oxidative and anti-inflammatory effects of polyphenols have also led researchers to propose that polyphenols can be used as an 'anti-ageing' treatment (Maurya et al., 2009).

#### **1.6.1. Polyphenols and sarcopenia**

Since beneficial effects of polyphenols have been shown in some diseases, research has focused on the effect of polyphenols on sarcopenia. An 8 week treatment of rats with catechins resulted in an increase in muscle mass and cross-sectional area which was associated with decreases in the levels of muscle specific ubiquitin ligases

Murf1 and Atrogin1 and therefore in proteasomal degradation (Meadori et al., 2015), as well as increasing protein synthesis (Mirza et al., 2014). Catechins also led to increased muscle mass following recovery of hind limb unloading in the rat plantaris through activation of satellite cells and a decrease in apoptosis (Alway et al., 2014). Treatment of old rats with green tea extract has prevented the acute loss of muscle mass and force generation and the decrease in cross-sectional area following hind limb suspension through an increase in satellite cell proliferation and differentiation coupled with a decrease in oxidative stress and apoptosis markers in the plantaris muscle (Alway et al., 2015). These data provide evidence for polyphenols as potential treatments for sarcopenia, since increased intake of polyphenols may be more achievable than other current treatments available such as exercise (Section 1.5).

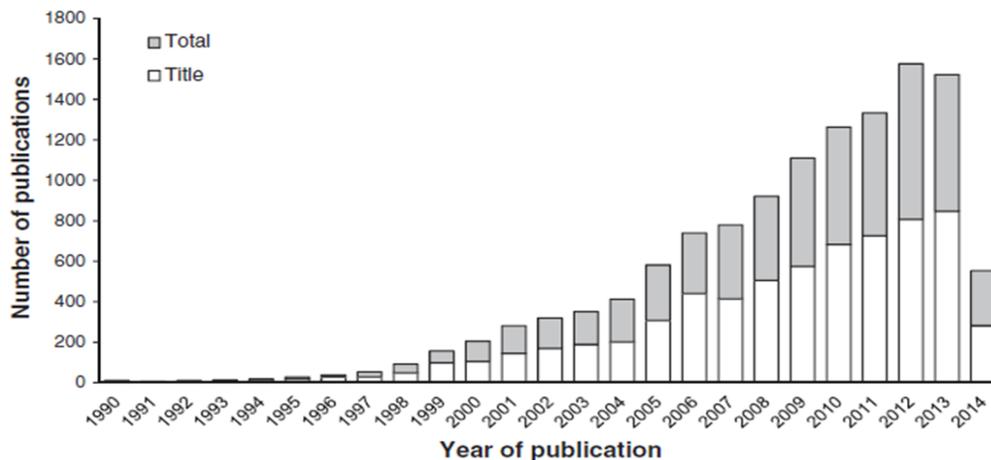
### **1.7. Resveratrol**

Resveratrol was first isolated from the roots of white hellebore (Takoka, 1940) and is found in high abundance in grapes and wine. Interest in resveratrol as a therapeutic agent began in 1992 when it was recognised as the beneficial component of wine that led to cardio-protective effects (Siemann et al., 1992). Resveratrol has also been linked with the 'French paradox'. The French paradox is the hypothesis that although the French nationals ingest high amounts of fat in their diet, the cardiovascular incidence does not represent the effects of this and this is thought to be due to the high intake of resveratrol through the high amount of wine the French consume (Renaud et al., 1992).

Despite this interest in resveratrol, research into the beneficial effects of resveratrol (Figure 1.11) only began once Jang et al (1997) identified that resveratrol could act as a chemo-protective agent at different stages of cancer progression (Jang et al., 1997) and in 2007 a cross-sectional study by Block et al (2007) showed that of all people who take regular supplements, 50% of people take resveratrol supplements (Block et al., 2007). Since research into resveratrol acting as a therapeutic agent began, resveratrol has been shown to be able to improve the symptoms of numerous diseases such as obesity (Baur et al., 2006), diabetes (Movahed et al., 2013; Asadi et al., 2015), cardiovascular diseases (Cheng et al., 2015), Alzheimer's disease (Porquet et al., 2014) and muscular dystrophy (Hori et al., 2011) and has been shown to increase memory (Witte et al., 2014). Thus, it is generally accepted that resveratrol has health benefits however the life extension properties of resveratrol are still unclear.

Evidence that resveratrol can increase lifespan has been shown in *Saccharomyces cerevisiae* (Howitz et al., 2003), *Caenorhabditis elegans*, *Drosophila melanogaster* (Wood et al., 2004) and *Nothobranchius furzeri* fish (Valenzano et al., 2006), however others show no difference in lifespan compared with controls (Bass et al., 2007; Pearson et al., 2008). Studies have shown life extension effects in organisms under oxidative stress but not normal conditions (Chen et al., 2013) and in mice on a high fat diet (Baur et al., 2006) suggesting the health status of the organism may be a determinant of the beneficial effects of resveratrol. For example Zou et al (2009) showed that the pro-longevity effects of resveratrol were dependent on calorie intake in the fruit fly (Zou et al., 2009). However, a meta-analysis of the ability of resveratrol

to cause life extension shows considerable variation between studies and few studies showed consistency within life extension properties thus it is concluded it is unlikely that resveratrol can increase lifespan (Hector et al., 2012).

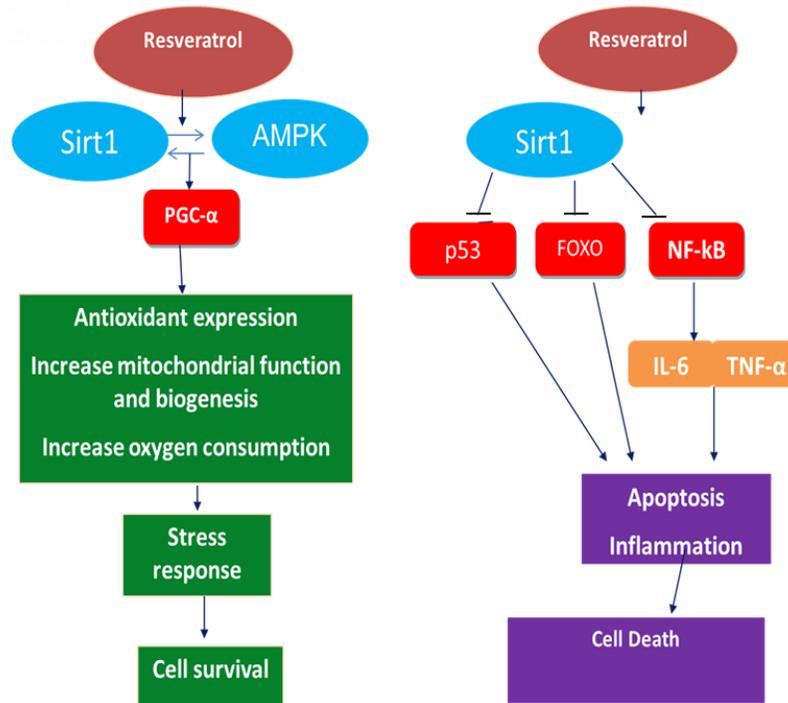


**Figure 1.11: Yearly publications with resveratrol in the title (1990–2014).** The original search with the chemical structure of RSV (CAS number 501-36-0) and the removal of duplicate articles which yielded 15,782 references which was accessed July 18, 2014 (Park et al., 2015).

### 1.7.1. Mechanisms of action of resveratrol

Similar to other polyphenols, resveratrol has also been shown to have antioxidant and anti-inflammatory properties and it is through these properties that resveratrol is proposed to offer beneficial effects to cells and tissues. Resveratrol is proposed to mimic calorie restriction (Wood et al., 2004; Pearson et al., 2008). These properties of resveratrol are thought to occur through direct activation of Sirt1 (Howitz et al., 2003; Wood et al., 2004; Borra et al., 2005; Lagouge et al., 2006; Cote et al., 2015). This is evidenced in transgenic mice with a Sirt1 deletion, where the effects of a resveratrol treatment were absent (Price et al., 2012).

Sirt1 is a member of the sirtuins family. Sirtuins are a conserved family of (NAD<sup>+</sup>) dependent deacetylases that are involved in the ageing process. Sirt1 resides mostly in the nucleus where it acts as a functional transcriptional repressor through histone deacetylation (Vaquero et al., 2004). One of the most repeatable and robust effects of resveratrol treatment is an increase in mitochondrial mass and biogenesis which is hypothesised to act through Sirt1 activation of the mitochondrial master regulator PGC1- $\alpha$  (peroxisome proliferator-activated receptor gamma co-activator 1 alpha) (Lagouge et al., 2006). Activation of PGC1- $\alpha$  will then lead to the activation of numerous transcription factors such as nuclear respiratory factor 1 (NRF1), PPAR $\gamma$  (peroxisome proliferator-activated receptor), ERR $\alpha$  (oestrogen-related receptor  $\alpha$ ) and Gabp $\alpha$  (GA binding protein transcription factor)/NRF2 (Wu et al., 1999; Mootha et al., 2004) which consequently leads to the increases in mitochondrial proteins such as COXIV, mitochondrial transcription factor A and cytochrome C (Wu et al., 1999) and in mitochondrial DNA. These increases in mitochondrial components lead to increases in total mitochondrial biogenesis, mass and function (Wu et al., 1999; Lehman et al., 2000). These changes in important mitochondria transcription factors also lead to increased antioxidant enzymes expression (Bellaver et al., 2014), increases in proteins responsible for alleviating stress from the endoplasmic reticulum (ER) and restoring homeostasis in the ER (Viswanathan et al., 2005), decreased apoptosis (Bournival et al., 2009) and thus protecting cells from oxidative damage and promoting cell survival (Figure 1.12).



**Figure 1.12 Postulated mechanism through which resveratrol carries out its antioxidant and anti-inflammatory effects.**

Despite the evidence for a role of Sirt1 activation in the cellular response to resveratrol there have been recent debates as to whether Sirt1 is activated by resveratrol (Beher et al., 2009; Guarente, 2012). These authors argue that evidence of Sirt1 activation following resveratrol treatment was a technical artefact of the fluorophore used (Beher et al., 2009). Furthermore, studies have shown no difference in Sirt1 activation following resveratrol treatment (Pacholec et al., 2010), others also believe AMPK (Dasgupta et al., 2007) or cAMP (Park et al., 2012) are the direct targets of resveratrol and these can consequently activate Sirt1. However, it is worth considering that the effects of resveratrol are likely to be dose dependent, with high and low doses of resveratrol having opposite effects (Calabrese et al., 2010). For

example <5mg/kg/day caused weight gain in mice on a high fat diet (Pearson et al., 2008) whereas treatment of mice with 400mg/kg/day resulted in weight loss in mice (Lagouge et al., 2006). Therefore, to date the mechanism of action of resveratrol is still highly controversial. This biphasic effect of resveratrol may account for some of the differences seen in studies and suggests that different concentrations may act through different pathways. This is an important for when considering resveratrol as a therapeutic agent, particularly when very high doses of resveratrol have been shown to have negative effects such as blunting beneficial cardiovascular effects of exercise (Gliemann et al., 2013), increasing the growth of mammary gland cancer (Castillo-Pichardo et al., 2013) and causing apoptosis in smooth muscle cells (Mnjoyan et al., 2003). Resveratrol also exacerbated multiple sclerosis (Sato et al., 2013a). High doses of resveratrol in human clinical trials have also caused diarrhoea, nausea and abdominal pain (Brown et al., 2010) and resveratrol has also been shown to cause cell senescence in dermal fibroblasts, however, where this occurred there was a down regulation of Sirt1 (Kilic Eren et al., 2015), suggesting that for the beneficial effects to occur Sirt1 must be activated. As well as a dose dependent effect, resveratrol has been shown to have the opposite effects when studied in cancerous cells compared to non-tumour cells (Baarine et al., 2011) suggesting that resveratrol also acts in a cell specific type manner.

Some of the downstream pathways shown to be affected by resveratrol include IGF-1 P13K/Akt (Jing et al., 2015), NRF2/ARE (Cheng et al., 2015), cAMP signalling (Zhang et al., 2015b), ERK (Wakabayashi et al., 2013) and Wnt signalling

(Zhou et al., 2015). Resveratrol has also been shown to directly scavenge ROS (Leonard et al., 2003; Ungvari et al., 2007).

### **1.7.2. Resveratrol and inflammation**

The anti-inflammatory effects of resveratrol are well established with resveratrol having the ability to decrease pro-inflammatory cytokines such as TNF- $\alpha$ , IL6, MCP-1, IL8 (Feng et al., 2002; Csaki et al., 2009; Wakabayashi et al., 2013) and increase anti-inflammatory cytokines (Song et al., 2014a) in many different cells and tissues and in the plasma.

The majority of cytokines mediate their effects through the transcription factor NF- $\kappa$ B and most anti-inflammatory agents diminish NF- $\kappa$ B activation in cells. The decreases in inflammatory markers by resveratrol have been shown occur through the suppression of NF- $\kappa$ B activation (Csaki et al., 2009). These anti-inflammatory effects of resveratrol have been shown in numerous cell types, as well as the ability of resveratrol to inhibit FOXO (Motta et al., 2004) and p53 (Eo et al., 2013), lead to a decrease in apoptosis and prevent cell death (Csaki et al., 2009). Resveratrol has also been shown to decrease inflammatory mediated production of ROS (Martinez et al., 2000). These decreases in inflammation have shown to attenuate inflammatory related diseases such as rheumatoid arthritis (Arend et al., 1995) and asthma (Royce et al., 2011).

## 1.8. Resveratrol and sarcopenia

Evidence for resveratrol as a therapeutic intervention for sarcopenia was provided with studies showing that resveratrol can be beneficial to skeletal muscle in number of ways. From a functional aspect, resveratrol prevented the loss of muscle mass and force and cross-sectional area in muscle in an unloaded model in rats (Jackson *et al.*, 2010; Dolinsky *et al.*, 2012). Resveratrol fed mice also had increased grip strength, increased time to fatigue and a decrease in biochemical markers of muscle fatigue such as ammonia and lactate (Wu *et al.*, 2013). Furthermore, resveratrol was able to prevent myotube atrophy following treatment of C2C12 cells with TNF- $\alpha$  (Wang *et al.*, 2014) and dexamethasone (Alamdari *et al.*, 2012) through the suppression of Murf1 and Atrogin1 and also ameliorated motor neuron loss, thus leading to an improved survival rate in an Amyotrophic Lateral Sclerosis (ALS) mouse model (Song *et al.*, 2014b).

Similar to other tissues, resveratrol is proposed to act through improved mitochondrial function (Lagouge *et al.*, 2006; Momken *et al.*, 2011; Joseph *et al.*, 2013a) thus, decreasing oxidative stress through increases in MnSOD and catalase activities, decreasing hydrogen peroxide production and lipid peroxidation and inhibiting apoptosis by decreasing p53, FOXO3, caspase 3 and 9, bax and increasing Bcl2 (Jackson *et al.*, 2010; Jackson *et al.*, 2011; Sin *et al.*, 2015). There is also evidence that resveratrol is able to prevent age-related changes in fibre type percentages (Jackson *et al.*, 2011; Zhang *et al.*, 2015a) and protein degradation (Russell *et al.*, 2006;

Wilson et al., 2015) as well as resulting in an increase synthesis of muscle contractile proteins (Lagouge et al., 2006).

The anti-inflammatory effects of resveratrol on other tissues are well known, however the anti-inflammatory effects of resveratrol on skeletal muscle are less clear. Resveratrol had no effect on CRP content in the blood of marathon runners (Laupheimer et al., 2011; Olesen et al., 2014). Whereas, in mdx mice, there was a decrease in inflammation cell infiltrates into the muscle following resveratrol treatment compared with non-treated mdx mice (Gordon *et al.*, 2014). Resveratrol also attenuated cancer mediated cachexia through the down regulation of NF- $\kappa$ B in muscle leading to a decrease in inflammation and a reduction in proteolysis of the sarcomere (Shadfara et al., 2011). However research into the anti-inflammatory effects of resveratrol on skeletal muscle is sparse and more work is needed in this area.

Resveratrol induced differentiation and hypertrophy in skeletal muscle cells (Montesano et al., 2013). Hypertrophy is likely caused by an increased IGF-1/Akt/mTOR pathway induction by resveratrol (Montesano *et al.*, 2013; Wang *et al.*, 2014). Resveratrol down-regulated microRNA 133, which is involved in muscle skeletal proliferation (Kaminskia et al., 2012b), and decreased cell proliferation, p21 and cyclins expression in C2C12 cultures following resveratrol treatment compared with untreated C2C12 cells. However resveratrol treatment also resulted in the increased of the myogenic regulators Myf-5, MyoD, myogenin and important structural proteins in C2C12 cells (Montesano et al., 2013). Importantly, this shows that it is unlikely that resveratrol is toxic to muscle cells and the decrease in cell number seen by Kaminskia

et al (2012) is likely due to the fusion of myoblasts into myotubes. These data suggest that resveratrol is potentially a differentiating agent, and may be beneficial during muscle regeneration. This has been further evidenced by treatment of C2C12 cells with low doses of resveratrol promoting muscle regeneration (Bosutti *et al.*, 2015) and *in vivo* (Bennett et al., 2013). Whether this improved regeneration was due to increases in satellite cells number (Bennett et al., 2013) or not (Ballak et al., 2015) is debateable, although increased Sirt1 has been shown to increase proliferation of satellite cells (Rathbone et al., 2009). Some studies have found that resveratrol was not able to protect against muscle wasting or fatigue (Busquetsa et al., 2007; Jackson et al., 2011), improve mitochondrial function (Joseph et al., 2013a) or have any beneficial effects on muscle regeneration (Rogers et al., 2015). Olesen et al (2014) showed that resveratrol was prevented the beneficial exercise-induced effects on protein oxidation and inflammation in older men (Olesen et al., 2014). However, treatment with resveratrol in senescence-accelerated prone (SAMP1) mice with resveratrol resulted in increased endurance and mitochondrial function-related enzymes (cytochrome oxidase III/IV, MCAD) (Murase et al., 2009).

Similar to other tissues, the mechanisms by which resveratrol was able to offer protection is debatable. Some studies showed that treatment with resveratrol resulted in an increase in muscle expression of Sirt1 (Joseph et al., 2013a) and PGC1- $\alpha$  (Kaminskia et al., 2012b) whereas others showed no change in the protein expression of Sirt1 (Ringholm et al., 2013), PGC1- $\alpha$  (Barger et al., 2008), mitochondrial genes or mitochondrial DNA content (Ringholm et al., 2013). In a similar manner to other

tissues, a lot of these discrepancies can possibly be explained by the difference in doses of resveratrol treatment, for example 10 $\mu$ M resveratrol was shown to be able to protect C2C12 muscle cells from oxidative damage, but higher doses were not (Bosutti *et al.*, 2015). Also a 25mg/kg/day dose of resveratrol was more beneficial at protecting muscle against fatigue than 50mg resveratrol/kg/day or 125mg resveratrol/kg/day (Wu *et al.*, 2013). There is considerable variability in doses and time courses of treatments with resveratrol within studies which reach contrasting conclusions.

Interestingly, although resveratrol was able to protect old mice from losing muscle mass and function during unloading, resveratrol had no effect on muscle mass or force generation in young mice (Jackson *et al.*, 2010) suggesting that the signalling pathways involved may be different during ageing. Some of these discrepancies may also be explained by to the different fibre type compositions of these muscles. For example, Sirt1 protein expression was increased in the white (type II) portion of the gastrocnemius following treatment with resveratrol, whereas there was no effect of resveratrol on Sirt1 protein expression in the red (type I) portion (Joseph *et al.*, 2013a). These data provides further evidence that the health status of the subject may be a major determinant of the outcome of resveratrol treatment, as it is type II fibres which are primarily affected during ageing. This would therefore explain why treatment with resveratrol could provide protection to type II fibres but not type I fibres.

These data suggests that resveratrol may be able to attenuate some of the effects of sarcopenia and may have the potential to be a therapeutic agent; however it is vital to validate the effects of different doses of resveratrol and to optimise the

length of treatment. Furthermore, the concentrations used in these studies, particularly *in vivo*, are extremely high and not achievable by diet; it is therefore a priority to identify whether physiological doses of resveratrol can provide these beneficial effects.

### **1.9. Aims and Objectives**

The main objectives of this study were to:

1. Establish an *in vitro* model to test the effects of resveratrol on primary muscle cells in culture and identify a concentration of resveratrol that was effective in both undifferentiated (myoblasts) and differentiated (myotubes) primary skeletal muscle cells.
2. Establish an *in vitro* model to study the effect of increased levels of IP10 on muscle cell atrophy and inflammation.
3. Examine whether a treatment of primary myotubes with resveratrol is able to protect against IP10 induced effects on skeletal muscle cell atrophy or inflammation.
4. Identify an effective dose of resveratrol in mice *in vivo* and examine the effect of this concentration on skeletal muscle force generation in adult and old mice.
5. Establish a 3D muscle construct model that could potentially be used to study the direct effects of compounds on skeletal muscle function *in vitro*.

The overall hypothesis of this thesis is that the increase in serum IP10 levels during ageing contributes to sarcopenia and causes an increase in markers of atrophy.

Due to the potential health benefits of resveratrol, a further hypothesis is that resveratrol is able to protect against some of these negative effects through its antioxidant and anti-inflammatory properties.

## **2. MATERIALS AND METHODS**

## 2.1. Project overview

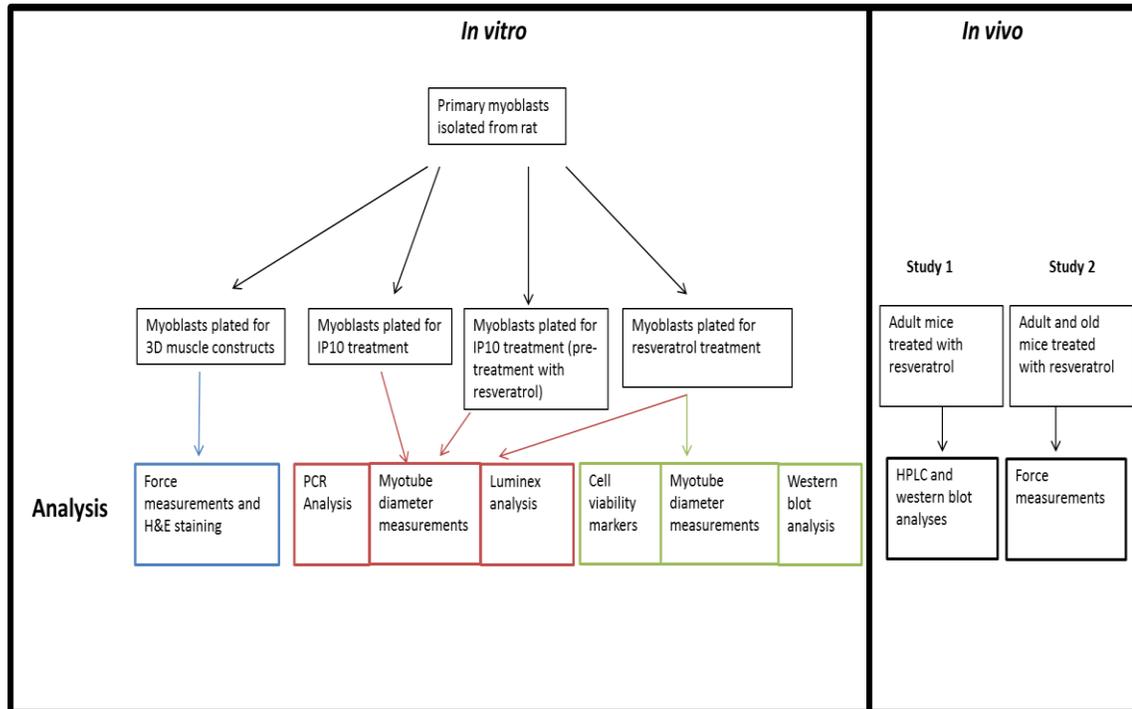


Figure 2.1 Schematic diagram showing overall experimental approach of thesis.

## 2.2. Animals

### 2.2.1. Animal husbandry & Home Office licence permissions

All experiments followed UK Home Office Animal Licensing Laws for the care and use of laboratory animals. All animals were housed in the specific pathogen free Biomedical Services Unit at the University of Liverpool where they were monitored on a daily basis. The animals had standardised lighting system (12 hr light cycles), controlled temperatures ( $22\pm 2^{\circ}\text{C}$ ) and access to food and water ad libitum.

### 2.2.2. Rat studies

3-4 week old wild type Wistar rats were used to generate primary muscle cells. Rats were used for these studies as it has not been possible to make 3D muscle

constructs (also known as myoids) using cells isolated from mice (Larkin Lab, University of Michigan, unpublished).

### **2.2.3. Mouse studies**

Young (approximately 6 months) and old (approximately 24 months) C57BL/6 male mice were used for the mouse studies.

## **2.3. Cell culture and treatment of primary cells isolated from rats**

### **2.3.1. Isolation of primary skeletal muscle cells**

#### **Reagents:**

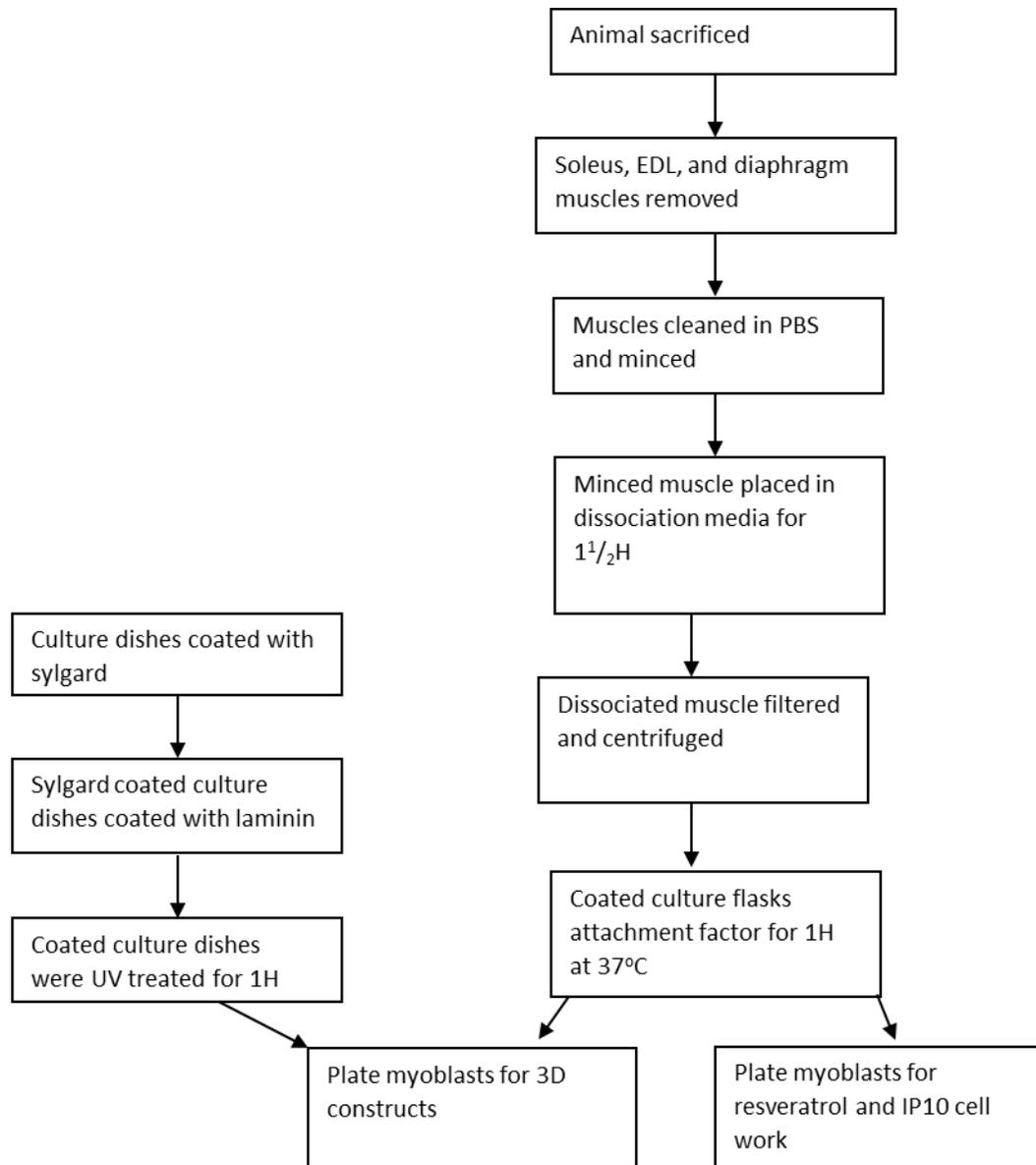
- Dissociation medium (for 200mg of tissue):
  - 0.4 units/mg Collagenase type IV (Invitrogen, Paisley, UK)
  - 239 units/mg Dispase (Invitrogen, Paisley, UK)
  - 35ml Hams-F12 (Invitrogen, Paisley, UK)
- Ethanol
- DPBS (Sigma Aldrich, Dorset, UK)
- Attachment factor protein (Invitrogen, Paisley, UK)
- Growth medium:
  - 60% v/v Hams F-12 nutrient mix (Invitrogen, Paisley, UK)
  - 25% v/v Dulbecco's modified eagle's medium (DMEM, Sigma Aldrich, Dorset, UK)
  - 15% v/v foetal bovine serum (FBS, Sigma Aldrich, Dorset, UK)

- 100µmol/ml penicillin and 0.1mg/ml streptomycin (Sigma Aldrich, Dorset, UK)

**Protocol:**

This method of rat primary cell isolation was adapted from a well-established protocol (Larkin et al., 2006) and results in a high myoblast to fibroblast ratio. Rats were sacrificed using CO<sub>2</sub>, cleaned with ethanol and the diaphragm and both the left and right soleus and EDL muscles were dissected and placed in DPBS.

The removed muscle was cleaned in ethanol and then washed in DPBS. Visible connective tissue was removed and the muscle minced in Hams F-12 nutrient mix using a razor blade. Approximately 200mg portion of muscle was placed in dissociation medium and another 15ml of Hams-F12 was added to each muscle portion to make a final volume of 35ml. The muscle was incubated for 1.5H at 37°C and was dissociated by pipetting every 15 minutes. After incubation, the solution was further dissociated by repetitive pipetting and the dissociated tissues were filtered through a 70µm mesh. The solution was centrifuged for 10 minutes at 250g and the pellet was re-suspended in growth medium. Cells were plated into a 25cm<sup>2</sup> tissue culture flask that had been previously coated with attachment factor for 1H to enhance the adhesion of the cells to the flask. Cells were left for 48H in a 37°C, 5% CO<sub>2</sub> incubator. A schematic representation of isolation of primary cells from skeletal muscle is shown in Figure 2.2.



**Figure 2.2** Flow diagram showing the dissociation and isolation of primary skeletal muscle cells. Cells are isolated, connective tissue removed and placed in dissociation medium for 1.5H. When cells reached 80% confluence, depending on the experiment, cells were then plated on laminin coated sylgard plates or were split for further culture.

### 2.3.2. Culture of primary muscle cells from rat

A large number of cells were required for several different experiments therefore, the cells were cultured until adequate numbers were reached. For each

primary cell isolation, cells were used between passage 1 and 6 as myoblasts did not differentiate into myotubes adequately if passaged further (data not shown).

**Reagents:**

- DPBS
- 0.025% Trypsin (Sigma Aldrich, Dorset, UK)
- Growth medium:
  - 60% v/v Hams F-12 nutrient mix
  - 25% v/v Dulbecco's modified eagle's medium
  - 15% v/v foetal bovine serum
  - 100µmol/ml penicillin and 0.1mg/ml streptomycin
- Differentiating medium:
  - 70% v/v Medium 199 (Invitrogen, Paisley, UK)
  - 24% v/v Dulbecco's modified eagle's medium
  - 6% v/v FBS (Sigma Aldrich, Dorset, UK)

All media were stored at 4°C until use and warmed to 37°C in a heated water bath for 20 minutes before use.

**Protocol:**

Forty eight hours after isolation, myoblasts were washed with DPBS and new growth medium was added to flasks. Once myoblast had reached 80% confluence, they were washed with DPBS and incubated in 3ml of trypsin at 37°C for 5 minutes to aid detachment from culture flasks. Detachment was confirmed by viewing the myoblasts

under the microscope. Myoblasts were re-suspended in approximately 1ml of growth medium (this volume varied depending on the size of the cell pellet) and 10µl of cell suspension was pipetted into a cell counter slide and counted using a TC-20 automatic cell counter (Biorad, Hertfordshire, UK) and plated at the appropriate density ( $2 \times 10^4$  per well) or split into adequate flasks (4 T25 flasks for each concentration at each time point).

#### **2.4. Treatment of rat primary muscle cells with resveratrol**

##### **Reagents:**

- Growth medium
- Resveratrol (Sigma Aldrich, Dorset, UK)

##### **Protocol:**

Following culture of myoblasts (Section 2.3.2), myoblasts were placed into 6 well plates at a density of  $2 \times 10^4$  for myoblast studies and  $5 \times 10^4$  for myotube studies and were left overnight to adhere. Resveratrol (Sigma Aldrich, Dorset, UK) that had been previously dissolved in distilled water and stored at  $-20^{\circ}\text{C}$  at a concentration of 5mM, was further diluted in growth medium or differentiation medium to reach a final concentration of 0.1, 1 or 10µM. For myoblast experiments, resveratrol was added to myoblasts for 3, 6, 12, 24 or 48H. For myotube experiments, myotubes were treated daily for 3, 5, 7 or 10 days. A detailed experimental plan is given in the appropriate chapter.

## **2.5. IP10 treatment of rat myotubes**

### **Reagents:**

- IP10 (Sigma Aldrich, Dorset, UK)

### **Protocol:**

Murine IP10 was reconstituted in dH<sub>2</sub>O and stored at -20°C at a concentration of 0.1mg/ml. IP10 was further diluted with differentiation medium to reach a final concentration of 150pg/ml or 200pg/ml. Recombinant IP10 was purified by the manufacturer and contained less than <0.2EU/ml.

## **2.6. Cell counts**

Trypan blue is routinely used in cell culture for analysing the viability of cells. Trypan blue is an impermeable dye and cannot penetrate the intact cell membranes of live cells, therefore will only stain unviable cells blue, allowing dead and live cells to be distinguished. The TC-20 automated cell counter (Biorad, Hertfordshire, UK) analyses multiple focal planes to identify the optimal plane of view of the cells. This optimal focal plane is then used for the cell counting algorithm to determine the number of cells. Using this automated machine is thought to improve the accuracy of cell counts by preventing the incorrect focal plane to be selected by user error. Automatic cell counters also allow users to set the width of the cell type to eliminate any artefacts that may be miscounted as cells.

**Reagents:**

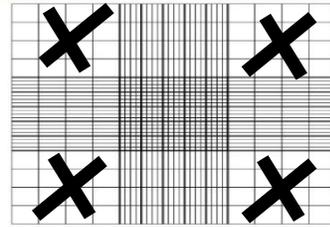
- DPBS
- 0.025% Trypsin containing EDTA (Sigma Aldrich, Dorset, UK)
- Growth Medium
- 0.4% Trypan Blue (Invitrogen, Paisley, UK)

**Protocol:**

Following the appropriate treatment and time points (Section 2.4), myoblasts were detached using trypsin (Section 2.3.2). Trypsin was neutralised with growth medium and cells were centrifuged at 100g for 5 minutes. Supernatant was removed and the cell pellet was re-suspended in 1ml of growth medium. Fifty microlitres of cell suspension was then mixed 1:1 with trypan blue. For manual counting, 10 $\mu$ l of cell suspension was mixed with trypan blue and added into each side of a cover slipped haemocytometer and 4 measurements were taken from each corner of the haemocytometer (Figure 2.3). For automatic cell counting, 10 $\mu$ l of sample and trypan blue mix was added into a cell counter slide and placed in the TC-20 automated counter and this repeated 4 times to gain 4 measurements for each sample.

For analysis of the effect of resveratrol on cell number, each measurement per well was averaged and all wells with the same treatment were then averaged to give an overall cell number per treatment. This was repeated for each time point.

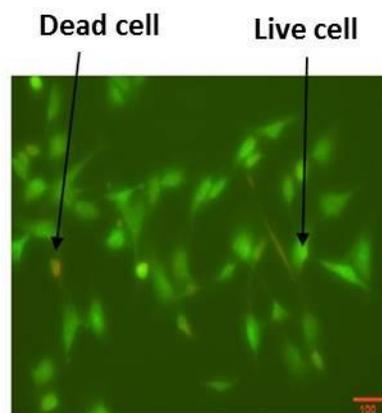
For all further experiments only TC-20 automatic cell counter was used to identify cell number.



**Figure 2.3 Hemacytometer: X marks where measurements were taken for each sample.**

### **2.7. Live and Dead assay**

Live and dead cells were visualised using a LIVE/DEAD<sup>®</sup> Reduced Biohazard Viability/Cytotoxicity Kit (Life technologies, Warrington, UK). The live and dead kit tests the viability of cells by distinguishing between the permeability of dead and live cell membranes. SYTO10 is a cell permeable dye which will penetrate all cell membranes staining all cells nuclei green. DEAD Red is a cell impermeable dye which can only stain cells with compromised membranes, and therefore will only stain the nuclei of dead cells (red). Thus, live cells stain green and dead cells stain red (Figure 2.4).



**Figure 2.4 Representative image of live and dead stained myoblasts. Scale bar, 100μm.**

**Reagents:**

- SYTO10 Green fluorescent Nucleic Acid Stain (Molecular Probes, Eugene, Oregon)
- DeadRed fluorescent nucleic acid stain (Molecular probes, Eugene, Oregon)
- Hepes Buffer Saline Solution (HBSS):
  - 135mM NaCl (Sigma Aldrich, Dorset, UK)
  - 5mM KCl (Sigma Aldrich, Dorset, UK)
  - 1mM MgSO<sub>4</sub> (BDH Prolabo, Leicestershire, UK)
  - 10mM Hepes (Sigma Aldrich, Dorset, UK)
  - 1.8mM CaCl<sub>2</sub> (Sigma Aldrich, Dorset, UK)
  - 4% glutaraldehyde (Sigma Aldrich, Dorset, UK)

**Protocol:**

Following treatment, cell media was removed and cells were washed in HBSS. Two microlitres of SYTO10 and 2µl of DeadRed was added to 1ml of HBSS (1:500 dilution of each) and mixed thoroughly by vortexing. Five hundred microlitres of the mixture was added to each well and incubated at room temperature for 15 minutes in the dark. Following incubation, media was removed and cells washed with HBSS. Cells were then fixed in 4% glutaraldehyde and placed at 4°C until viewing with the Nikon Eclipse TE2000 (Nikon, Kingston upon Thames, UK).

## **2.8. Measurement of myotube diameter**

Myotubes were viewed using a Nikon Eclipse TE2000 (Nikon, Kingston upon Thames, UK) at X20 magnification and 6 images taken from 6 different areas in each well. Each image was then analysed using ImageJ (US National Institutes of Health, Maryland, USA). For each image, every myotube was measured 3 times along its length to give an average diameter per myotube, each myotube was then pooled to give an average per well. The average per well was then used to determine an average for the treatment groups.

## **2.9. Harvesting of cells for protein extraction**

### **Reagents**

- DPBS
- 1% SDS (Sigma Aldrich, Dorset, UK)
- Protease inhibitors (Sigma Aldrich, Dorset, UK)

### **Protocol:**

Cell media was removed and myoblasts were washed and scraped in PBS and centrifuged at 14000g for 5 minutes at 4°C. The supernatant was aspirated and cells were re-suspended in 50µl of 1% SDS containing protease inhibitors. Cells were sonicated on ice for 15 seconds and centrifuged at 14000g for 5 minutes at 4°C. The pellet of the sonicated samples was discarded and a BCA assay was carried out on the supernatant to determine protein concentration.

## 2.10. Analysis of protein content of samples using the bicinchoninic acid assay

Bicinchoninic acid assay (BCA) (Smith et al., 1985) is an assay used to detect the concentration of proteins in a sample and is based on the method developed by Smith et al (1985). The principle of the BCA assay is based on  $\text{Cu}^{2+}$  protein complex formation. The reduction of  $\text{Cu}^{2+}$  to  $\text{Cu}^{1+}$  is proportional to protein content of the sample, resulting in the formation of a purple coloured complex with  $\text{Cu}^{1+}$  which can be quantified using a spectrophotometer (BMG laboratories, Buckinghamshire, UK)

### Reagents:

- Bicinchoninic acid (BCA) solution (Sigma Aldrich, Dorset, UK, Dorset UK)
- Copper sulphate (Sigma Aldrich, Dorset, UK, Dorset UK)
- Distilled water

### Protocol:

The BCA assay was carried out using a 96 well plate. A standard curve was generated from a 1mg/ml stock solution of protein standard (Sigma Aldrich, Dorset, UK, Dorset UK) as shown in Table 2.1.

**Table 2.1 Typical concentrations of a standard curve for protein determination using the BCA assay.**

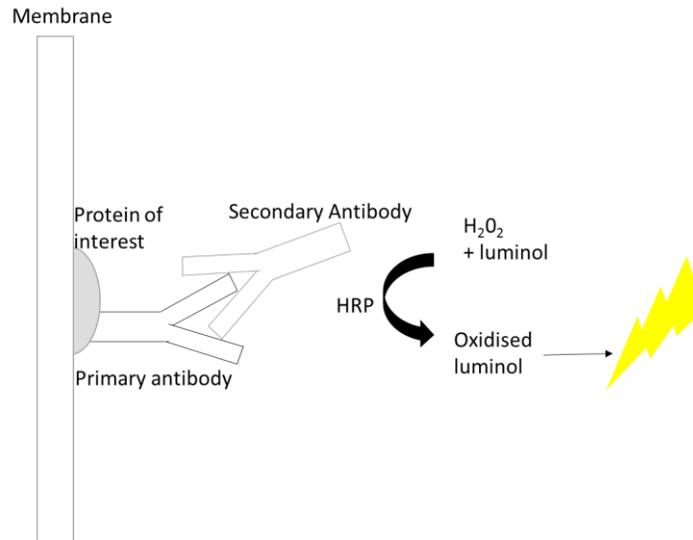
Standard	Concentration
1	500 $\mu\text{g}/\text{ml}$
2	250 $\mu\text{g}/\text{ml}$
3	200 $\mu\text{g}/\text{ml}$
4	150 $\mu\text{g}/\text{ml}$
5	100 $\mu\text{g}/\text{ml}$
6	50 $\mu\text{g}/\text{ml}$
7	25 $\mu\text{g}/\text{ml}$
B	DH <sub>2</sub> O

**Protocol:**

Twenty microlitres of each standard and samples that had been previously diluted with  $\text{DH}_2\text{O}$  was added to a well. Immediately before use, copper sulphate was diluted 50x with BCA solution and 200 $\mu\text{l}$  of this added to each well. The plate was then incubated at 37°C for 30 minutes before measuring the absorbance at 570nm on the spectrophotometer (BMG laboratories, Buckinghamshire, UK). The protein content was calculated from the standard curve. Each standard and sample was analysed in duplicate.

**2.11. Analysis of MnSOD, catalase and Sirt1 protein content of samples using SDS-PAGE and western blotting**

SDS-PAGE and western blotting is a technique commonly used in research to separate and identify proteins in a sample. Proteins are separated in polyacrylamide gels and transferred on to a membrane for visualisation. Membranes are firstly blocked in milk and then incubated with the antibody for the protein of interest; membranes are then incubated with the secondary antibody which has a horseradish peroxidase (HRP) label. This secondary antibody will bind to the primary antibody. Membranes are then placed in an ECL solution; the HRP will form a fluorescent complex that allows visualisation with a chemiluminescence imager that will convert the fluorescent signal from the HRP-ECL binding into a digital image (Figure 2.5).



**Figure 2.5 ECL Western blot detection method summary. Primary antibody binds to the antigen of the protein of interest. The secondary antibody is conjugated to the enzyme (HRP) and binds to the primary antibody. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) activates HRP which will lead to the oxidation of luminol, which releases light that can be detected by the chemiluminescence imaging system.**

### 2.11.1. Preparation of polyacrylamide gels

#### Reagents:

- Stock acrylamide solution:
  - 30% acrylamide, 0.8% bisacrylamide cross-link, in dH<sub>2</sub>O (Protogel, National Diagnostics, USA)
- Resolving buffer:
  - 1.5M Tris/HCl, 0.384% SDS, pH 8.8 (Protogel, National Diagnostics, USA)
- Stacking Buffer:
  - 0.5M Tris/HCl, 0.4% SDS, pH 6.8 (Protogel, National Diagnostics, USA)

- 12% acrylamide solution:
  - 8ml stock acrylamide solution
  - 5ml resolving buffer
  - 6.8ml DH<sub>2</sub>O
- 4% acrylamide solution:
  - 1.3ml stock acrylamide solution
  - 2.5ml stacking buffer
  - 6.1ml DH<sub>2</sub>O
- 10% ammonium persulphate solution (APS, Sigma Aldrich, Dorset, UK, Dorset UK)
- NNN'N-tetramethylethylene-diamine (TEMED, Sigma Aldrich, Dorset, UK, Dorset UK)
- 2-propanol (Sigma Aldrich, Dorset, UK, Dorset UK)

**Protocol:**

Twelve percent and 4% polyacrylamide gels were prepared as described above. For the 12% acrylamide solution, gel formation was catalysed by the addition of 200µl of 10% aqueous ammonium persulphate solution (APS) and 20µl of NNN'N-tetramethylethylene-diamine (TEMED). Following the addition of APS and TEMED, the solution was immediately poured between 2 gel plates (8 x 10cm) with 1.5mm spacers, 200µl of 2-propanol was added to the top of the gel to ensure the gel was even and allowed to set. Following the crosslinking of the 12% gel, 2-propanol was poured off. A 4% acrylamide solution was prepared, with the addition of 100µl of 10% APS and 25µl

of TEMED and was added on top of the 12% resolving gel and a well comb placed in position to allow sample loading.

### **2.11.2. SDS PAGE**

#### **Reagents:**

- Molecular weight marker (17-225kDA) (Amersham, Buckinghamshire, UK)
- Running buffer:
  - 10X Tris/Glycine/ SDS (w/v) (National Diagnostics, Hull, UK)
  - Distilled water

#### **Protocol:**

Pre-prepared gels were loaded into gel tanks and 1x running buffer added. Protein was loaded into the wells in the pre-prepared acrylamide gels and electrophoresis carried out. Electrophoresis was carried out using an electrophoresis tank (Gene flow, Litchfield, UK) with a power pack (Biorad, Hertfordshire, UK) with a constant current of 12mA per gel until the samples had run through the 4% stacking gel. The current was then increased to 20mA per gel until the bromophenol blue dye had reached the bottom of the resolving gel (2-3 hours). The separated proteins were then transferred from the acrylamide gel on to a nitrocellulose membrane by western blotting.

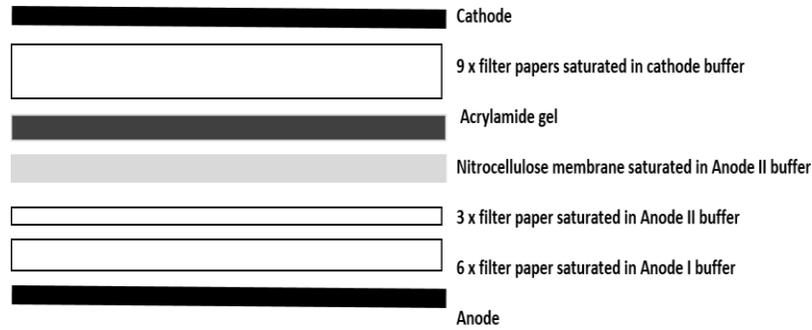
### 2.11.3. Western blotting

#### Reagents:

- Anode 1 buffer: 0.3M Tris (Sigma Aldrich, Dorset, UK) in a 20% Methanol (Sigma Aldrich, Dorset, UK) solution, pH 10.4
- Anode 2 buffer: 25mM Tris in a 20% methanol solution, pH 10.4
- Cathode buffer: 40mM 6-amino n hexanoic acid (Sigma Aldrich, Dorset, UK) in a 20% methanol solution, pH 7.6.
- Ponceau S (Sigma Aldrich, Dorset, UK)

#### Protocol:

The Geneflow blotting system (Gene flow, Litchfield, UK), consists of two graphite plate electrodes with Whatman No1 filter paper used as a buffer reservoir (Figure 2.1). Following electrophoresis, the acrylamide gel was removed from the glass plates and the 4% stacking gel was discarded. The 12% resolving gel was placed on top of the nitrocellulose membrane and sandwiched as shown in Figure 2.1 and a current of 100mA per gel was applied to the system for a minimum of 1H. Following transfer, membranes were stained with Ponceau S (Sigma, Dorset, UK) to ensure equal loading and transfer of proteins.



**Figure 2.6 Schematic of western blotting-setup.**

#### **2.11.4. Processing and development of nitrocellulose membrane**

##### **Reagents:**

- PBS tween solution: 0.005% (v/v) polyoxyethylene-sorbitan monolaurate (Tween-20) (Sigma Aldrich, Dorset, UK) in PBS solution
- Blocking solution: 5% milk powder in 0.005% PBS Tween
- Primary antibody solution: 1% of milk powder in 0.005% PBS Tween
- Secondary antibody solution: 3% of milk powder in 0.005% PBS Tween
- Antibodies (Table 2.2)
- Chemiluminescence reagent kit (ECL, Thermochemical, Cheshire, UK)

##### **Protocol:**

Following electroblotting, membranes were placed in 10ml of a blocking solution consisting of 5% milk for 1H. The membrane was washed with PBS tween and incubated overnight at 4°C with the appropriate primary antibody (Table 2.2). Membranes were washed for 3x5 minute with PBS-Tween and then incubated at room

temperature for 1H with the appropriate secondary antibody (Table 2.2). After 3x5 minute washes, membranes were developed with a chemiluminescence reagent kit (ECL, Thermochemical, Cheshire, UK), placed between acetate sheets and analysed using a chemiluminescence imager (Biorad, Hertfordshire, UK).

#### **2.11.5. Primary antibodies:**

##### **2.11.5.1. MnSOD**

Rabbit polyclonal antibody (Enzo, Exeter, UK) reconstituted in sodium phosphate buffer containing 15mg/ml BSA, 0.09% sodium azide, and 50% glycerol. Detects a band at approximately 25kDa.

##### **2.11.5.2. Catalase**

Mouse polyclonal antibody (Sigma Aldrich, Dorset, UK) reconstituted in 0.01M phosphate buffered saline, pH7.4, containing 15 mM sodium azide. Detects a band at approximately 60kDa.

##### **2.11.5.3. Sirt1**

Mouse polyclonal antibody (Abcam, Cambridge, UK) reconstituted in 1% BSA containing 0.02% Sodium Azide. Detects a band at approximately 120kDa.

##### **2.11.5.4. Acetylation**

Rabbit polyclonal antibody (Cell signalling, Boston, USA) reconstituted in 10mM sodium HEPES, 150mM NaCl, 100µg/ml BSA and 50% glycerol.

**Table 2.2 Specific antibody information used for analysis of MnSOD, catalase, Sirt1 and acetylation proteins by western blotting**

<b>Protein</b>	<b>Source</b>	<b>Catalogue Number</b>	<b>Amount of protein loaded (µg)</b>	<b>Final Primary Antibody Concentration (Dilution Used)</b>	<b>Primary diluent</b>	<b>Final Secondary Antibody Concentration (Dilution Used)</b>	<b>Secondary diluent</b>	<b>Primary host</b>
MnSOD	Enzo	ADI-SOD-111	10	2ng/ml (1 in 1000)	1% Milk	0.2µg/ml (1 in 5000)	3% Milk	Rabbit
Catalase	Sigma	C-0979	10	2ng/ml (1 in 1000)	1% Milk	0.2µg/ml (1 in 5000)	3% Milk	Mouse
Sirt1	Abcam	Ab7343	25	1µg/ml (1 in 1000)	5% BSA	0.2µg/ml (1 in 2000)	1% BSA	Mouse

#### **2.11.6. Western blotting analysis**

Following exposure of membrane, the intensity of each band on the nitrocellulose membrane was measured using ImageJ (US National Institutes of Health, Maryland, USA). This was then normalised to the intensity of the Ponceau S stain (Appendix Section 10.1). This was done by measuring the average intensity of Ponceau S across the whole sample.

#### **2.12. Determination of Atrogin1 and Murf1 expression by quantitative PCR**

Quantitative polymerase chain reaction (qPCR) is a modification of polymerase chain reaction (PCR) and is used for the measurement of mRNA levels. For this to occur, RNA is made into complimentary DNA (cDNA). cDNA is then added to a master mix containing non-specific fluorescent dye and primers. Primers are sequences that are complimentary to the gene of interest; each master mix contains both a forward and reverse primer, so both the sense and antisense of cDNA will be amplified. qPCR is then carried out in a thermal cycler. Firstly cDNA is denatured, the primers are annealed and finally extended and this is repeated for a number of cycles. As each cycle is carried out the non-specific fluorescent dye is intercalated with the double stranded DNA, i.e. when primers have annealed to target sequences, causing a fluorescence that is detected by the thermo cycler. This provides each sample with a cycle threshold (CT) value. The CT value is the number of cycles at which the fluorescent signal from the sample exceeds that of the background fluorescence, so the lower the CT value, the more mRNA of the gene of interest is present in the

sample. The CT value is then normalised to housekeeping genes CT values to give a relative expression value which can then be compared to other samples.

### **2.13. Amplex red assay for determining hydrogen peroxide concentrations**

The enzymatic detection of hydrogen peroxide concentrations can be determined using Amplex red (N-acetyl-3, 7-dihydroxyphenoxazine), which has been shown to be highly specific and sensitive to hydrogen peroxide (Zhou et al., 1997). Amplex Red is a colourless solution that, in the presence of horse radish peroxidase (HRP), reacts with hydrogen peroxide at a 1:1 stoichiometry. This reaction of Amplex Red and hydrogen peroxide in the presence of HRP produces the highly fluorescent resorufin where the intensity of fluorescence can then be detected.

#### **Reagents:**

- **Buffer Z:**
  - 110mM K-Mes (2-(N-morpholino) ethanesulfonic acid Sigma Aldrich, Dorset, UK)
  - 35mM KCl (BDH Prolabo, Leicestershire, UK)
  - 1mM EGTA (Sigma Aldrich, Dorset, UK)
  - 3mM MgCl<sub>2</sub> (BDH Prolabo, Leicestershire, UK)
  - 10mM K<sub>2</sub>HPO<sub>4</sub> (BDH Prolabo, Leicestershire, UK)
- **Amplex Red Solution:**
  - 50µl SOD (Molecular Probes, Eugene, Oregon)
  - 200µl Amplex Red (Molecular Probes, Eugene Oregon)
  - 10µl of HRP (Molecular Probes, Eugene Oregon)

### 2.13.1. Standard curve for optimisation of settings for Amplex Red analysis

Standards were generated as shown in Table 2.3. A 2.5 $\mu$ M stock solution of hydrogen peroxide was prepared and nine standards prepared using serial dilutions. One hundred microlitres of each standard was then added to a black 96 well plate (Starlab, Milton Keynes UK) in duplicates. The plate was then read immediately on the luminescence reader (BMG laboratories, Buckinghamshire, UK) to set the optimum gains to achieve optimum signal to noise for that batch of Amplex Red for further experiments to determine concentration of hydrogen peroxide in the media of resveratrol treated cells.

**Table 2.3 Concentrations used for construction of standard curve for Amplex Red**

Standard	1	2	3	4	5	6	7	8	9	10
Concentration of H <sub>2</sub> O <sub>2</sub>	2.5 $\mu$ M	1.25 $\mu$ M	750nM	375nM	187.5nM	93.75nM	46.88nM	23.44nM	11.72nM	5.86nM
Amplex Red solution ( $\mu$ l)	250	250	250	250	250	250	250	250	250	250

### 2.13.2. Determination of H<sub>2</sub>O<sub>2</sub> concentration from media of myotubes treated with resveratrol

Fifty microlitres of differentiating media was added to 50 $\mu$ l of Amplex Red solution in a black 96 well plate. The plate was read immediately on the luminescence reader. The end point value was then normalised to the total protein content of the sample. Total protein from cells was determined using the BCA assay (Section 2.10).

## **2.14. Harvesting of cells for RNA extraction**

### **Reagents:**

- Tri-Reagent (Life technologies, Warrington, UK)
- Chloroform (Sigma Aldrich, Dorset, UK)
- Isopropanol (Sigma Aldrich, Dorset UK)
- Ethanol (Sigma Aldrich, Dorset, UK)

### **Protocol:**

Myotubes were scraped in 500µl of Tri-Reagent and 200µl of chloroform was added. Tri-Reagent and chloroform were thoroughly mixed and samples centrifuged at 14,000g for 15 minutes. Centrifugation resulted in phase separation and the top clear layer containing RNA was transferred to new eppendorfs and 500µl of isopropanol was added. Samples were left at room temperature for 10 minutes and centrifuged for 30 minutes at 14,000g at 4°C. The isopropanol was removed and 200µl of 80% ethanol was added and samples centrifuged for a further 2 minutes. The ethanol was then removed and RNA pellets were re-suspended in RNase-free water. One microlitre of sample was then added to the Nanodrop (Thermoscientific, Cheshire, UK) to determine RNA concentration and purity.

## **2.15. cDNA synthesis**

### **Reagents:**

- Random Hexamers (Sigma Aldrich, Dorset, UK)
- RNA

- RNase free water (Sigma, Dorset, UK)
- Superscript II (Invitrogen, Paisley, UK)
- Deoxynucleotides (dNTP's, Invitrogen, Paisley, UK)
- Dithiothreitol (DTT, Invitrogen, Paisley, UK)

**Protocol:**

One hundred nanograms of RNA from each sample were placed in 96 well plates and 1µl of random hexamers was added to each sample. RNA was incubated at 42°C for 10 minutes and placed on ice. Following incubation, 8µl of a master mix consisting of 4µl of buffer, 2µl of DTT, 1µl of superscript II and 1µl of dNTPs and appropriate amount of RNase free water to make a final volume of 20µl, was added to each well and placed at 65°C for 1H. cDNA was then stored at -20°C.

**Table 2.4 Make-up of master mix for cDNA synthesis.**

Component	Volume
Random Hexamers	1µl
First strand 5x buffer	4µl
DTT	2µl
dNTP	1µl
RNase free water	variable
Total	20µl

**2.16. Designing of primers**

Primers (Table 2.5) were designed using NCBI primer blast.

**Table 2.5 Primer sequences for S29 Murf1 and Atrogin1.**

Target gene	Primer sequence (forward)	Primer sequence (reverse)
S29	TCCTTTTTCCTCCTGGGCG	TTAGAGCAGACGCGGCAAGA
Murf1	GCTGGTGAAAACATCATTGACAT	CATCGGGTGGCTGCCTTT
Atrogin1	GCAGAGAGTCGGCAAGTC	CAGGTCGGTGATCGTGAG

## 2.17. Qualitative PCR

### Reagents:

- cDNA
- Sybrgreen Master mix (Thermoscientific, Cheshire, UK)
- Primers (Sigma Aldrich, Dorset, UK)

### Protocol:

cDNA was diluted 1 in 100 with RNase free water and 10µl of diluted cDNA was added into 96 well plates. A master mix consisting of 25µl of Sybrgreen, 2.5µl of forward primer and 2.5µl of reverse primer (Table 2.6) was prepared for each sample and 30µl were added to cDNA). Plates were then sealed, pulse centrifuged for 5 seconds and placed in the thermal cycler (Biorad, Hertfordshire, UK) for thermo cycling (Table 2.7) for Atrogin1, S29 and Murf1. Forty five cycles were performed.

**Table 2.6 Reaction mix for qPCR analysis of s29, murf1 and atrogin1**

Component	Volume
Sybrgreen	25µl
Primer (forward)	2.5µl
Primer (reverse)	2.5µl
cDNA	10µl
Total Volume	50µl

**Table 2.7 Cycle run for qPCR**

Temperature °C	95	95	60	72
Time (mm:ss)	10:00	00:10	00:15	00:20

Samples were placed at 95°C for 10 minutes and 10 seconds for denaturing, 60°C for 15 seconds for annealing and 72°C for 20 seconds for extension.

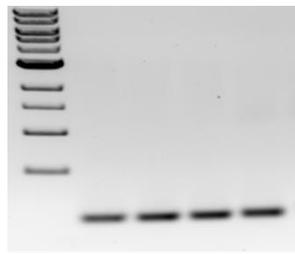
### 2.17.1. Validation of qPCR products

#### Reagents:

- 1% agarose (Sigma Aldrich, Dorset, UK) in TAE Buffer (Life technologies, Warrington, UK)
- Loading dye (Life technologies, Warrington, UK)
- Midori green (Geneflow, Litchfield, UK)

#### Protocol:

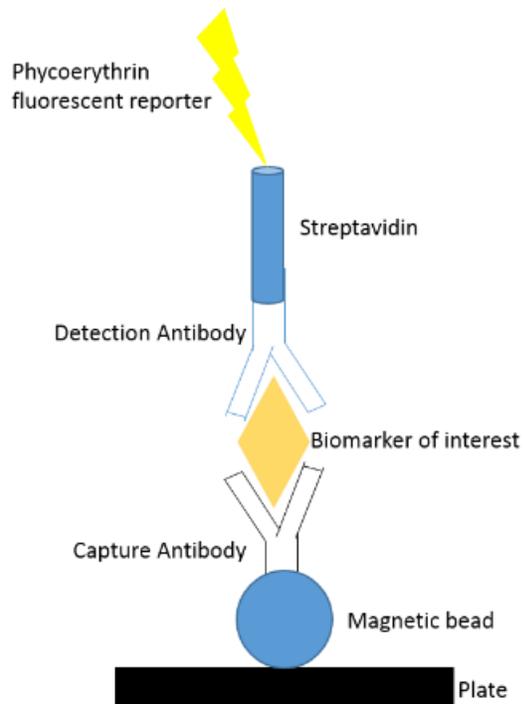
To ensure that the qPCR had amplified the correct products and no primer dimers had formed, qPCR products were separated on a 1% agarose gel containing 20µl of midori green and were visualised using a UV transilluminator light on the chemilumiscence (Biorad, Hertfordshire, UK).



**Figure 2.7 Example agarose gel showing products of PCR following amplification.**

### **2.18. Analysis of cytokine content of cell culture media with luminex assay**

Luminex is a magnetic bead based colour coded multiplex assay that measures multiple cytokines, chemokines and growth factors. The luminex assay forms a sandwich complex with a magnetic bead containing the capture antibody for the biomarker of interest. The biomarker of interest in the sample binds to the capture antibody. A biotinylated detection antibody, specific for the biomarker of interest, then binds to the complex. Detection is then allowed with addition of the streptavidin-phycoerythrin (PE) conjugate. The magnetic beads are detected on a bio-plex system. The bio-plex system is a dual-laser flow based detected instrument containing two lasers, one that classifies the bead and determines which analyte is being detected. The secondary laser defines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound (Figure 2.8).



**Figure 2.8: Brief overview of luminex assay. Magnetic beads are attached to the plate and the biomarker of interest will bind to this through the capture antibody. The detection antibody will then bind to the streptavidin-phycoerythrin complex and the fluorescence detected by the bio-plex system.**

**Reagents:**

- Biorad 24 plex rat cytokine assay (Biorad, Hertfordshire, UK):
  - Standards
  - Magnetic beads
  - Antibodies
  - Streptavidin-PE
  - Wash buffer
  - Assay buffer
- Differentiation medium

**Protocol:**

The Bio-Plex system was calibrated using calibration beads (Biorad, Hertfordshire, UK) before preparation of the plate. Standards were prepared as per manufacturer’s instructions; the standard was reconstituted in differentiation medium and vortexed thoroughly. Eppendorfs were labelled and the appropriate amount of diluent placed in each eppendorf (Table 2.8). Serial dilutions of the reconstituted standard were then made as shown in Table 2.8 with 128µl of reconstituted standard placed into standard 1 and vortexed thoroughly, 50µl of standard 1 transferred to standard 2 and this repeated to get a series of dilutions. Differentiation medium was used as a diluent.

**Table 2.8. Serial dilutions of Bioplex standards**

Standard	1	2	3	4	5	6	7	8	blank
Volume of diluent (ul)	72	150	150	150	150	150	150	150	150

Magnetic beads containing capture antibodies were re-suspended in assay buffer and 50µl added to each well to place magnetic beads on the plate. All wells were then washed twice with 100µl of wash buffer to remove excess beads. Fifty microlitres of each sample was pipetted into wells. This was repeated with the standards. Plates were then protected from light and left shaking at approximately 300g for 30 minutes. All wells were washed 3 times with 100µl of wash buffer. Detection antibody was reconstituted and 25µl added to each well. Plates were then protected from light and left shaking at approximately 300g for 30 minutes, following incubation; wells were washed 3 times with 100µl of wash buffer. Streptavidin-PE was re-suspended ensuring it

was protected from light at all times and 50µl was added to each well and plates were left shaking at approximately 300g for 10 minutes. Plates were washed for a final 3 times with 100µl of wash buffer. The plate was then placed on a vortex for 30 seconds at approximately 1100g to re-suspend the magnetic beads. Plates were then read at High RP1 on the Bio-Plex system (Biorad, Hertfordshire, UK). Plates were protected from light at all times.

### **2.19. 3D muscle construct studies**

Interest in the measurement of muscle in culture began in the early 90's when Vandeburgh et al constructed "organoids" (Vandeburgh et al., 1991), as a solution to measure functional properties of muscle in cell culture. Force was measured using a lateral loading and displacement arrangement with a calibrated micro needle. However, measurement of force in organoids was criticised due to the possibility of lateral loading changing the sarcomere lengths within the organoid (Dennis et al., 2000). More recently the Tissue Engineering Laboratory at the University of Michigan has furthered this technique (Dennis et al., 2000; Dennis et al., 2001; Kosnik et al., 2001; Baker et al., 2003) to allow functional measurements of 3D muscle constructs known as "myoids" and have further aimed to use this technique as a tool for the restoration of muscle after acute injury, surgery, or disease.

### **2.19.1. Preparation of culture dishes for 3D constructs**

#### **Reagents:**

- Sylgard elastomer base (Dow Chemical Corporation, Stockport, UK)
- Sylgard elastomer curer (Dow Chemical Corporation, Stockport, UK)
- Ethanol
- DPBS (Sigma Aldrich, Dorset, UK)
- Laminin (Invitrogen, Paisley, UK)
- Growth Medium

#### **Protocol:**

Sylgard was made by mixing sylgard elastomer base with elastomer curer at a 1 in 10 dilution. Each 60-mm plate was coated with 5ml of Sylgard and left for 3 weeks before use. Once ready to use, sylgard coated dishes were soaked in 70% ethanol for 10 minutes and washed in DPBS. The DPBS was aspirated off and the plates coated with a solution of 4ml DPBS containing  $2.0\mu\text{g}/\text{cm}^2$  laminin. The plates were left to dry overnight in a cell culture hood. Once the laminin was dry, dishes were rinsed with DPBS for 10 minutes and each plate was covered with growth medium and stored in a  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  incubator until use.

### **2.19.2. Preparation of 3D constructs**

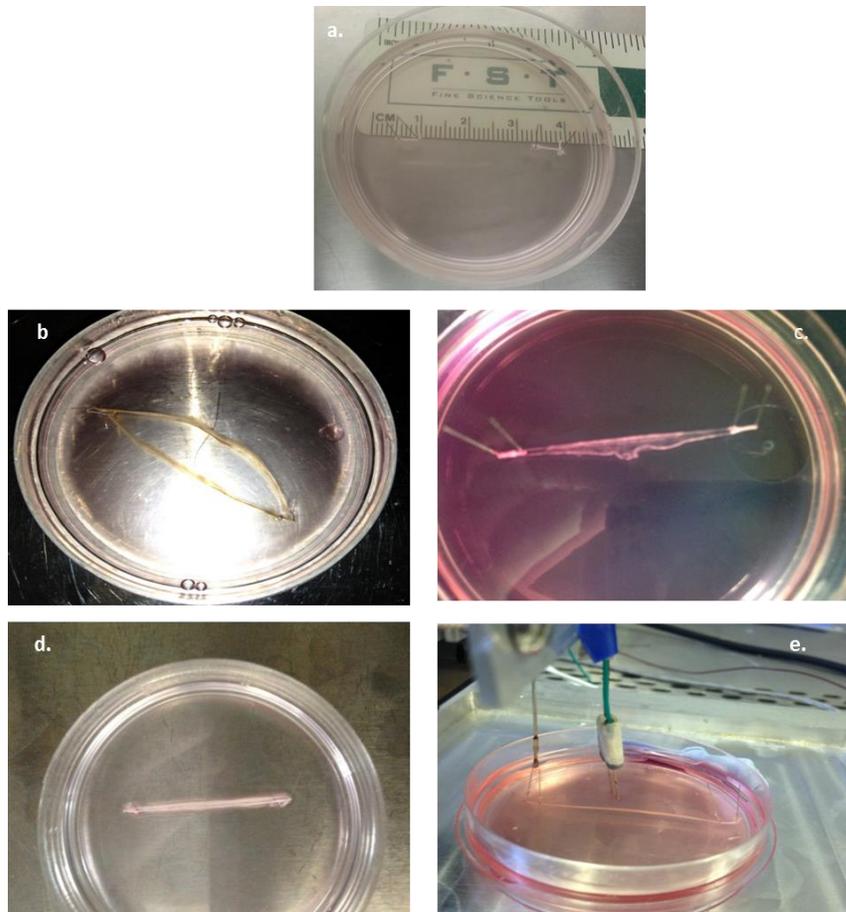
#### **Reagents:**

- Growth medium (GM)
- Differentiation medium (DM)

- 0.025% Trypsin containing EDTA (Sigma Aldrich, Dorset, UK)
- DPBS
- Tendon from rat tail

**Protocol:**

Myoblasts were counted and plated at a density of approximately  $4 \times 10^5$  on a pre-prepared dish in 5ml of growth medium. Once myoblasts had reached 80% confluence, the tendon from a rat tail was cut into 3mm sections and two pieces were pinned 30mm apart. Media was changed from growth medium to differentiation medium and differentiation medium 'topped up' every 2 days. Within a few days (3-5 days) myotubes would then begin to delaminate and roll around the pinned tendon. For constructs that did not completely roll up spontaneously, the monolayer was manually teased away from the culture dish using a pipette tip to encourage the rolling up.



**Figure 2.9 Stages of 3D construct (a) Cells are plated and once confluent pins were added to culture dish. (b and c) Following 3-5 days, cells began to roll up and (d) form a 3D construct. This was then attached to a force transducer (e) and force generation was measured.**

### **2.19.3. Measurement of force production by a 3D construct**

Contractile properties were measured the following day after the formation of a 3D construct. The pin on one end of the 3D construct was released from the Sylgard and attached to a force transducer with wax. Platinum wire electrodes were positioned on either side of the construct to allow stimulation of the whole construct. Passive baseline force was measured as the average baseline force before the onset of stimulation. Twitches were elicited using a single 1.2-ms pulse at 10V and maximum

tetanic force was determined at 10, 20, 40, 80 100 and 200Hz. Maximum specific force was determined by normalising peak tetanic force to the cross sectional area of the 3D construct that was determined by measuring the diameter of the 3D construct measured using Nikon TE2000 microscope (Nikon, Kingston upon Thames, UK).

#### **2.19.4. Haematoxylin and eosin staining of 3D constructs**

Haematoxylin and Eosin use the chemistry of the tissue to differentially colour various components of the cell. When in a complex with aluminium salts, Haematoxylin is cationic and reacts with negatively charged, basophilic cell components, such as nucleic acids in the nucleus. As a consequence of this reaction, these basophilic cell components will stain blue. Eosin is anionic and reacts with positively charged, acidophilic components in the tissue, for example amino groups in proteins in the cytoplasm. As a result of this reaction, these acidophilic components will stain pink.

#### **Reagents:**

- Tissue embedding medium
- Liquid nitrogen
- Haematoxylin (Leica, Milton Keynes, UK)
- Eosin (Leica, Milton Keynes, UK)
- Acetic acid
- Ethanol
- DPX mountant (Sigma Aldrich, Dorset, UK)

**Protocol:**

3D constructs were placed on a wooden stick and were covered in tissue embedding medium and placed into liquid nitrogen. Frozen constructs were stored at -80°C until use. Once ready to section, 3D constructs were placed in the cryostat (Leica, Milton Keynes UK) for at least 1H before commencing of sectioning. Ten micron sections were cut and left to dry for approximately 1H. Sections were placed into running tap water for 30 seconds followed by 4 minute incubation in haematoxylin. Sections were placed under running tap water 30 seconds, followed by 30 seconds in 1% acid alcohol. Sections were placed under running tap water for 3 minutes and incubated in eosin for up to 4 minutes. Sections were then placed under running water for 3 minutes. Sections were then dehydrated in 70% ethanol for 3 minutes followed by 3 minutes in 90% ethanol. Sections were then placed in 100% ethanol for a further 3 minutes. A coverslip was then placed over the sections with DPX mountant and left overnight to dry. The following day sections were placed at 4°C until viewing with the Nikon Eclipse TE2000 (Nikon, Kingston upon Thames, UK).

**2.20. *In vivo* studies****2.20.1. Mouse studies to establish an effective resveratrol treatment****Reagents:**

- Resveratrol (Sigma Aldrich, Dorset, UK)
- 20% DMSO (Sigma Aldrich, Dorset, UK)

**Protocol:**

Resveratrol was dissolved in 20% DMSO and mice were given 25mg resveratrol/kg/day or 125mg resveratrol/kg/day or the equivalent 20% DMSO carrier by oral gavage every day for 14 days an additional group of untreated mice was studied. On day 15, mice were sacrificed by overdose with pentobarbitone (60mg i.p injection) and gastrocnemius muscles collected for HPLC and western blotting analysis (Section 2.11.2 and 2.11.3).

**2.20.2. Analysis of resveratrol and metabolites by HPLC**

Gastrocnemius muscles were ground under liquid nitrogen and then sent to University of Parma on dry ice where samples underwent HPLC analysis for resveratrol and metabolites as described (Bresciani et al., 2014).

**2.20.3. Analysis of MnSOD and Sirt1 in mouse muscle****Reagents:**

- DPBS
- Liquid Nitrogen
- 1% SDS
- Protease inhibitors
- BCA and copper sulphate
- Protein loading buffer (Geneflow, Litchfield, UK)

**Protocol:**

Gastrocnemius muscles were ground under liquid nitrogen and placed in 1% SDS containing protease inhibitors. Muscles were then homogenised, sonicated and

centrifuged at 14000g for 5 minutes at 4°C and supernatants were used for the determination of protein content by the BCA method (Section 2.10) and subsequent SDS-PAGE (Section 2.11.2) and western blotting (Section 2.11.3) for detection of MnSOD and Sirt1 (Section 2.11.5).

#### **2.20.4. Mouse studies focusing on the effect of resveratrol on muscle force generation**

##### **Reagents:**

- Resveratrol
- DMSO
- Ketamine
- Domitor
- OCT (Merck, UK)
- Isopentane (Sigma Aldrich, Dorset, UK)

##### **Protocol:**

Resveratrol was dissolved in 20% DMSO and mice fed with 125mg/kg/day or the equivalent DMSO control by oral gavage every day for 14 days. On day 15 mice were anaesthetised with an intraperitoneal injection of 0.66mg/kg of ketamine and 0.55mg/kg of domitor.

#### **2.20.5. Muscle force measurements *in vivo***

##### **Protocol:**

Force measurements were performed according to McArdle *et al* (2004) with assistance from Dr Kasia Whysall. Briefly, the distal tendon was exposed and attached

to the lever arm of a servomotor (Cambridge Technology, UK). The lever served as a force transducer. The peroneal nerve was exposed, and electrodes placed across the exposed nerve. Stimulation voltage and muscle length were adjusted to produce maximum twitch force. With the muscle at optimum length, the maximum force was determined during 300 msec of voltage stimulation. The maximum force was identified by increasing the frequency of stimulation at 2 min intervals until the maximum force reached a plateau. Muscle fibre length (Lf) and cross-sectional area were calculated to determine specific force of muscle (McArdle et al., 2004).

Following force measurements mice were culled, the EDL and gastrocnemius muscles dissected and weighed. The gastrocnemius muscle was collected for western blotting analysis (Section 2.20.3).

### **3. EFFECT OF RESVERATROL ON MYOBLAST VIABILITY *IN VITRO***

### 3.1 Introduction

Resveratrol is a well-studied polyphenol shown to have potential health benefits in numerous diseases such as cancer (Harati et al., 2015) and diabetes (Brasnyó et al., 2011). There is some evidence that resveratrol may improve skeletal muscle strength. For example, Dolinsky et al (2012) found that resveratrol treatment resulted in an increased twitch and tetanic force of the soleus and anterior tibialis muscles in 22 week old rats (Dolinsky et al., 2012).

Considerable work has been carried out examining the effect of resveratrol on cell viability and cell number. These studies have shown that the effects of resveratrol seems to be cell type specific with resveratrol treatment preserving cell viability following serum deprivation in embryonic fibroblasts (Ulakcsai et al., 2015) but causing cell death in numerous cancer lines (Ming-Huan et al., 2006; Gomeza et al., 2013). The effects of resveratrol have also been shown to be dose dependent; for instance low concentrations (1-10 $\mu$ M) of resveratrol led to cell proliferation in the prostate cancer cell line PC3 (Chang et al., 2013) whereas higher concentrations (10-100 $\mu$ M) resulted in decreased cell viability and an increased percentage of apoptotic cells (Sheth et al., 2012). Another variable of the effect of resveratrol appears to be whether the organism is under stress. A number of studies have suggested that resveratrol may only be effective when there is a pre-existing condition for example, obesity (Lagouge et al., 2006), but may have little effect in healthy animals (Rogers et al., 2015).

Resveratrol has been proposed to protect against sarcopenia and a number of studies have focused on examining the effect of resveratrol on skeletal muscle viability using the skeletal muscle cell line C2C12. These studies have resulted in conflicting results. Several studies using C2C12 cells have shown a decrease in cell number following treatment of 0.1 and 25 $\mu$ M resveratrol, (Montesano et al., 2013), whereas others have shown that resveratrol does not affect myoblast viability until concentrations reach 50 $\mu$ M (Higashida et al., 2013). Similar to other cell lines, myoblast sensitivity to resveratrol has been shown to be dose dependent. Thus, treatment of cells with 10 $\mu$ M of resveratrol resulted in an increase in cell motility and fusion, however this effect was inhibited by increasing concentrations of resveratrol (20 $\mu$ M, 40 $\mu$ M and 60 $\mu$ M) (Bosutti *et al.*, 2015). No work has been undertaken looking at the effect of resveratrol on primary myoblast viability despite primary myoblasts being a more physiologically relevant model to study muscle *in vitro*.

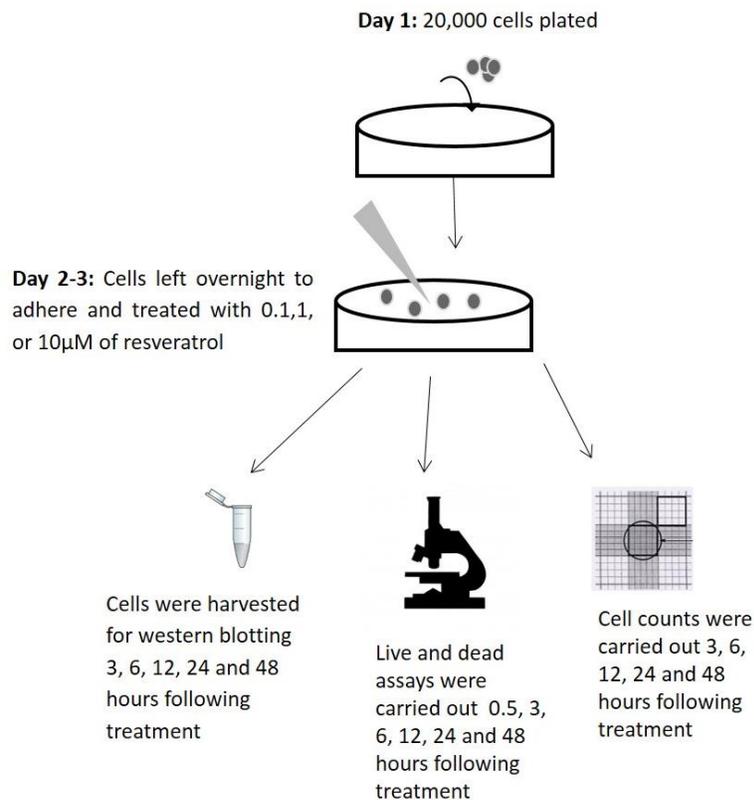
One of the most common and reproducible effects of resveratrol is an increase in mitochondrial biogenesis through the upregulation of PGC1- $\alpha$ . The mechanism by which this occurs is still unclear. There is some evidence that resveratrol directly activates the NAD<sup>+</sup> dependent deacetylase Sirt1. Sirtuins are a conserved mammalian family of NAD<sup>+</sup> dependent deacetylases and ADP-ribosyltransferases that are involved in numerous processes such as regulation of metabolism, repair of DNA and the silencing of genes (Rahman et al., 2011).

The direct activation of Sirt1 by resveratrol is supported by the evidence that the effects of resveratrol are abolished in cells pre-treated with shRNAs targeted against Sirt1 and this work is further supported by studies using mice with a Sirt1 deletion (Price et al., 2012).

This increase in Sirt1 activity has also been implicated in the resveratrol-induced increase in MnSOD and catalase protein content and activity (Jackson et al., 2011) through increasing PGC1- $\alpha$  and subsequently NRF-2 (nuclear respiratory factor 2), leading to increases in mitochondria function. MnSOD and catalase are both antioxidant defence enzymes which play a role in the protection of cells against ROS (Ji, 1993).

The aim of this chapter was to identify a concentration of resveratrol that would be functional and non-toxic in single skeletal muscle cells (primary myoblasts) but was also physiologically achievable by changes in diet. Myoblasts were used to determine the effect of resveratrol on myoblast proliferation and further experiments will use these data to further optimise a resveratrol treatment to be effective during differentiation of muscle (Chapter 4).

## 3.2 Methods



**Figure 3.1** A brief overview of the methodology used. Myoblasts were treated with different concentrations of resveratrol for different lengths of time and were either harvested for western blotting or used for cell counts and live and dead assays.

### 3.2.1 Treatment of myoblasts with resveratrol

Rat primary myoblasts were isolated, cultured and maintained in growth medium as described in Section 2.3.1. Myoblasts were plated at a density of  $2 \times 10^4$  per well in 6 well plates. All myoblasts were allowed to adhere overnight and then treated with 0, 0.1, 1 or 10µM of resveratrol for 3, 6, 12, 24 or 48H. Myoblasts were treated at passage 1-6.

### **3.2.2 Counting of myoblasts following resveratrol treatment**

Myoblasts were detached from the flask using trypsin and re-suspended in 1ml of growth medium. Twenty microlitres of cell suspension was added to 20 $\mu$ l of trypan blue and 10 $\mu$ l added to each side of the cell counter for use in the automatic cell counter (Biorad, Hertfordshire, UK) or 10 $\mu$ l was added to each side of the haemocytometer. Myoblasts were counted with both a haemocytometer and automatic counter (Biorad, Hertfordshire, UK) to ensure accurate counting of cell number. Four measurements were taken from the cell counting slide and 4 measurements taken from each side of the haemocytometer. All values for each well were averaged and the 6 wells pooled together to produce an average value for each treatment group.

### **3.2.3 Live and Dead staining of resveratrol treated and control myoblasts**

Myoblasts were washed with HBSS and incubated for 15 minutes in a 1ml solution with 1 in 500 dilution of both Syto10 and DeadRed (Invitrogen, Paisley, UK) as instructed by the manufacturers. Myoblasts were then washed with HBSS, fixed in a 4% glutaraldehyde solution for 1H and placed at 4°C until viewing with the Nikon Eclipse TE2000 microscope (Nikon Kingston upon Thames, UK).

### **3.2.4 Harvesting of resveratrol treated and control cells for western blotting**

Media was removed and cells washed with PBS. Cells were scraped in PBS and centrifuged at 14000g for 5 minutes at 4°C and re-suspended in 50 $\mu$ l of 1% SDS containing protease inhibitors (Sigma Aldrich, Dorset, UK). Cells were sonicated for 15 seconds and centrifuged at 14000g for 5 minutes. The pellet was discarded and a BCA

assay (Section 2.1.1) was carried out on the supernatant to determine protein concentration. Samples were then diluted 1:1 with protein loading buffer (National Diagnostics, Hull, UK) in preparation for detection of protein content of MnSOD, catalase and Sirt1 (Section 2.12). Following exposure of membranes, the intensity of each band was measured using ImageJ (US National Institutes of Health, Maryland, USA) and this was normalised to total protein content using Ponceau S (Sigma Aldrich, Dorset, UK) staining (Appendix Section 10.1).

### **3.2.5 Analysis of MnSOD, catalase and Sirt1 protein concentrations by SDS PAGE and western blotting**

Samples were loaded into acrylamide gels and proteins resolved. Following transfer of proteins to a nitrocellulose membrane using the Geneflow blotting system (Section 2.2.3), membranes were blocked in 5% milk and incubated with primary antibodies (Table 2.2) at 4°C overnight. Membranes were washed 3x5 minutes and incubated with the secondary antibody (Table 2.2) for 1H at room temperature. Membranes were then washed for 3x5 minutes and proteins were visualised using ECL with a Chemidoc (Biorad, Hertfordshire, UK).

### **3.2.6 Statistical analysis**

Graphpad 5 (Graphpad Software, San Diego, USA) was used to perform a One-way ANOVA followed by a Dunnett's post-test to identify significant differences. Data are represented as mean  $\pm$  SEM.

### **3.3 Results**

#### **3.3.1 Effect of increasing concentrations of resveratrol on number, proliferation and viability of primary myoblasts**

##### **3.3.1.1 Effect of increasing concentrations of resveratrol on myoblast number and cell proliferation**

Myoblast number following treatment with resveratrol is shown in Figures 3.2-3.6. Treatment of myoblasts with 0.1, 1 and 10 $\mu$ M of resveratrol resulted in decreased myoblast number compared with control myoblasts at 3H (Figure 3.2  $p<0.01$ ), 6H ( $p<0.01$ ), 12H ( $p<0.05$ ) and 24H ( $p<0.05$ ) following resveratrol treatment. This was accompanied by a premature elongation of resveratrol treated myoblasts compared with untreated myoblasts (Figure 3.6). A significant increase in myoblast number compared with control was seen 48H following treatment with all concentrations of resveratrol ( $p<0.05$ ). Growth curves show that treatment of myoblasts with resveratrol resulted in increased myoblast proliferation (Figure 3.3). This data was validated using two methods of cell counting, the automatic cell counter and manual counting, an example of this is shown in Figure 3.2.

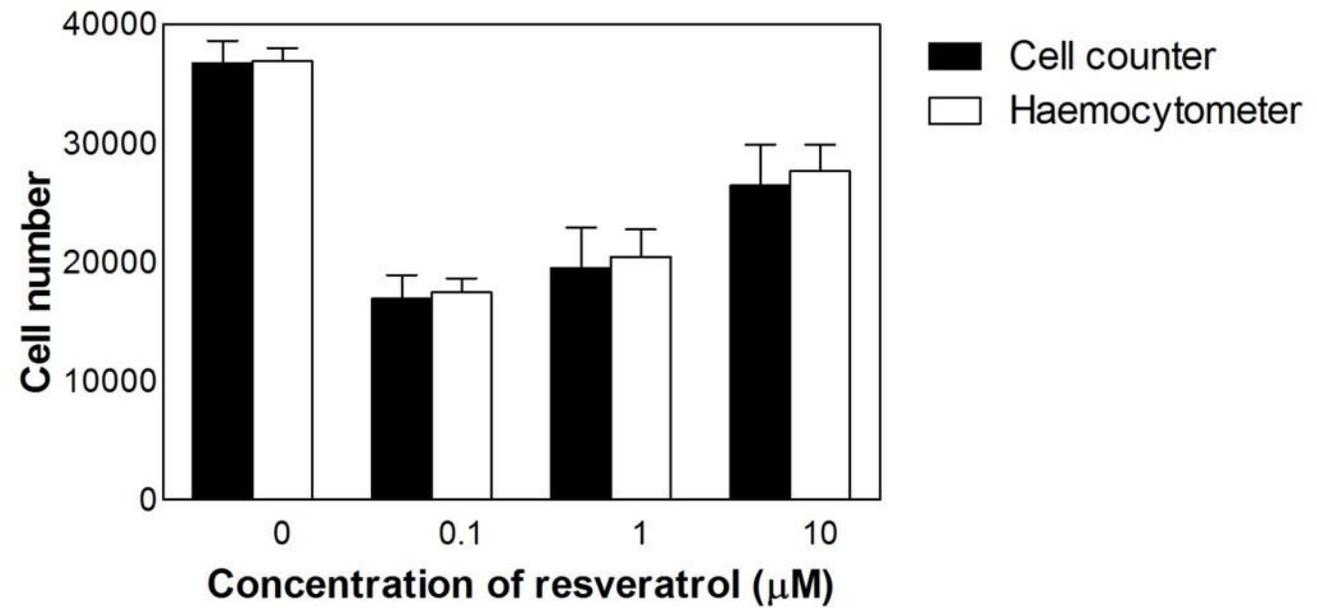


Figure 3.2 Myoblast number at 3H following resveratrol treatment of myoblasts. Myoblast number using a haemocytometer and cell counter and (c) a comparison of the two techniques is shown. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with untreated control by One-way ANOVA. Values are mean  $\pm$  SEM ( $n \geq 3$ ).

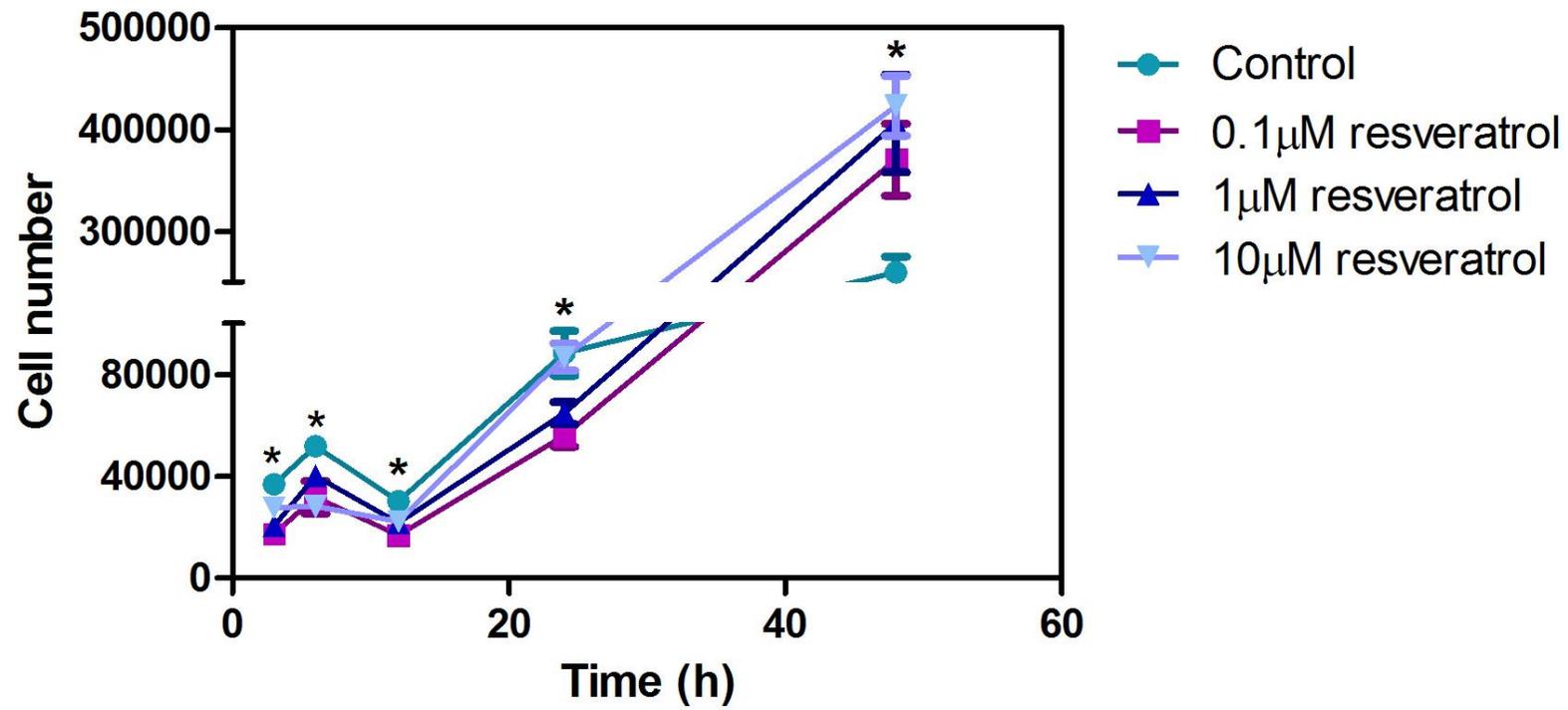
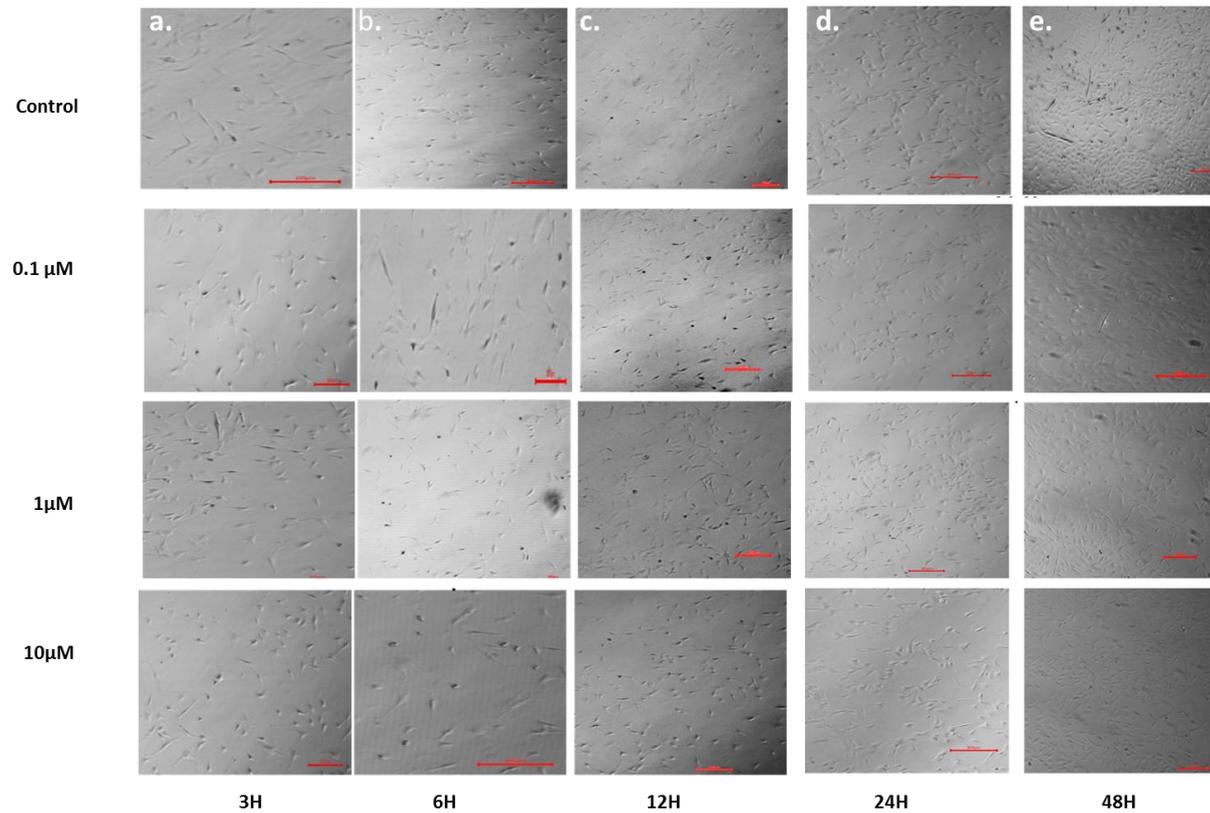


Figure 3.3 Effect of increasing amounts of resveratrol over a period of time on myoblast number. Myoblast numbers were established using a cell counter. \* $p < 0.05$  compared with resveratrol treated myoblasts at same time point. One way ANOVA.



**Figure 3.4** Representative images of myoblasts at (a) 3H (b) 6H (c) 12H (d) 24H and (e) 48H following treatment with 0.1, 1 or 10 μM of resveratrol compared with control myoblasts. Scale bar is 100 μM.

### **3.3.1.2 Effect of resveratrol on cell viability of myoblasts**

Myoblast viability following treatment with increasing amounts of resveratrol is shown in Figure 3.5 and Figure 3.6. Treatment of myoblasts with 0.1 $\mu$ M of resveratrol resulted in a reduction in cell death at 3H ( $p < 0.05$ ), 6H ( $p < 0.01$ ) and 12H ( $p < 0.001$ ) following treatment compared with untreated control myoblasts. Treatment of myoblasts with 1 $\mu$ M of resveratrol also resulted in a decrease in cell death at 3H ( $p < 0.05$ ), 6H ( $p < 0.001$ ) and 12H ( $p < 0.001$ ) following treatment compared with untreated control myoblasts. No difference cell death was identified in myoblasts treated with 0.1 $\mu$ M and 1 $\mu$ M of resveratrol at 24H or 48H following treatment compared with untreated myoblasts. Treatment of myoblasts with 10 $\mu$ M of resveratrol significantly decreased cell death 3H ( $p < 0.05$ ), 6H ( $p < 0.01$ ), 12H ( $p < 0.01$ ), 24H ( $p < 0.01$ ) and 48H ( $p < 0.01$ ) following treatment compared with untreated control myoblasts.

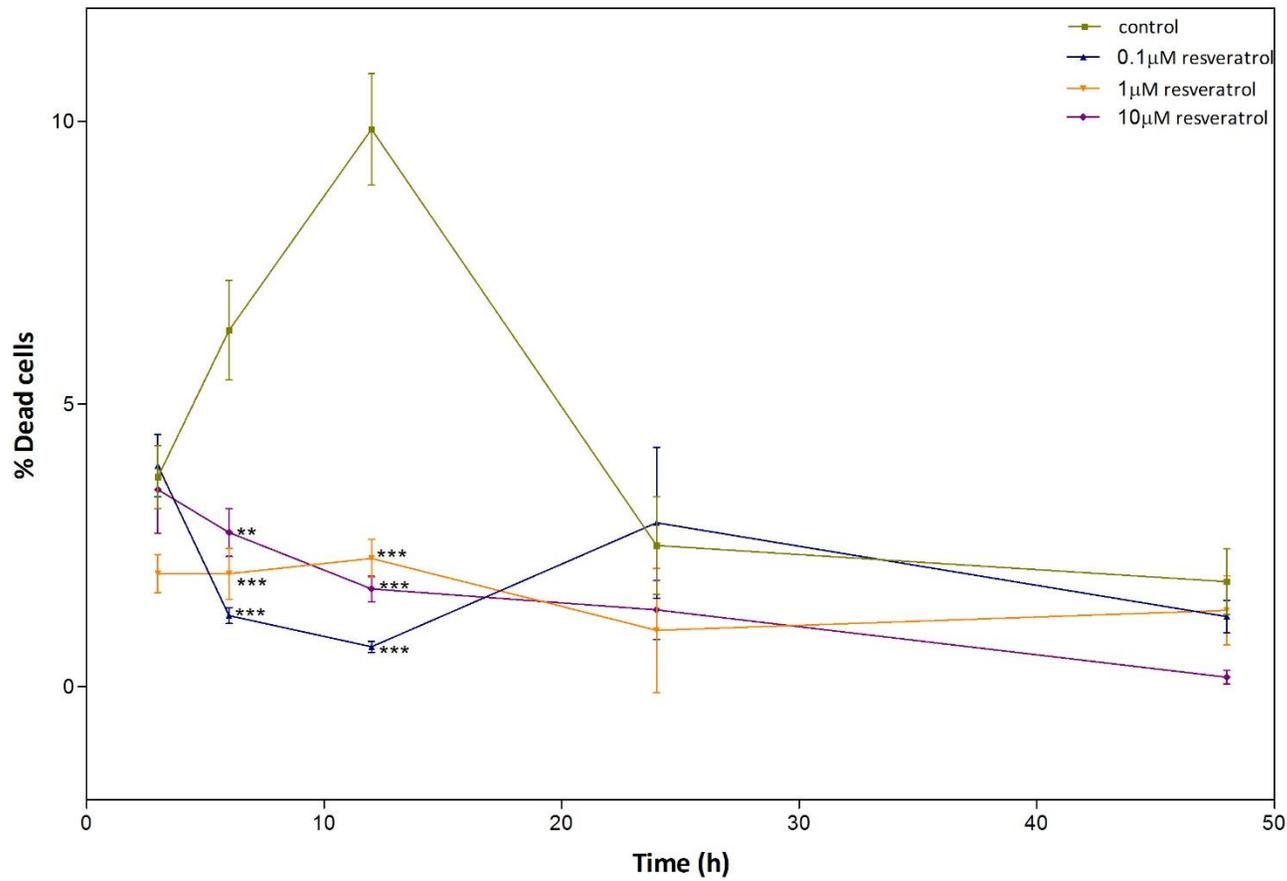
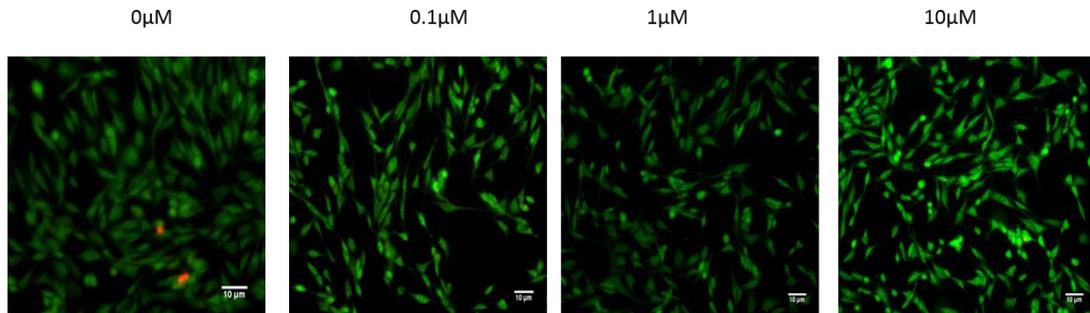


Figure 3.5 Effect of increasing amounts of resveratrol overtime on myoblast viability over a 48H time course. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with time matched untreated control ( $0 \mu\text{M}$ ) of resveratrol. Values are mean  $\pm$  SEM ( $n \geq 3$ ). One-way ANOVA.

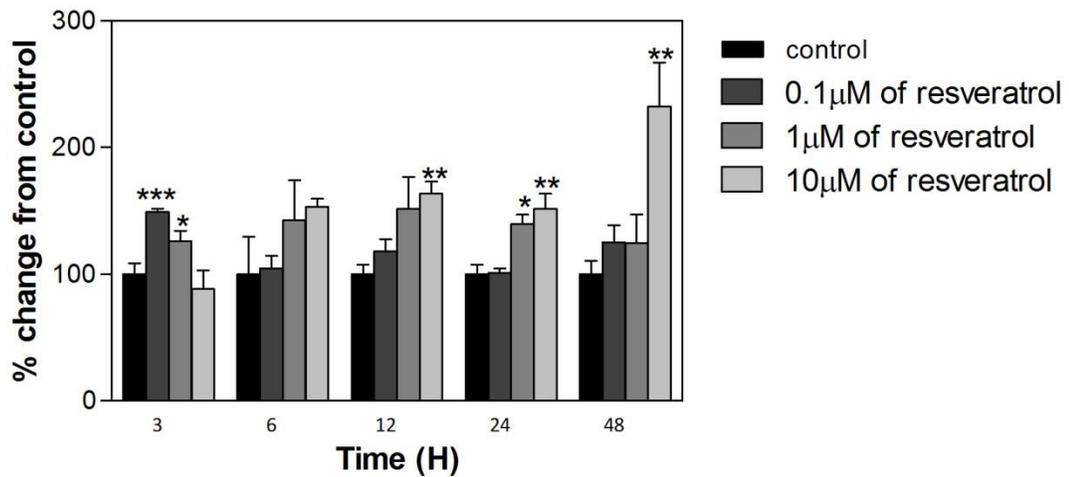


**Figure 3.6.** Representative images of live and dead analyses of myoblasts following a 48H time course of treatment with 0, 0.1, 1 and 10 $\mu$ M of resveratrol. Green cells represent live myoblasts and red cells are dead myoblasts. Scale bar=10 $\mu$ m.

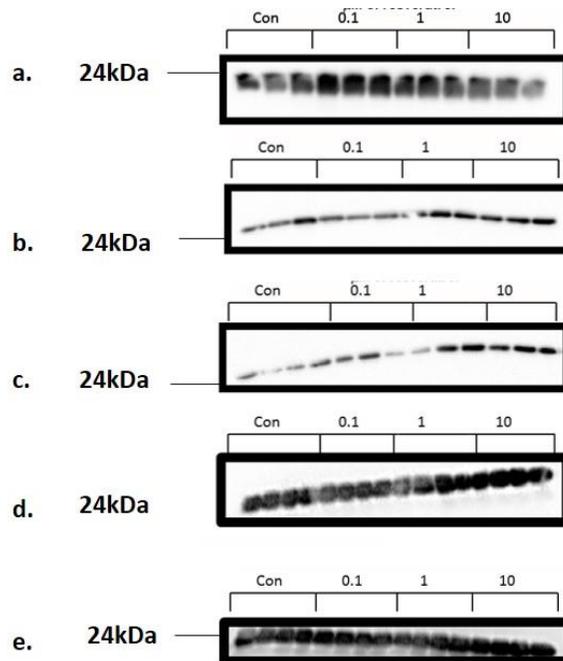
### **3.3.2 Effect of increasing concentrations of resveratrol on MnSOD, catalase and Sirt1 protein content in primary myoblasts**

#### **3.3.2.1 Effect of resveratrol on MnSOD protein content of myoblasts**

MnSOD protein content in myoblasts following treatment with increasing concentrations of resveratrol is shown in Figures 3.11 and 3.12. With the exception of a 3H time point (6H-48H) there was a suggestion of a concentration dependent increase of MnSOD protein content in myoblasts compared with untreated control myoblasts following treatment with 0.1 $\mu$ M, 1  $\mu$ M and 10  $\mu$ M of resveratrol (Figure 3.8a  $p < 0.05$ ). This increase in MnSOD protein content reached significance following treatment of myoblasts with 1 $\mu$ M of resveratrol at 3H ( $p < 0.05$ ) and 24H post-treatment (Figure 3.8  $p < 0.05$ ), as well as at 12H ( $p < 0.01$ ), 24H ( $p < 0.01$ ) and 48H ( $p < 0.05$ ) following treatment of myoblasts with 10 $\mu$ M of resveratrol (Figure 3.8). Treatment of myoblasts with 0.1 $\mu$ M ( $p < 0.001$ ) of resveratrol increased MnSOD protein content compared with untreated control myoblasts at 3H following treatment (Figure 3.8).



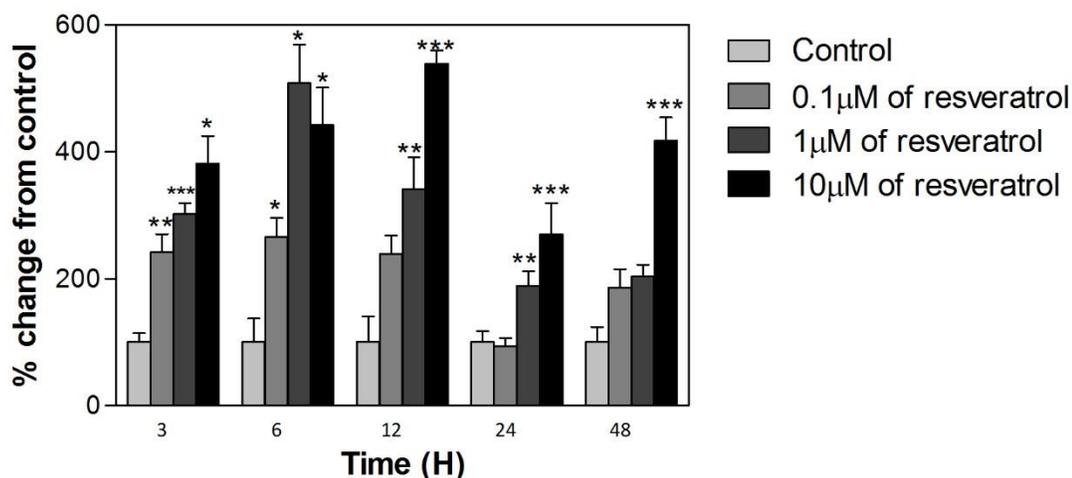
**Figure 3.7** MnSOD protein content expressed as percentage change from untreated control myoblasts following treatment with increasing concentrations of resveratrol over a 48H time course. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with untreated control. Values are mean  $\pm$  SEM (n=3-4) One-way ANOVA.



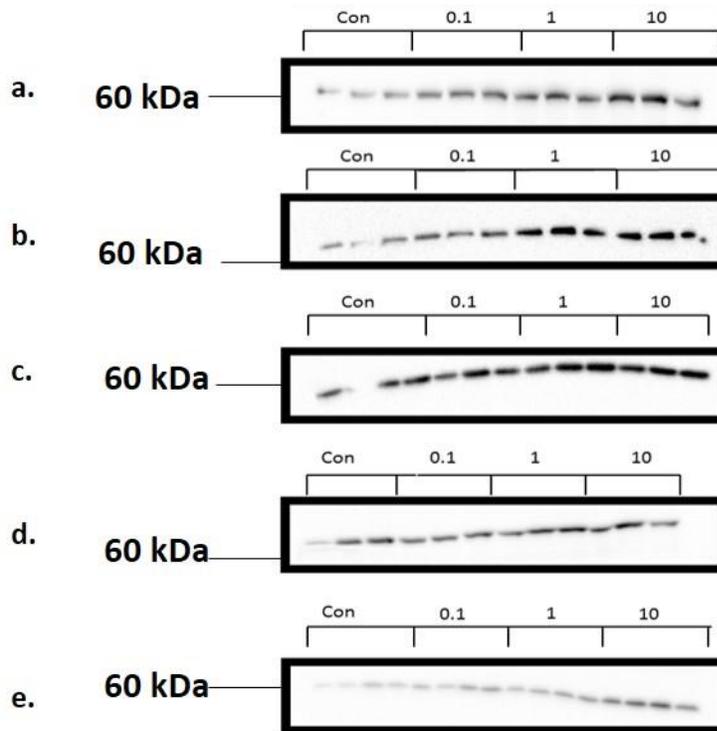
**Figure 3.8** MnSOD protein content in myoblasts at (a) 3H (b) 6H (c) 12H (d) 24H (e) and 48H following treatment with 0, 0.1, 1 and 10 μM of resveratrol.

### 3.3.2.2 Effect of resveratrol on catalase protein content of myoblasts

Catalase protein content of myoblasts following treatment with increasing concentrations of resveratrol is shown in Figure 3.9 and Figure 3.10. Following treatment of myoblasts with 0.1, 1 and 10 $\mu$ M of resveratrol, an approximate concentration dependent increase in catalase protein content was observed compared with untreated control myoblasts. This reached significance up to 6H following treatment with 0.1 $\mu$ M of resveratrol ( $p < 0.05$ ) and up to 24H following 1 $\mu$ M of resveratrol ( $p < 0.05$ ). This increase in catalase protein content was seen at 48H following a treatment of myoblasts with 10 $\mu$ M of resveratrol ( $p < 0.05$ ) compared with untreated myoblasts.



**Figure 3.9 Catalase protein content expressed as percentage change from untreated control following treatment with increasing concentrations of resveratrol over a 48H time course. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with untreated control. Values are mean  $\pm$ SEM (n=3-4) One-way ANOVA.**

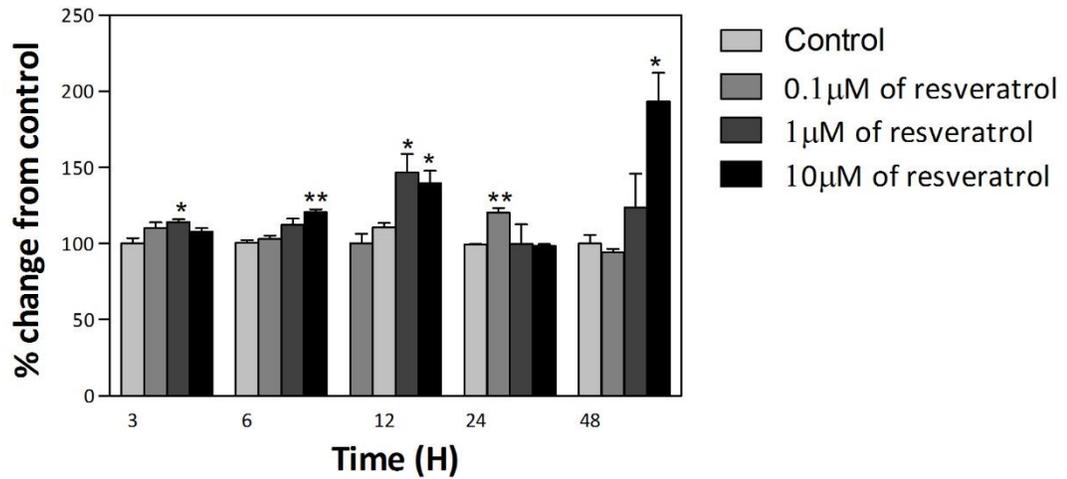


**Figure 3.10** Catalase protein content in myoblasts (a) 3H (b) 6H (c) 12H (d) 24H (e) and 48H following treatment with 0, 0.1, 1 and 10 $\mu$ M of resveratrol.

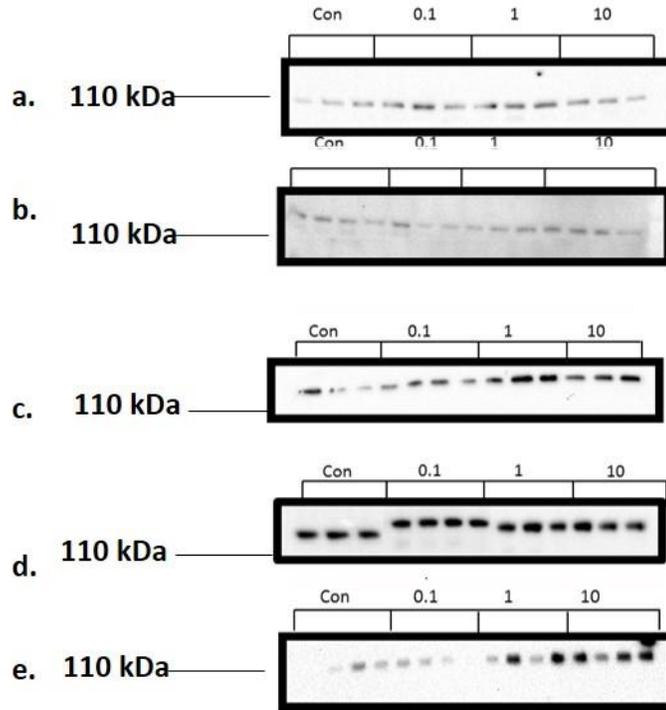
### 3.3.2.3 Effect of resveratrol on Sirt protein content of myoblasts

Sirt1 protein content in myoblasts following treatment with increasing concentrations of resveratrol is shown in Figure 3.11 and Figure 3.12. Treatment of myoblasts with 0.1, 1 and 10 $\mu$ M of resveratrol resulted in a transient increase in Sirt1 protein content compared with untreated control; this increase was significant 24H (Figure 3.12  $p < 0.01$ ) following treatment of 0.1 $\mu$ M resveratrol. Treatment of myoblasts with 1 $\mu$ M of resveratrol increased Sirt1 protein content 3H (Figure 3.12  $p < 0.05$ ) and 12H (Figure 3.12  $p < 0.05$ ) following treatment compared with untreated control myoblasts. Treatment of myoblasts with 10 $\mu$ M of resveratrol resulted in an increase in Sirt1 protein content at 6H (Figure 3.12  $p < 0.01$ ), 12H (Figure 3.12  $p < 0.05$ )

and 48H (Figure 3.12  $p < 0.01$ ) following treatment compared with untreated control myoblasts.



**Figure 3.11** Sirt1 protein content expressed as percentage change from untreated control following treatment with increasing concentrations of resveratrol over a 48H time course. \* $p < 0.05$ , \*\* $p < 0.01$  compared with untreated control. Values are mean  $\pm$ SEM (n=3-4) One-way ANOVA.



**Figure 3.12** Sirt1 protein content in myoblasts (a) 3H (b) 6H (c) 12H (d) 24H (e) and 48H following treatment with 0, 0.1, 1 and 10 $\mu$ M of resveratrol.

### 3.4 Discussion

The aim of this Chapter was to examine the effects of increasing concentrations of resveratrol on number and viability of myoblasts and examine the effect of resveratrol on the protein content of the predicted target of resveratrol, Sirt1, MnSOD and catalase. This would allow identification of a non-toxic, and functional concentration of resveratrol to then optimise treatment of differentiated myoblasts (myotubes) in future experiments. Data presented here showed that untreated myoblasts show some cell death shortly after plating and treatment of myoblasts with resveratrol prevented some of this cell death. This increase in viability of myoblasts treated with resveratrol was coupled with an initial reduction in cell number that

eventually led to an increase in proliferation rate compared with control myoblasts. Treatment of myoblasts with resveratrol also resulted in an increase in MnSOD and catalase protein content. The effect of resveratrol on Sirt1 protein content varied with concentration and time.

#### **3.4.1 Effect of treatment of myoblasts with resveratrol on cell number**

Data demonstrated a decrease in myoblast number for up to 24H following treatment with 0.1, 1 and 10 $\mu$ M of resveratrol compared with untreated myoblasts, although this was not evident with 10 $\mu$ M of resveratrol at 24H following treatment. In contrast, a dose dependent increase in myoblast number was seen following treatment of myoblasts with 0.1, 1 and 10 $\mu$ M of resveratrol at 48H after treatment.

The decrease in myoblast number observed following treatment with resveratrol at all time points up to 24H following treatment suggests that resveratrol is initially damaging to myoblasts; an increase in myoblast number in both untreated control and resveratrol treated myoblasts was then seen at 48H following resveratrol treatment. The difference in percentage change in myoblast number between the 24H and 48H time point was higher in the resveratrol treated myoblasts (586 $\pm$ 33 (0.1 $\mu$ M), 754 $\pm$ 33 (1 $\mu$ M), 612 $\pm$ 37 (10 $\mu$ M)) compared with the untreated cells (382 $\pm$ 24), indicating that in the long term resveratrol treatment did not negatively affect the replicative capacity of myoblasts. The acute decrease in myoblast number following resveratrol treatment may be due to resveratrol acting to initiate the differentiation of myoblasts into myotubes rather than cell replication, as previously suggested

(Kaminskia et al., 2012a). This may be due to the ability of resveratrol to control the cell cycle regulators, cyclins, p21 (Montesano et al., 2013) and p16 (Rathbone et al., 2009) allowing early exit from the cell cycle.

Despite resveratrol treatment resulting in a decrease in myoblast number, representative images of myoblasts at each time point appeared to demonstrate premature elongation in resveratrol treated myoblasts compared with untreated control myoblasts. This has been previously shown 6H following resveratrol treatment of C2C12 myoblasts (Kaminskia et al., 2012a). However, it is unlikely that resveratrol would initiate differentiation after just a 3H treatment, nor would this explain why the effect on myoblast number was reversed at 48H following treatment. Treatment of cells with resveratrol has been shown to result in a short term decrease in metabolic rate in other cell types, suggesting that resveratrol may inhibit oxidative phosphorylation, however, this suppression of cellular metabolism was not maintained with a longer term treatment (Robb et al., 2006).

The increase in myoblast number at 48H following treatment with resveratrol compared with untreated control myoblasts suggests that longer term treatment with resveratrol increases the proliferative rate of myoblasts, the mechanisms by which this occurs is unclear.

### **3.4.2 Effect of resveratrol on viability of myoblasts**

Treatment of myoblasts with resveratrol resulted in a decrease in myoblast number compared with untreated control myoblasts at 24H following treatment.

Despite this, cell viability assays undertaken showed that, compared with untreated control myoblasts, treatment of myoblasts with 0.1, 1 and 10 $\mu$ M of resveratrol resulted in a significant decrease in myoblast death. It is worth noting that the live and dead assay requires removal of the cell media and numerous washing steps. As myoblasts are adhesive, any dead myoblasts will eventually lift off the culture dishes and would not be seen in the subsequent live and dead assay. Therefore it is possible that the lack of evidence of gross myoblast death together with a decrease in myoblast number, despite myoblasts initially being seeded at the same density, could be due to rapid toxicity of resveratrol which may decrease the adhesiveness of myoblasts. One reason for this may be increased migration and fusion of cells that has been previously shown as an effect of resveratrol in skeletal muscle cells (Bosutti *et al.*, 2015).

Data from cell viability assays agrees with previous studies that have shown a decrease in cell number but an absence of apoptosis, (Kaminskia *et al.*, 2012a), a decrease in the pro-apoptotic p53 and caspase 3 and 9 activities as well as an increase in the anti-apoptotic Bcl-2 following treatment of muscle with resveratrol (Jackson *et al.*, 2010). Resveratrol has also demonstrated the ability to inhibit the activity of the apoptosis related transcription factor, FOXO (Wang *et al.*, 2014). These studies provide a potential mechanism by which resveratrol may be able to prevent apoptosis in myoblasts.

### **3.4.3 Effect of resveratrol on Sirt1, MnSOD and catalase protein content of myoblasts**

An established effect of resveratrol is an increase in the antioxidants MnSOD and catalase expression and activity in muscle (Ryan et al., 2010; Jackson et al., 2011) and in other cells types (Robb et al., 2006).

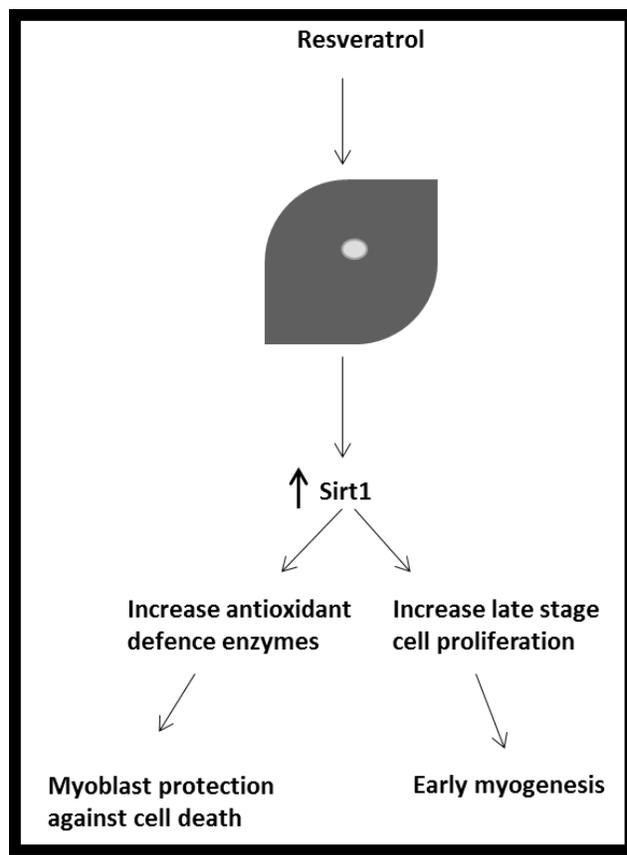
Data in this Chapter demonstrated a dose dependent effect of resveratrol, resulting in an increased MnSOD protein content, whereby 0.1 $\mu$ M of resveratrol resulted in a significant upregulation of MnSOD protein content in myoblasts at 3H following resveratrol treatment. Resveratrol treatment of myoblasts also increased MnSOD protein content at 3H and 24H following treatment with 1 $\mu$ M resveratrol and at 12H, 24H and 48H following treatment with 10 $\mu$ M resveratrol. Catalase protein content was very sensitive to resveratrol treatment and generally the increase seen was dose dependent, whereby treatment with 0.1 $\mu$ M of resveratrol resulted in an increase in catalase protein content for up to 6H. Treatment of myoblasts with 1 $\mu$ M treatment of resveratrol resulted in increased catalase protein content for up to 24H. Treatment of cells with 10 $\mu$ M was able to increase catalase protein content for up to 48H following treatment.

It has been proposed that the increase in MnSOD and catalase may be through indirect actions of resveratrol. For example, resveratrol has been shown to increase mitochondrial density (Lagouge et al., 2006) and it is hypothesised that the increase in catalase may be due to the addition of polyphenols to cell culture media. The addition of polyphenols has been shown to react with constituents of the cell culture media

such as pyruvate, leading to the oxidation of the polyphenol and the production of hydrogen peroxide (Long et al., 2010). However, unpublished work from our laboratory and other studies (Long et al., 2010) have shown that resveratrol treatment does not cause an increased hydrogen peroxide in the media from cells. Thus, it is likely that resveratrol has a functional effect in myoblasts and the increase in MnSOD and catalase production is in direct response to treatment of myoblasts with resveratrol and not as a response to the production of hydrogen peroxide in the cell culture media.

The mechanism through which resveratrol results in an increase in MnSOD and catalase protein content in myoblasts is thought to occur through the upregulation of the NAD-dependent deacetylase, Sirt1 (Kitada et al., 2011). Despite this, Sirt1 involvement in mediating the effects of resveratrol is controversial with some data showing that increases in Sirt1 activity following resveratrol treatment is an artefact of the fluorophore used in these studies (Behr et al., 2009; Pacholec et al., 2010). Data presented here showed the potential involvement of Sirt1 in resveratrol mediated effects in myoblasts. However, data also shows some temporal effects on Sirt1 when myoblasts were treated with different concentrations of resveratrol and at different time points. This is in agreement with data suggesting that different concentrations of resveratrol may act through different pathways, for example AMPK has also been proposed as a target for resveratrol (Centeno-Baez et al., 2011; Higashida et al., 2013), and may explain the lack of any clear dose dependent response in Sirt1 protein content of myoblasts following treatment with resveratrol. However, in support of a role of Sirt1 in the mechanism of action of resveratrol in myoblasts whereby overexpression of

Sirt1 in rat myoblasts resulted in increased myoblast proliferation which was accompanied by an early G1 to S phase progression of myoblasts (Rathbone et al., 2009) suggesting that Sirt1 may be involved in resveratrol-mediated increases in myoblast proliferation. However as this was not seen until later time points, it may suggest that Sirt1 is not a direct target of resveratrol but a part of a downstream pathway (Figure 3.13).



**Figure 3.13. Potential mechanism of the action of resveratrol through Sirt1 activation in primary myoblasts.**

### **3.5 Conclusions**

Concentrations of resveratrol that are achievable through diet were initially damaging to myoblasts but these effects were not long lasting. Resveratrol conferred some protective effects against cell death and in the longer term resulted in increased myoblast proliferation and potentially early myogenesis. Physiological doses of resveratrol resulted in an increase in MnSOD and catalase protein contents of myoblasts, indicating functionality of resveratrol in myoblasts. Data suggested that resveratrol acted, at least in part, through Sirt1.

## **4. EFFECT OF RESVERATROL ON VIABILITY AND HYPERTROPHY OF MYOTUBES *IN VITRO***

#### 4.1 Introduction

Data presented in the previous Chapter demonstrated that treatment of primary myoblasts with 1 $\mu$ M resveratrol resulted in significant changes in Sirt1, catalase and MnSOD protein content of myoblasts at 24H following treatment, suggesting that at this concentration and time point, resveratrol had functional effects on primary myoblasts. Myoblasts are committed muscle precursor cells that are generated from muscle stem cells, satellite cells. Myoblasts proliferate and eventually fuse with other myoblasts when in close proximity, and these multinuclear cells further differentiate to become multinucleated myotubes (Mauro, 1961; Reznik, 1969). This process is known as myogenesis and is controlled by myogenic regulatory factors (MRFs) such as MyoD, Mrf5 and myogenin. Myotubes are immature muscle fibres and are commonly used in tissue culture studies as a model of skeletal muscle.

Resveratrol has been shown to promote elongation of myoblasts and premature differentiation of C2C12 cells (Kaminskia et al., 2012a; Montesano et al., 2013) and this is supported by data presented in Chapter 3. Resveratrol has also been shown to increase the myoblast protein content of muscle specific proteins, MRFs and cycle regulators associated with early cell cycle arrest (Montesano et al., 2013).

Resveratrol has been shown to have differential effects in type I and type II skeletal muscle fibres, where resveratrol treatment in rats *in vivo* resulted in a decrease in the protein content of the antioxidant defence enzyme, CuZnSOD in primarily type II EDL muscle fibres and increased the content of MnSOD protein content in the primarily type I soleus muscle in rats (Chang et al., 2014). Furthermore,

resveratrol has previously been shown to affect undifferentiated and differentiated cells differently. For example, treatment of the rat adrenal gland cell line, PC12 cells, with resveratrol at concentrations of up to 100µM resulted in an increase in cell death in undifferentiated cells, but protected against cell death in differentiated PC12 cells (Hayakawa et al., 2013). Therefore, it is possible that there may be different effects of resveratrol on primary myoblasts and myotubes. To my knowledge; no work on the effect of resveratrol on cell viability in primary myotubes has been previously been undertaken.

Skeletal muscle hypertrophy is the process of increased muscle fibre size and occurs when muscle is regularly exposed to work that is beyond its existing capacity. Hypertrophy can be achieved by an activation of Akt, which leads to the initiation of protein synthesis (Bodine et al., 2001a). Studies have shown that resveratrol can increase Akt phosphorylation and activation (Wang *et al.*, 2014) as well as increasing the myotube diameter (Montesano et al., 2013) in C2C12 myotubes. Treatment of mice with resveratrol resulted in increased maximum force production by the soleus and anterior tibialis muscles in rodents (Dolinsky et al., 2012; Wu et al., 2013). Although treatment with resveratrol has been shown to result in an increase Akt phosphorylation in humans platelet cells (Brasnyó et al., 2011) and microarray analysis of human muscle biopsies following supplementation with resveratrol demonstrated a change in the expression of 489 genes (Timmers et al., 2011), no studies have examined the effect of resveratrol on muscle mass and function in humans.

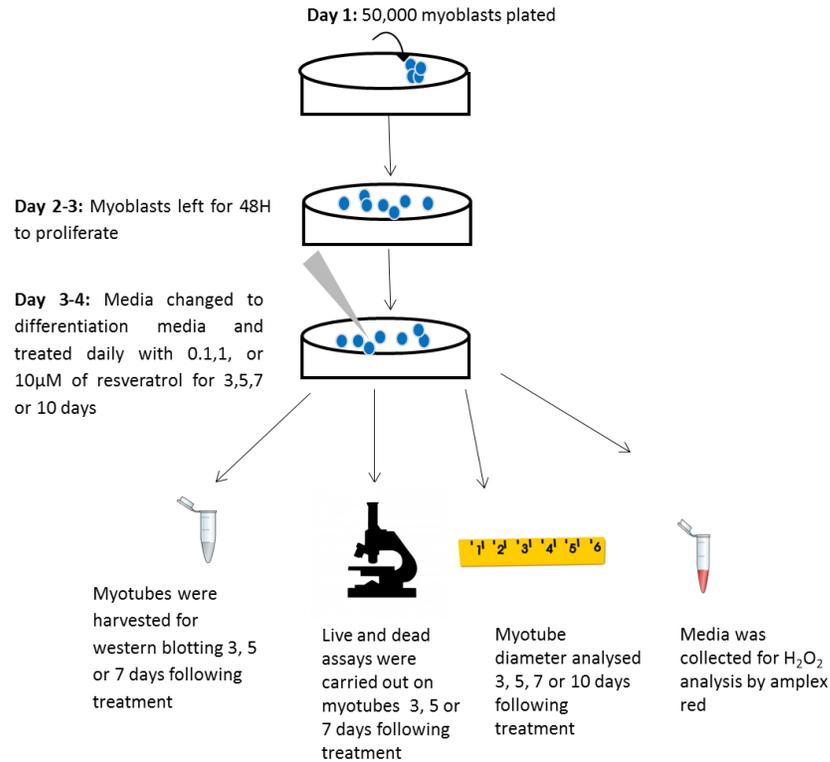
Resveratrol has also been shown to prevent the activation of atrophic pathways in TNF- $\alpha$  treated C2C12 myotubes (Wang *et al.*, 2014) in cachexia models (Shadfara *et al.*, 2011), in a mouse model of muscular dystrophy (Hori *et al.*, 2011) and following unloading in rats (Momken *et al.*, 2011). In contrast, there was no effect of resveratrol on weight loss in rodent models of cancer (Busquetsa *et al.*, 2007) or on muscle mass in healthy adult mice (Menziés *et al.*, 2013). The majority of studies that have examined the effect of resveratrol on muscle mass and function have focused on the ability of resveratrol to prevent atrophy within a pre-existing condition or following a stress rather than the ability to activate hypertrophy in a healthy muscle.

Data from Chapter 3 and from published studies (Jackson *et al.*, 2010; Ryan *et al.*, 2010; Jackson *et al.*, 2011) indicated that antioxidant defence enzymes such as catalase and MnSOD are upregulated in myoblasts following treatment with resveratrol. Studies concluded that resveratrol can modify the redox status of cells by the upregulation of antioxidants (Robb *et al.*, 2008; Jackson *et al.*, 2011).

Sirt1 is a NAD-dependent deacetylase, which removes acetyl groups from proteins resulting in gene silencing. There is evidence for a role for Sirt1 in the effects of resveratrol (Jackson *et al.*, 2011; Morita *et al.*, 2012). However, data observed in the previous Chapter showed that the changes in protein content of Sirt1 after treatment of myoblasts with resveratrol were transient and another study found no difference in Sirt1 protein content or activity in the plantaris muscle of rats following resveratrol treatment (Bennett *et al.*, 2013).

The aim of this Chapter was to optimise a concentration and time frame of resveratrol treatment of myotubes that would be non-toxic, achievable with diet and functional, to examine the effect of resveratrol on myotubes *in vitro*. The hypothesis was that resveratrol would lead to an increase in the protein content of MnSOD and catalase through the upregulation of the deacetylase Sirt1 and that this would also result in a decrease in the overall acetylation status of proteins in the resveratrol treated cells.

## 4.2 Methods



**Figure 4.1** A brief overview of the methodology used. Myotubes were treated with different concentrations of resveratrol for different lengths of time and were either harvested for western blotting, analysed for cell viability by a live and dead assay or analysed for myotube diameter measurements. Media was analysed at 3 days following resveratrol treatment for H<sub>2</sub>O<sub>2</sub> analysis by Amplex Red.

### 4.2.1 Culturing of rat primary cells

Rat primary myoblasts were isolated, cultured and maintained in growth medium as described in Section 2.3.1. Cells were seeded on to 6 well plates that had previously been treated with 500  $\mu$ l of attachment factor (Invitrogen, Paisley, UK) for 1H at a density of approximately  $5 \times 10^4$  per well.

#### **4.2.2 Treatment of myotubes with resveratrol**

Once cells had reached 80% confluence, media was changed to differentiation media to initiate differentiation into myotubes and differentiation media was supplemented with 0, 0.1, 1 or 10 $\mu$ M of resveratrol daily for 3,5, 7 or 10 days.

#### **4.2.3 Live and Dead staining of resveratrol treated myotubes**

Media was removed and myotubes were washed with HBSS. Myotubes were incubated for 15 minutes in a 1ml solution containing Syto10 and DeadRed (Invitrogen, Paisley, UK). Stained myotubes were then washed with HBSS, fixed in a 4% glutaraldehyde solution for 1H and placed at 4 $^{\circ}$ C until imaging using a Nikon Eclipse TE2000 fluorescent microscope (Nikon, Kingston upon Thames, UK). Images were analysed and percentage of dead myotubes was calculated using ImageJ (US National Institutes of Health, Maryland, USA).

#### **4.2.4 Harvesting of resveratrol treated myotubes for western blotting**

Media was removed and myotubes were washed with PBS. Myotubes were scraped in PBS and centrifuged at 14000g for 5 minutes at 4 $^{\circ}$ C and the cell pellet was re-suspended in 50 $\mu$ l of 1% SDS containing protease inhibitors. Cells were sonicated on ice for 15 seconds and centrifuged at 14000g for 5 minutes. The pellet was discarded and a BCA assay (Section 2.11) was carried out on the supernatant to determine protein concentration. Samples were then diluted 1:1 with protein loading buffer in preparation for detection of protein levels of MnSOD, catalase and Sirt1 with SDS-PAGE western blotting (Section 2.12). Following exposure of membranes, the intensity of

each band was measured using ImageJ (US National Institutes of Health, Maryland, USA) and this was normalised to total protein content using Ponceau S staining (Appendix Section 10.1).

#### **4.2.5 Analysis of MnSOD, catalase and Sirt1 protein concentrations by SDS-PAGE and western blotting**

Samples were loaded onto a pre-prepared acrylamide gel (Section 2.11.1) and proteins were resolved. Following transfer onto a nitrocellulose membrane using the GeneFlow blotting system (Section 2.11.3), membranes were incubated in blocking solution (Table 2.2) and then incubated with primary antibodies (Table 2.2) at 4°C overnight. The following day, membranes were washed 3x5 minutes and the secondary antibody (Table 2.2) was added for 1H at room temperature. Membranes were then washed for 3x5 minutes, placed in ECL solution and viewed using a Chemidoc (Biorad, Hertfordshire, UK).

#### **4.2.6 Measurement of myotube diameter**

Control and resveratrol treated myotubes were viewed using a Nikon TE2000 microscope (Nikon, Kingston upon Thames, UK). Up to 6 random areas from each well were imaged. Images were analysed with ImageJ (US National Institutes of Health, Maryland, USA). Three measurements were taken at three points along each myotube; an average was then obtained for each myotube and then for each treatment group.

#### **4.2.7 Measurement of H<sub>2</sub>O<sub>2</sub> production by myotubes following**

The media of myotubes treated with daily doses of 0.1, 1 and 10 $\mu$ M of resveratrol for 3 days was subjected to an Amplex Red assay (Section 2.13) to determine the effect resveratrol treatment on hydrogen peroxide production of myotubes.

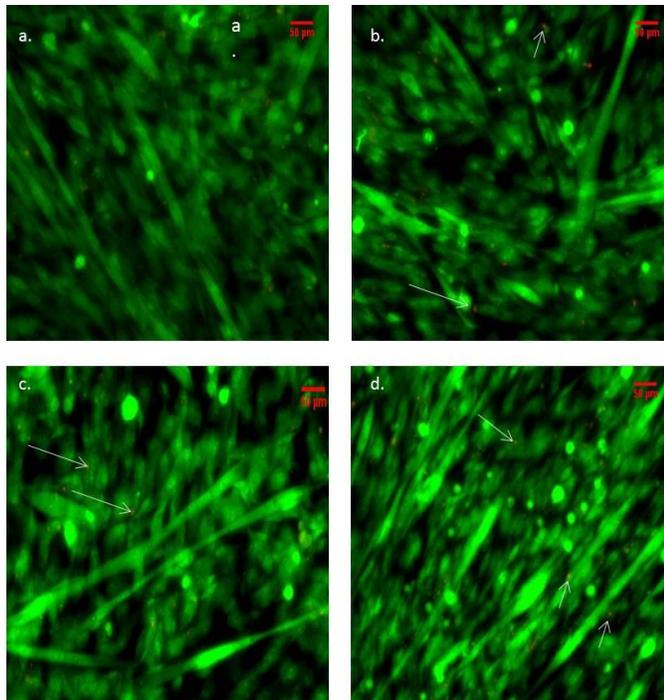
#### **4.2.8 Statistical analysis**

Graphpad 5 (GraphPad software, San Diego, USA) was used to perform One-way ANOVA followed by a Dunnett's post-test to identify significant differences. Data are represented as mean  $\pm$  SEM.

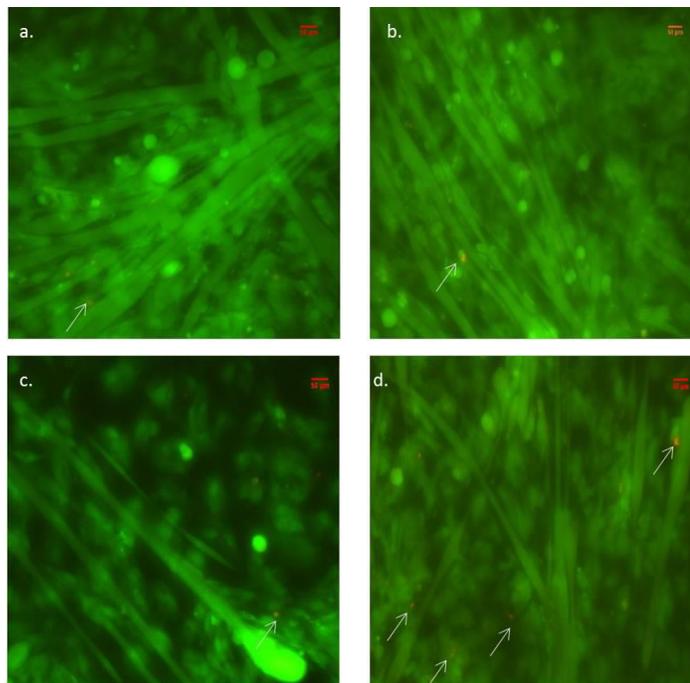
### **4.3 Results**

#### **4.3.1 Effect of increasing treatment of myotubes with resveratrol on myotube viability**

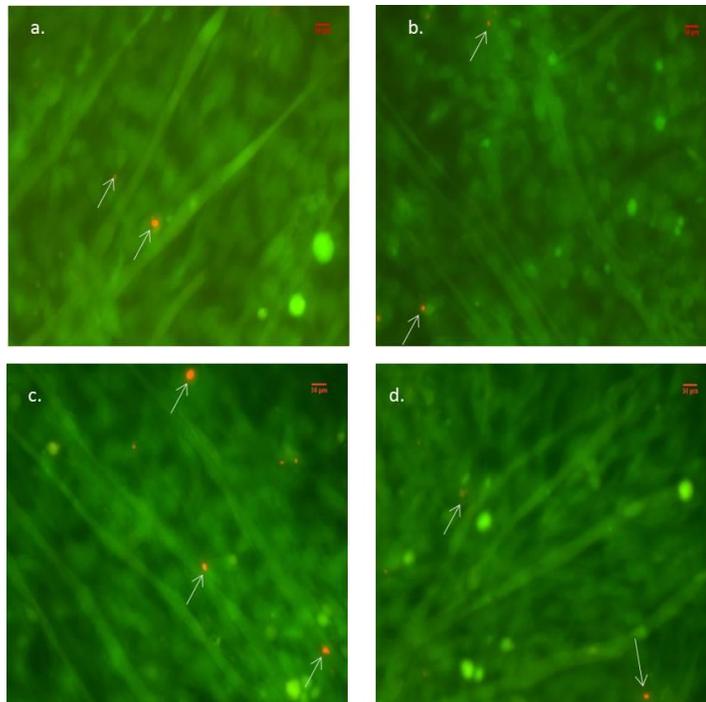
Myotube viability following treatment of myotubes with resveratrol is shown in Figure 4.2-4.5. Treatment of myotubes with 0.1, 1, and 10 $\mu$ M resveratrol resulted in a concentration dependent increase in cell death at 3 days of treatment ( $p < 0.01$ ) compared with untreated control myotubes. Treatment of myotubes with 10 $\mu$ M of resveratrol caused an increase in cell death for up to 5 days of treatment (Figure 4.3  $p < 0.05$ ). The induction of cell death by resveratrol treatment on myotubes was abolished following a 7 day treatment (Figure 4.4).



**Figure 4.2** Representative images of myotubes treated for 3 days (a) Untreated control cells (b) 0.1 $\mu$ M, (c) 1 $\mu$ M and (d) 10 $\mu$ M of resveratrol. Scale bar 50 $\mu$ M. White arrows indicate dead cells.



**Figure 4.3** Representative images of myotubes treated for 5 days (a) Untreated control cells (b) 0.1 $\mu$ M, (c) 1 $\mu$ M and (d) 10 $\mu$ M of resveratrol. Scale bar 50 $\mu$ M. White arrows indicate dead cells.



**Figure 4.4** Representative images of myotubes treated for 7days (a) Untreated control cells (b), 0.1 $\mu$ M (c) 1 $\mu$ M (d) and 10 $\mu$ M of resveratrol. Scale bar 50 $\mu$ M. White arrows indicate dead cells.

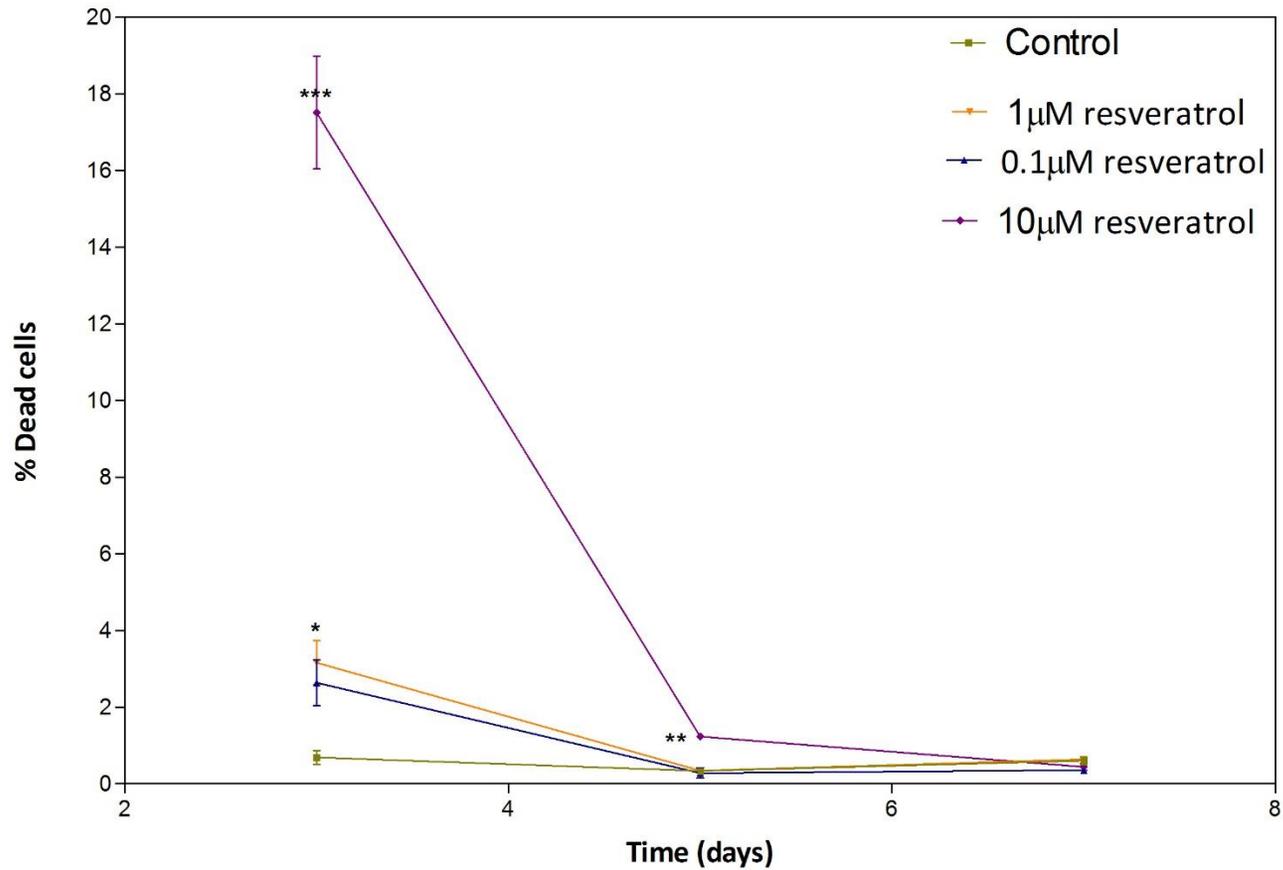
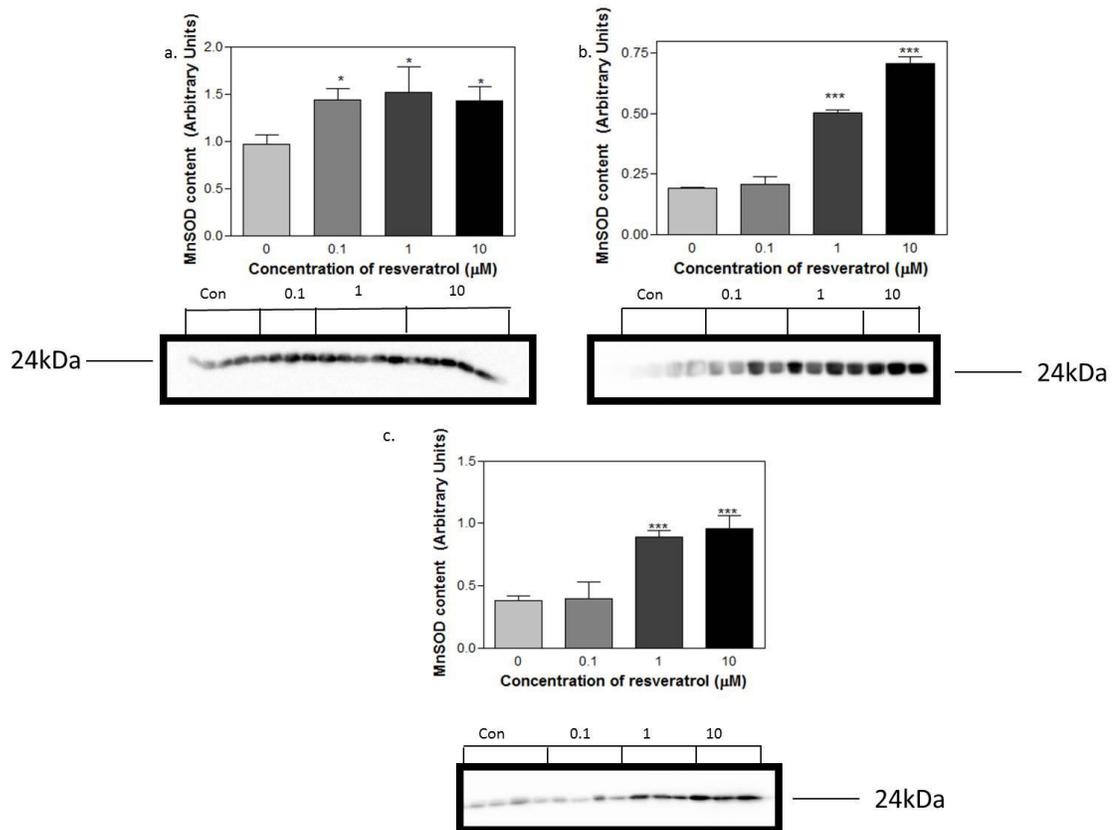


Figure 4.5. Viability of myotubes following treatment with increasing concentrations of resveratrol over a period of time. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared with untreated control myotubes. Values are mean  $\pm$  SEM ( $n \geq 3$ ).

### 4.3.2 Effect of treatment of myotubes with resveratrol on MnSOD, catalase and Sirt1 protein content

#### 4.3.2.1 Changes in MnSOD protein content of primary myotubes following treatment with increasing concentrations of resveratrol

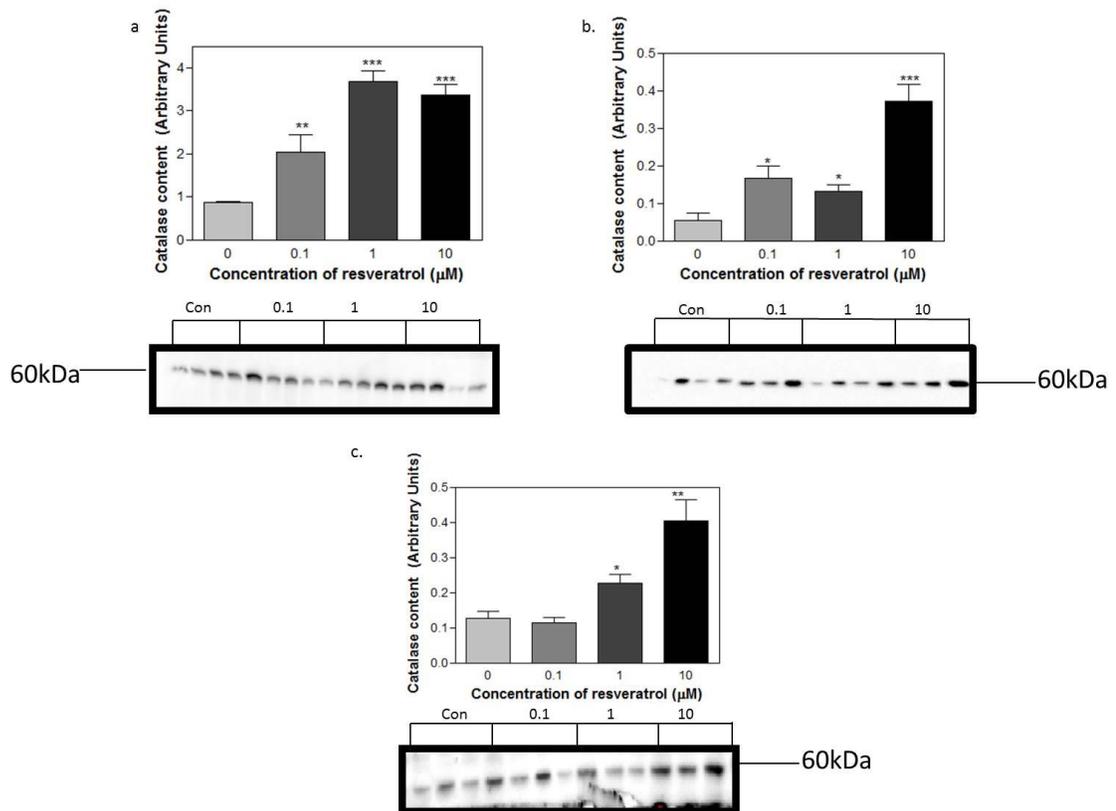
MnSOD protein content in myotubes following resveratrol treatment is shown in Figure 4.6. MnSOD protein content in myotubes was significantly following treatment with 0.1, 1 and 10 $\mu$ M resveratrol daily for 3 days. This increase was maintained for up to 7 days of treatment with 1 $\mu$ M and 10 $\mu$ M resveratrol.



**Figure 4.6** MnSOD content and representative western blots for MnSOD protein content of control myotubes and myotubes following resveratrol treatment with 0, 0.1, 1 or 10 $\mu$ M of myotubes for (a) 3 days (b) 5 days and (c) 7 days. Values are mean  $\pm$  SEM ( $n \geq 3$ ). \*  $p < 0.05$ , \*\*\* $p < 0.001$  compared with untreated control myotubes One-way ANOVA.

#### 4.3.2.2 Changes in catalase protein content of primary myotubes following treatment with increasing concentrations of resveratrol

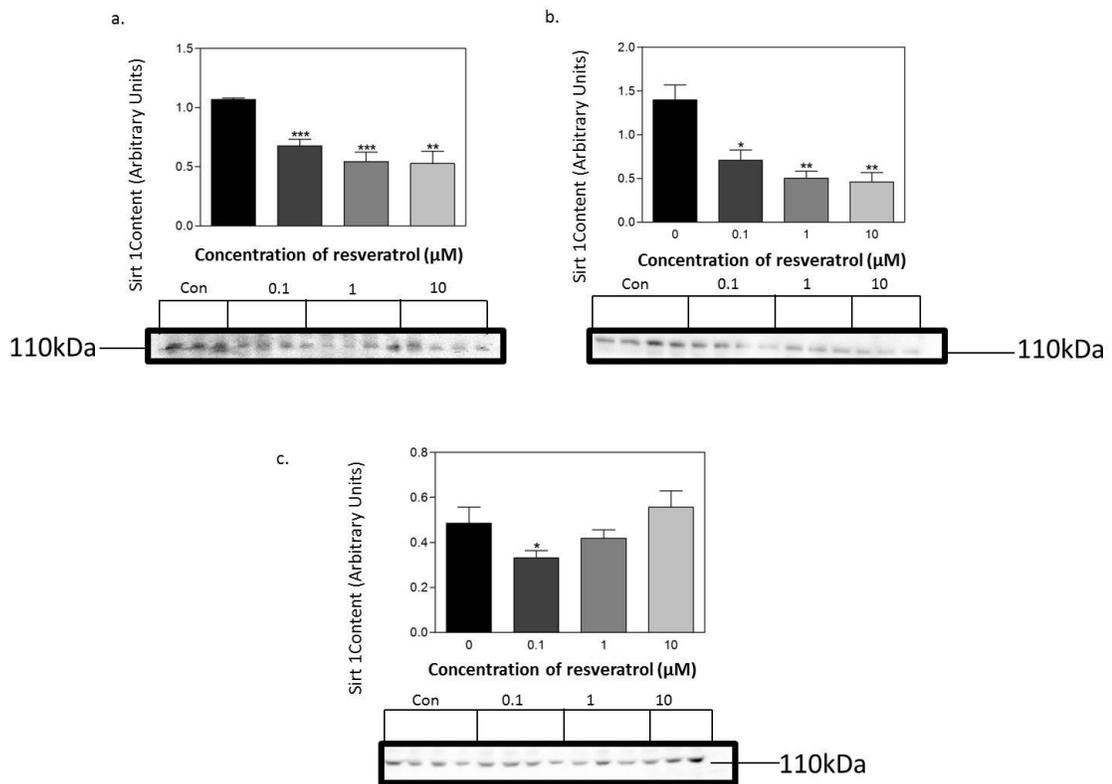
Catalase protein content of myotubes following treatment with resveratrol is shown in Figure 4.7. Treatment of myotubes with 0.1, 1 and 10 $\mu$ M of resveratrol resulted in significant increases in catalase protein content for 3 and 5 ( $p < 0.05$ ) days of daily treatments. This increase in catalase protein content in myotubes was maintained with 7 days of treatment with 1 $\mu$ M ( $p < 0.05$ ) and 10 $\mu$ M ( $p < 0.05$ ) of resveratrol.



**Figure 4.7** Catalase content and representative western blots for catalase protein content of control myotubes and myotubes following treatment with 0, 0.1, 1 or 10 $\mu$ M resveratrol for (a) 3 days (b) 5 days and (c) 7 days. Values are mean  $\pm$ SEM ( $n \geq 3$ ). \*  $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with untreated control myotubes, One-way ANOVA.

### 4.3.2.3 Changes in Sirt1 protein content of primary myotubes following treatment with increasing concentrations of resveratrol.

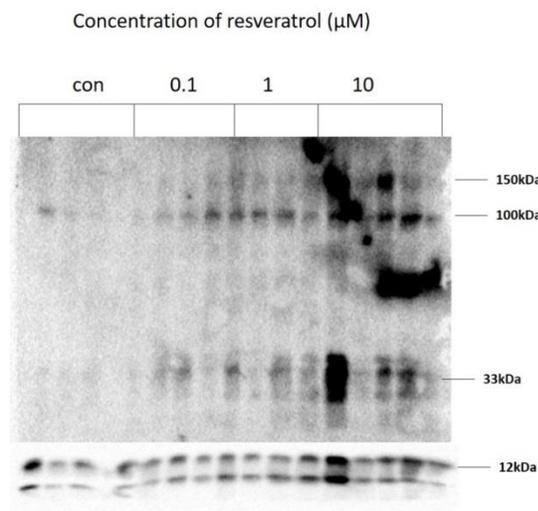
Sirt1 protein content of myotubes following resveratrol treatment is shown in Figure 4.8. Sirt1 protein content of myotubes was decreased at 3 and 5 days following daily treatment of myotubes with 0.1 $\mu$ M, 1 $\mu$ M and 10 $\mu$ M of resveratrol ( $p < 0.05$ ). This decrease of Sirt1 protein content of myotubes was maintained for 7 days of treatment of myotubes daily with 0.1 $\mu$ M of resveratrol ( $p < 0.05$ ).



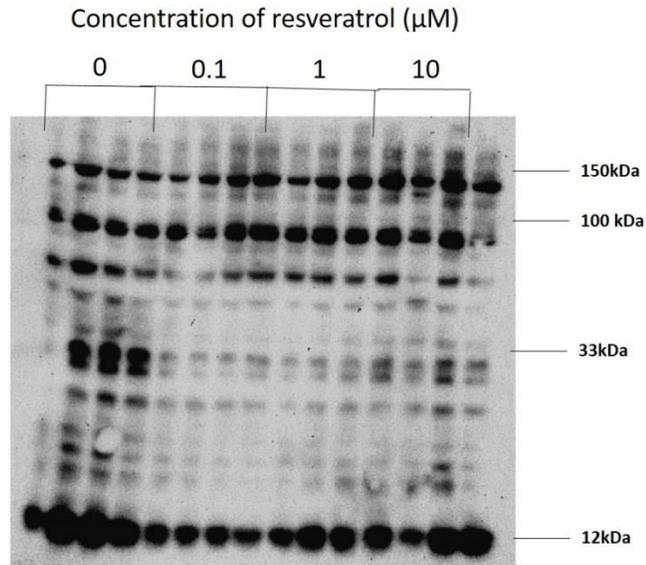
**Figure 4.8** Representative western blots for Sirt1 protein content following resveratrol treatment with 0, 0.1, 1 or 10 $\mu$ M of myotubes for (a) 3 days (b) 5 days and (c) 7 days. Values are mean  $\pm$ SEM ( $n \geq 3$ ). \*  $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with untreated control myotubes One-way ANOVA.

### 4.3.3 Effect of treatment of myotubes with increasing concentrations of resveratrol on acetylation of cellular proteins

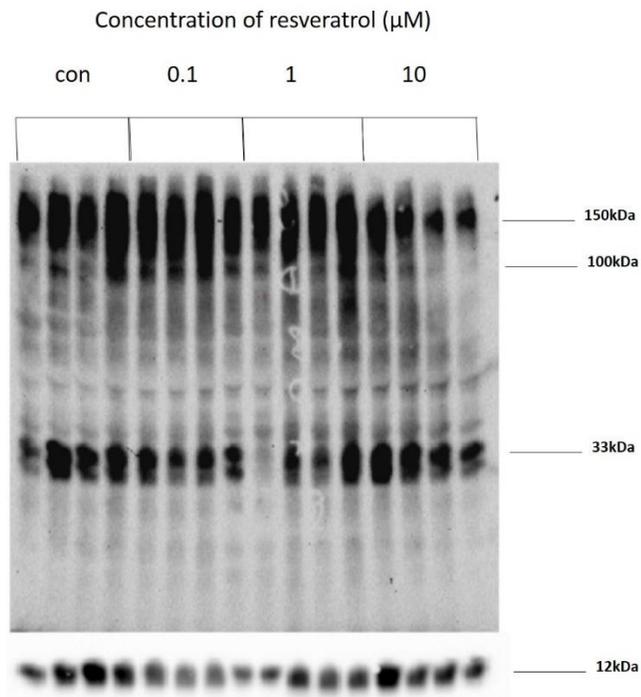
The protein acetylation status of myotubes following daily treatment with 0.1, 1 and 10 $\mu$ M resveratrol at 3, 5 and 7 days is shown in Figure 4.9-Figure 4.11. Treatment of myotubes with resveratrol resulted in an increase in protein acetylation compared with untreated control myotubes following daily treatment with resveratrol for 3 days (Figure 4.9). Treatment of myotubes with resveratrol resulted in a decrease in protein acetylation in higher molecular weights following daily treatment with resveratrol for 5 days. Treatment of myotubes with resveratrol had no effect on the acetylation profile following 7 days of daily treatment. The acetylation profile was less defined in myotubes following 3 days of treatment (Figure 4.9) than following 5 and 7 days of resveratrol treatment (Figure 4.10 and Figure 4.11). Histones are typically 11-20kDa, data from these images show it is unlikely that the acetylation status of the histones has been modified.



**Figure 4.9** Representative western blot of acetylation of proteins in untreated control myotubes and in myotubes following treatment with resveratrol for 3 days.



**Figure 4.10** Representative western blot of acetylation of proteins in untreated control myotubes and in myotubes following treatment with resveratrol for 5 days.

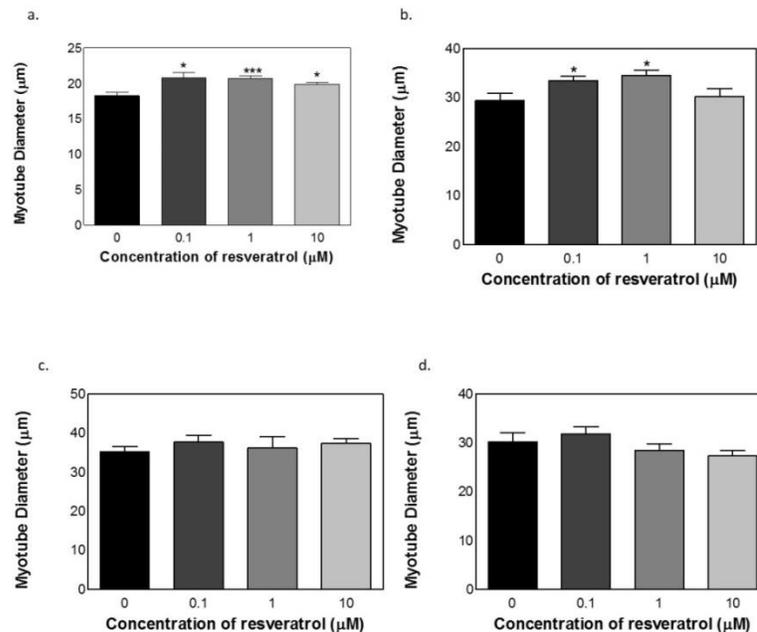


**Figure 4.11** Representative western blot of acetylation of proteins in untreated control myotubes and in myotubes following treatment with resveratrol for 7 days.

#### 4.3.4 Effect of treatment of myotubes with increasing concentrations of resveratrol on myotube diameter

##### 4.3.4.1 Changes in myotube diameter following resveratrol treatment

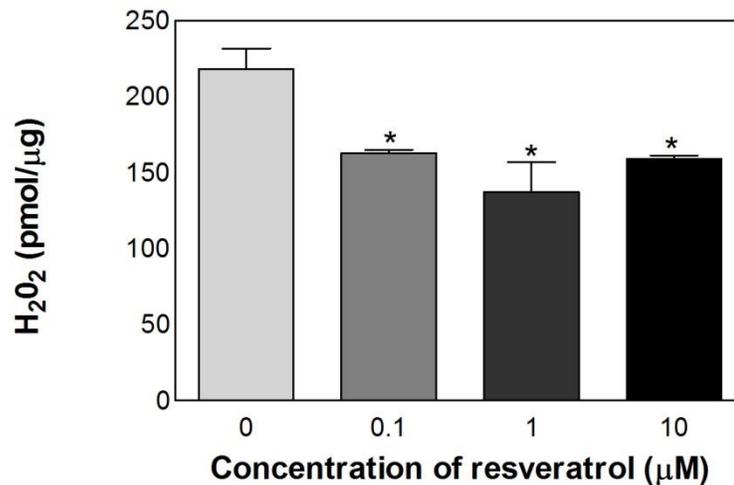
Myotube diameter following daily treatment with resveratrol for 3, 5, 7 and 10 days is shown in Figure 4.12. Treatment of myotubes with 0.1, 1 and 10 $\mu$ M of resveratrol resulted in an increase in the diameter of myotubes following daily treatments for 3 days ( $p < 0.05$ ). This increase in diameter was maintained following 5 days of treatment with 0.1 $\mu$ M and 1 $\mu$ M ( $p < 0.05$ ) of resveratrol but this effect was lost at later time points.



**Figure 4.12.** Myotube diameter following treatment with increasing concentrations of resveratrol following (a) 3 day (b) 5 day (c) 7 day and (d) 10 day treatment. Values are mean  $\pm$ SEM ( $n \geq 3$ ). \* $p < 0.05$ , \*\*\* $p < 0.001$  compared with untreated control myotube. One-way ANOVA.

#### 4.3.4.2 Effect of treatment of myotubes with increasing concentrations of resveratrol on hydrogen peroxide production

Hydrogen peroxide content of the media of myotubes treated daily for 3 days with resveratrol is shown in Figure 4.13. Treatment of myotubes with 0.1, 1 and 10 $\mu$ M resveratrol for 3 days resulted in a decrease in hydrogen peroxide content of in the media of myotubes ( $p < 0.05$ ).



**Figure 4.13** Hydrogen peroxide production in media of myotubes following 3 days of increasing concentrations of resveratrol.

#### 4.4 Discussion

The aim of this Chapter was to examine the effects of resveratrol on the viability of myotubes and the protein content of Sirt1, MnSOD and catalase. This study aimed to identify whether resveratrol led to hypertrophy or atrophy of myotubes. Furthermore this study aimed to examine whether resveratrol affected the production of hydrogen peroxide by myotubes, in order to identify if the observed increases in

catalase protein content following resveratrol treatment were as a direct or indirect effect of resveratrol.

Data demonstrated an acute minor toxic effect of resveratrol by an increase in death of myotubes at 3 days of treatment. This cell death was not evident at later time points. In myotubes treatment with resveratrol resulted in a significant increase in MnSOD and catalase protein content and a decrease in Sirt1 as well as an initial increase in myotube diameter, an effect that was lost at later time points. Treatment of myotubes with resveratrol also resulted in a decrease in hydrogen peroxide release from myotubes.

#### **4.4.1 Effect of resveratrol treatment on myotube viability**

To examine the effects of resveratrol on viability of myotubes, a Live and Dead assay was undertaken at different time points following resveratrol treatment. Data demonstrated that myotubes were more susceptible in the long term to cell death from resveratrol treatment than myoblasts, with resveratrol causing transient increases in myotube death compared with control myotubes. Any differences in effect could be due to uptake of resveratrol by myotubes being more efficient than by myoblasts as previously shown in C2C12 cells (Kaminskia et al., 2012a) and therefore being exposed to higher levels of resveratrol. Despite the initial cell death the remaining myotubes were able to overcome these toxic effects suggesting this cell death may not be a major concern with chronic treatment.

#### **4.4.2 Effect of treatment of myotubes with resveratrol on MnSOD, catalase and Sirt1 protein content in myotubes**

Studies have shown that MnSOD and catalase activity and protein content are upregulated following treatment of muscle with resveratrol (Ryan et al., 2010; Jackson et al., 2011) both *in vivo*, (Robb et al., 2008) and *in vitro* (Inglés et al., 2014) as well as in other cells and tissues (Morita et al., 2012). Others have also shown the involvement of Sirt1 in these resveratrol-induced changes in MnSOD and catalase (Price et al., 2012). Therefore the protein content of MnSOD, catalase and Sirt1 following resveratrol treatment was studied as an indication of resveratrol having an transcriptional effect in myotubes. This study has demonstrated that all 3 concentrations of resveratrol resulted in an increase in MnSOD and catalase protein content following a 3 day treatment. Treatment of myotubes for 5 and 7 days with 1 $\mu$ M and 10 $\mu$ M of resveratrol resulted in an upregulation of MnSOD and catalase. This is in agreement with other studies suggesting that resveratrol can act to increase the cellular antioxidant defences and alleviate oxidative stress (Jackson et al., 2011).

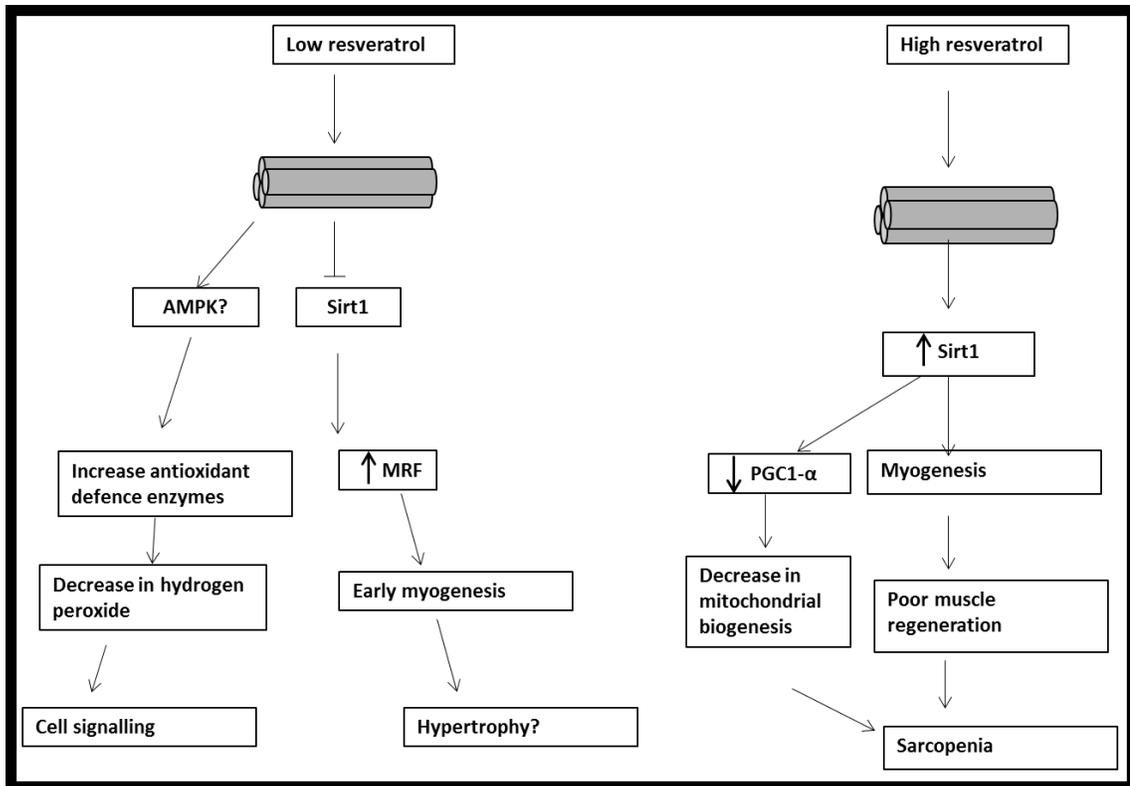
Studies have shown modest increases of Sirt1 protein level and activity in muscle (Jackson et al., 2011) and in other tissues following treatment with resveratrol (Morita et al., 2012). In contrast, the current study demonstrated a decrease in Sirt1 protein content following treatment of myotubes with resveratrol for 3-5 days. Treatment of myotubes with lower concentrations of resveratrol resulted in a decrease in Sirt1 protein content after 7 days of treatment. These data agree with other published data that suggest Sirt1 is not a direct target of resveratrol (Higashida et al.,

2013) but provides further evidence that resveratrol may cause early onset of myogenesis by down regulation of Sirt1 since Sirt1 has been shown to increase the proliferation of satellite cells (Rathbone et al., 2009). Overexpression of Sirt1 has been shown to increase proliferation of satellite cells and myoblasts (Rathbone et al., 2009). Sirt1 negatively regulates myogenesis by inhibiting MRFs and expression of muscle structural proteins. Furthermore, myoblasts overexpressing Sirt1 had impaired differentiation (Fulco et al., 2003). This impairment, was reversible with treatment of C2C12 cells and human myocytes with shRNA or a NAM (NAD antagonist) (Fulco et al., 2003). Thus, to facilitate muscle differentiation, decreasing the  $\text{NAD}^+/\text{NADPH}$  ratio in the myotube prevents Sirt1 activation and allows myogenesis to occur to its full potential (Fulco et al., 2003).

This study found that the lowest concentration (0.1 $\mu\text{M}$ ) of resveratrol was the most efficient at reducing Sirt1 activation. Sirt1 is thought to be beneficial in a number of instances. In particular, Sirt1 has been proposed to be life extending (Howitz et al., 2003; Porquet et al., 2013). However, Sirt1 activity has been shown to be upregulated in muscle of old mice (Jackson et al., 2011), suggesting that increased Sirt1 activity may not be advantageous to muscle, as higher levels of Sirt1 has been shown to inhibit myogenesis (Fulco et al., 2003). Therefore there is a possibility that the increase in Sirt1 activity in muscle with age may contribute to the poor regenerative potential in the muscle of old animals (Gutmann et al., 1976; McArdle et al., 2004). Furthermore, treatment of C2C12 myotubes with high concentrations of resveratrol resulted in decreased PGC1- $\alpha$  activity, leading to a decline in mitochondria number (Higashida et

al., 2013). The difference in dose effects of resveratrol is particularly important, as it seems common practice in the majority of studies to use extremely high, non-physiological concentrations of resveratrol, which cause an increase in Sirt1 protein content and activity. Although the high doses of resveratrol and subsequent upregulation of Sirt1 may show benefits in other tissues, this may be detrimental to muscle, particularly for muscle repair. Resveratrol treatment has been shown to be beneficial in diseases such as muscular dystrophy by improving muscle mass and partially preventing the infiltration of non-muscular tissue into the muscles (Hori et al., 2011), further indicating the importance of understanding the effect of different doses of resveratrol. This different dose effects could be one of the reasons for the discrepancies reported in Sirt1 activation in muscle following resveratrol treatment (Jackson et al., 2011; Bennett et al., 2013). To my knowledge, these are the first data suggesting a role of resveratrol in decreasing Sirt1 protein content in muscle, as other studies have found increases (Jackson et al., 2011) or no effect on protein content of Sirt1 (Bennett et al., 2013) in muscle following resveratrol treatment. It is worth noting that a number of these studies were undertaken *in vivo*, which could also account for the differences in results for several reasons including the differences in the muscle environment or differential effects of the degradation products of resveratrol *in vivo* following degradation in the gut.

Data presented here suggests that all three doses of resveratrol used in the current study have a physiological effect in myotubes and the proposed mechanisms of action are shown in Figure 4.14.



**Figure 4.14 Hypothesised differences in mechanisms of resveratrol in myotubes following high and low doses of resveratrol.**

#### **4.4.3 Effect of resveratrol treatment on the acetylation status of proteins in myotubes**

As protein content does not necessarily translate to a change in activity, the acetylation status of the proteins in myotubes following resveratrol treatment was also examined. In agreement with the decrease observed in Sirt1 protein content following resveratrol treatment in myotubes, there was an indication that acetylation was increased in the proteins from resveratrol treated myotubes, particularly at early time points following resveratrol treatment. This would suggest that there is a higher deacetylase activity in the untreated myotubes compared with resveratrol treated cells. It is important to note that there were differences in acetylation states between

the higher and lower molecular weight proteins, with high molecular weight proteins seemingly highly sensitive to resveratrol and lower molecular weight proteins relatively insensitive. These discrepancies could be explained by the presence of a larger number of targets on the higher molecular weight proteins. However, previous work has shown resveratrol increased acetylation, inhibited acetylation and had no effect on different proteins, suggesting that Sirt1 deacetylase activity following resveratrol treatment may be substrate selective (Lakshminarasimhan et al., 2012).

#### **4.4.4 Effect of resveratrol treatment on myotube diameter**

Treatment of myotubes with resveratrol resulted in some acute cell death in myotubes, therefore the aim was to identify whether this increase in cell death was associated with myotube atrophy. Treatment of myotubes with resveratrol resulted in an increase in myotube diameter following 3 and 5 days of daily treatments, however, there was no difference in myotube diameter between untreated and resveratrol treated myotubes at later time points. Although resveratrol has been shown to induce muscle hypertrophy (Montesano et al., 2013), these data provide evidence that instead of causing hypertrophy directly, resveratrol may act by initiating early myogenesis, causing treated myoblasts to fully differentiate more rapidly and for this reason there was an earlier peak in myotube diameter that was absent in the later time points. Therefore it would be interesting to investigate at a later time point, when myogenesis was complete, in both the control and resveratrol-treated cells, whether the myotube protein content of Sirt1 still differ between the untreated control and resveratrol-treated myotubes.

#### **4.4.5 Effects of resveratrol treatment on H<sub>2</sub>O<sub>2</sub> production by myotubes**

This study aimed to further examine whether the changes in catalase protein content in myotubes induced by treatment of resveratrol affected hydrogen peroxide release by myotubes. These data has demonstrated that treatment of myotubes with all three concentrations of resveratrol resulted in a decrease in hydrogen peroxide production by myotubes compared with untreated control myotubes. These data are in agreement with the protein content data, which showed that all 3 concentrations of resveratrol resulted in increased catalase protein content of myotubes. The data also provide evidence that the increase in catalase is likely to be a direct result of the resveratrol treatment, resulting in the decrease in hydrogen peroxide generation rather than vice versa. This is particularly important as there is increasing amounts of evidence that low amounts of ROS act as signalling molecules and the production of hydrogen peroxide may be chronically increased in old mammals. Resveratrol may be effective in decreasing the levels of ROS. However, the pathway through which resveratrol achieves this in muscle is unclear, since data has shown that resveratrol treatment also resulted in a decrease in Sirt1 protein content of myotubes.

Data demonstrated that treatment of myotubes with 1µM of resveratrol for 3 days was sufficient to increase antioxidant defence enzymes content, myotube diameter and decreased levels of hydrogen peroxide release. Therefore this concentration of resveratrol that was chosen for use in future experiments.

#### **4.5 Conclusion**

Achievable concentrations of resveratrol were initially toxic to myotubes. Despite this toxicity, treatment of myotubes with resveratrol initially caused hypertrophy and decreased hydrogen peroxide production by myotubes compared with control myotubes. Treatment of myotubes with resveratrol resulted in an increase in the myotube content of MnSOD and catalase and a decrease in Sirt1 protein as well as an increase in overall protein acetylation compared with untreated control myotubes. These data suggest that resveratrol may cause pre-mature myogenesis and indirectly offers antioxidant protection to myotubes.

**5. THE EFFECT OF RESVERATROL ON  
INFLAMMATION INDUCED ATROPHY OF  
MYOTUBES *IN VITRO***

## 5.1 Introduction

In 1979 Rosenberg defined “sarcopenia” as the decline in muscle mass and function (Rosenberg, 1989) and due to the ever increasing life expectancy, it is becoming more important to find efficacious therapeutic avenues for prevention and treatment of sarcopenia. The development of an effective treatment for or prevention of sarcopenia is still a major challenge as the mechanisms responsible for skeletal muscle ageing are still undefined and it is likely that there is a multitude of important factors involved. One of the major factors proposed to play a role in sarcopenia is increases in both systemic circulating pro – inflammatory cytokines such as TNF- $\alpha$  and IL6 (Fagiolo et al., 1993) as well a local production in myokines (cytokines released by the muscle) such as MCP-1, IL8 (Caldow et al., 2013) and IL1 $\beta$  (Borge et al., 2009).

The imbalance in important systems such as the proteasomal degradation system and autophagy in skeletal muscle during ageing leads to detrimental cellular effects; there is an inability of muscle to regenerate as well as usual due to a decrease in the number (Gibson et al., 1983) and capacity of satellite cells to self-replenish the reserve population, (Shefer et al., 2006; Day et al., 2010). These changes in satellite cells can lead to the replacement of muscle fibres with connective tissue (fibrosis) (Brack et al., 2007) and intramuscular adipose tissue (IMAT) (Borkan et al., 1983; Visser et al., 2005). There is a decrease in muscle cross-sectional area type II fibres and furthermore a decrease in the ratio of fast type II fibres to slow type I fibres (Lee et al., 2006), as well as a decrease in the number of motor neurons (Rowan et al., 2012) and denervation of

neuromuscular junctions (Deschenes et al., 2010), all of which subsequently contribute to the decrease in muscle mass and force that is seen in sarcopenia

Studies have shown improvements in satellite cell function and the ability of muscle to regenerate when old muscle is placed in a young environment, signifying the importance of the environment of the muscle (Carlson et al., 1989; Brack et al., 2007). Furthermore, changes have been seen in important inflammatory markers such as NF- $\kappa$ B (Cai et al., 2004; Hunter et al., 2004; Vasilaki et al., 2006), TNF- $\alpha$  and IL-6 (Pedersen et al., 2003) in muscle during ageing. Interferon induced protein 10 (IP10), is a 8.7kDa pro-inflammatory chemokine involved in early recruitment of T-helper type 1 cells to sites of inflammation (Luster *et al.*, 1985) and is upregulated in response to many inflammatory agents such as IFN- $\gamma$  and TNF- $\alpha$  (Crescioli et al., 2012). Unpublished data from our laboratory and other studies have shown an increase in IP10 in the old compared with younger counterparts both in rodents (Chen et al., 2003a) and humans (Bonfante et al., 2015) although the role of IP10 in ageing associated conditions remains unclear.

Only a small amount of work has been done examining the effects of IP10 on skeletal muscle although it is thought that the role of IP10 is pivotal to changes to muscle in several situations. A reduction of IP10 has been shown to decrease inflammation in muscle in myositis (Kim et al., 2014) and IP10 has shown to be upregulated in situations where neuromuscular junction defects are evident (Feferman et al., 2005). IP10 was also shown to be linked to the regulatory of the central metabolism of genes in skeletal muscle in COPD patients, as well as in a guinea pig

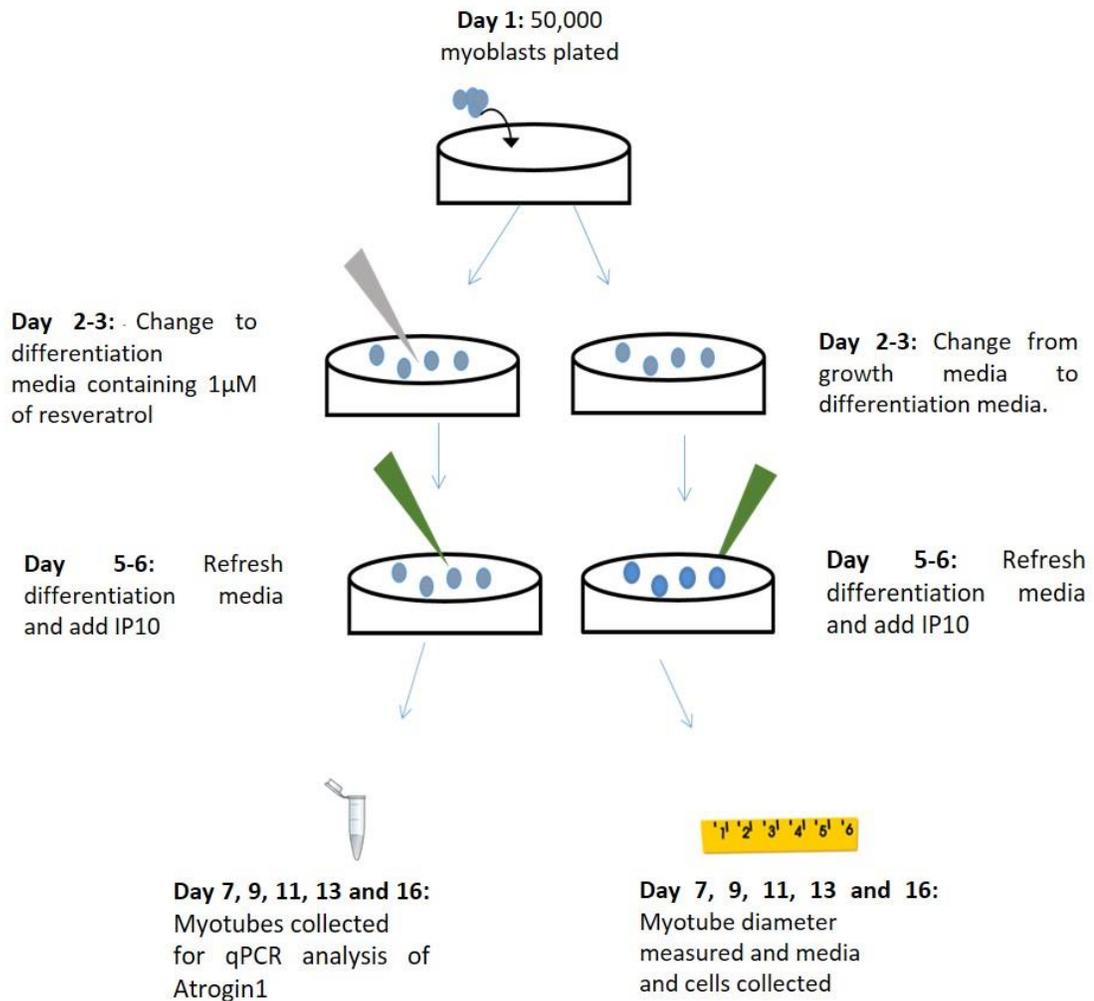
model of COPD (Davidsen et al., 2014). Intrinsic changes that occur within the muscle fibre have been shown to also exacerbate systemic ageing (Demontis et al., 2013). Therefore, IP10 has the potential to be a therapeutic target for sarcopenia and other muscle wasting diseases (Crescioli et al., 2012). No work has been published examining the role of IP10 in the development of sarcopenia.

“Atrogenes” are atrophy related genes, and this term was created from a study which identified genes which are changed during various types of atrophy (Sacheck et al., 2007). Of all of the established atrogenes, Atrogin1 (muscle atrophy F box/MAFbx) and muscle ring finger 1 (Murf1) are two of those most changed during atrophy. Atrogin1 and Murf1 are muscle specific ubiquitin ligases involved in the ubiquitin-proteasome machinery (Section 1.2.3) and are therefore used as indicators of protein degradation and thus, muscle atrophy (Gomes et al., 2001; Cohen et al., 2009; Fjällström et al., 2014). Atrogin1 and Murf1 are activated during the inflammatory response (Cohen et al., 2009; Wu et al., 2011) however, the effect of IP10 on Atrogin1 and Murf1 expression in skeletal muscle has not been studied.

Resveratrol is a polyphenol that has been shown to have anti-inflammatory properties in many different cell lines and tissues (Hwa Kanga et al., 2009; Zhu et al., 2011; Amcaoglu Rieder et al., 2012; Tomé-Carneiro et al., 2012), as well as in skeletal muscle (Pearson et al., 2008; Centeno-Baez et al., 2011). Thus, resveratrol has been proposed as a potential treatment for sarcopenia. Although resveratrol has been shown to decrease TNF- $\alpha$  induced Atrogin1 and Murf1 upregulation in skeletal muscle, the specific effect of resveratrol on IP10 induced atrophy has not been examined.

The aim of this study was to examine the effects that different concentrations of IP10 found in the plasma of healthy old compared with adult subjects had on myotubes and whether resveratrol was able to attenuate any atrophic effects of IP10. The hypothesis was that treatment of myotubes with the relatively high concentrations of IP10 seen in serum of old individuals would result in activation of atrophic processes in comparison with treatment of myotubes with lower levels of IP10, more consistent with the levels seen in younger individuals. In addition, prior treatment of myotubes with resveratrol would prevent the atrophic effects of IP10.

## 5.2. Methods



**Figure 5.1** A brief overview of the methodology used. Myotubes were treated with or resveratrol for 3 days. Myotubes were then treated with 150 or 200pg/ml of IP10. Myotube diameter was measured and myotubes used for qPCR analysis of Atrogin1 at 1,3,5,7 and 10 days following treatment with IP10.

### 5.2.1. IP10 preparation

IP10 was dissolved in distilled water at a concentration of 0.1mg/ml and was stored at -20°C until use.

### **5.2.2. Treatment of myotubes with IP10 +/- resveratrol**

Skeletal muscle cells isolated from Wistar rats were grown in culture until they reached 80% confluence; cells were counted using TC-20 automatic cell counter (Biorad, Hertfordshire, UK) and seeded in 6 well plates at a density of approximately  $5 \times 10^4$  per well. Once cells had reached 80% confluence, media was changed to differentiating media containing 0 or  $1 \mu\text{M}$  of resveratrol. Resveratrol was added daily for 3 days. Following resveratrol treatment, myotubes were treated with 0, 150pg/ml or 200pg/ml of IP10 and visualised for 1, 3, 5, 7 or 10 days following treatment using the Nikon TE2000 microscope (Nikon, Kingston upon Thames, UK). Myotube diameter was measured using ImageJ (US National Institutes of Health, Maryland, USA). For each image, every visible myotube was measured 3 times along its length to produce an average diameter per myotube, each myotube was then pooled to give an average myotube diameter per well. The average myotube diameter per well was then used to determine an average for the treatment groups.

### **5.2.3. Determination of gene expression in myotubes treated with IP10 +/- resveratrol using qPCR**

Myotubes were harvested in TriReagent, RNA extracted from myotube lysates (Section 2.14) and 100ng of RNA synthesised into cDNA (Section 2.15). PCR arrays were carried out to examine the expression of Atrogin1 as described in Section 2.12. All gene CT values were normalised to S29 CT values.

#### **5.2.4. Statistics**

Graphpad 5 (Graphpad software, San Diego, USA) was used to perform One-way ANOVA followed by a Dunnett's post-test to identify significant differences. Data are represented as mean  $\pm$  SEM.

### **5.3. Results**

#### **5.3.1. Effect of IP10 and resveratrol on myotube diameter over time.**

##### **5.3.1.1. The effect of IP10 on myotube diameter**

Myotube diameter following treatment of IP10 is shown Figure 5.2. There was a transient decrease in myotube diameter following treatment of myotubes with 150pg/ml IP10 at 3 days following treatment ( $p < 0.05$ ). There was no effect of treatment with 150pg/ml IP10 on myotube diameter at 1, 5, 7 or 10 days following treatment (Figure 5.2) compared with untreated control myotubes. In contrast, treatment of myotubes with 200pg/ml of IP10 resulted in a significant and persistent decrease in myotube diameter (Figure 5.2  $p < 0.05$ ), leading to a 33% reduction in myotube diameter compared with untreated control myotubes by 10 days post-treatment. Treatment of myotubes with 200pg/ml of IP10 resulted in a significant reduction in myotube diameter compared with a treatment of myotubes with 150pg/ml of IP10 at all time points.

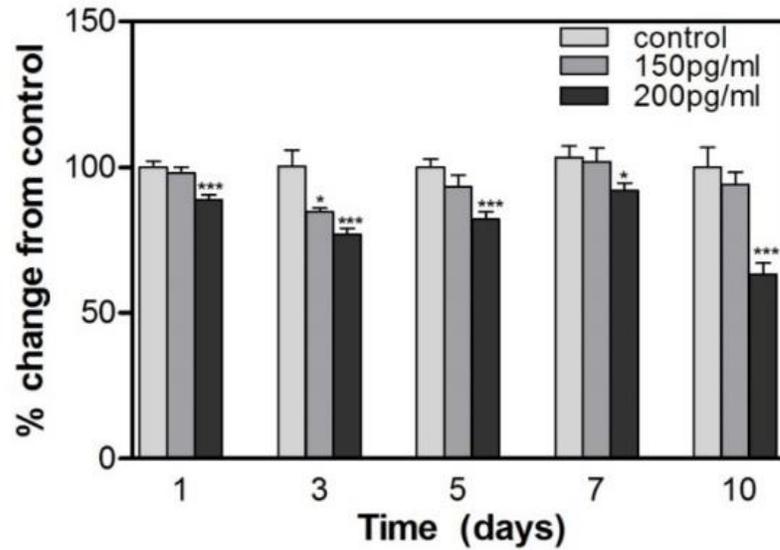


Figure 5.2 Myotube diameter expressed as percentage change from untreated control myotubes at 1, 3, 5, 7 and 10 days following treatment with 150pg/ml and 200pg/ml IP10 \*p<0.05, \*\*\*p<0.001 compared with untreated control myotubes One-way ANOVA. Values are myoblasts mean  $\pm$ SEM (n=3-4).

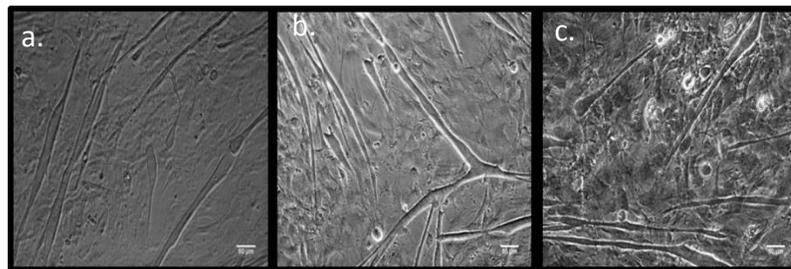
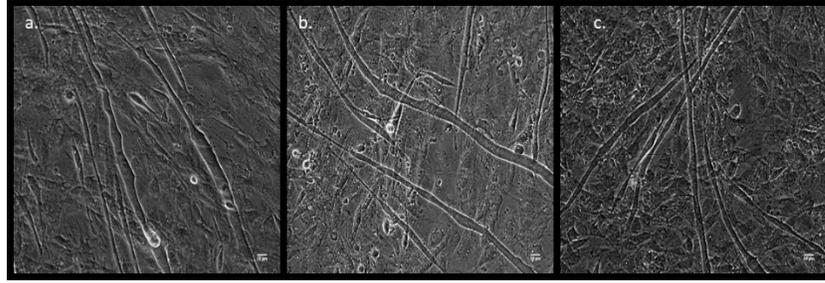
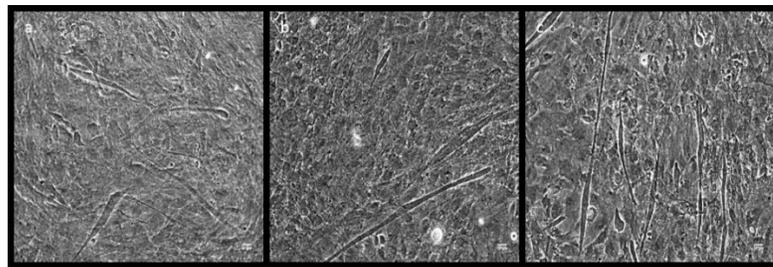


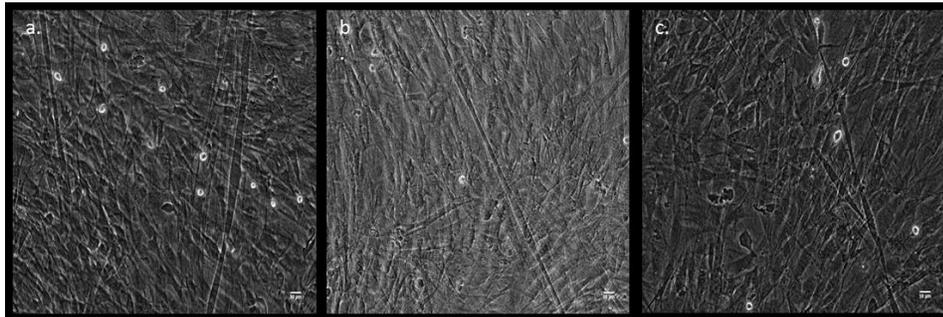
Figure 5.3 Myotube diameter at 1 day following IP10 treatment. Bright field images showing (a) untreated control (b) myotubes treated with 150pg/ml treated or (c) myotubes treated with 200pg/ml IP10.



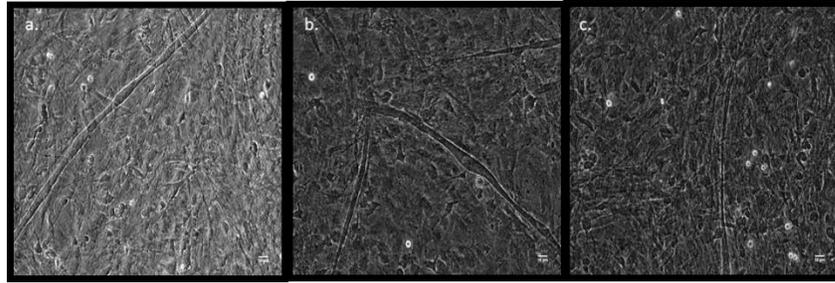
**Figure 5.4 Myotube diameter at 3 days following IP10 treatment. Bright field images showing (a) untreated control (b) myotubes treated with 150pg/ml IP10 or (c) myotubes treated with 200pg/ml IP10.**



**Figure 5.5 Myotube diameter at 5 days following IP10 treatment. Bright field images showing (a) untreated control (b) myotubes treated with 150pg/ml treated or (c) myotubes treated with 200pg/ml IP10.**



**Figure 5.6 Myotube diameter at 7 days following IP10 treatment. Bright field images showing (a) untreated control (b) myotubes treated with 150pg/ml treated or (c) myotubes treated with 200pg/ml IP10.**



**Figure 5.7 Myotube diameter at 10 days following IP10 treatment. Bright field images showing (a) untreated control (b) myotubes treated with 150pg/ml treated or (c) myotubes treated with 200pg/ml IP10.**

#### **5.3.1.2. Effect of resveratrol on IP10-induced atrophy**

Myotube diameter following a 3 day pre-treatment with 1 $\mu$ M of resveratrol followed by the addition of 150pg/ml or 200pg/ml of IP10 at 1, 3, 5, 7 or 10 days following IP10 treatment is shown in Figure 5.8 and Figure 5.9. There was a gradual decrease in myotube diameter following treatment with 200pg/ml IP10 for up to 10 days following treatment, whereas treatment of 150pg/ml only transiently decreased myotube diameter compared with untreated control myotubes at 3 days following IP10 treatment (Figure 5.2). Pre-treatment of myotubes with resveratrol resulted in protection against the atrophic effects of treatment of myotubes with both 150pg/ml and 200pg/ml of IP10 at all time points following treatment.

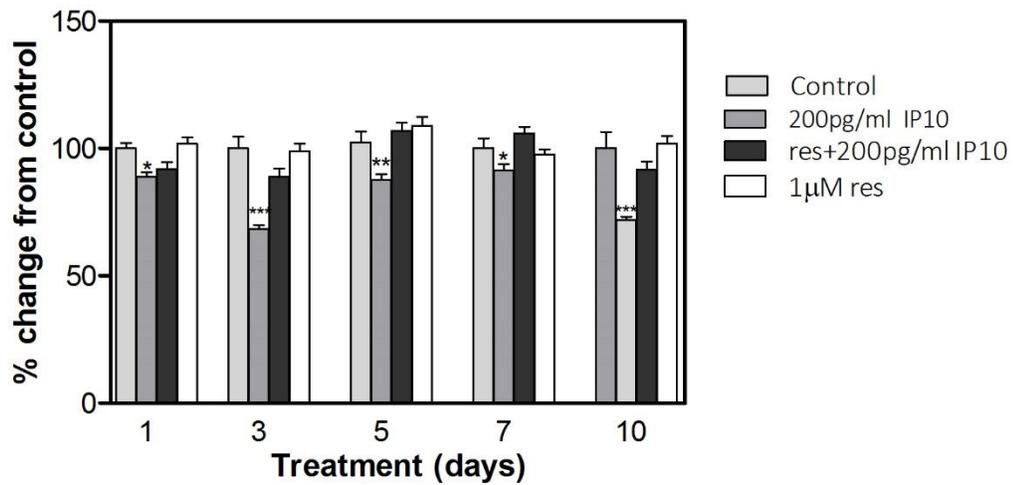


Figure 5.8 Myotube diameter expressed as percentage change from untreated control myotubes following a pre-treatment of resveratrol followed by 200pg/ml IP10 at 1, 3, 5, 7 or 10 days or a treatment with resveratrol alone \*p<0.05, \*\*p<0.01 \*\*\*p<0.001 compared with untreated control myotubes. One-way ANOVA. Values are mean ±SEM (n=3-4).

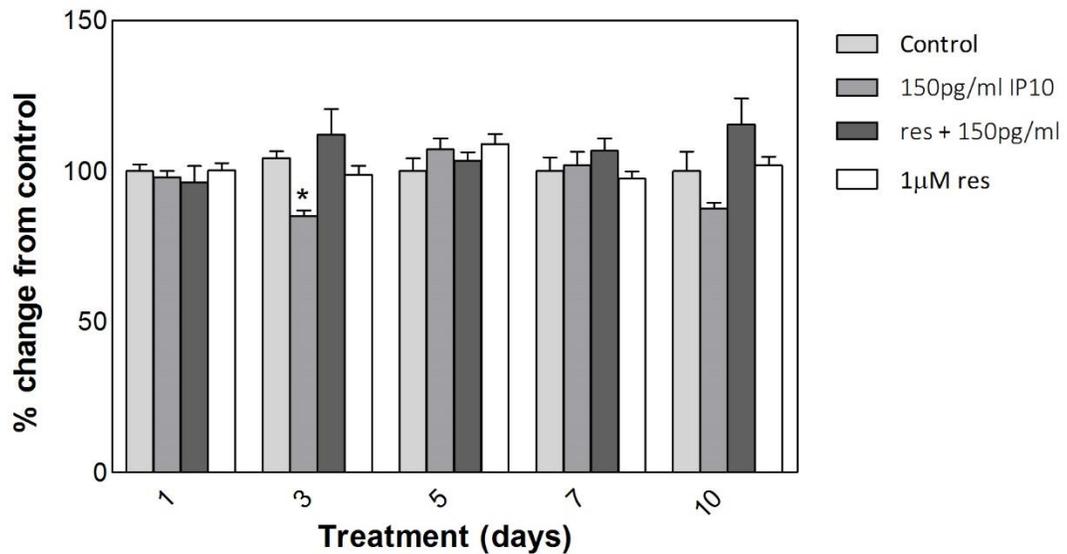
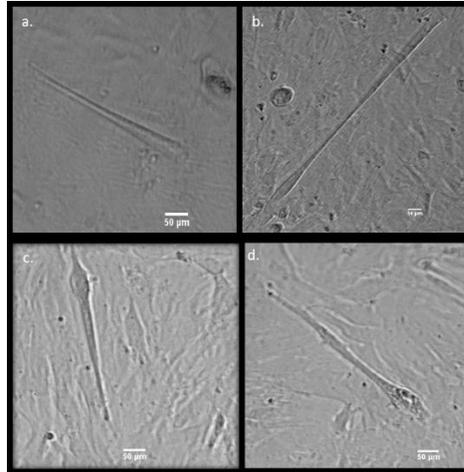
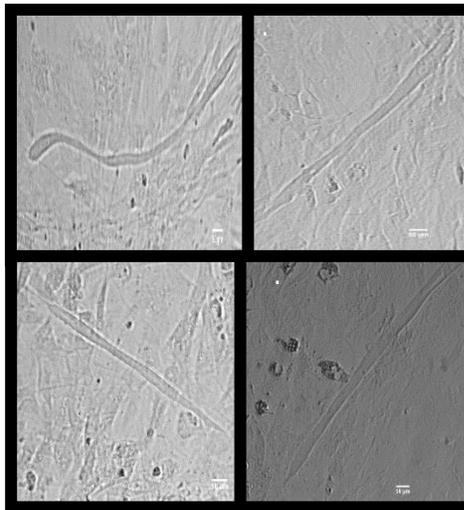


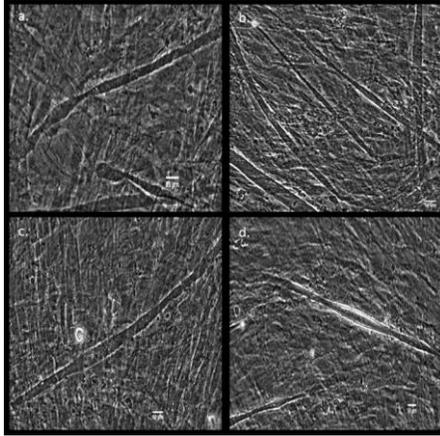
Figure 5.9 Myotube diameter expressed as percentage change from untreated control myotubes following a pre-treatment of resveratrol followed by 150pg/ml IP10 at 1, 3, 5, 7 or 10 days or a treatment with resveratrol alone \*p<0.05, \*\*p<0.01 \*\*\*p<0.001 compared with untreated control myotubes One-way ANOVA. Values are mean ±SEM (n=3-4).



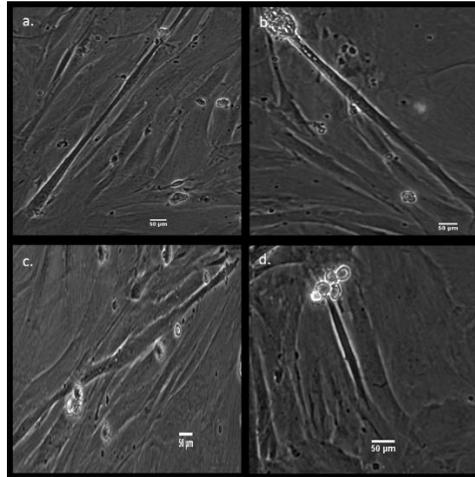
**Figure 5.10** Representative images of myotubes pre-treated with resveratrol on changes in myotube diameter induced 1 day following IP10. Bright field images showing (a) untreated control myotubes and myotubes treated with (b) resveratrol + 150pg/ml IP10 (c) resveratrol + 200pg/ml IP10 (d) 1µM of resveratrol alone. Scale bar is 50µm.



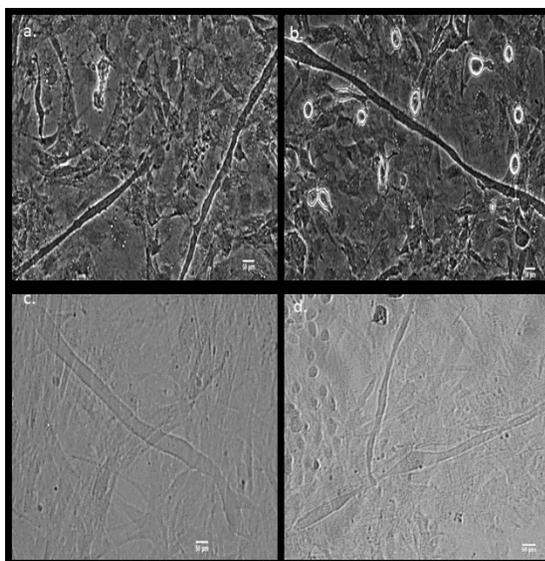
**Figure 5.11** Representative images of myotubes pre-treated with resveratrol on changes in myotube diameter induced 3 day following IP10. Bright field images showing (a) untreated control myotubes and myotubes treated with (b) resveratrol + 150pg/ml IP10 (c) resveratrol + 200pg/ml IP10 (d) 1µM of resveratrol alone. Scale bar is 50µm.



**Figure 5.12** Representative images of myotubes pre-treated with resveratrol on changes in myotube diameter induced 5 day following IP10. Bright field images showing (a) untreated control myotubes and myotubes treated with (b) resveratrol + 150pg/ml IP10 (c) resveratrol + 200pg/ml IP10 (d) 1µM of resveratrol alone. Scale bar is 50µm.



**Figure 5.13** Representative images of myotubes pre-treated with resveratrol on changes in myotube diameter induced 7 day following IP10. Bright field images showing (a) untreated control myotubes and myotubes treated with (b) resveratrol + 150pg/ml IP10 (c) resveratrol + 200pg/ml IP10 (d) 1µM of resveratrol alone. Scale bar is 50µm.



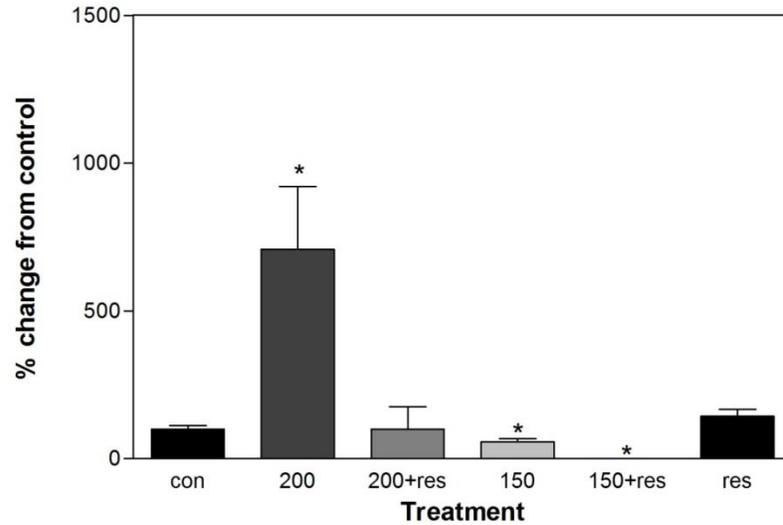
**Figure 5.14** Representative images of myotubes pre-treated with resveratrol on changes in myotube diameter induced 10 day following IP10. Bright field images showing (a) control myoblasts and myoblasts treated with (b) resveratrol + 150pg/ml IP10 (c) resveratrol + 200pg/ml IP10 (d) 1µM of resveratrol alone. Scale bar is 50µm.

### **5.3.2. Effects of treatment of myotubes with IP10 on Atrogin1 mRNA expression and effect of pre-treatment with resveratrol**

Atrogin1 levels in myotubes following treatment with 150pg/ml and 200pg/ml of IP10 and the effect of a pre-treatment with resveratrol is shown in Figure 5.15- Figure 5.17. Atrogin1 levels in myotubes were decreased at 1 day following treatment of myotubes with 150pg/ml of IP10 ( $p < 0.05$ ) compared with untreated myotubes. Treatment of myotubes with 200pg/ml of IP10 resulted in a significant increase in Atrogin1 mRNA levels at 1 ( $p < 0.05$ ) and 7 days ( $p < 0.05$ ) following treatment and there was a tendency for Atrogin1 mRNA levels to remain elevated 10 days following treatment of myotubes with IP10 when compared with untreated myotubes.

Any increase in Atrogin1 mRNA levels in myotubes induced by IP10 treatment was generally prevented with a pre-treatment of myotubes with resveratrol. However,

Atrogin1 mRNA levels were increased in myotubes pre-treated with resveratrol and treated with 150pg/ml of IP10 at 10 days following treatment compared with 150pg/ml IP10 treatment ( $p < 0.05$ ). Atrogin1 mRNA levels were not changed from untreated control myotube levels following a treatment with resveratrol alone.



**Figure 5.15** Changes in Atrogin1 mRNA levels in myotubes compared with untreated control myotubes (con) at 1 days following IP10 treatment with 150pg/ml (150) or 200pg/ml (200) of IP10 +/- a pre-treatment (200+res/150+res) with 1 $\mu$ M of resveratrol or treatment resveratrol alone (res) ( $n \geq 3$ ) \* $p < 0.05$  compared with untreated control myotubes. One way ANOVA.

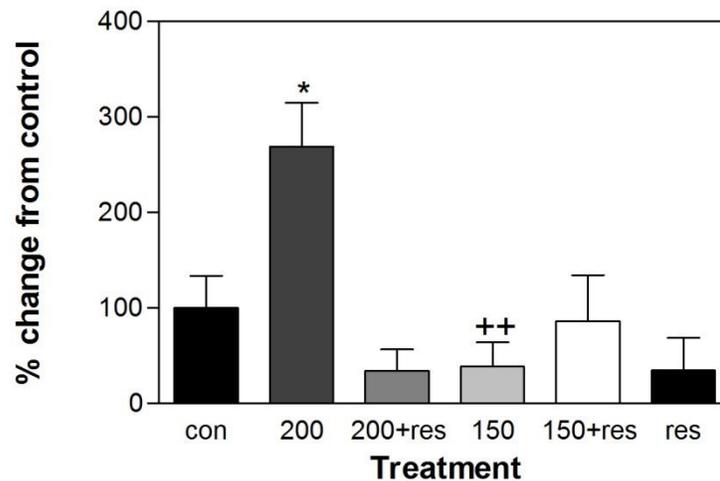


Figure 5.16 Changes in Atrogin1 mRNA levels in myotubes compared with untreated control myotubes (con) at 7 days following IP10 treatment with 150pg/ml (150) or 200pg/ml (200) of IP10 +/- a pre-treatment (200+res/150+res) with 1 $\mu$ M of resveratrol or treatment resveratrol alone (res) (n $\geq$ 3) \*p<0.05 compared with untreated control myotubes, ++p<0.01 compared with 200pg/ml. One-way ANOVA.

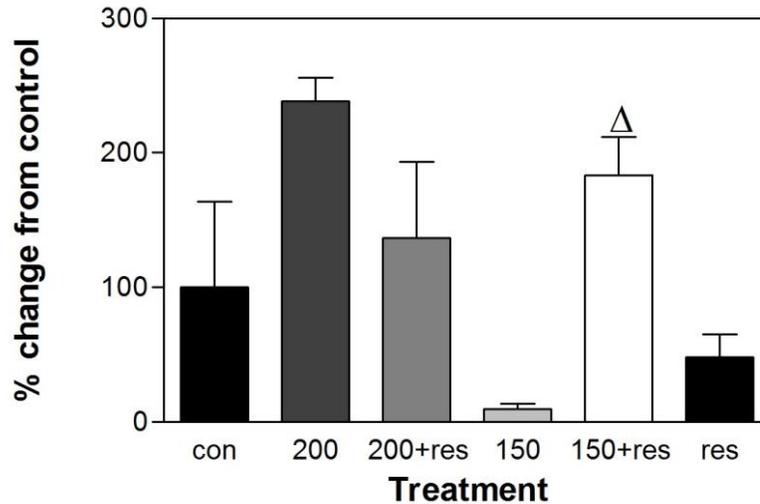


Figure 5.17 Changes in Atrogin1 mRNA levels in myotubes compared with untreated control myotubes (con) at 10 days following IP10 treatment with 150pg/ml (150) or 200pg/ml (200) of IP10 +/- a pre-treatment (200+res/150+res) with 1 $\mu$ M of resveratrol or treatment resveratrol alone (res) (n $\geq$ 3)  $\Delta$ p<0.01 compared with 150pg/ml. One-way ANOVA.

#### 5.4. Discussion

The aim of this Chapter was to examine the effects of IP10 on myotubes and to identify whether there were differential effects of different concentrations of circulating IP10 found in either old (200pg/ml) or adult (150pg/ml) healthy individuals. A secondary aim of the study was to identify whether resveratrol was able to prevent any negative effects of IP10 treatments in myotubes. Data showed different effects of the different concentrations of IP10 on myotubes. Treatment of myotubes with a

concentration of 200pg/ml of IP10 resulted in a chronic decrease in myotube diameter, whereas treatment of myotubes with 150pg/ml resulted in a transient decrease in myotube diameter detected only at 3 days following treatment. Pre-treatment of myotubes with resveratrol generally prevented any atrophic effects of both concentrations of IP10. Data from myotube diameter were reflected in data examining the effect of IP10 and resveratrol on the mRNA levels of the atrogene, Atrogin1. Treatment of myotubes with 200pg/ml of IP10 resulted in increased mRNA expression of Atrogin1 at 1 and 7 days and a pre-treatment of myotubes with resveratrol prevented this increase. Treatment of myotubes with 150pg/ml of IP10 resulted in a decrease in Atrogin1 expression 1 day following treatment. A pre-treatment of myotubes with resveratrol followed by a 1 day treatment of 150pg/ml of IP10 further decreased Atrogin1 expression, making levels undetectable. Pre-treatment of myotubes with resveratrol prior to treatment with 150pg/ml IP10 resulted in an increased Atrogin1 expression at 10 days following treatment above the levels seen in 150pg/ml IP10 alone. Treatment of myotubes with resveratrol alone had no effect on Atrogin1 mRNA expression. Furthermore, neither IP10 nor resveratrol had an effect on Murf1 mRNA expression (data shown in appendix Section 10.2).

#### **5.4.1. Effects of IP10 on myotube diameter**

Sarcopenia is the loss of muscle mass and function as we age. One major contributor to the development of sarcopenia is the atrophy of individual muscle fibres in older individuals. Therefore, some aspects of this can be studied in cell cultures using a decrease in myotube diameter as an indicator of atrophy. Studies of different

atrophic situations have shown that mechanisms through which myotubes decrease myotube diameter include a lower fusion index, increased expression of the ubiquitin ligase Atrogin1 as well a decrease in protein synthesis through the IGF-1/Akt pathway (Stitt et al., 2004; Pomies et al., 2015). IP10 is a chemokine involved in the early stages of the inflammatory response and has been shown to be increased with age in various tissues (Chen et al., 2003a; Bonfante et al., 2015). Studies from our laboratory have demonstrated that levels of IP10 in plasma of older individuals are approximately 200pg/ml and 150pg/ml in the plasma of healthy younger individuals (Ford et al, unpublished). In the current study, treatment of myotubes with 200pg/ml of IP10 resulted in a chronic decrease in myotube diameter, suggesting an atrophic effect of this concentration of IP10. Treatment of myotubes with 150pg/ml had little effect on myotube diameter, although a transient decrease in myotube diameter was seen 3 days following treatment, suggesting an acute but reversible effect. It is well known that as we age there is an increase in the inflammatory state, and it is widely hypothesised that this plays an important role in both systemic ageing and sarcopenia. The contrasting effects of different levels of IP10 offers further evidence that the environment may play an important role in the onset of age-related muscle weakness and for the first time provides potential evidence for a role of serum changes of IP10 in sarcopenia.

Very little work has been carried out examining the role of IP10 in the development of sarcopenia with a PubMed search for IP10/CXCL0 and sarcopenia returning 0 results. Data in this Chapter suggests that treatment of myotubes with IP10

at the levels found in old individuals, decrease in myotube diameter is persistent, suggesting that at these levels IP10 may contribute to sarcopenia. IP10 has been shown to be undetectable in untreated muscle and other cells (Torvinen et al., 2007; Crescioli et al., 2012) and low levels of IP10 have been shown to inhibit numerous types of cancer (Zhang *et al.*, 2005; Wang *et al.*, 2013), induce migration and proliferation of smooth muscle cells (Wang et al., 1996) and pericytes (Bonacchi et al., 2001) and have been shown to play an important role in the uptake of pluripotent stem cells following liver injury (Chan et al., 2012), suggesting that low levels of IP10 can be beneficial to cells and may play important roles in signalling pathways. The absence of long term effects of 150pg/ml of IP10 on myotubes suggests that these beneficial effects may extend to skeletal muscle. Further data show that IP10 is upregulated in early myogenesis (Griffin et al., 2010) suggesting that lower levels IP10 may also be required for myogenesis and muscle regeneration. However, the persistent atrophic effects caused by higher concentrations of IP10 suggest that IP10 may be involved in sarcopenia and provides further evidence of the importance of understanding the dysregulation of IP10, as increased IP10 levels are correlated with numerous diseases such as asthma (Medoff et al., 2002), infection (Tanaka et al., 2009) and lung cancer (Siva et al., 2014). Thus, a study in patients with Hepatitis C has shown that serum levels of  $\leq 150$ pg were considered low level risk with no requirement for treatment (Grebel et al., 2013) these data are particularly interesting as they suggest a new potential target for sarcopenia rather than the more commonly studied TNF- $\alpha$  and IL6 (Visser et al., 2002; Pedersen et al., 2003).

#### **5.4.2. Effects of IP10 on markers of atrophy**

Atrogin1 and Murf1 are muscle specific ligases, that have been shown to be upregulated in numerous types of atrophy (Gomes et al., 2001) and are part of a group of genes known as atrogenes – atrophy related genes. Preliminary experiments were performed to examine mRNA expression of Atrogin1 and Murf1 at 1 day following IP10 treatment as a potential mechanism for the observed decrease in myotube diameter. Treatment of myotubes with 150pg/ml of IP10 had no effect on Atrogin1 mRNA levels, whereas treatment of myotubes with 200pg/ml of IP10 resulted in an increase in Atrogin1 expression. None of the treatment groups had any effect on Murf1 mRNA expression (appendix Section 10.2). The difference in effects on of IP10 treatment Atrogin1 and Murf1 mRNA expression were not surprising as this has been shown to be the case in other studies (Stefanetti et al., 2014). Due to this lack of effect of IP10 and resveratrol on Murf1 expression further work focused on the effect of IP10 on Atrogin1 expression.

Treatment of myotubes with 200pg/ml of IP10 resulted in an increase Atrogin1 expression both 1 day and 7 days following treatment when myotube atrophy was evident. In contrast, treatment of myotubes with 150pg/ml of IP10 decreased Atrogin1 mRNA levels from untreated control myotubes 1 day following treatment, and Atrogin1 levels in myotubes were significantly decreased compared with the level in myotubes following treatment with 200pg/ml of IP10 7 days following treatment. The increase in Atrogin1 mRNA levels following treatment of myotubes with 200pg/ml of IP10 is in agreement with the decrease in myotube diameter established in this study

and further supports the hypothesis that the levels of IP10 found in old individuals may contribute to sarcopenia. This data also provides a possible mechanism through which the subsequent upregulation of Atrogin1 occurs leading to increased degradation of muscle proteins and thus atrophy. Due to the absence of detection of Atrogin1 in untreated control myotubes and in myotubes at 3 and 5 days following of IP10 treatments, these data suggests that there may be an acute and chronic response to IP10 treatment. The absence of the atrophic effects of treatment of myotubes with 150pg/ml of IP10 provides evidence for IP10 involvement in sarcopenia.

As discussed only a sparse amount of work on IP10 during atrophy has been undertaken, therefore the mechanism responsible for IP10 increases in Atrogin1 mRNA levels remains unknown. TNF- $\alpha$  is a strong inducer of IP10 expression (Fang et al., 2012) and therefore it is likely that IP10 is a downstream effector of TNF- $\alpha$ . TNF- $\alpha$  treatment of C2C12 myotubes demonstrated an increase Atrogin1 expression through the inhibition of the FOXO/mTOR/AKT/S6k pathway (Wang *et al.*, 2014). Furthermore, IFN- $\gamma$ , a known activator of IP10, also resulted in a decrease in the Akt activation leading to increased Atrogin1 levels in C2C12 myotubes (Smith et al., 2007). Therefore it may be speculated that IP10 acts through the same pathway. However further investigation is required.

#### **5.4.3. Effects of resveratrol on IP10 induced atrophy**

Pre-treatment of myotubes with resveratrol was able to prevent the decrease in myotube diameter and increase in Atrogin1 mRNA expression caused by treatment of myotubes with 200pg/ml dose of IP10. Interestingly, the increase in myotube

diameter induced by daily treatments of myotubes for 3 days with resveratrol seen in Chapter 4 was not evident 1 day after the cessation of resveratrol treatment, suggesting the effects of resveratrol, are not long lasting. This is not unexpected as resveratrol is known to be metabolised very fast in humans following oral ingestion (Wenzel *et al.*, 2005; Almeida *et al.*, 2009) and in cell cultures of liver cells (Lançon A *et al.*, 2007) and therefore it is likely that the half-life of resveratrol in myotube cultures is also short. Resveratrol alone had no effect on Atrogin1 expression. These data suggest that resveratrol prevents the atrophic effects of IP10 by preventing the increase in Atrogin1. Resveratrol has been shown to increase Akt activation in C2C12 myotubes and Akt is able to inhibit Atrogin1 (Stitt *et al.*, 2004; Wang *et al.*, 2014), suggesting a potential mechanism by which resveratrol may prevent an increase in Atrogin1, however further work is needed.

The absence of effects on Atrogin1 by resveratrol alone offers further evidence that resveratrol is only effective when there is an existing dysfunction within the cells (Lagouge *et al.*, 2006). Furthermore, the increase in Atrogin1 mRNA levels in myotubes at 10 days following treatment with 150pg/ml of IP10 which had been pre-treated with resveratrol also offers support to previous work that has shown that resveratrol may exacerbate some (Sato *et al.*, 2013a) and may be potentially harmful or at least ineffective in healthy subjects. These data suggest caution when considering the use of resveratrol in healthy individuals and thus suggesting that resveratrol would only be useful as a treatment for sarcopenia and not as a preventative measure.

## **5.5. Conclusions**

Evidence of atrophy in myotubes following treatment with an IP10 concentration similar to that found in older healthy individuals has provided evidence for a potential role of increased IP10 levels in the development of sarcopenia. IP10 induced increases in the ubiquitin ligase Atrogin1 suggest this as a potential mechanism by which IP10 induces atrophy. Pre-treatment of myotubes with resveratrol was able to prevent the atrophic effects of IP10, providing a potential treatment for muscle fibre atrophy developed sarcopenia. However, the induction of atrophic pathways following the compounded effect of treatment of myotubes with 150pg/ml IP10 when pre-treated with resveratrol signifies a need for caution when using resveratrol, particularly in young, healthy subjects.

## **6. THE EFFECT OF RESVERATROL ON CYTOKINE PRODUCTION BY MYOTUBES *IN VITRO***

## 6.1. Introduction

The inflammatory response is a series of cellular processes, characterised by the secretion of inflammatory mediators such as IL6, C-reactive protein (CRP), TNF- $\alpha$  (Ballou et al., 1992; Bartlett et al., 2012) and other cytokines, from damaged, infected or immune cells which result in the chemotaxis of immune cells, in an attempt to achieve resolution of the disease state. The inflammatory response is vital for the protection of cells against foreign invading bodies. However, as we age it is well known that there is an overall decrease in the production of anti-inflammatory cytokines coupled with an increase in circulating pro-inflammatory cytokines that leads to a chronic low grade systemic inflammation in the plasma of older people (Miles et al., 2008; Cartier et al., 2009). This will potentially have serious detrimental effects on many different systems in the body. This phenomena has been termed 'inflamm-ageing' (Franceschi et al., 2000) and is supported by heterochronic parabiosis studies that show that some aspects of tissue ageing can be reversed when placed into the environment of a younger host (Ruckh et al., 2012; Villeda et al., 2014). The increase in the baseline cytokine levels has been correlated with numerous age-related pathologies such as obesity-related diabetes (Xu et al., 2003), Alzheimer's disease (Leea et al., 2008), cardiovascular disease (Cuff et al., 2001) and sarcopenia (Schaap et al., 2006).

The underlying mechanisms responsible for sarcopenia are still poorly understood, however, it is proposed that an increase in cytokine levels is likely to play a role in the age-related loss of muscle mass and function (Degens, 2010; Lightfoot et al.,

2014), particularly now as it is recognised that muscles are also able to release cytokines, named myokines (Sorby Borge *et al.*, 2009).

Interest in myokines first began during the late 1990's when it was noted that 'factors' that had a functional importance were released from muscle following exercise. Interleukin 6 was the first myokine to be described following contraction (Ostrowski *et al.*, 1998). Furthermore, there is evidence that contracting muscle can act as an endocrine organ with data showing muscle secretion of numerous different cytokines such as IL15 and IL8 (Pedersen, 2011). Myokine release has been shown to be involved in hypertrophic processes and myokines are proposed to play a role in some of the beneficial effects of exercise (Jung *et al.*, 2015). However, myokine secretion has also been shown in atrophy, for example; IL6 has been shown to cause muscle atrophy (Haddad *et al.*, 2005). However, this is controversial as IL6 has also been shown to be beneficial to muscle with evidence of a role in myogenesis and satellite cell proliferation (Serrano *et al.*, 2008). Thus the role of myokines in muscle atrophy and sarcopenia is sparsely studied and unclear.

A potential role for changes in myokine and cytokine levels in sarcopenia is supported by heterochronic parabiosis studies that showed improved proliferation capacity and regeneration capability of satellite cells in muscles of old mice when exposed to a younger environment (Conboy *et al.*, 2005). The foundations for this were demonstrated by seminal transplantation studies in the 1980s, which indicated that the age of the host determines the outcome of muscle regeneration and function. Specifically, muscle from old rats transplanted into a young rat displayed successful

regeneration, whereas muscles taken from young rats transplanted into an old host did not (Carlson et al., 1989). These findings exemplify the importance of the environment in skeletal muscle regeneration and atrophy and have provided further evidence for a role of inflamm-ageing as an important mediator of sarcopenia. Furthermore, the deposition of adipose tissue into muscle is an additional hallmark of ageing (Song et al., 2004) and is reported to play a compounding role in the age-related loss of muscle mass and function (Visser et al., 2005). Adipocytes are able to secrete a number of different cytokines, such as TNF- $\alpha$  (Hotamisligil et al., 1995; Sopasakis et al., 2005) therefore it is hypothesised that this intramuscular adipose tissue (IMAT) may contribute to age-related increases in local skeletal muscle production of cytokines. This is evidenced in a study by Schapp et al (2009) showing that when adjusted for fat mass, systemic inflammation is attenuated (Schaap et al., 2009) and in other studies which show muscle IL6 levels correlate with IMAT (Addison et al., 2014).

Increases in levels of cytokines are a good indicator of a risk for functional decline in skeletal muscle; IL6 shows a linear association with loss in muscle strength (Schaap et al., 2006) and is correlated with muscle atrophy (Haddad et al 2005). Higher levels of TNF- $\alpha$ , CRP and IL1 are correlated with sarcopenia and muscle related disorders such as insulin sensitivity (Marques-Vidal et al., 2013), aerobic fitness (Kohut et al., 2006), strength and physical functionality (Visser et al., 2002).

Studies on systemic and muscle ageing have focused on common cytokines such as CRP, IL6 and TNF- $\alpha$ . Unpublished work from our laboratory and work from other groups (Bonfante et al., 2015) has found an age-related increase in plasma levels

of the pro-inflammatory chemokine IP10. Furthermore, a study that took into consideration other co-morbidities showed that plasma levels of IP10 were still significantly upregulated in the blood of older people, whereas many cytokines that are usually associated with ageing were not (Miles et al., 2008). These findings suggest that IP10 may play a role in ageing, thus it may be reasonable to hypothesise that other cytokines which are described in the context of “inflamm-ageing” may be derived from confounding comorbidities.

Chapter 5 demonstrated that treatment of myotubes with concentrations of IP10 found in the plasma of healthy old subjects (200pg/ml) resulted in a decrease in myotube diameter coupled with an increase in the mRNA expression of the atrogene, Atrogin1 (Chapter 5). There was no effect on myotube diameter or Atrogin1 expression when myotubes were treated with concentrations of IP10 generally found in the plasma of young adults (150pg/ml). These results suggested higher concentrations of IP10 are associated with atrophic effects and therefore suggest a potential role of IP10 in sarcopenia.

Considerable work has focused on the effect of exercise on inflammation as a potential therapeutic intervention to sarcopenia and studies have shown that different types of exercise are successful in decreasing inflammation in skeletal muscle (Gielen et al., 2003; Zanchi et al., 2010; Lightfoot et al., 2016). However, exercise may not be well tolerated or appropriate and some older people are reluctant to implement exercise protocols whereas others are physically unable to carry out such activities.

Recent research has moved towards developing potential pharmacological or nutritional interventions to combat sarcopenia.

Polyphenols are plant extracts that have been shown to have beneficial effects in many different tissues and disease states (Klein et al., 2002; Sies et al., 2005; Porquet et al., 2014). Resveratrol is a commonly studied and used polyphenol and work in Chapter 5 demonstrated that pre-treatment of myotubes with a low concentrations of resveratrol (1 $\mu$ M) was able to prevent some of the atrophic effects of IP10 on myotubes. The prevention of both the decrease in myotube diameter and the increase in the ubiquitin ligase Atrogin1, suggests that resveratrol may have a role as a potential therapeutic agent against sarcopenia. However the mechanisms by which these effects occur remain unclear.

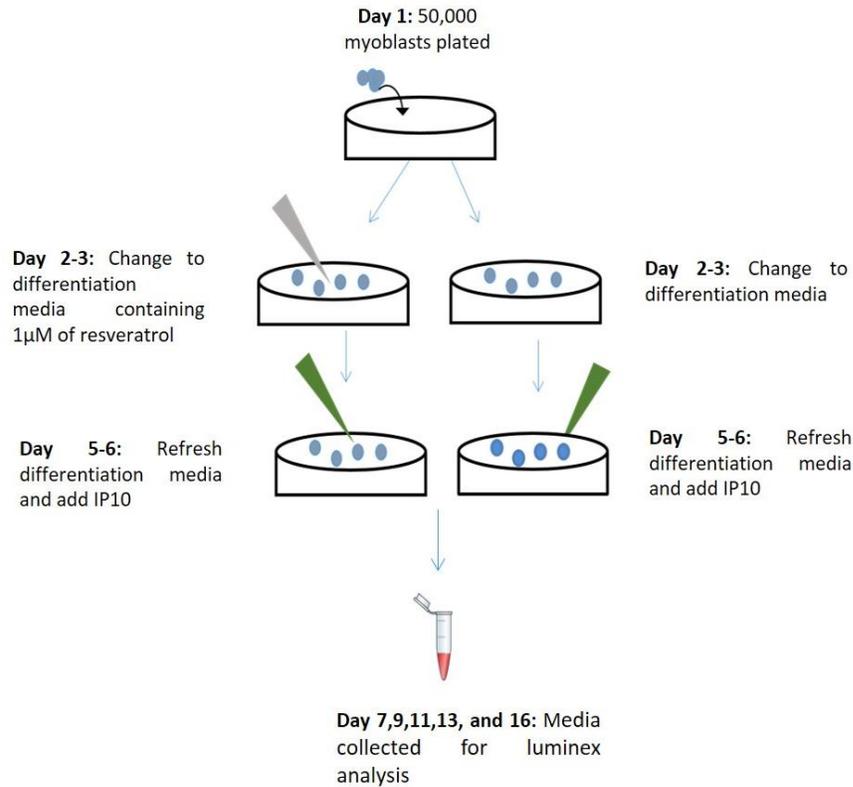
Resveratrol has been shown to have anti-inflammatory effects through decreasing pro-inflammatory cytokines and inflammatory cell infiltration as well as increasing anti-inflammatory cytokines (Bereswill et al., 2010), both systemically (Yong et al., 2014) and in tissues (Palmieri et al., 2011; Lv et al., 2015). A decrease in IP10 levels was seen in human epithelial cells (Abengózar-Vela et al., 2015) and mouse macrophages (Chung et al., 2011) that had been treated with resveratrol. Some studies have shown that resveratrol has anti-inflammatory effects in skeletal muscle (Bereswill et al., 2010), as well as decreasing inflammatory cell infiltrates in the muscles of MDX model of muscle dystrophy (Gordon et al., 2013). However other studies have shown that even when resveratrol was able to attenuate muscle wasting, there was no effect

on cytokine levels in the muscle (Momken et al., 2011), suggesting that another mechanism may be involved.

The ubiquitin ligase, Atrogin1, is upregulated in muscle during the inflammatory response (Ramirez et al., 2011); therefore it is possible that a resveratrol-mediated decrease in Atrogin1 mRNA levels downstream of elevated cytokines is a potential mechanism by which resveratrol provides protection.

The aim of this Chapter was to examine the effects of IP10 at concentrations found in the older (200pg/ml) and younger population (150pg/ml) on the content of 24 cytokines released by myotubes into the media and whether a pre-treatment of myotubes with resveratrol (Chapter 4) had any effect on IP10 induced changes in the media content of these cytokines (Table 6.2).

## 6.2. Methods



**Figure 6.1** A brief overview of experiments; Myotubes were pre-treated daily with 1µM or 0µM (control) of resveratrol for 3 days and then treated with 0, 150 or 200pg/ml of IP10 and luminex analyses carried out on media of myotubes.

### 6.2.1. IP10 preparation

Murine recombinant IP10 was dissolved in distilled water at a concentration of 0.1mg/ml and was stored at -20°C.

### 6.2.2. Treatment of myotubes with IP10 +/- resveratrol

Skeletal muscle myoblasts were isolated from Wistar rats and cultured until myoblasts had reached 80% confluency; cells were counted using TC-20 automatic cell counter (Biorad, Herefordshire, UK) and plated in 6 well plates at a density of

approximately  $5 \times 10^4$ . Once cells had reached 80% confluency, media was changed to differentiation media alone (control) or containing  $1 \mu\text{M}$  of resveratrol. Resveratrol was added every 24H for 3 days. Myotubes were then treated with 0, 150pg/ml or 200pg/ml and media examined 1, 3, 5, 7 or 10 days following treatment.

**Table 6.1 Timetable of treatment of myotubes**

Treatment	Day 1	Days 3-5	Day 6	Day 7 -16
Untreated control	50,000 myoblasts plated	Only differentiation media	Only differentiation media	Media collected at specified time points
Resveratrol	50,000 myoblasts plated	Differentiation media containing $1 \mu\text{M}$ of resveratrol every 24H	Only differentiation media	Media collected at specified time points
IP10	50,000 myoblasts plated	Only differentiation media	Differentiation media containing 150pg/ml or 200pg/ml of IP10	Media collected at specified time points
IP10 and Resveratrol	50,000 myoblasts plated	Differentiation media containing $1 \mu\text{M}$ of resveratrol every 24H	Differentiation media containing 150pg/ml or 200pg/ml of IP10	Media collected at specified time points

### 6.2.3. Luminex analysis for determination of cytokine levels following IP10 and resveratrol treatment

The concentration of a panel of cytokines (Table 6.2) in media from primary rat myotubes treated with IP10 +/- resveratrol was determined using Bioplex multibead analysis as described in Section 2.18.

**Table 6.2 List of cytokine examined and their inflammatory function**

<b>Cytokine</b>	<b>Pro or Anti inflammatory</b>
Erythropoietin (EPO)	- (stimulant for blood cells and bone marrow)
Granulocyte macrophage colony-stimulating factor (GM-CSF)	Pro inflammatory
Granulocyte-colony stimulating factor (GCSF)	Pro inflammatory
Keratinocyte chemo attractant (GRO-KC)	Pro inflammatory
Interferon gamma (IFN- $\gamma$ )	Pro inflammatory
Interleukin 6 (IL6)	Pro and anti inflammatory
Interleukin 1 $\alpha$ (IL $\alpha$ )	Pro inflammatory
Interleukin 1 $\beta$ (IL $\beta$ )	Pro inflammatory
Interleukin 2 (IL2)	Anti inflammatory
Interleukin 4 (IL4)	Anti inflammatory
Interleukin 5 (IL5)	Pro inflammatory
Interleukin (IL7)	Pro inflammatory
Interleukin 10 (IL10)	Anti inflammatory
Interleukin 12P (IL12p)	Pro inflammatory
Interleukin 13 (IL13)	Anti inflammatory
Interleukin 17 (IL15)	Pro inflammatory
Interleukin 18 (IL18)	Pro inflammatory
Monocyte chemo attractant protein 1 (MCP-1)	Pro inflammatory
Macrophage colony stimulating factor (M-CSF)	Pro inflammatory
Macrophage inflammatory protein 1 (MIP1)	Pro inflammatory
Macrophage inflammatory protein 3a (MIP3a)	Pro inflammatory
Rantes	Pro inflammatory
Tumour Necrosis Factor (TNF- $\alpha$ )	Pro inflammatory
Vascular endothelial growth factor (VEGF)	- (Angiogenesis stimulant)

#### **6.2.4. Statistics**

Graphpad 5 (Graphpad Software, San Diego, USA) was used to perform One-way ANOVA test followed by a Dunnett's post-test to identify significant differences between data with 3 or more groups; a T Test was used to identify significant differences between data between 2 groups. Data are represented as mean  $\pm$  SEM.

### **6.3. Results**

#### **6.3.1. Effect of IP10 and resveratrol on cytokine content in the media of myotubes**

Cytokine content into the media by myotubes following treatment of IP10 and resveratrol is shown in Figure 6.2-Figure 6.13. Overall treatment of myotubes with both concentrations of IP10 led to significant changes in the inflammatory cytokine profile in the media of myotubes and the addition of resveratrol reduced some of the effects of the IP10 treatments. Of the 24 cytokines studied, 11 (EPO, GM-CSF, GCSF, IFN- $\gamma$ , IL-1 $\alpha$ , IL-1B, IL-4, IL-10, IL12p, IL-18, Rantes) were not detected in the media either prior to or following treatment of myotubes and there was no difference in media content on M-CFS before or after treatments.

##### **6.3.1.1. Effect of resveratrol and IP10 on VEGF content of the media of myotubes**

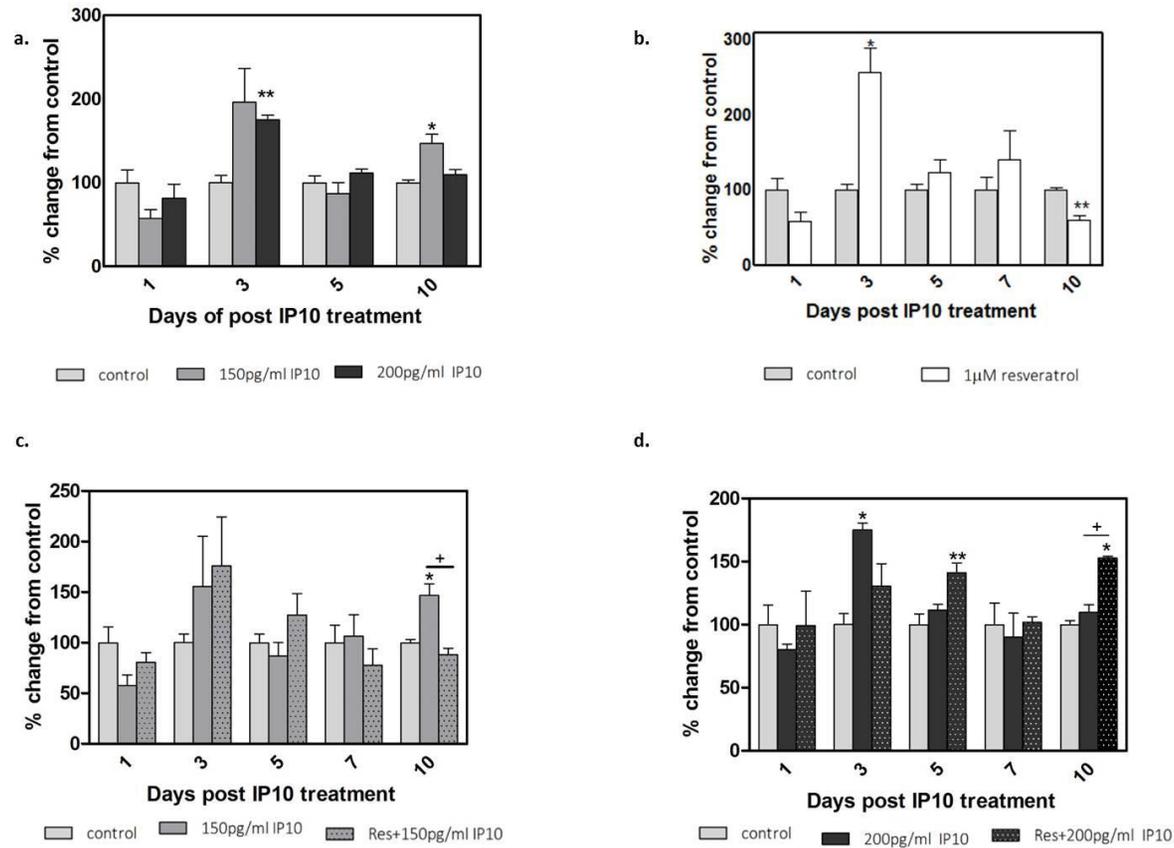
VEGF content of the media of myotubes at 1-10 days following treatment with IP10+/- resveratrol is shown in Figure 6.2. Ten days following treatment of myotubes with 150pg/ml of IP10, VEGF content in the media of myotubes was significantly increased ( $p < 0.05$ ); a pre-treatment of myotubes with resveratrol prevented this increase (Figure 6.2c). A treatment of myotubes with 200pg/ml of IP10 increased VEGF

content in the media of myotubes at 3 days following treatment ( $p < 0.01$ ). A pre-treatment of resveratrol prevented this increase, but caused an increase in VEGF content in the media of myotubes 5 ( $p < 0.05$ ) and 10 days ( $p < 0.05$ ) following treatment (Figure 6.2d). Resveratrol alone increased the content of VEGF in the media of myotubes 3 days following treatment ( $p < 0.05$ ) and decreased VEGF content in the media of myotubes 10 days after treatment ( $p < 0.01$  Figure 6.2b).

**Table 6.3 Summary of effects of IP10 and resveratrol on VEGF content of media of myotubes.**

Days post IP10 treatment	Average levels in control myotubes (pg/ml)	Effect of 150pg/ml compared with untreated control?	Effect of 150pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of 200pg/ml compared with untreated control	Effect of 200pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of resveratrol alone compared with untreated control
1	1152	X	N/A	X	N/A	X
3	1710	X	N/A	↑	Y	↑
5	1117	X	N/A	X	N/A (↑)	X
7	1903	X	N/A	X	X	X
10	3060	↑	Y	X	N/A(↑)	↓

**X denotes no effect compared with untreated control, arrows denote effect of treatment compared with untreated control. Y (yes) denotes to the ability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects and N (no) denotes to the inability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects.**



**Figure 6.2** VEGF content in the media of myotubes following treatment of myotubes with (a) 150pg/ml and 200pg/ml of IP10, (b) 1µM of resveratrol (c) treatment with resveratrol prior to treatment with 150pg/ml of IP10 and (d) treatment with resveratrol prior to treatment with 200pg/ml of IP10. \* $p < 0.05$ , \*\* $p < 0.01$ , + $p < 0.05$  compared with pre-treatment with resveratrol (One-way Anova/T Test). Values are presented as mean  $\pm$  SEM.

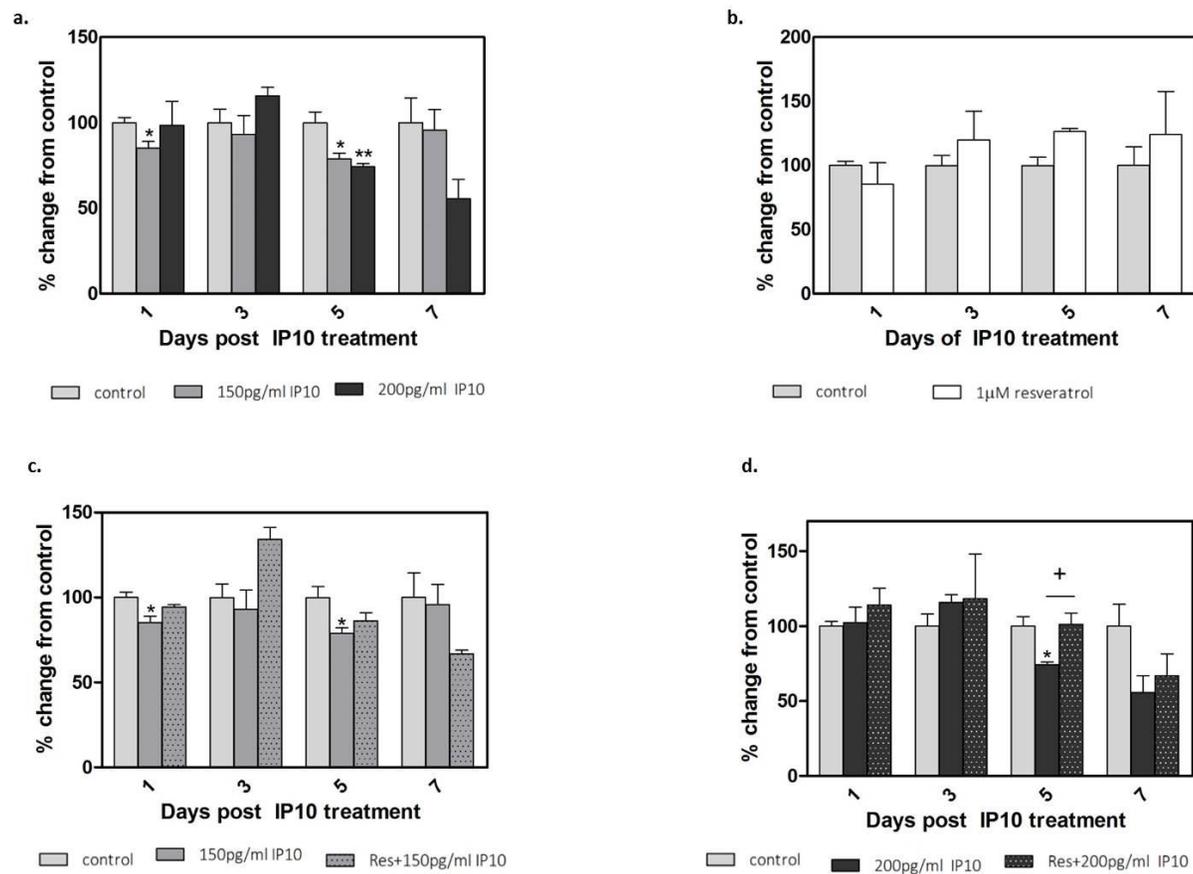
**6.3.1.2. Effect of resveratrol and IP10 on GRO-KC content of the media of myotubes**

GRO-KC content in the media of myotubes at 1-10 days following treatment with IP10+/- resveratrol is shown in Figure 6.3. Treatment of myotubes with 150pg/ml of IP10 resulted in a decrease in GRO-KC content in the media of myotubes at 1 ( $p<0.05$ ) and 5 days ( $p<0.05$ ) following treatment. Pre-treatment of myotubes with resveratrol prevented this decrease (Figure 6.3c). Treatment of myotubes with 200pg/ml of IP10 resulted in a decrease in GRO-KC content in the media of myotubes at 5 days following treatment and this was prevented by a pre-treatment of myotubes with resveratrol (Figure 6.3d). Treatment of myotubes with resveratrol alone had no effect on GRO-KC content of the media of myotubes.

**Table 6.4 Summary of effects of IP10 and resveratrol of GRO-KC content in media of myotubes.**

Days following IP10 treatment	Average levels in control myotubes (pg/ml)	Effect of 150pg/ml compared with untreated control?	Effect of 150pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of 200pg/ml compared with untreated control	Effect of 200pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of resveratrol alone compared with untreated control
1	467	↓	Y	X	X	X
3	372	X	N/A	X	X	X
5	451	↓	Y	↓	Y	X
7	89	X	N/A	X	X	X
10	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected

**X denotes no effect compared with untreated control, arrows denote effect of treatment compared with untreated control. Y (yes) denotes to the ability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects and N (no) denotes to the inability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects.**



**Figure 6.3** GRO-KC content of the media of myotubes following treatment of myotubes with (a) 150pg/ml and 200pg/ml of IP10, (b) 1µM of resveratrol (c) treatment with resveratrol prior to treatment with 150pg/ml of IP10 and (d) treatment with resveratrol prior to treatment with 200pg/ml of IP10. \* $p < 0.05$ , \*\* $p < 0.01$  compared with untreated controls, + $p < 0.05$  compared with IP10 treatment (One-way Anova/T Test). Values are presented as mean  $\pm$  SEM.

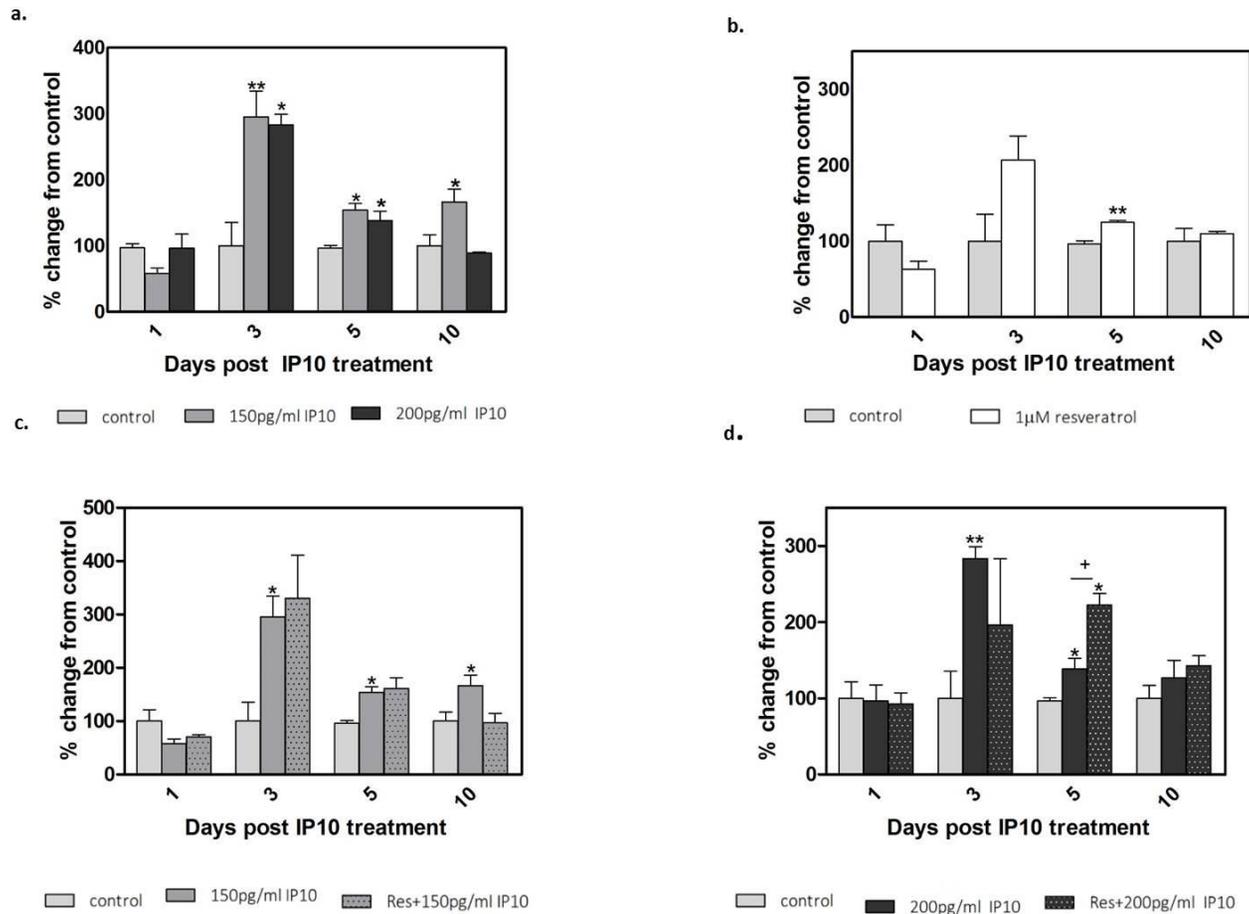
### 6.3.1.3. Effect of resveratrol and IP10 of IL6 content of the media of myotubes

IL6 content in the media of myotubes at 1-10 days following treatment with IP10+/- resveratrol is shown in Figure 6.4. Treatment of myotubes with 150pg/ml of IP10 resulted in an increase in the IL6 content of the media of myotubes at 3, 5 and 10 days following treatment ( $p < 0.05$ ). Pre-treatment of myotubes with resveratrol generally reduced this increase (Figure 6.4c). Treatment of myotubes with 200pg/ml of IP10 resulted in an increase in IL6 content in the media of myotubes at 3 ( $p < 0.01$ ) and 5 days ( $p < 0.05$ ) following treatment. Pre-treatment of resveratrol prevented this increase at 5 days following treatment, ( $p < 0.05$  Figure 6.4d) but not at 3 days. Treatment of myotubes with resveratrol alone resulted in an increase in IL6 content of the media of myotubes at 5 days following treatment ( $p < 0.01$  Figure 6.4b).

**Table 6.5 Summary of effects of IP10 and resveratrol on IL6 content of media of myotubes.**

Days following IP10 treatment	Average levels in control myotubes (pg/ml)	Effect of 150pg/ml compared with untreated control?	Effect of 150pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of 200pg/ml compared with untreated control	Effect of 200pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of resveratrol alone compared with untreated control
1	700	X	N/A	X	N/A	X
3	370	↑	Y	↑	Y	X
5	564	↑	Y	↑	N (↑)	↑
7	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
10	950	↑	Y	X	X	X

**X denotes no effect compared with untreated control, arrows denote effect of treatment compared with untreated control. Y (yes) denotes to the ability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects and N (no) denotes to the inability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects.**



**Figure 6.4** IL6 content in the media of myotubes following treatment of myotubes with (a) 150pg/ml and 200pg/ml of IP10, (b) 1μM of resveratrol (c) treatment with resveratrol prior to treatment with 150pg/ml of IP10 and (d) treatment with resveratrol prior to treatment with 200pg/ml of IP10. \* $p < 0.05$ , \*\* $p < 0.01$  compared with untreated controls, + $p < 0.05$  compared with IP10 treatment (One-way Anova/T Test). Values are presented as mean  $\pm$  SEM.

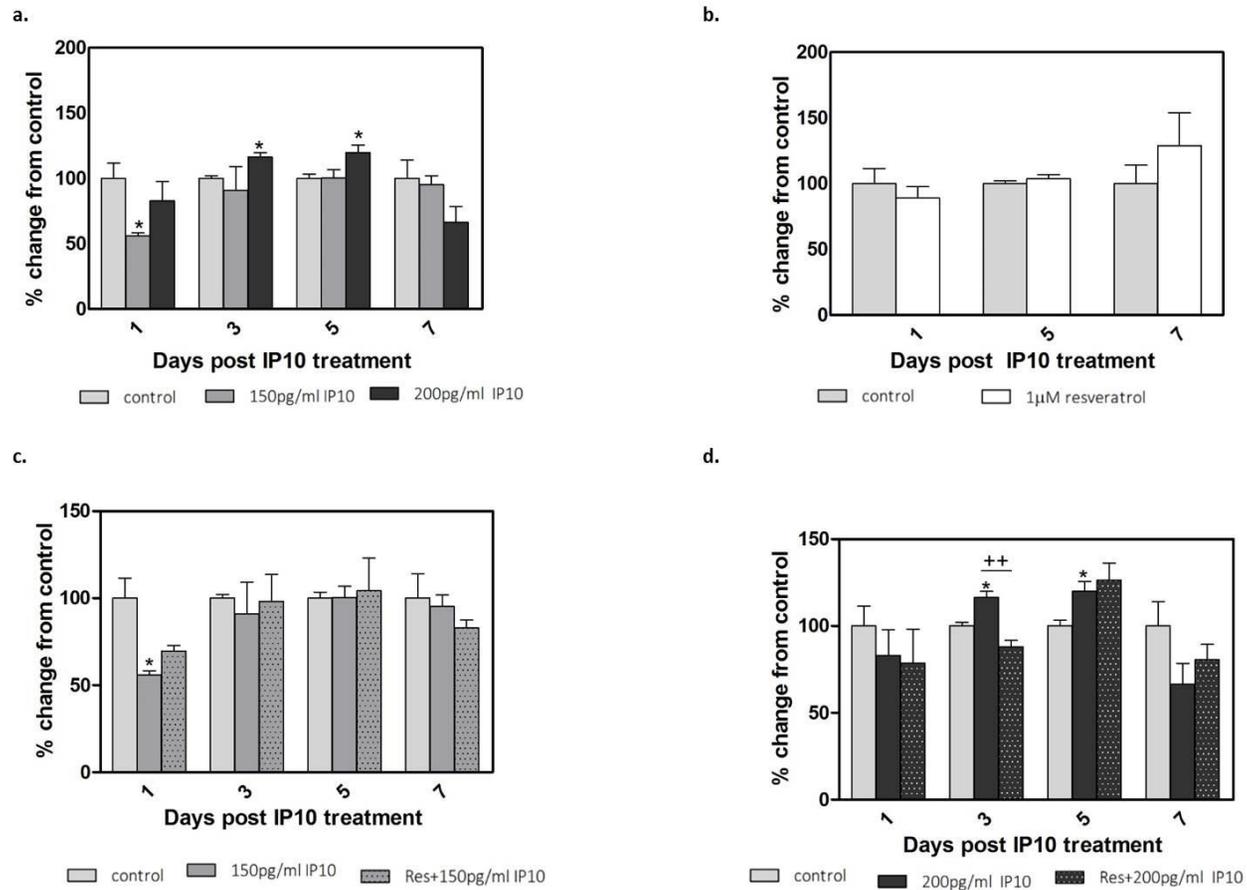
**6.3.1.4. Effect of resveratrol and IP10 of MCP-1 content of the media of myotubes**

MCP-1 content in the media of myotubes at 1-10 days following treatment with IP10+/- resveratrol is shown in Figure 6.5. Treatment of myotubes with 150pg/ml of IP10 resulted in a decrease in MCP-1 content in the media of myotubes at 1 day following treatment ( $p < 0.05$ ) and this decrease was prevented by a pre-treatment of myotubes with resveratrol (Figure 6.5c). Treatment of myotubes with 200pg/ml of IP10 resulted in an increase in MCP-1 at 3 and 5 days following treatment and this increase was prevented with a pre-treatment of myotubes with resveratrol ( $p < 0.05$  Figure 6.5d). MCP-1 was undetectable in myotubes treated with resveratrol alone 3 days following (Figure 6.5).

**Table 6.6 Summary of effects of IP10 and resveratrol on MCP-1 content of media of myotubes.**

Days following IP10 treatment	Average levels in control myotubes (pg/ml)	Effect of 150pg/ml compared with untreated control?	Effect of 150pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of 200pg/ml compared with untreated control	Effect of 200pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of resveratrol alone compared with untreated control
1	11839	↓	Y	X	N/A	X
3	9069	X	N/A	↑	Y	Not detected
5	9341	X	N/A	↑	Y	X
7	1181	X	N/A	X	N/A	X
10	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected

**X denotes not effect compared with untreated control, arrows denote effect of treatment compared with untreated control. Y (yes) denotes to the ability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects and N (no) denotes to the inability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects.**



**Figure 6.5** MCP-1 content of the media of myotubes following treatment of myotubes with (a) 150pg/ml and 200pg/ml of IP10, (b) 1µM of resveratrol (c) treatment with resveratrol prior to treatment with 150pg/ml of IP10 and (d) treatment with resveratrol prior to treatment with 200pg/ml of IP10. \* $p < 0.05$ , \*\* $p < 0.01$  compared with untreated controls ++ compared with IP10 treatment (One-way Anova/T Test). Values are presented as mean  $\pm$  SEM.

### 6.3.1.5. Effect of resveratrol and IP10 of IL2 content in the media of myotubes

IL2 content in the media of myotubes at 1-10 days following treatment with IP10+/- resveratrol is shown in Figure 6.6. Treatment of myotubes with 150pg/ml of IP10 resulted in an increase in IL2 content in the media of myotubes at 10 days following treatment ( $p < 0.001$ ). A pre-treatment with resveratrol prevented this increase ( $p < 0.05$  Figure 6.6). Treatment of myotubes with 200pg/ml IP10 had no effect on IL2 content in the media of myotubes. Treatment of myotubes with resveratrol alone resulted in an increase in IL2 content at 3 days following treatment ( $p < 0.01$  Figure 6.6b).

**Table 6.7: Summary of effects of IP10 and resveratrol on IL2 content in the media of myotubes**

Days following IP10 treatment	Average levels in control myotubes (pg/ml)	Effect of 150pg/ml compared with untreated control?	Effect of 150pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of 200pg/ml compared with untreated control	Effect of 200pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of resveratrol alone compared with untreated control
1	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
3	6.1	X	N	X	N/A	↑
5	23	X	N	X	N/A	X
7	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
10	21	↑	N (↑)	X	N/A	Not detected

X denotes no effect compared with untreated control, arrows denote effect of treatment compared with untreated control. Y (yes) denotes to the ability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects and N (no) denotes to the inability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects.

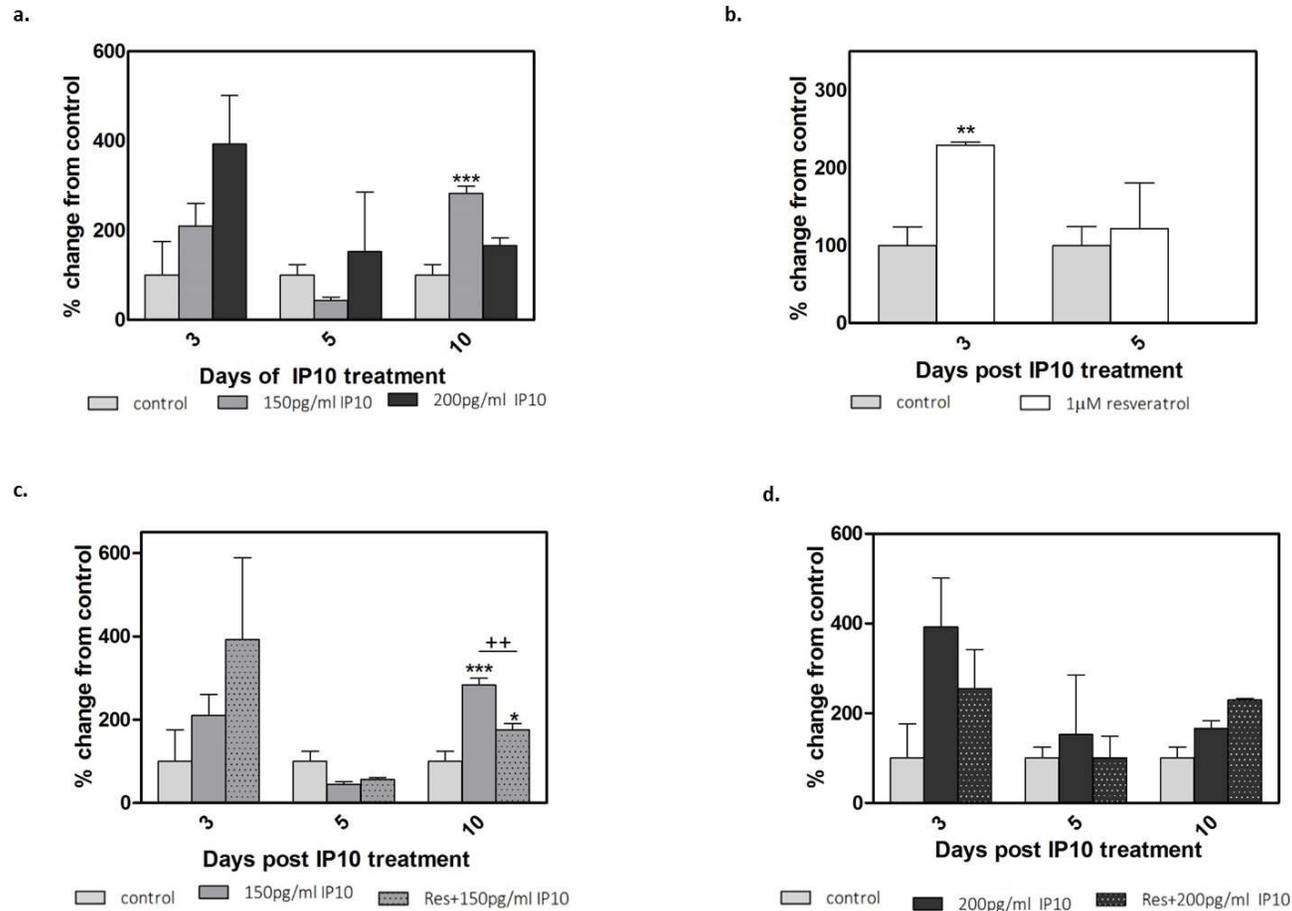


Figure 6.6 IL2 content in the media of myotubes following treatment of myotubes with (a) 150pg/ml and 200pg/ml of IP10, (b) 1µM of resveratrol (c) treatment with resveratrol prior to treatment with 150pg/ml of IP10 and (d) treatment with resveratrol prior to treatment with 200pg/ml of IP10. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with untreated control, ++ $p < 0.01$  compared with IP10 treated (One-way Anova/T Test). Values are presented as mean  $\pm$  SEM.

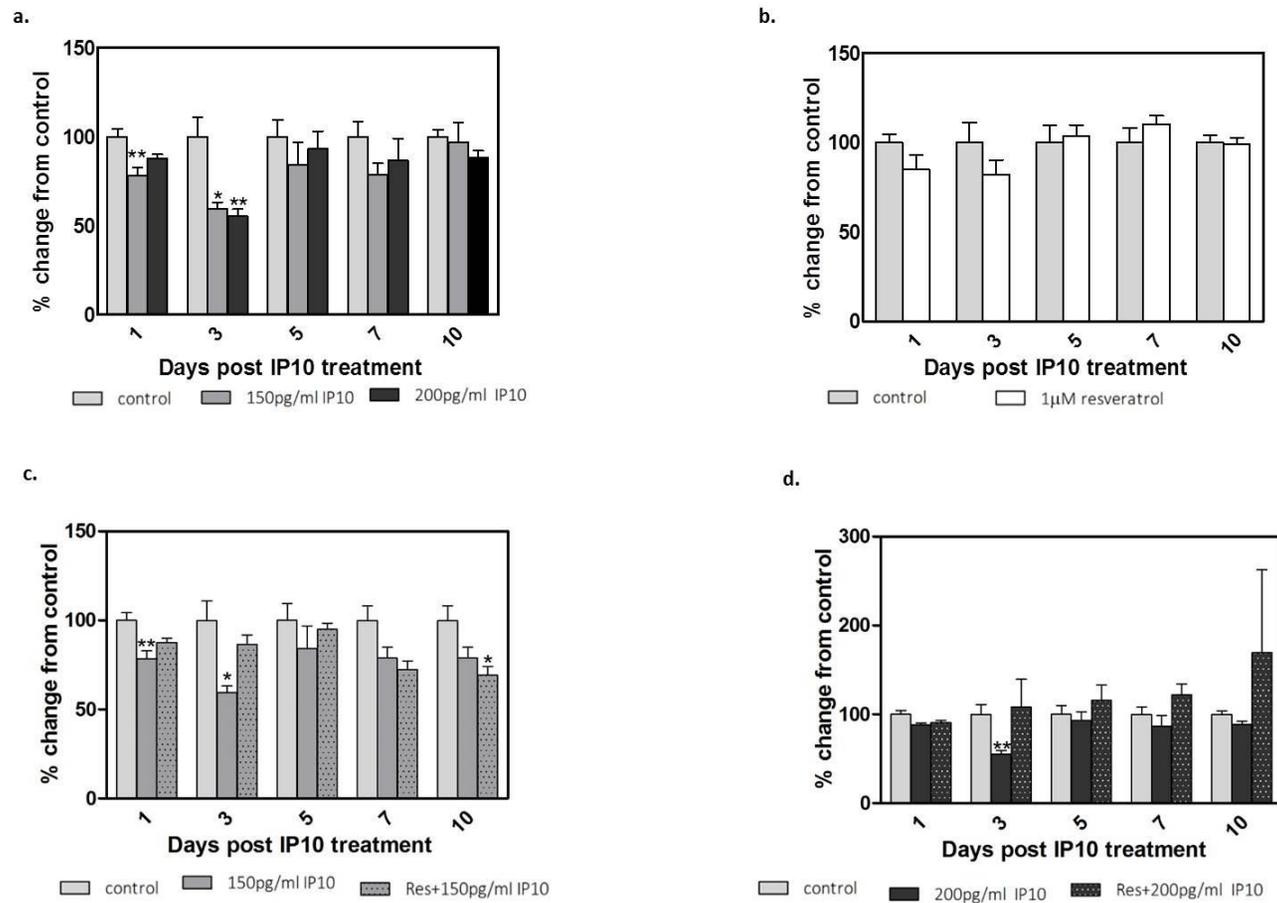
### 6.3.1.6. Effect of resveratrol and IP10 of IL5 content of the media of myotubes

IL5 content of the media of myotubes at 1-10 days following treatment with IP10+/- resveratrol is shown in Figure 6.7. Treatment of myotubes with 150pg/ml of IP10 resulted in a decrease in the IL5 content of the media of myotubes at 1 ( $p<0.01$ ) and 3 ( $p<0.05$ ) days following treatment. Pre-treatment with resveratrol prevented this decrease, but also resulted in a decrease in IL5 content of the media of IP10 treated myotubes at 10 days following treatment ( $p<0.05$  Figure 6.7c). Treatment of myotubes with 200pg/ml of IP10 resulted in a transient decrease in IL5 content in the media of myotubes at 3 days following treatment ( $p<0.01$ ) and this decrease was prevented by pre-treatment of myotubes with resveratrol (Figure 6.7d). Treatment of myotubes with resveratrol alone had no effect on the content of IL5.

**Table 6.8 Summary of effects of IP10 and resveratrol on IL5 content of media of myotubes.**

Days following IP10 treatment	Average levels in control myotubes (pg/ml)	Effect of 150pg/ml compared with untreated control?	Effect of 150pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of 200pg/ml compared with untreated control	Effect of 200pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of resveratrol alone compared with untreated control
1	203	↓	Y	X	N/A	X
3	232	↓	Y	↓	Y	X
5	171	X	N/A	X	N/A	X
7	107	X	N/A	X	N/A	X
10	176	X	N/A(↓)	X	N/A	X

**X denotes no effect compared with untreated control, arrows denote effect of treatment compared with untreated control. Y (yes) denotes to the ability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects and N (no) denotes to the inability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects.**



**Figure 6.7** IL5 content of the media of myotubes following treatment of myotubes with (a) 150pg/ml and 200pg/ml of IP10, (b) 1µM of resveratrol (c) treatment with resveratrol prior to treatment with 150pg/ml of IP10 and (d) treatment with resveratrol prior to treatment with 200pg/ml of IP10. \* $p < 0.05$ , \*\* $p < 0.01$  compared with untreated control (One-way Anova/T Test). Values are presented as mean  $\pm$  SEM.

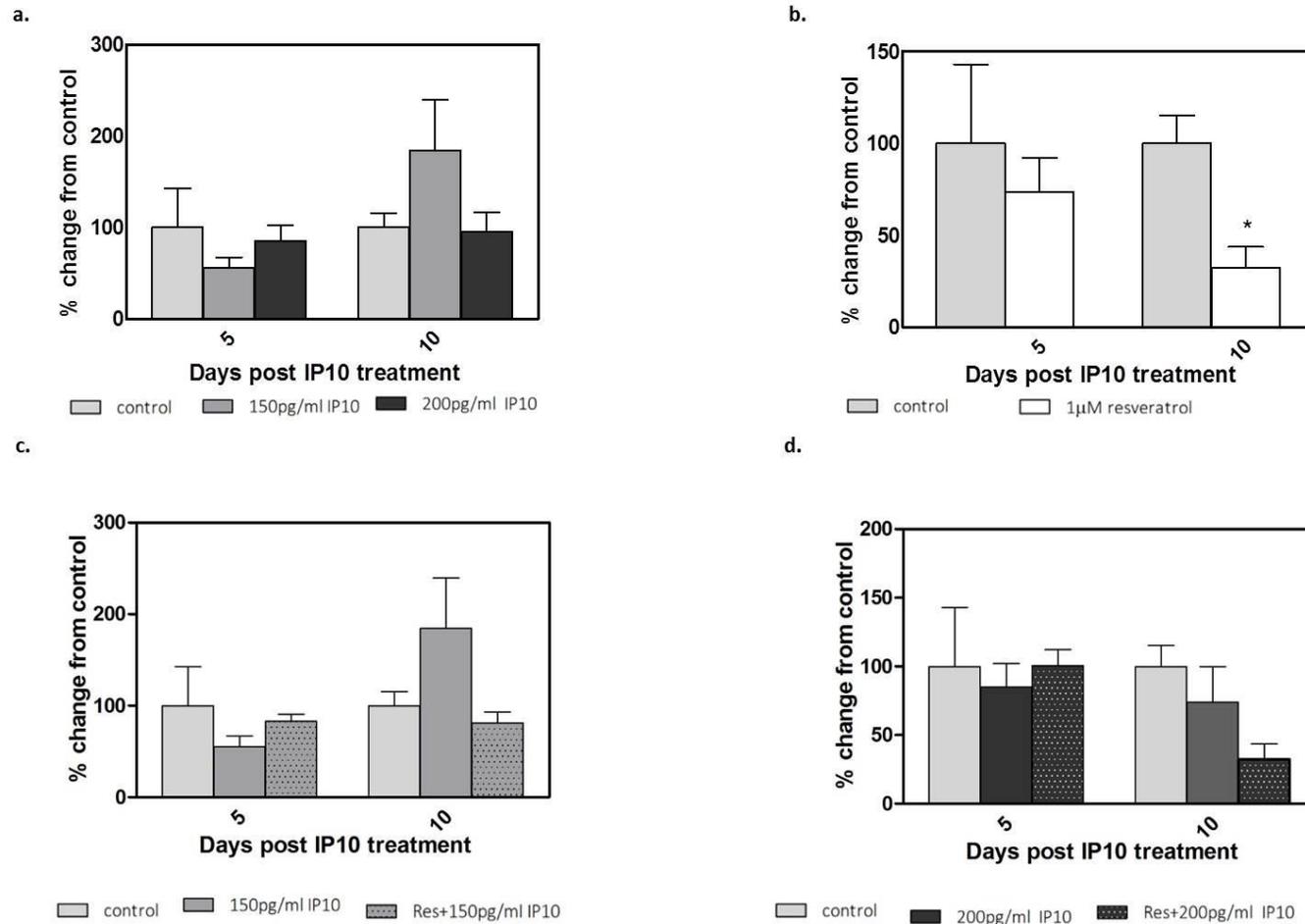
**6.3.1.7. Effect of resveratrol and IP10 of IL7 content in the media of myotubes**

IL7 content in the media of myotubes at 1-10 days following treatment with IP10+/- resveratrol is shown in Figure 6.8. IL7 levels were low and not detected in the media of myotubes at 1, 3 or 7 days following treatment. There was no significant difference between treatment groups, with the exception of resveratrol alone treatment which resulted in a significant decrease in IL7 content of media at 10 days following treatment (Figure 6.8b  $p < 0.01$ ).

**Table 6.9 Summary of effects of IP10 and resveratrol on IL7 content of media of myotubes.**

Days following IP10 treatment	Average levels in control myotubes (pg/ml)	Effect of 150pg/ml compared with untreated control?	Effect of 150pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of 200pg/ml compared with untreated control	Effect of 200pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of resveratrol alone compared with untreated control
1	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
3	33	Not detected	Not detected	Not detected	Not detected	Not detected
5	47	X	N/A	X	N/A	X
7	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
10	52	X	N/A	X	N/A	↓

**X denotes no effect compared with untreated control, arrows denote effect of treatment compared with untreated control. Y (yes) denotes to the ability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects and N (no) denotes to the inability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects.**



**Figure 6.8** IL7 content in the media of myotubes following treatment of myotubes with (a) 150pg/ml and 200pg/ml of IP10, (b) 1µM of resveratrol (c) treatment with resveratrol prior to treatment with 150pg/ml of IP10 and (d) treatment with resveratrol prior to treatment with 200pg/ml of IP10. \* $p < 0.05$ , compared with untreated controls (One-way Anova/T Test). Values are presented as mean  $\pm$  SEM.

#### **6.3.1.8. Effect of resveratrol and IP10 of IL13 content in the media of myotubes**

IL13 content of the media of myotubes at 1-10 days following treatment with IP10+/- resveratrol is shown Figure 6.9. IL13 levels were relatively low and not detectable in any treatment group at 1 and 7 days following treatment. Treatment of myotubes with 150pg/ml of IP10 resulted in an increase in IL13 content of the media of myotubes at 3 days following treatment ( $p < 0.01$ ) and IL13 was undetectable in the media of myotubes at 5 days following treatment. Pre-treatment of myotubes with resveratrol prevented both of these effects (Figure 6.9c). Treatment of myotubes with 200pg/ml of IP10 resulted in IL13 being undetectable in the media of myotubes at 5 and 10 days following treatment. Pre-treatment of myotubes with resveratrol prevented this decrease in IL13 in the media of myotubes and also resulted in a reduction in IL13 levels at 3 days following treatment (Figure 6.9d). Treatment of myotubes with resveratrol alone resulted in an increase in IL13 content of the media of myotubes at 5 days following treatment which became undetectable by 10 days following treatment (Figure 6.9b).

**Table 6.10 Summary of effects of IP10 and resveratrol on IL13 content of media of myotubes.**

Days following IP10 treatment	Average levels in control myotubes (pg/ml)	Effect of 150pg/ml compared with untreated control?	Effect of 150pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of 200pg/ml compared with untreated control	Effect of 200pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of resveratrol alone compared with untreated control
1	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
3	5	↑	Not detected	X	Not detected	X
5	4	Not detected	N/A	Not detected	N/A	↑
7	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
10	8	X	Not detected	Not detected	N/A	Not detected

**X denotes no effect compared with untreated control, arrows denote effect of treatment compared with untreated control. Y (yes) denotes to the ability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects and N (no) denotes to the inability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects.**

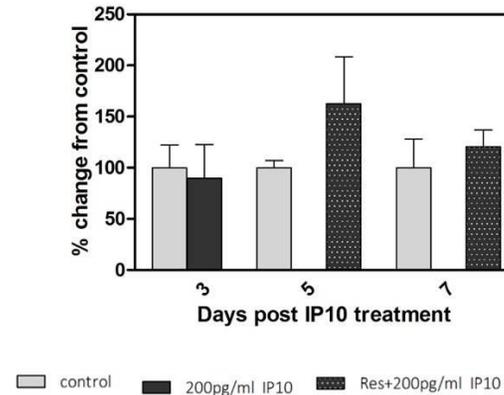
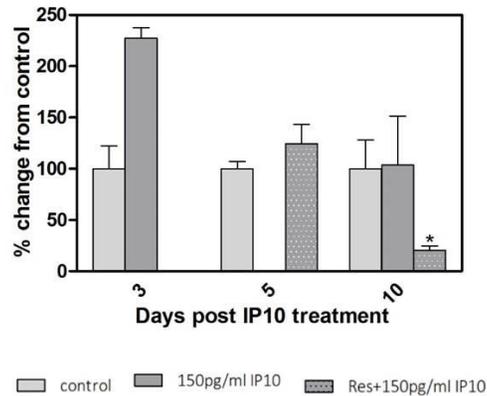
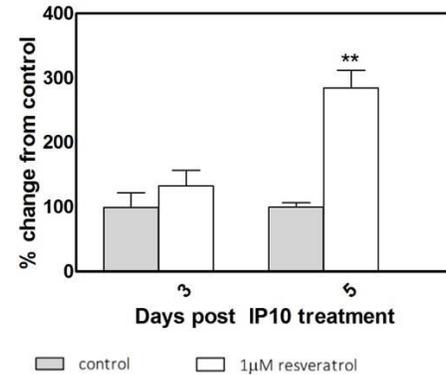
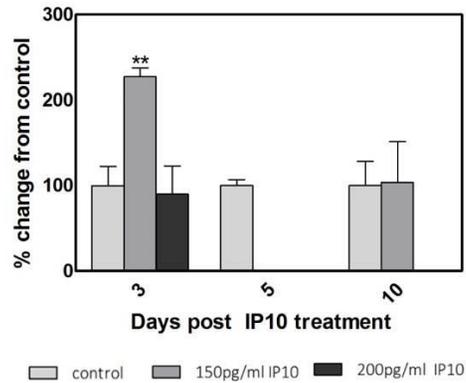


Figure 6.9 IL13 content of the media of myotubes following treatment of myotubes with (a) 150pg/ml and 200pg/ml of IP10, (b) 1µM of resveratrol (c) treatment with resveratrol prior to treatment with 150pg/ml of IP10 and (d) treatment with resveratrol prior to treatment with 200pg/ml of IP10. \*p<0.05, \*\*p<0.01 compared with untreated controls (One-way Anova/T Test). Values are presented as mean ±SEM.

### 6.3.1.9. Effect of resveratrol and IP10 of IL17 content of the media of myotubes

IL17 content in the media of myotubes at 1-10 days following treatment with IP10 +/- resveratrol is shown in Figure 6.10. Treatment of myotubes with 150pg/ml of IP10 resulted in a decrease in IL17 content of the media of myotubes at 5 and 10 days following treatment ( $p < 0.05$ ). This was prevented by a pre-treatment of myotubes with resveratrol ( $p < 0.05$  Figure 6.10c). A treatment of myotubes with 200pg/ml of IP10 resulted in a decrease in IL17 content in the media of myotubes at 5 and 10 days following treatment ( $p < 0.05$ ), but this was not prevented by a pre-treatment of myotubes with resveratrol ( $p < 0.05$  Figure 6.10d). Treatment of myotubes with resveratrol alone had no effect on IL17 content (Figure 6.10b)

**Table 6.11 Summary of effects of IP10 and resveratrol on IL17 content of media of myotubes.**

Days following IP10 treatment	Average levels in control myotubes (pg/ml)	Effect of 150pg/ml compared with untreated control?	Effect of 150pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of 200pg/ml compared with untreated control	Effect of 200pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of resveratrol alone compared with untreated control
1	18	X	X	X	X	X
3	33	X	X	X	X	X
5	48	↓	Y	X	N/A	X
7	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
10	52	X	N/A(↓)	↓	N(↓)	X

**X denotes not effect compared with untreated control, arrows denote effect of treatment compared with untreated control. Y (yes) denotes to the ability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects and N (no) denotes to the inability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects.**

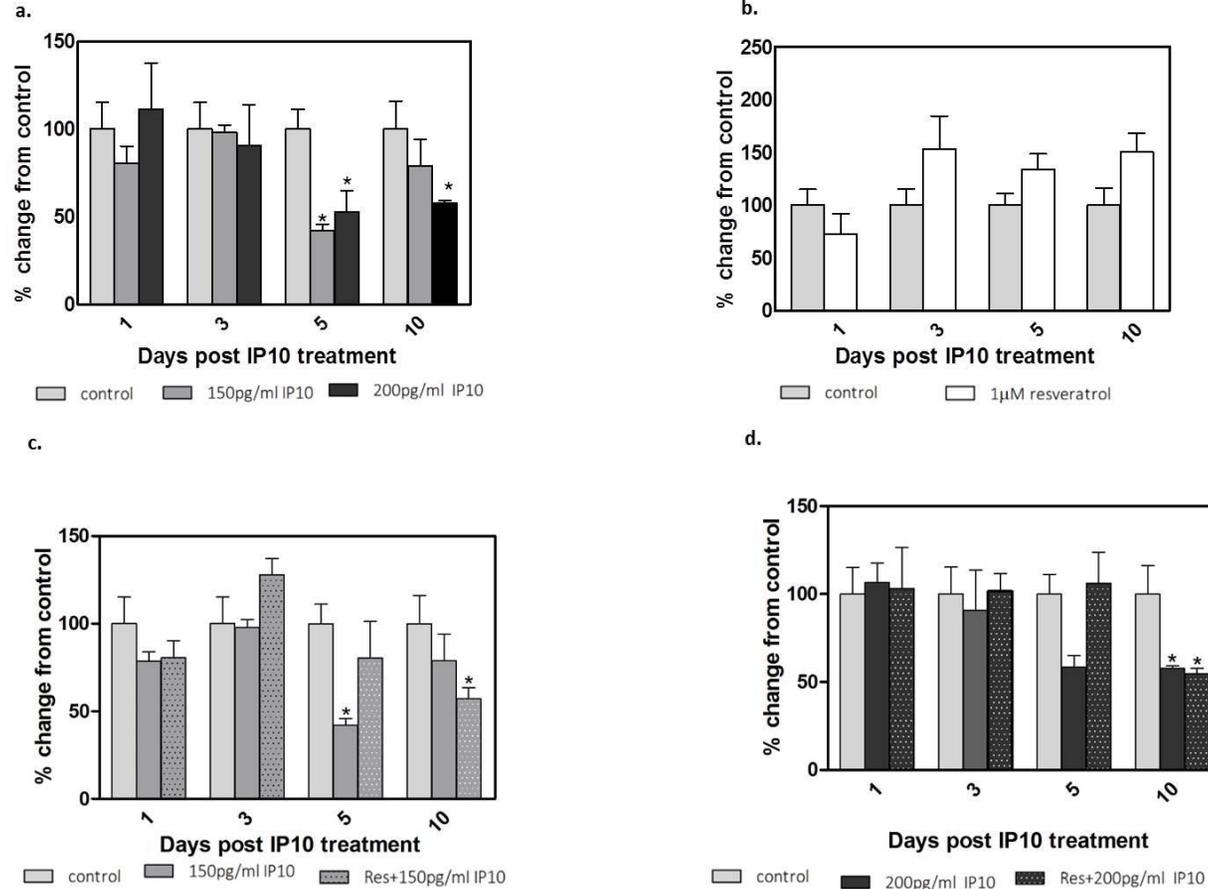


Figure 6.10 IL17 content in the media of myotubes following treatment of myotubes with (a) 150pg/ml and 200pg/ml of IP10, (b) 1µM of resveratrol (c) treatment with resveratrol prior to treatment with 150pg/ml of IP10 and (d) treatment with resveratrol prior to treatment with 200pg/ml of IP10. \* $p < 0.05$ , compared with untreated controls (One-way Anova/T Test). Values are presented as mean  $\pm$  SEM.

#### **6.3.1.10. Effect of resveratrol and IP10 of Mip-1 $\alpha$ content of the media of myotubes**

Mip-1 $\alpha$  content of the media of myotubes at 1-10 days following with IP10+/- resveratrol is shown in is shown Figure 6.11. Treatment of myotubes with 150pg/ml of IP10 had no significant effect on Mip-1 $\alpha$  content of the media but a pre-treatment of myotubes with resveratrol resulted in an transient but substantial increase in Mip-1 $\alpha$  content of the media of myotubes at 3 days following treatment ( $p < 0.05$  Figure 6.11c). Treatment of myotubes with 200pg/ml of IP10 resulted in a decrease in Mip-1 $\alpha$  content of the media of myotubes at 5 days following treatment ( $p < 0.05$ ). This effect was prevented by pre-treatment of myotubes with resveratrol (Figure 6.11d). Treatment of myotubes with resveratrol alone had little effect on media content of Mip-1 $\alpha$  (Figure 6.11b).

**Table 6.12 Summary of effects of IP10 and resveratrol on content of Mip-1 $\alpha$  media of myotubes**

Days following IP10 treatment	Average levels in control myotubes (pg/ml)	Effect of 150pg/ml compared with untreated control?	Effect of 150pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of 200pg/ml compared with untreated control	Effect of 200pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of resveratrol alone compared with untreated control
1	2	X	N/A	X	N/A	X
3	8	X	N/A( $\uparrow$ )	X	N/A	X
5	2	X	N/A	$\downarrow$	Y	X
7	1.6	X	N/A	Not detected	N/A	Not detected
10	22	X	N/A	X	N/A	X

X denotes no effect compared with untreated control, arrows denote effect of treatment compared with untreated control. Y (yes) denotes to the ability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects and N (no) denotes to the inability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects.

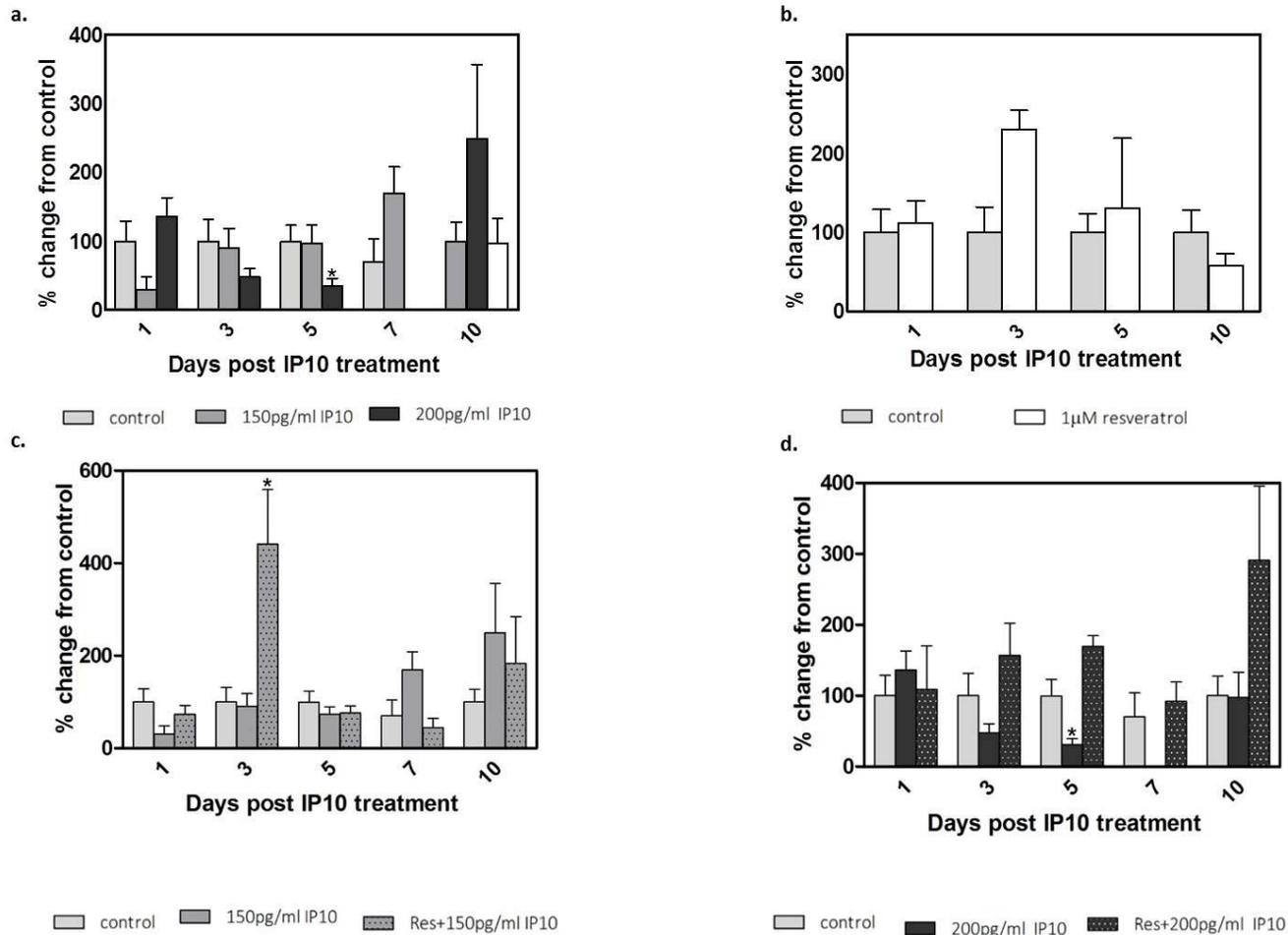


Figure 6.11 Mip-1 $\alpha$  content of the media of myotubes following treatment of myotubes with (a) 150pg/ml and 200pg/ml of IP10, (b) 1 $\mu$ M of resveratrol (c) treatment with resveratrol prior to treatment with 150pg/ml of IP10 and (d) treatment with resveratrol prior to treatment with 200pg/ml of IP10. \* $p < 0.05$ , compared with untreated controls (One-way Anova/T Test). Values are presented as mean  $\pm$  SEM.

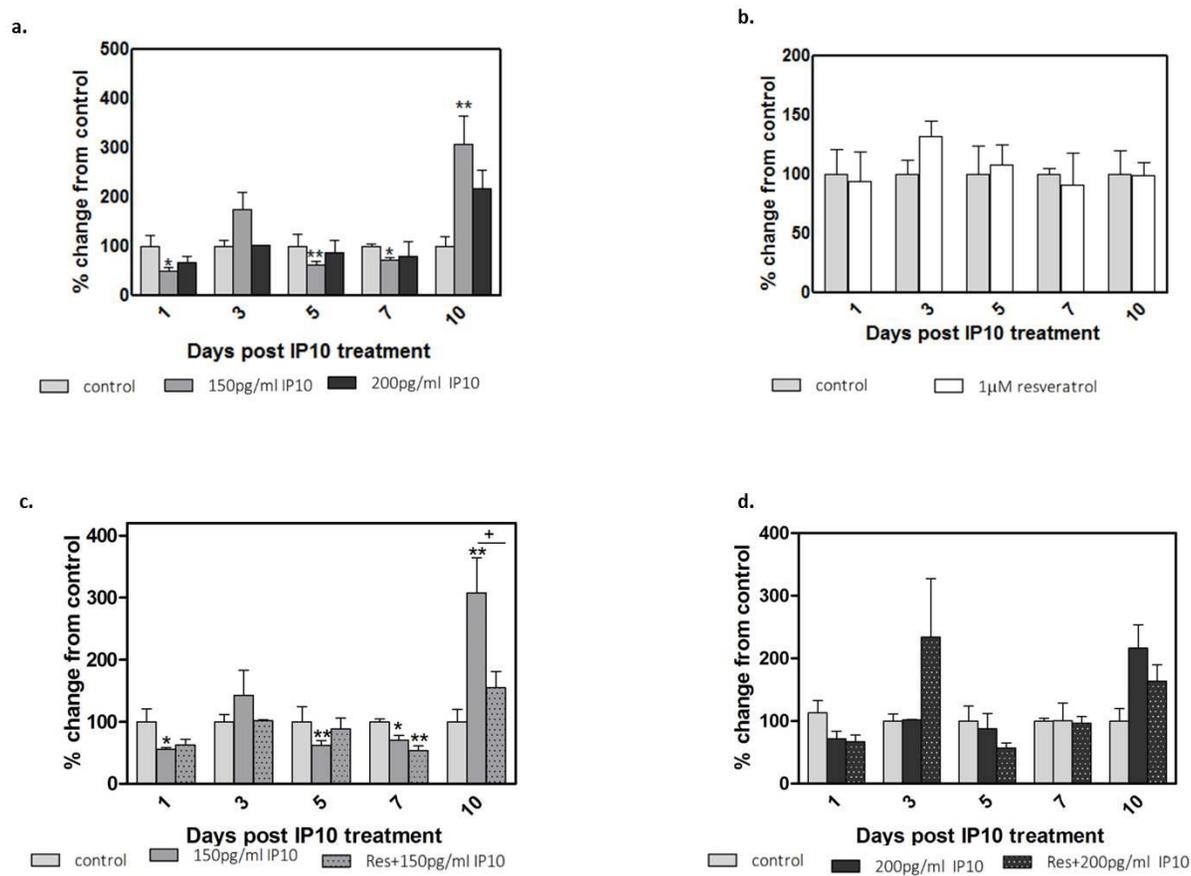
**6.3.1.11. Effect of resveratrol and IP10 of Mip-3 $\alpha$  content in the media of myotubes**

Mip-3 $\alpha$  content of the media of myotubes at 1-10 days following treatment of myotubes with IP10+/- resveratrol is shown in Figure 6.12. Treatment of myotubes with 150pg/ml of IP10 resulted in a small but significant decrease in Mip-3 $\alpha$  content of the media of myotubes at 1 ( $p<0.05$ ), 5 ( $p<0.01$ ) and 7 ( $p<0.05$ ) days following treatment. Treatment of myotubes with 150pg/ml of IP10 also resulted in an increase in the content of Mip-3 $\alpha$  in the media of myotubes 10 days following treatment. These effects were generally prevented by a pre-treatment of myotubes with resveratrol ( $p<0.01$  Figure 6.12c). Treatment of myotubes with 200pg/ml with or without resveratrol or treatment of myotubes with resveratrol alone had no effect on Mip-3 $\alpha$  content of the media of myotubes (Figure 6.12b/d).

Table 6.13 Summary of effects of IP10 and resveratrol on content of Mip-3 $\alpha$  media of myotubes

Days following IP10 treatment	Average levels in control myotubes (pg/ml)	Effect of 150pg/ml compared with untreated control?	Effect of 150pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of 200pg/ml compared with untreated control	Effect of 200pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of resveratrol alone compared with untreated control
1	2	↓	Y	X	N/A	X
3	9	X	N/A	X	N.A	X
5	3	↓	Y	X	N/A	X
7	2	↓	N(↓)	X	N/A	X
10	22	↑	Y	X	N/A	X

X denotes no effect compared with untreated control, arrows denote effect of treatment compared with untreated control. Y (yes) denotes to the ability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects and N (no) denotes to the inability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects.



**Figure 6.12** Mip-3 $\alpha$  content in the media of myotubes following treatment of myotubes with (a) 150pg/ml and 200pg/ml of IP10, (b) 1 $\mu$ M of resveratrol (c) treatment with resveratrol prior to treatment with 150pg/ml of IP10 and (d) treatment with resveratrol prior to treatment with 200pg/ml of IP10. \* $p$ <0.05, \*\* $p$ <0.01 compared with untreated controls, + $p$ <0.05 compared with IP10 treated (One-way Anova/T Test). Values are presented as mean  $\pm$ SEM.

#### **6.3.1.12. Effect of resveratrol and IP10 of TNF- $\alpha$ content in the media of myotubes**

TNF- $\alpha$  content of the media of myotubes at 1-10 days following treatment of myotubes with IP10+/- resveratrol is shown in Figure 6.13. TNF- $\alpha$  levels were extremely low throughout the study. Treatment of myotubes with 150pg/ml of IP10 resulted in a decrease in TNF- $\alpha$  content of the media of myotubes at 1 day following treatment ( $p < 0.05$ ) and this was not prevented by pre-treatment of myotubes with resveratrol ( $p < 0.01$  Figure 6.13c). Treatment of myotubes with 200pg/ml of IP10 had no effect on TNF- $\alpha$  content in the media of myotubes however pre-treatment of myotubes with resveratrol led to a reduction in the TNF- $\alpha$  content of media at 1 day following treatment ( $p < 0.05$  Figure 6.13d). Treatment of myotubes with resveratrol alone resulted in a decrease in TNF- $\alpha$  at content of media at 1 day following treatment ( $p < 0.01$  Figure 6.13b).

**Table 6.14 Summary of effects of IP10 and resveratrol on TNF- $\alpha$  content of media of myotubes**

Days following IP10 treatment	Average levels in control myotubes (pg/ml)	Effect of 150pg/ml compared with untreated control?	Effect of 150pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of 200pg/ml compared with untreated control	Effect of 200pg/ml IP10 modified by pre-treatment with resveratrol?	Effect of resveratrol alone compared with untreated control
1	2	↓	N(↓)	X	-(↓)	↓
3	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
5	20	X	N/A	X	N/A	X
7	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
10	21	X	N/A	X	N/A	X

**X denotes no effect compared with untreated control, arrows denote effect of treatment compared with untreated control. Y (yes) denotes to the ability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects and N (no) denotes to the inability of a pre-treatment of myotubes with resveratrol to prevent IP10 induced effects.**

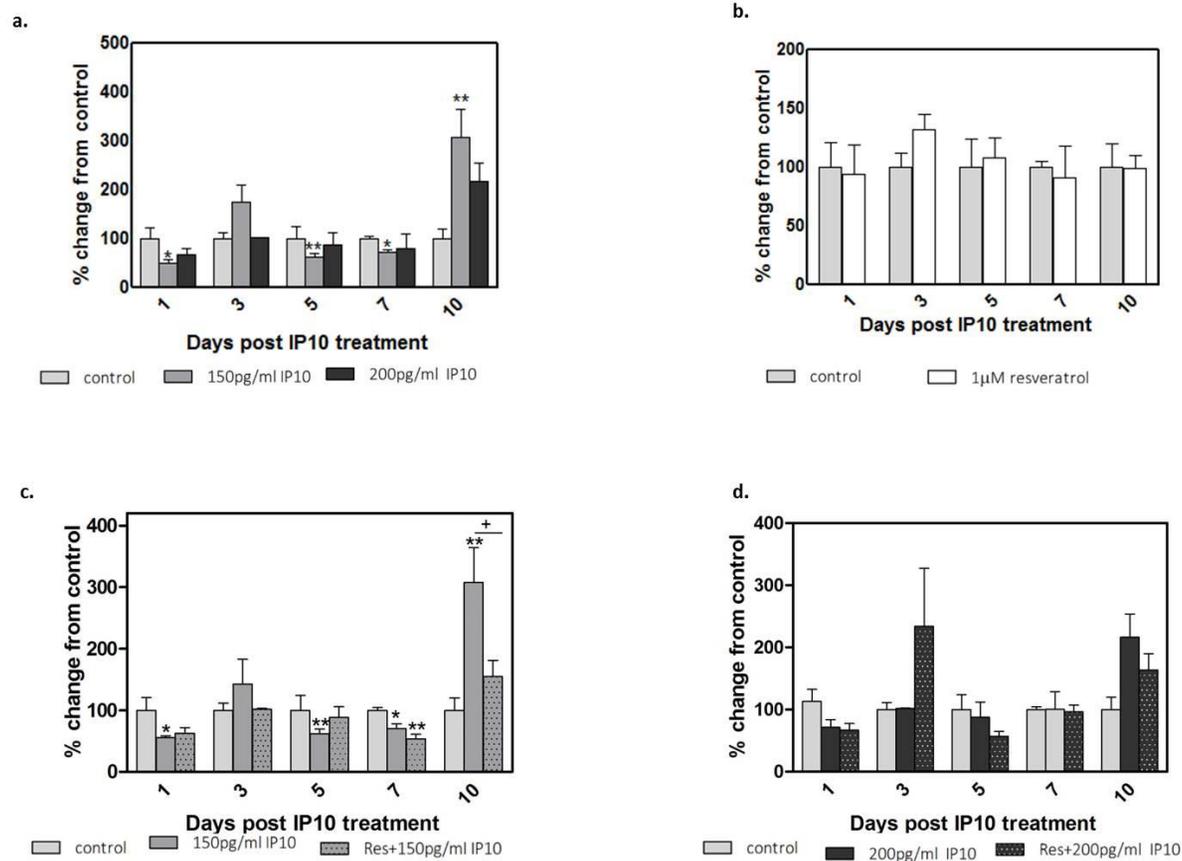
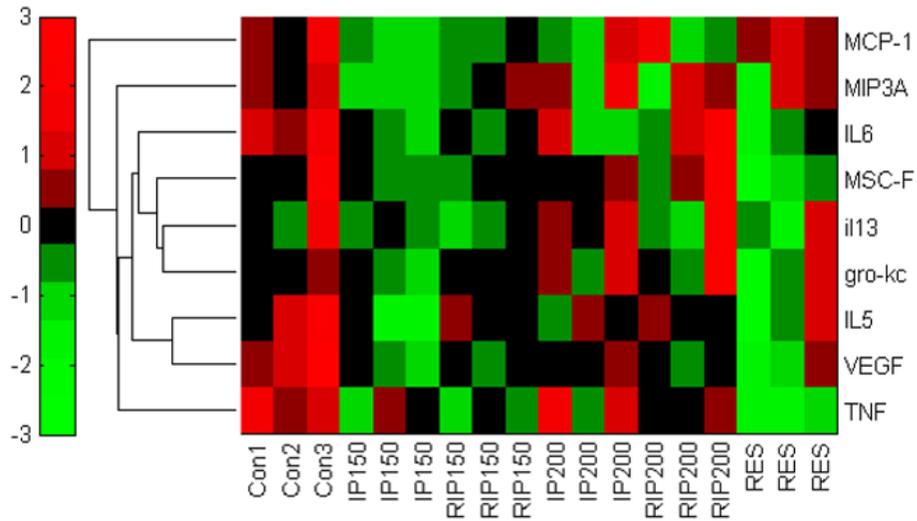


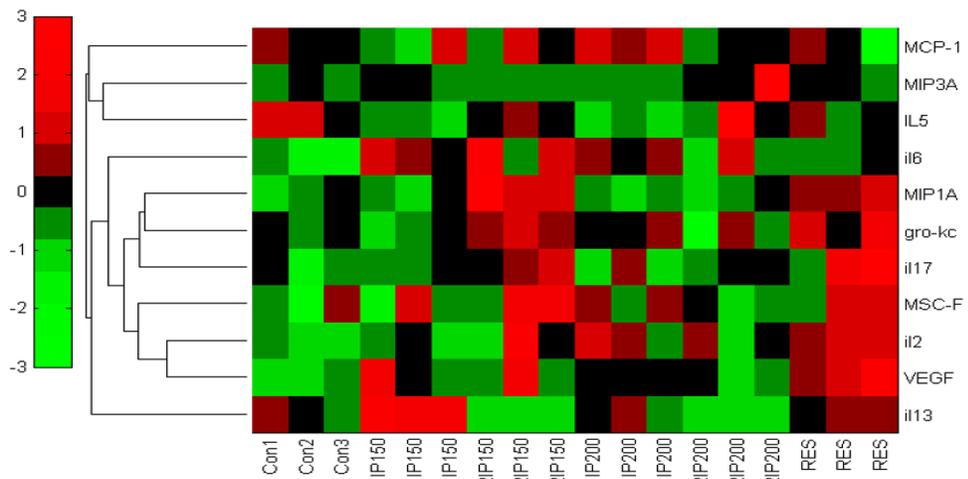
Figure 6.13 TNF $\alpha$  content of the media of myotubes following treatment of myotubes with (a) 150pg/ml and 200pg/ml of IP10, (b) 1 $\mu$ M of resveratrol (c) treatment with resveratrol prior to treatment with 150pg/ml of IP10 and (d) treatment with resveratrol prior to treatment with 200pg/ml of IP10. \*p<0.05, compared with untreated controls (One-way Anova/T Test). Values are presented as mean  $\pm$  SEM.

### **6.3.2. Effect of IP10 and resveratrol on cytokine clustering**

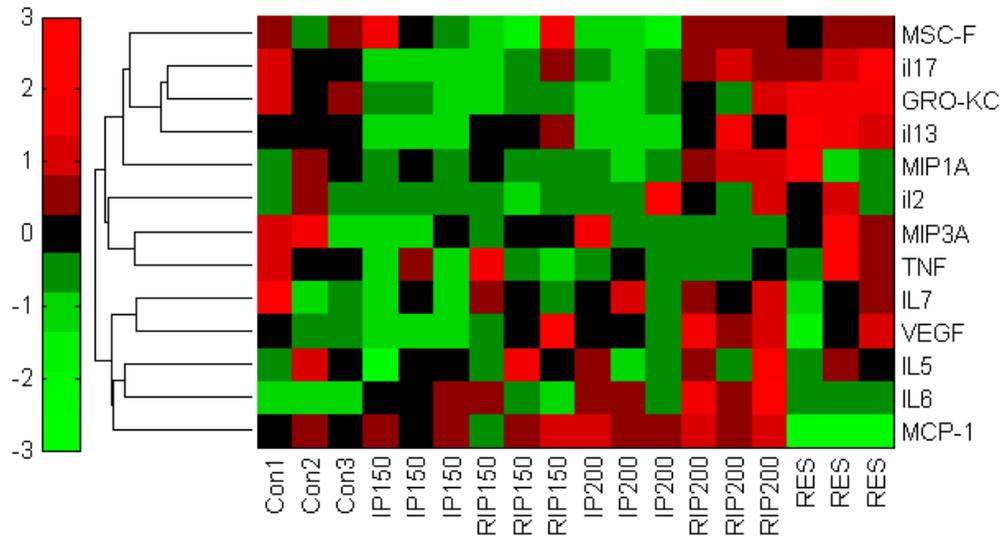
Clustergrams were produced from the cytokine data identifying hierarchical clustering of cytokine profiles in the media from primary rat myotubes treated with IP10+/- resveratrol and are shown in Figure 6.14 - Figure 6.18. Clustergrams were produced using MATLAB, which uses a non-biased mathematical algorithm based on Eisen et al (1998) and is a common approach in several genome wide expression studies (Eisen et al., 1998). Clustergrams function as an output of hierarchical clustering. Overall there was not any clear pattern in the clustering, however TNF- $\alpha$  and MIP-3 $\alpha$  were continually clustered together in the presence of IL7 at day 5 and 10 following treatment of myotubes with IP10 and resveratrol (Figure 6.16 and Figure 6.18). IL5 and VEGF were clustered at days 1, 5 and 7 following treatment of myotubes with IP10 and resveratrol (Figure 6.14 - Figure 6.16). GRO-KC and IL13 also showed some correlations in the media content of myotubes at days 1 and 5 following treatment of myotubes with IP10 and resveratrol (Figure 6.16).



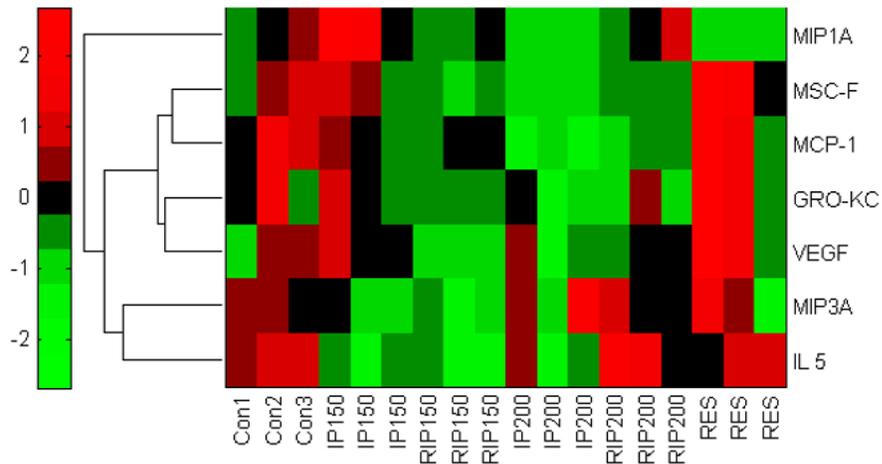
**Figure 6.14** Clustergrams of cytokine content in the media of untreated control myotubes (con), and myotubes treated with 150pg/ml of IP10 with (RIP150) and without (IP150) resveratrol, 200pg/ml of IP10 with (RIP200) and without (IP200) resveratrol and resveratrol treatment alone (res) at 1 day following treatment.



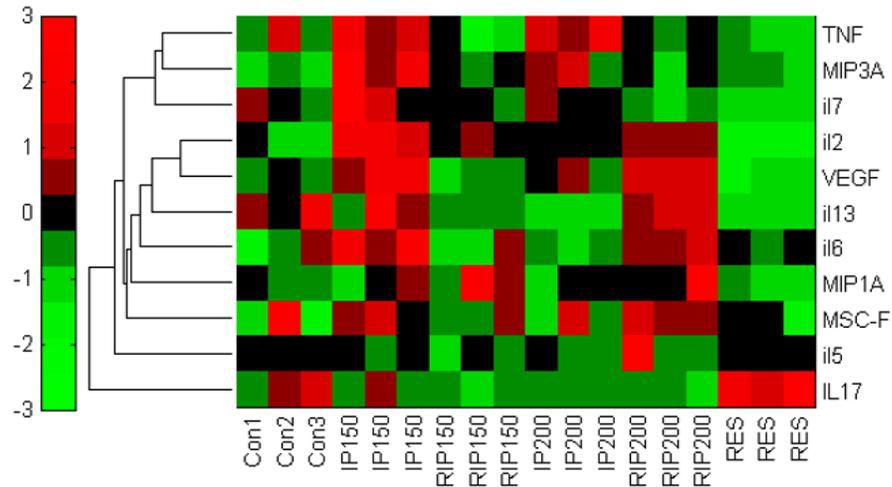
**Figure 6.15** Clustergrams of cytokine content in the media of untreated control myotubes (con), and myotubes treated with 150pg/ml of IP10 with (RIP150) and without (IP150) resveratrol, 200pg/ml of IP10 with (RIP200) and without (IP200) resveratrol (IP200) resveratrol treatment alone (res) at 3 days following treatment.



**Figure 6.16** Clustergrams of cytokine content in the media of untreated control myotubes (con), and myotubes treated with 150pg/ml of IP10 with (RIP150) and without (IP150) resveratrol, 200pg/ml of IP10 with (RIP200) and without (IP200) resveratrol and resveratrol treatment alone (res) at 5 days following treatment.



**Figure 6.17** Clustergrams of cytokine content in the media of untreated control myotubes (con), and myotubes treated with 150pg/ml of IP10 with (RIP150) and without (IP150) resveratrol (IP150), 200pg/ml of IP10 with (RIP200) and without (IP200) resveratrol and a lone resveratrol treatment (res) at 7 days following treatment.



**Figure 6.18** Clustergrams of cytokine content in the media of untreated control myotubes (con), and myotubes treated with 150pg/ml of IP10 with (RIP150) and without (IP150) resveratrol, 200pg/ml of IP10 with (RIP200) and without (IP200) resveratrol and resveratrol treatment alone (res) at 10 days following treatment.

#### 6.4. Discussion

The aim of this Chapter was to identify whether the different levels of IP10 found in serum of the older and younger human population altered the secretion of cytokines by myotubes (myokines) and to identify whether the beneficial effects of resveratrol seen in myotubes (Chapter 5) were due to changes in cytokine production by myotubes. Data from this Chapter has demonstrated no overall trend but suggest that production of cytokines by skeletal muscle is a very complex process.

##### 6.4.1. Cytokines not released or not affected by IP10 and resveratrol

Data demonstrated that 11 of the cytokines measured were not present in the media of either treated or untreated control myotubes; EPO, GM-CSF, GCSF, IFN- $\gamma$ , IL-1 $\alpha$ , IL-1, IL-4, IL-10, IL12p, IL-18, and Rantes and there was no effect of treatment of the content of M-CFS. This was not unexpected as skeletal muscles have been shown

to secrete a limited range of cytokines (Nagaraju *et al.*, 1998; Bortoluzzi *et al.*, 2006; Sorby Borge *et al.*, 2009). For example, it has been proposed that IL10 and IFN- $\gamma$  are not expressed in skeletal muscle (Nagaraju *et al.*, 1998), IL4 is proposed to be involved in myoblast migration and fusion and so is unlikely to play a major role in differentiated myotubes (Horsley *et al.*, 2003), although it may be elevated acutely if damage has occurred and myoblasts are activated for repair. In contrast, Rantes, IL1 $\alpha$  and IL $\beta$  are quite commonly reported in skeletal muscle; in particular IL1 $\alpha$  has been shown to have negative effects on skeletal muscle protein synthesis and cell proliferation (Cooney *et al.*, 1999; Neustrom *et al.*, 2009), and so the lack of evidence of secretion of some of these cytokines from myotubes into the media was surprising. However, some studies have shown that these cytokines are not constitutively present in muscle and were only detected in skeletal muscle following the addition of TNF- $\alpha$  or IFN- $\gamma$  (De Rossi *et al.*, 2000; Lightfoot *et al.*, 2015). GM-CSF has been previously shown to be present in low basal levels in skeletal muscle and was only upregulated following a pro-inflammatory stimuli (Nagaraju *et al.*, 1998). These data suggest that the addition of IP10 may not be a strong enough stimulus to increase secretion of these cytokines from myotubes. A review by Peake *et al.* (2015) demonstrates the differential release of cytokines between humans, other species and skeletal muscle cell lines (Peake *et al.*, 2015), which may also explain some of the discrepancies in the data. Furthermore Lightfoot *et al.* (2015) demonstrated that myotubes produced from C2C12 cells released substantial amounts of Rantes (Lightfoot *et al.*, 2015); however Rantes was not present in the media of primary rat myotubes in the current study. There is also the possibility that

the considerable increases in cytokines seen in some *in vivo* studies may be due to release from other cells present such as the adipocytes and fibroblasts.

#### **6.4.2. Effects of resveratrol on cytokine secretion by myotubes by myotubes**

Treatment of myotubes with resveratrol alone resulted in some beneficial changes in the inflammatory profile in the media of myotubes. For example, there was an increase in the media content of the anti-inflammatory cytokines IL13 and IL2 and a decrease in the pro-inflammatory cytokine IL7. Resveratrol treatment of myotubes also resulted in an increase in IL6 which has been shown to have both pro and anti-inflammatory effects. Prior treatment of myotubes with resveratrol was able to decrease TNF- $\alpha$  in the media in the short term, this is in contrast to other studies which have shown that resveratrol can lead to long term decreases in TNF- $\alpha$  and protect against TNF- $\alpha$  induced damage (Zhu *et al.*, 2011; Wang *et al.*, 2014). M-CSF is a cytokine which has been shown to have anti-inflammatory effects in muscle and accelerate muscle regeneration more rapidly leading to a larger muscle fibre (Dumont *et al.*, 2013). Treatment of myotubes with resveratrol led to decreases in M-CSF, paired with TNF- $\alpha$  data this gives further evidence that, without a pre-existing condition, resveratrol may not act as a major anti-inflammatory agent in muscle (Rogers *et al.*, 2015) and may be detrimental (Gliemann *et al.*, 2013).

#### **6.4.3. Effect of treatment of myotubes with IP10 on cytokine content of media of myotubes**

Treatment of myotubes with IP10 resulted in significant improvements in the profile of the pro-inflammatory cytokines including VEGF, KC, IL2, IL5, IL17 and Mip3- $\alpha$

as well as a transient increase in the anti-inflammatory IL13, suggesting IP10 may be protective in skeletal muscle cells. In contrast, treatment of myotubes with IP10 resulted in significant increases in the content of several pro-inflammatory cytokines in the media of myotubes. These included Mip-3 $\alpha$ , MCP-1 and IL6. However, with respect to MCP-1, greater effects were produced by treatment with 200pg/ml compared with 150pg/ml of IP10. With respect to Mip-3 $\alpha$ , treatment of myotubes with 150pg/ml IP10 resulted in an acute significant decrease in the release by myotubes of this pro-inflammatory cytokine, and increases at a later time point. In contrast, no effect was seen on Mip-3 $\alpha$  release following treatment with 200pg/ml of IP10. With respect to IL6, treatment of myotubes with IP10 resulted in increased release of IL6 from myotubes into the media with little evidence of any major differential effects of the two concentrations of IP10 used.

#### **6.4.4. Effect of pre-treatment of resveratrol on IP10 effects on content of cytokines of the media of myotubes**

Treatment of myotubes with an optimised concentration of resveratrol prior to IP10 treatment prevented some of the beneficial effects that were seen following IP10 treatment. For example, resveratrol prevented the decrease in the content of pro-inflammatory cytokines VEGF, GRO-KC, IL5, and IL6 as well as preventing the increase in the anti-inflammatory cytokine IL13. In contrast, pre-treatment of myotubes with resveratrol also prevented some of the negative effects of IP10 on the cytokine profile in the media of myotubes. These included IL6, IL5 and Mip-3 $\alpha$  and also resulted in a decrease in IL17. Pre-treatment of myotubes with resveratrol also resulted in transient

increases in the content of the pro inflammatory cytokines Mip-1 $\alpha$  in the media of myotubes suggesting some pro-inflammatory effects.

The data discussed above shows that, in terms of inflammation, IP10 treatment at concentrations of 150pg/ml and 200pg/ml have generally similar overall but different temporal effects, with some differences changes occurring with resveratrol pre-treatment. Whether or not resveratrol was pro or anti-inflammatory in myotubes was inconclusive from these results as resveratrol treatment showed characteristics of both. Furthermore, the lack of changes in cytokine content between 150pg/ml and 200pg/ml of IP10 suggest that changes in IP10 levels that occur with age are not a master regulator of the age-related changes in cytokine levels that are seen in sarcopenia.

Importantly, the majority of cytokines have both anti- and pro-inflammatory properties depending on the context they are in (Cavaillon, 2001). For example, both concentrations of IP10, were able to upregulate IL6, which is known to have both anti and pro inflammatory effects (Sugama et al., 2012) and has also been shown to be vital for satellite cell activation (Serrano et al., 2008) and myoblast proliferation (Serrano et al., 2008; Zhang et al., 2013); suggesting that the increased secretion caused by IP10 may be beneficial for skeletal muscle. Similarly, MCP-1, is generally considered a pro-inflammatory cytokine and is increased in muscle in response to cardiotoxin injury (Hirata et al., 2003). However, MCP-1 has also been shown to increase myoblast proliferation and promote growth (Yahiaoui et al., 2008). MCP-1 content was

decreased at 1 day following 150pg/ml treatment and treatment of myotubes with 200pg/ml increased MCP-1 secretion at 3 and 5 days following treatment. Therefore, whether this translates to 150pg/ml being harmful to muscle (i.e. decreases myoblast proliferation) and 200pg/ml being beneficial (i.e. increases myoblast proliferation), or alternatively that 150pg/ml reduced inflammation, whereas 200pg/ml resulted in an increase in inflammation is unclear from these data.

VEGF (vascular endothelial factor) is a molecule with a well known role in angiogenesis (Connolly et al., 1989), that had temporal changes in content depending on the treatment administered. VEGF mRNA has been shown to be upregulated in skeletal muscle 4 hours following training (Breen et al., 1996) in rats as well as after electrical stimulation (Jensen et al., 2004) of primary rat skeletal muscle as well as in C2C12 cells (Kanno et al., 1999) and C2C12 constructs (Park et al., 2008) following contraction, suggesting VEGF is present in the muscle following a stress. Therefore it suggests that maybe the effects of the different treatments required different amounts of time to have an effect, depending on the stress they put on the myotubes. Resveratrol has been shown to prevent increases in VEGF following exercise in humans (Gliemann et al., 2014) and in the current study the induction of VEGF with treatment of 150pg/ml was prevented by prior resveratrol treatment, but not following treatment with 200pg/ml of IP10 or treatment with resveratrol alone which also caused an increase, giving further evidence that a lower treatment of IP10 may be beneficial for skeletal muscle and is possibly able to mimic exercise which has previously been proposed (Momken et al., 2011).

#### **6.4.5. Clustergram of cytokine secretion**

Due to the ambiguity of the changes in media content of the different cytokines, hierarchical clustering (Eisen et al., 1998) of the different cytokines was carried out to examine whether there was a relationship between the changes in any of the cytokines studied that could give further insight into the effects of both IP10 and resveratrol.

Data from cluster analysis of content of cytokines in the media of myotubes at 1 day following treatment showed that changes in MCP-1 content in the media of myotubes were not related to any other cytokine changes. MCP-1 plays a major role in systemic inflammatory and in skeletal muscle regeneration (Hirata et al., 2003) by recruiting macrophages. These data suggests that MCP-1 may be an early regulator of the regeneration process and acts as one of the master regulators of the inflammatory response. This is further evidenced by a gradual reduction in the content in media of myotubes at 7 days following treatment and the subsequent absence of the cytokine at 10 days following treatment. Similarly to MCP-1, the changes in IL6 content in the media was not related to any other cytokines at 3 days following treatment, again this was not surprising since IL6 has been shown to have a prominent role in inflammation and has been shown to regulate many of the cytokines studied.

IL5 and VEGF were also clustered together at day 1 and 5 following treatment. Correlated expression of these two molecules has been seen in models of pneumonia (Nishigaki et al., 2003; Choi et al., 2009), providing evidence that increases in IL5 and VEGF may have synergistic roles in various tissues including skeletal muscle.

There were also correlations between IL13 and GRO-KC content in the media of myotubes at day 1, 3 and 5 following treatment. IL13 and GRO-KC have been shown to be involved in myoblast proliferation (Jacquemin et al., 2007; Heredia et al., 2013; Iwasaki et al., 2013). A role for IL13 and GRO-KC in proliferation is supported by the heat map element of the clustergram analysis that showed there was no overall change in the content of IL13 and GRO-KC in the media of untreated control myotubes. Furthermore, the heat map also showed an increase in GRO-KC and IL13 content in the media of myotubes treated only with resveratrol, this may suggest an increase in proliferation of myoblasts by a resveratrol treatment, supporting the findings from Chapter 3.

TNF- $\alpha$  and Mip-3 $\alpha$  are well known pro-inflammatory cytokines and were also correlated at day 5 and 10 following treatment of myotubes. This only occurred in the presence of IL7. IL7 is a pro-inflammatory cytokine that has been shown to effect myoblast migration (Haugen et al., 2010). There are no studies which have previously reported correlations of these cytokines; however, these data suggest this triplet may play a synergistic role in myogenesis. Considering IL7 has been shown to be involved in myoblast migration (Haugen et al., 2010) treatment of myotubes with resveratrol alone as well as a pre-treatment of myotubes with resveratrol followed by a treatment with 200mg/ml IP10 resulted in a decrease in IL7 content at 10 days following treatment. Furthermore, no differences were seen between any of the other treatment groups. These data disagrees with previous data that has shown resveratrol to increase motility and migration of myoblasts (Bosutti *et al.*, 2015).

Previous data from Chapters 3 and 4 provided evidence that resveratrol may cause early myogenesis; therefore this suggests that following the different treatments myotubes may be at different stages of maturity and may explain some of the temporal changes within the treatment groups. This hypothesis is supported by another study which showed differences in cytokine responses in myoblasts and myotubes (Podbregar et al., 2013). Furthermore, there is a lot of variance within treatment groups, which would be expected in primary cell cultures, particularly here when there are some fibroblasts also present that may also contribute to cytokine secretion, particularly as some of the cytokines found in the media of myotubes have not previously been identified as myokines and further work is warranted to clarify this.

It is important to note that although initially the same number of cells was plated for each treatment group, experimental design did not allow for protein content to be measured and therefore cytokine secretion was not normalized to total protein content. Furthermore, data in Chapter 5 showed that Atrogin1 levels were upregulated in IP10 treatment groups at days 1, 7 and 10. As Atrogin1 is an ubiquitin ligase that has been shown to be upregulated in many models of atrophy, it is possible that this could mean there was an increase in cell death; some evidence of this has been shown in cardiac muscle where correlations between Atrogin1 and apoptosis were found (Zeng et al., 2013). This could mean that there were fewer cells in the 200pg/ml of IP10 treated group and therefore cytokine secretion may have been under estimated. However, to my knowledge, no link between Atrogin1 and apoptosis has been shown in skeletal

muscle and as we were using myotubes, it is unlikely that levels of apoptosis reached were that high of an increase that there would be significant difference in myotube number.

## **6.5. Conclusions**

The data presented in this Chapter suggest that the role of IP10 treatment is dose and time dependent. The lack of any major effects of IP10 on the content of cytokine in the media of myotubes also suggests that IP10 is not a major regulator in the age-related increase in cytokine production by muscle. Furthermore, these data and data from other studies (Momken *et al.*, 2011) suggest that there is little evidence that resveratrol has major anti-inflammatory properties in muscle. This further suggests that beneficial effects of resveratrol on skeletal muscle described in Chapters 2-4 may not be due to local anti-inflammatory effects of resveratrol on muscle.

**7. THE EFFECT OF RESVERATROL ON MUSCLE  
FORCE GENERATION IN ADULT AND OLD MICE *IN  
VIVO* AND OPTIMISATION OF 3D MUSCLE  
CONSTRUCTS *IN VITRO***

## 7.1. Introduction

Data presented in Chapters 3-6 showed that treatment of primary muscle cells isolated from rat with resveratrol resulted in an increase in myoblast proliferation, hypertrophy of myotubes and protection of myotubes from IP10 induced atrophy. Other studies have also shown beneficial effects of resveratrol in muscle such as hypertrophy as well as a reduction in oxidative stress (Jackson *et al.*, 2011), decrease in and protection from inflammation (Gordon *et al.*, 2013; Wang *et al.*, 2014) and well characterised increases in mitochondrial biogenesis and function (Price *et al.*, 2012).

The effect of resveratrol on skeletal muscle function is less clear. *In vivo* studies have shown varied effects of increased resveratrol intake on the ability of skeletal muscle to generate force. For example Dolinsky *et al* (2012) showed that, feeding 22-week old rats chow supplemented with resveratrol for 12 weeks; resulted in an increase in both the twitch and maximum tetanic force generated by the soleus and tibialis anterior muscles (Dolinsky *et al.*, 2012). These effects of resveratrol were similar to the effects of exercise (Dolinsky *et al.*, 2012), suggesting that resveratrol may act as an exercise mimetic. Oral treatment of 6-week old mice for 21 days with resveratrol resulted in an increase in grip strength as well as protection from physical fatigue (Wu *et al.*, 2013). Treatment of rats with resveratrol has also been shown to prevent unloading induced atrophy (Jackson *et al.*, 2010; Momken *et al.*, 2011; Gordon *et al.*, 2014). In contrast, other studies show little effects, for example, resveratrol had no effect on the maximum peak or twitch force in the anterior crural muscles of 21-month old mice that were fed chow supplemented with resveratrol for 7 weeks (Baumann *et*

*al.*, 2015). Furthermore, treatment of rats for 10 months with resveratrol resulted in improved markers of oxidative stress but had no effect on the force generation of the plantaris muscle (Jackson et al., 2011). In addition, resveratrol treatment prior to barium chloride injections had no effect on the force generation by the anterior tibialis either before or after injection (Russell et al., 2015). The variable effects of resveratrol treatment on muscle function could be due to differences in the methods of administration, length of treatments and species and muscles used. The age and health status of the subject may also been important in the outcome on benefits of resveratrol treatment (Baur et al., 2006).

The ability to measure muscle cell function/ force generation in cell culture would be ideal for a number of reasons. This would allow the effects of a treatment on isolated muscle cells to be studied without any confounding factors such as the presence of other cells. It would also remove some of the variables that may affect the outcome of treatment as discussed above, such as route of administration and systemic metabolism of the compound. A major benefit of the establishment of a 3D muscle construct is the ability to measure force generation. An additional benefit of the use of a muscle 3D construct is the ability to directly test the effect of the parent compound, such as resveratrol, on muscle function rather than this being complicated by the potential breakdown of the compound in the gut. It also allows identification of the functional effect of treatments directly on the isolated muscle cells. Ethically, it would considerably reduce the number of animals needed for experimental work.

The use of 3D cultures to measure force generation *in vitro* was carried out by Vandeburgh in avian skeletal muscle cells and these were termed “organoids”. (Vandeburgh et al., 1991) The use of lateral loading of the muscle in this model which could potentially affect the length of the sarcomere and so give inaccurate force measurements meant that this model was met with criticism. Later, 3D constructs were also designed using artificial matrix (Okano et al., 1997; Shansky et al., 1997). Again, these constructs, when used for functional measurements, were criticised as the interference of the scaffold has been shown to introduce a large resting tension (Delvoe et al., 1991). Since then, 3D constructs made with primary cells from rat skeletal muscle without scaffolds have been relatively well established and characterised (Kosnik et al., 2001).

The use of 3D skeletal muscle constructs (also known as myoids) as implants offer great promise for treatment of muscular diseases such as muscular dystrophy and for regenerative medicine. Studies have already been carried out using implantation of skeletal muscle constructs into rodents and this had led to restoration of function (Machingal et al., 2011). Despite the benefits for using 3D constructs in research, 3D cell culture models are still only minimally used.

Thus, the use of 3D constructs in culture and studies *in vivo* provide complimentary approaches to determine the effects of supplementation on muscle function whereby 3D constructs can be used to determine the direct effects of treatment and *in vivo* studies incorporate the potential interactions of the compound with other tissues, including the gut.

The aim of this Chapter is twofold:

1) To identify a dose of resveratrol which results in known transcriptional response in muscle and then to determine the effects of this dose on muscle function in adult (6 months old) and old (24 months old) mice *in vivo* and

2) To optimise the formation of 3D muscle constructs that could be used to measure force generation *in vitro* for future studies.

## **7.2. Methods**

### **7.2.1. Preparation of resveratrol**

Resveratrol was dissolved in 20% DMSO solution and stored at 4°C.

### **7.2.2. Mice**

C57BL6/J adult and old mice were used and housed as described in Section 2.2.3.

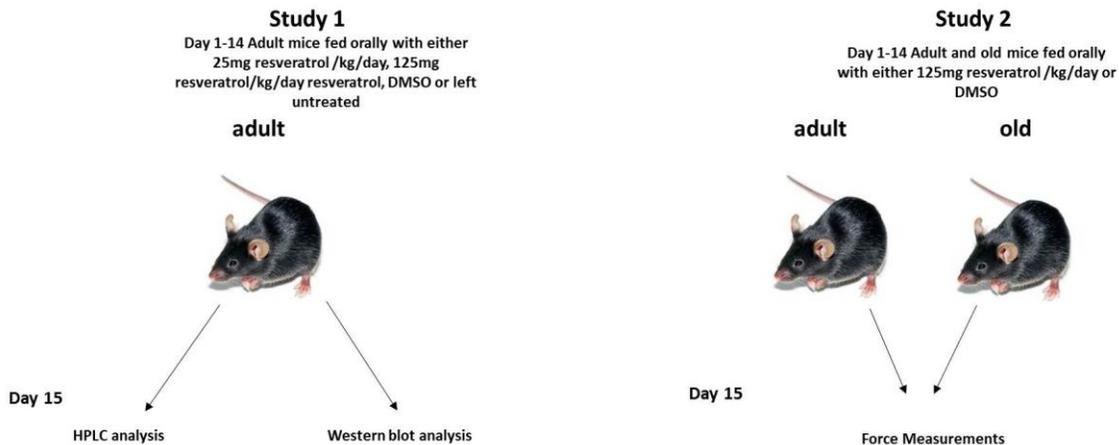
### **7.2.3. Treatment of mice with resveratrol or DMSO**

A pilot study was undertaken to potentially inform a larger study to determine the effects of supplementation of adult and old mice with resveratrol on muscle function.

**Study 1:** Male mice were split into four groups:

Group 1, treated with 25mg resveratrol/kg/day by oral gavage daily for 14 days (n=5), group 2, treated with 125mg resveratrol/kg/day by oral gavage daily for 14 days (n=3), group 3, treated with DMSO vehicle by oral gavage daily for 14 days. (n=5) and group 4 , an untreated control group (n=3).

**Study 2:** Once a dose of resveratrol which had resulted in changes in Sirt1 and MnSOD content of muscle was identified from Study 1, adult and old mice were split into two groups each; one group was treated with 125 resveratrol (n=5) mg/kg/day and the other treated with DMSO vehicle (n=6) by oral gavage daily for 14 days.



**Figure 7.1 Overview of studies 1 and 2, to determine the effect of resveratrol on biochemical markers and muscle function in adult and old mice *in vivo*.**

#### 7.2.4. HPLC analysis (Study 1)

Twenty four hours following the final resveratrol treatment, mice were culled with overdose of anaesthetic and gastrocnemius muscles were removed, frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis. One of the gastrocnemius muscles was ground under liquid nitrogen and sent to Dr Bresciani at the University of Parma, Italy for HPLC analysis for determination of content of resveratrol and its metabolites; dihydroresveratrol aglycone, dihydroresveratrol sulfate, dihydroresveratrol glucuronide, resveratrol disulfate, and dihydroresveratrol sulfo-glucoronide. The other gastrocnemius muscle was subjected to western blot analysis for determination of Sirt1 and MnSOD protein content (Section 2.11).

#### 7.2.5. Preparation of skeletal muscle for SDS-PAGE and western blotting

Muscle samples were ground under liquid nitrogen and were sonicated on ice for 15 seconds and centrifuged at 14000g for 5 minutes. The pellet was discarded and a

BCA assay (Section 2.10) was carried out on the supernatant to determine total protein concentration. Fifty micrograms of protein was diluted 1:1 with protein loading buffer in preparation for SDS-PAGE (Section 2.11). Western blotting for MnSOD and Sirt1 contents was performed as described in Sections 2.11.3 and 2.11.4.

#### **7.2.6. Determination of the effect of resveratrol on force production by EDL muscles of mice *in vivo* (Study 2)**

Following treatment of mice with resveratrol or DMSO carrier, force generation by the EDL muscles was measured as described in Section 2.20.4. Briefly, adult and old mice were anaesthetised. The knee of the right hind-limb was fixed. The distal tendon was exposed and attached to the lever arm of a servomotor (Cambridge Technology, UK). The lever served as a force transducer. The peroneal nerve was exposed, and electrodes were placed across the nerve. Stimulation voltage and muscle length were each adjusted to produce maximum twitch force. The optimal length for maximum twitch force is also the optimal length ( $L_0$ ) for the development of maximum tetanic force ( $P_0$ ) (Brooks et al., 1988). Optimal length was determined by increasing the length of the muscle until optimal twitch force was reached. With the muscle at  $L_0$ , the  $P_0$  was determined during 500 msec of voltage stimulation. The  $P_0$  was identified by increasing the frequency of stimulation at 2-min intervals until the maximum force plateaued. Muscle fibre length ( $L_f$ ) and cross-sectional area were calculated (Brooks et al., 1988). Following the final measurement, mice were killed by cervical dislocation. The EDL and gastrocnemius muscles were removed and weighed. The specific  $P_0$  was calculated as the absolute  $P_0$ /total muscle cross-sectional area (Brooks et al., 1988).

### 7.2.7. Preparation of 3D muscle constructs using primary rat myotubes

Following isolation and expansion in culture (Section 2.3), myoblasts were plated on pre-prepared laminin coated sylgard plates at a density of approximately  $4 \times 10^5$ . Once myoblasts had reached 80% confluence, small pieces of tendon from rat tail were placed into the petri dish (Section 2.19) and media changed to differentiation media to initiate myotube formation. Within 3-5 days, myotubes began to delaminate and roll around the pinned tendon. A full 3D construct was formed approximately 7 days following plating of myoblasts (Figure 7.2).

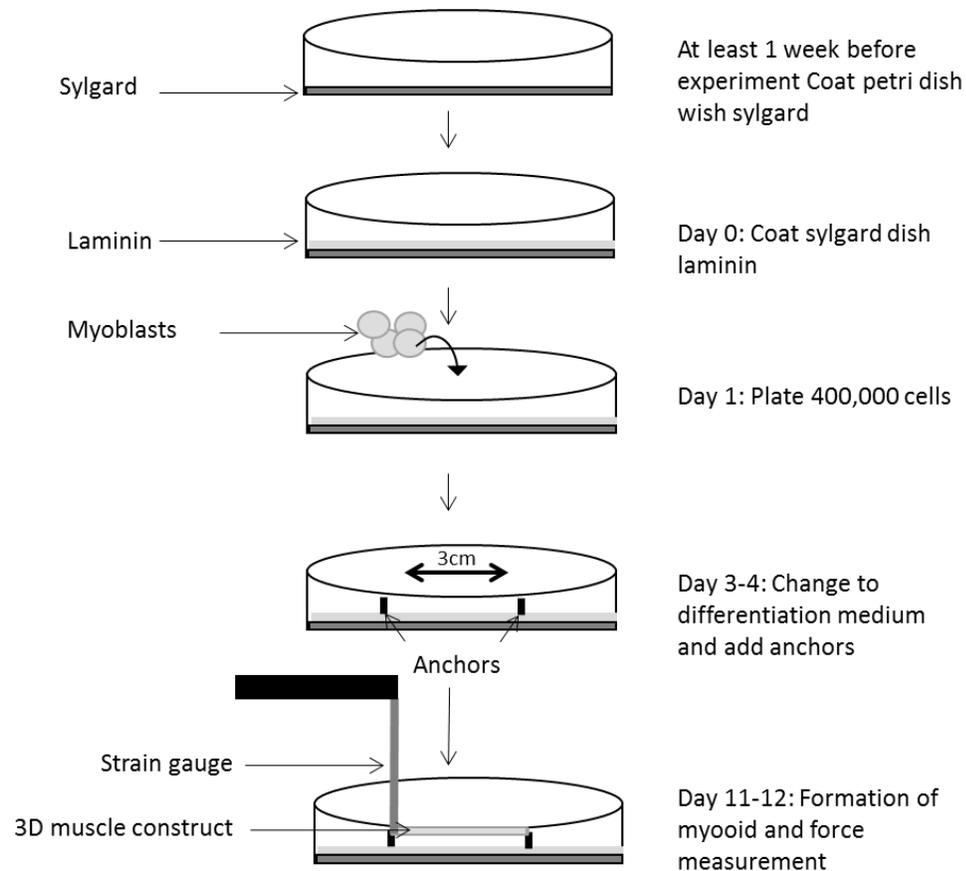


Figure 7.2 Stages of construction of a 3D muscle construct.

### **7.2.8. Measurement of force generation by 3D muscle constructs**

Contractile properties were measured the following day after the formation of a 3D construct. The anchor of one end of the 3D construct was released from the Sylgard and attached to a force transducer with wax. Platinum wire electrodes were positioned on either side of the construct to allow stimulation of the whole construct. Passive baseline force was measured as the average baseline force before the onset of stimulation. Twitches were elicited using a single 1.2-ms pulse at 10V and maximum tetanic force was determined at 10, 20, 40, 80 100 and 200Hz. Maximum specific force was determined by normalising peak tetanic force to the cross sectional area of the 3D construct that was determined by measuring the diameter of the 3D construct measured using Nikon TE2000 microscope (Nikon, Kingston upon Thames, UK).

### **7.2.9. Statistics**

Graphpad 5 (Graphpad Software, San Diego, USA) was used to perform One-way ANOVA test followed by a Dunnett's post-test to identify significant differences between data in Study 1. A two-way ANOVA followed by a Bonferonni post-test was used to identify significant differences between data in Study 2. Data are represented as mean  $\pm$  SEM.

## **7.3. Results**

### **7.3.1. Study 1: HPLC analysis of muscle for resveratrol and its metabolites.**

Resveratrol or resveratrol metabolites were undetectable in the gastrocnemius of DMSO and resveratrol treated mice (data not shown).

### 7.3.2. Study 1: Effect of treatment of resveratrol on MnSOD and Sirt1 protein content of *in vivo*

MnSOD and Sirt1 protein contents of the gastrocnemius muscle of mice following treatment with resveratrol with either 25 or 125mg resveratrol/kg/day are shown in Figure 7.3 and Figure 7.4. MnSOD (Figure 7.3  $p < 0.01$ ) and Sirt1 (Figure 7.4  $p < 0.01$ ) protein content was significantly increased following treatment with 125mg resveratrol/kg/day compared with the DMSO vehicle group. The protein contents of MnSOD and Sirt1 in untreated mice and mice treated with 25mg resveratrol/kg/day did not show any changes compared with the DMSO vehicle mice.

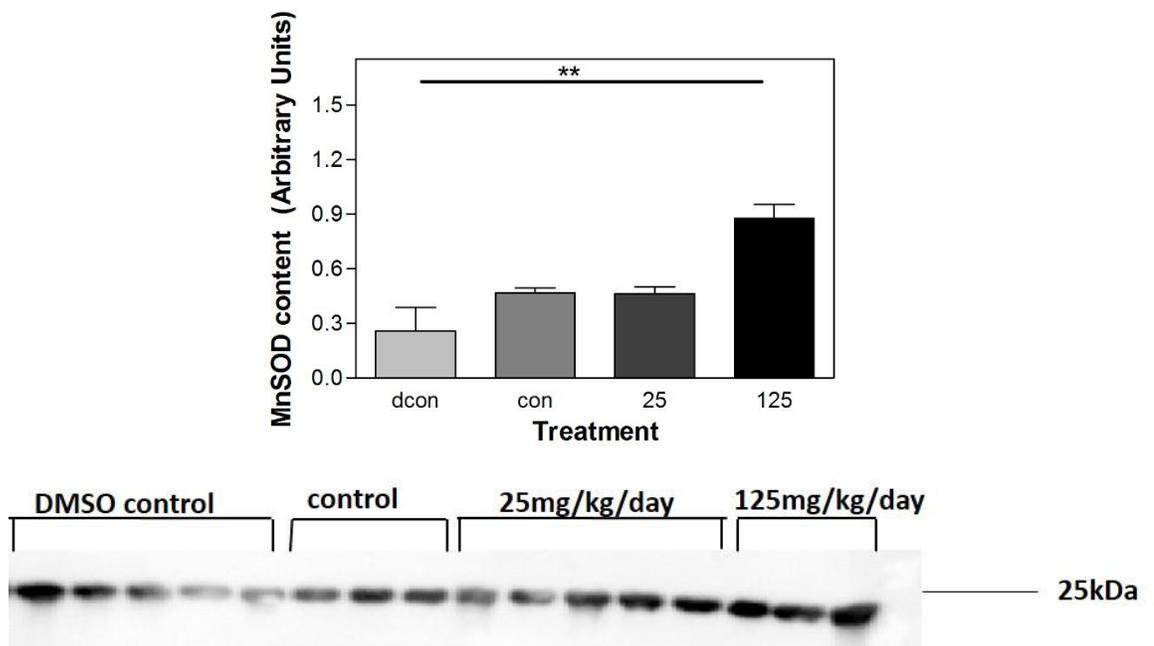
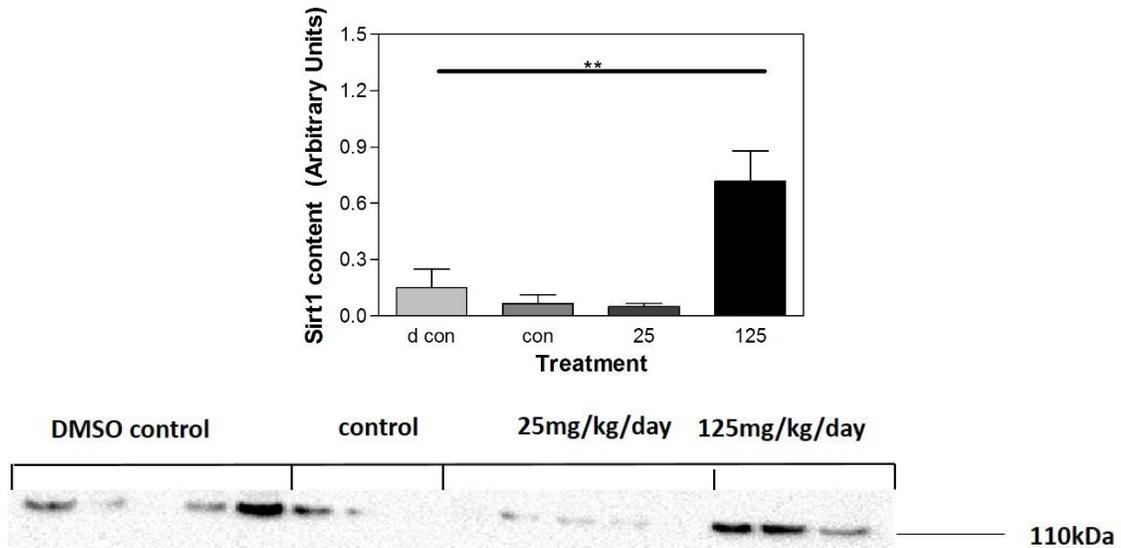


Figure 7.3 MnSOD content of the gastrocnemius muscles of adult mice following treatment with 125mg resveratrol/kg/day (125), 25mg resveratrol /kg/day (25), compared with untreated control (con) and DMSO vehicle treated control (dcon). \*\* $p < 0.01$  One-way ANOVA. Values are presented as mean  $\pm$  SEM.



**Figure 7.4** Sirt1 content of the gastrocnemius muscles of adult mice following treatment with 125mg resveratrol/kg/day (125), 25mg resveratrol /kg/day (25), compared with untreated control (con) and DMSO vehicle treated control (dcon). \*\* $p < 0.01$  One-way ANOVA. Values are presented as mean  $\pm$  SEM.

### 7.3.3. Effect of treatment of adult and old mice with resveratrol on muscle mass of EDL and gastrocnemius muscles

Data for twitch force, maximum force, muscle masses and CSA and maximum specific force, of muscles of adult and old mice following treatment with 125mg resveratrol/kg/day is shown in Figure 7.5-Figure 7.13. Thus, no significant effect of age was seen on absolute muscle mass (Figure 7.5 and Figure 7.7) although a significant decline was seen when muscle mass was expressed as a percentage of body weight (Figure 7.6 and Figure 7.8). There was a non-significant  $\sim 20\%$  reduction in maximum tetanic force (Figure 7.11), which became significant when muscle CSA was accounted for in the specific force data (Figure 7.13).

No difference in maximum tetanic force was seen in EDL muscles of mice treated with resveratrol although a small but significant reduction in muscle CSA was seen in adult mice treated with resveratrol compared with DMSO controls ( $p > 0.05$  Figure 7.12). Similar patterns were seen in muscles of old mice treated with resveratrol compared with DMSO treated old mice. Thus, no effect of resveratrol was seen on maximum tetanic force, CSA or maximum specific force generation between old mice treated with resveratrol and old mice treated with DMSO vehicle (Figure 7.11, Figure 7.12 and Figure 7.13).

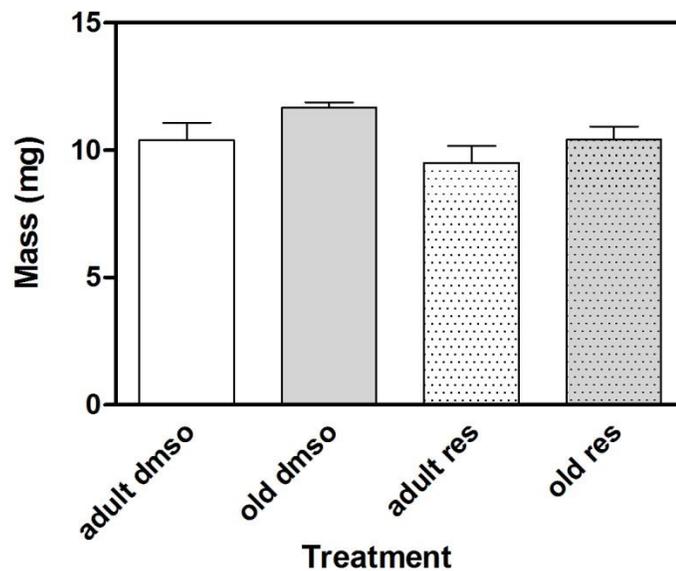


Figure 7.5 Mass of EDL muscles from adult and old mice treated with 125mg resveratrol/kg/day or DMSO for 14 days. Values are presented as mean  $\pm$  SEM.

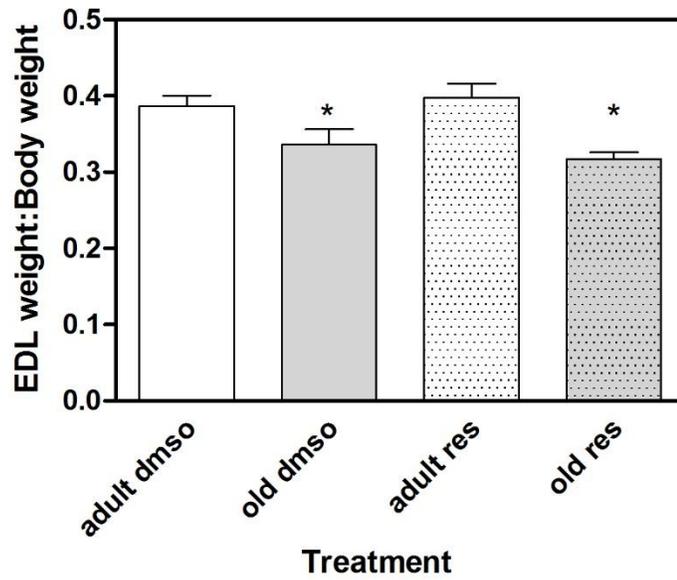


Figure 7.6 Mass of EDL muscles normalised to body mass of adult and old mice treated with 125mg resveratrol/kg/day or DMSO for 14 days. \* $p < 0.05$  compared with same treatment in adult. Two way ANOVA. Values are presented as mean  $\pm$  SEM.

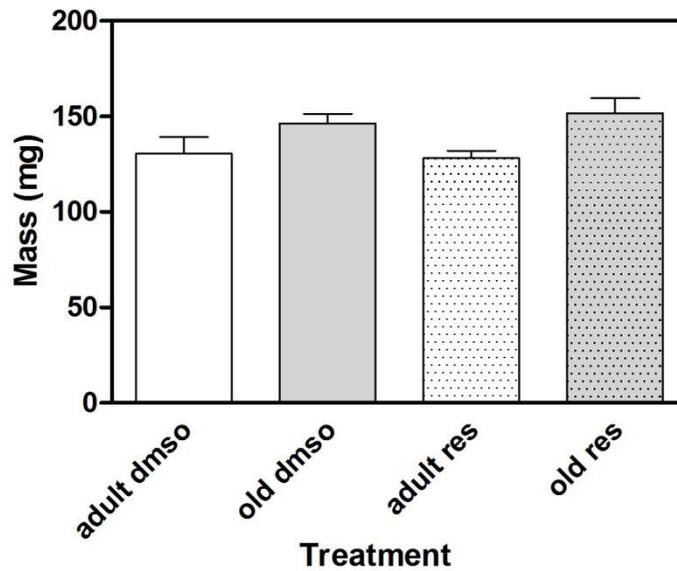


Figure 7.7 Mass of gastrocnemius muscles of adult and old mice treated with 125mg resveratrol/kg/day or DMSO for 14 days. Values are presented as mean  $\pm$  SEM.

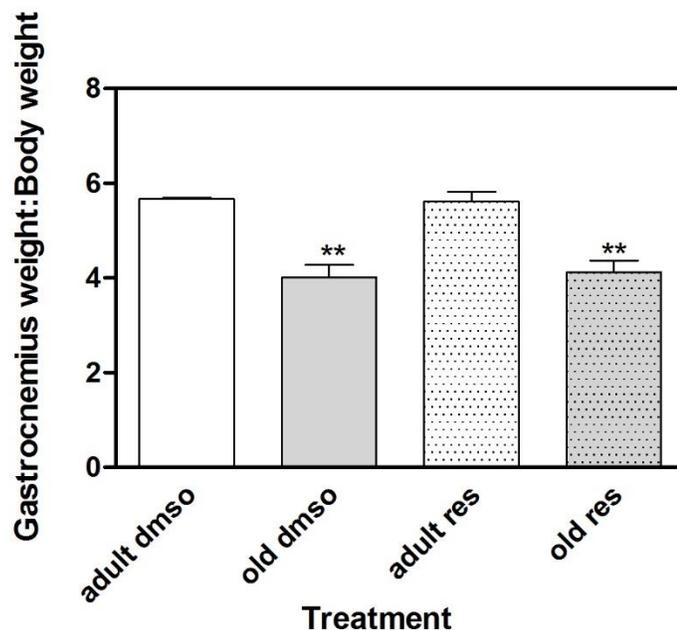


Figure 7.8 Mass of gastrocnemius muscles normalised to body mass of adult and old mice treated with 125mg resveratrol/kg/day or DMSO for 14 days. \*\* $p < 0.01$  compared with same treatment in adult. Two-way ANOVA. Values are presented as mean  $\pm$  SEM.

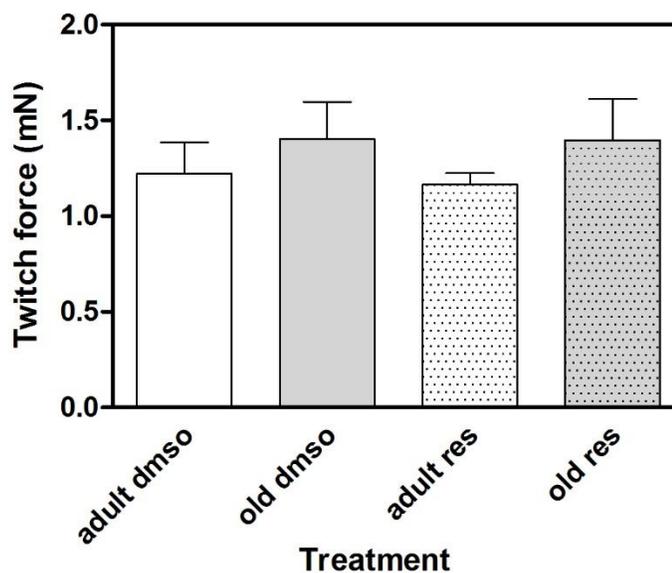


Figure 7.9 Maximum twitch force of EDL muscles from adult and old mice treated with 125mg resveratrol/kg/day or DMSO for 14 days. Values are presented as Mean  $\pm$  SEM.

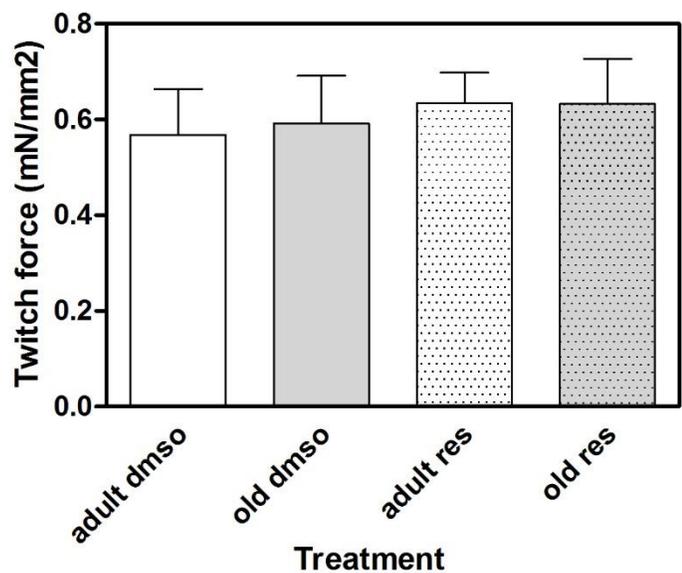


Figure 7.10 Maximum twitch force normalised to CSA of EDL muscles from adult and old mice treated with 125mg resveratrol/kg/day or DMSO for 14 days. Values are presented as mean  $\pm$ SEM.

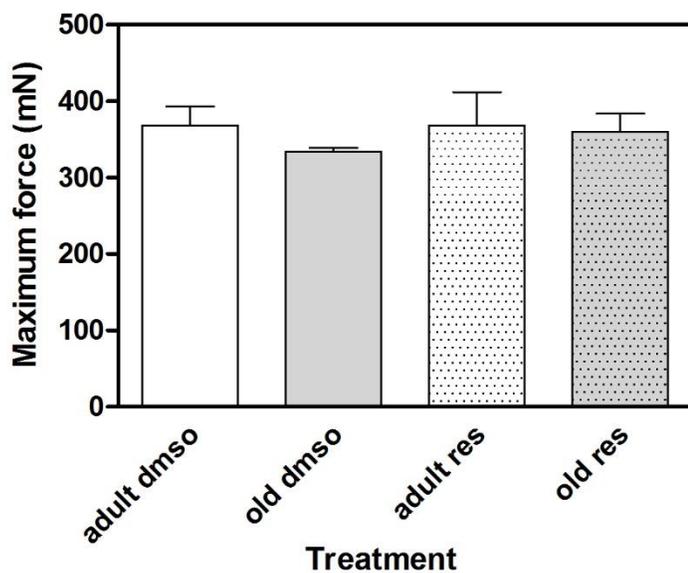


Figure 7.11 Maximum tetanic force generation by EDL muscles from adult and old mice treated with 125mg resveratrol/kg/day or DMSO for 14 days. Values are presented as mean  $\pm$ SEM.

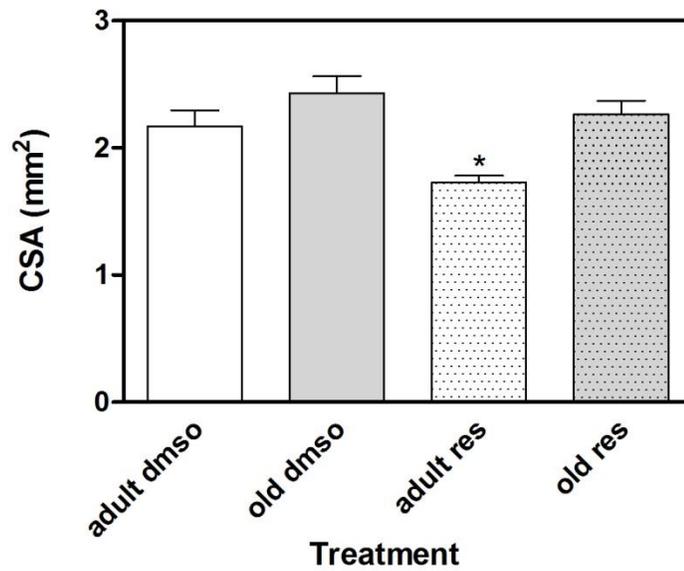


Figure 7.12 Cross sectional area of EDL muscles of adult and old mice treated for 14 days with 125mg resveratrol /kg/day or DMSO. \*\*p<0.01 compared with adult +p<0.05 compared with DMSO control. Two-way ANOVA. Values are presented as mean ±SEM.

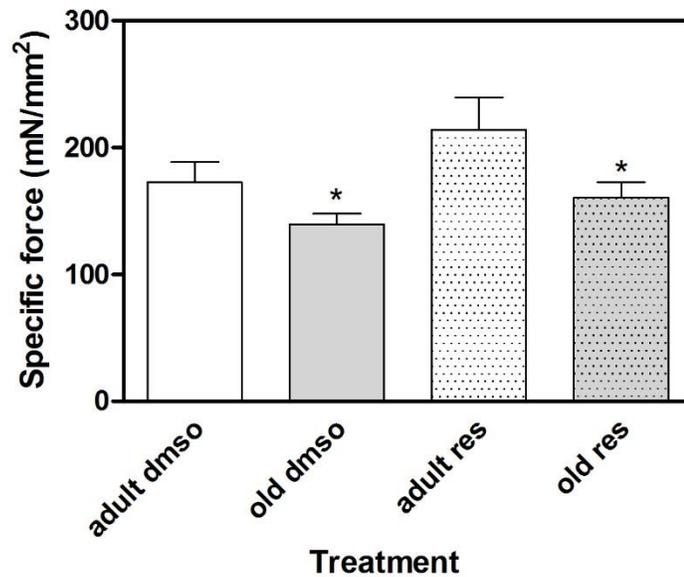
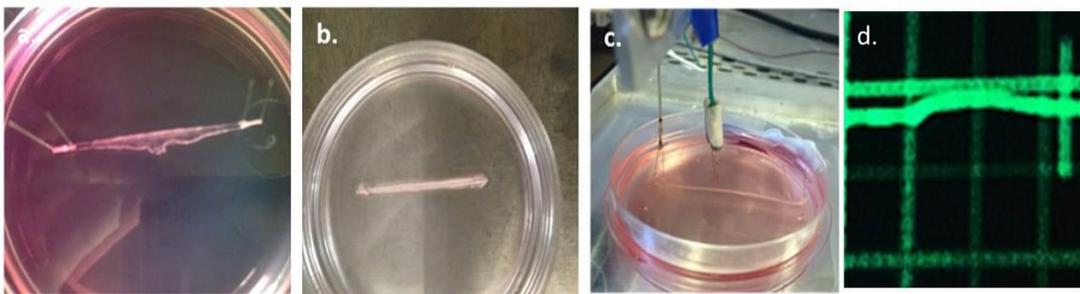


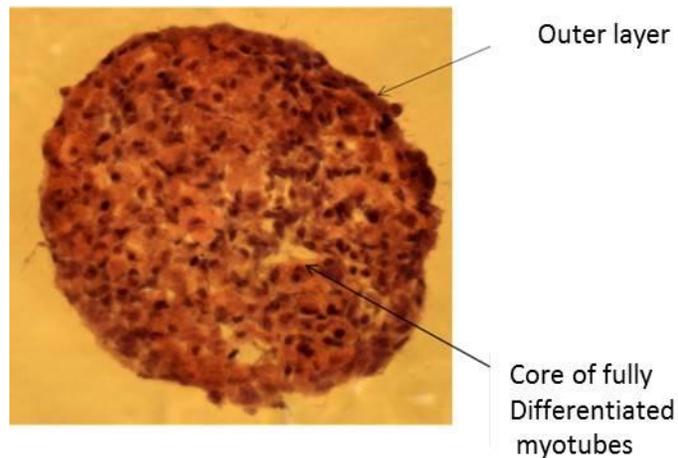
Figure 7.13 Specific force generation of EDL muscles from adult and old mice treated with 125mg resveratrol/kg/day or DMSO for 14 days. Values are presented as mean ±SEM Two-way ANOVA.

### 7.3.4. Establishment of 3D muscle construct

Representative images of 3D constructs are shown in Figure 7.14 and Figure 7.15. The maximum and specific force generation of a single 3D construct was 76.4 $\mu$ N and 10.9N/mm<sup>2</sup> respectively. Haematoxylin staining of 3D constructs revealed the congregation of fibroblasts around the outer layer with differentiated myotubes occupying the middle of the construct (Figure 7.15).



**Figure 7.14** Representative images at different stages of 3D construct formation; (a) following plating of cells and insertion of anchors, cells start to delaminate (b) and a 3D construct will be fully formed within approximately 1 week. (c) Following formation of 3D constructs, (d) forces are measured using a force transducer.



**Figure 7.15** Cross section of 3D construct stained with H&E. Fibroblasts occupy the outside layer of construct and the middle area consists of myotubes.

#### 7.4. Discussion

Data from this Chapter showed that resveratrol and its metabolites were undetectable in the gastrocnemius muscle of adult mice treated with resveratrol for 14 days. Despite this, resveratrol was able to have a transcriptional effect in the muscle of mice, demonstrated by the upregulation of the muscle protein content of MnSOD and Sirt1. This increase in MnSOD and Sirt1 protein content was seen following a treatment with a 125mg resveratrol/kg/day whereas treatment of mice with 25mg resveratrol/kg/day had no effect on the muscle protein content of Sirt1 or MnSOD. Consequently, 125mg resveratrol/kg/day was chosen as the optimum treatment for a pilot study (Study 2) aiming to determine the effects of this dose of resveratrol on muscle function in adult and old mice *in vivo*.

Data comparing muscles of adult and old mice showed a reduction in CSA and specific force generation and in the gastrocnemius muscle when muscle mass was expressed as a percentage of body weight. This is in agreement with other studies suggesting a significant reduction in lean body mass and muscle function.

No significant effects were observed on maximum tetanic force generation, CSA and specific force generation when examining the effect of resveratrol on muscles of adult or old mice, or when comparing muscles of adult mice treated with resveratrol with adult mice treated with DMSO vehicle or between old mice treated with resveratrol and old mice treated with DMSO vehicle. Treatment of mice with resveratrol resulted in a significant decrease in muscle CSA in muscles of both old and adult mice. However, there was no major effect on tetanic force generation, this

resulted in an apparent (non-significant) increase in specific force generation in both adult (approximately 25%) and old (approximately 15%) mice when compared with the adult and old mice treated with DMSO vehicle, suggesting that caution is required when interpreting such data since treatment with resveratrol seems to have induced some muscle atrophy, particularly in adult mice. In addition, treatment of old mice with resveratrol had no effect on the gastrocnemius mass when normalised to body weight, further suggesting that resveratrol was not effective in preserving muscle mass in older mice.

Therefore these data suggest that resveratrol treatment at this dose has little beneficial effect on muscle function in adult and old mice.

#### **7.4.1. Optimisation of resveratrol treatment *in vivo***

Resveratrol has been shown to have numerous health benefits *in vitro*. However, due to the rapid absorption and break down of the compound, HPLC studies have shown that only 1% of ingested resveratrol reaches the plasma with the rest being metabolised (Wenzel *et al.*, 2005). Therefore whether the effects that have been seen in cell culture studies are achievable *in vivo* is debatable (Boocock *et al.*, 2007).

Concentrations used as well as length and route of administration of resveratrol vary widely between studies. Therefore, this work focused on identifying a treatment of mice with resveratrol that would have known transcriptional effects, demonstrating that resveratrol, or its metabolites had reached the muscle. Based on published data, mice were treated with two different concentrations of resveratrol;

25mg resveratrol/kg/day and 125mg resveratrol/kg/day for 14 days where resveratrol treatment resulted in increased grip strength and decreased markers of fatigue (Wu et al., 2013). Resveratrol was administered via oral gavage to ensure that all mice received equal doses.

Resveratrol or resveratrol metabolites were not detected by HPLC in the gastrocnemius muscle from mice treated with either 25mg resveratrol/kg/day or 125mg resveratrol/kg/day. Muscles were removed at 24H following the final resveratrol treatment, therefore the absence of resveratrol in the muscle may be due to the fast metabolism of resveratrol (Wenzel *et al.*, 2005). Resveratrol has been shown to be present in muscle following administration of 5.9mg/kg/day in the pig (Azorin-Ortuno et al., 2011). However, this study collected muscle at 6 hours following treatment and resveratrol was also administered intragastrically. A study by Andres-Lacueva et al (2012) detected a small number of resveratrol metabolites in muscle following a 6-week treatment of rats with 6, 30 or 60mg resveratrol/kg/day in their normal chow however others were undetectable, (Andres-Lacueva et al., 2012). This indicates that skeletal muscle may not be very efficient at absorbing resveratrol and in order to determine resveratrol absorption by muscle, collection of tissue should be undertaken over a time course of less than 24 hours. These data also suggest that, in particular, different routes of administration and studies in different species may also affect the outcome of the resveratrol study. Despite the lack of detection of resveratrol or its metabolites in muscle, known transcriptional targets of resveratrol, MnSOD and Sirt1 protein content in the muscle were increased following treatment of mice with

125mg resveratrol/kg/day. These data showed that although resveratrol was undetectable by HPLC analysis, treatment of mice with resveratrol at a higher dose was still able to have an effect on translation of known targets in muscle. Sirt1 as a direct target for resveratrol is controversial (Beher et al., 2009). Some studies have shown that Sirt1 is activated following resveratrol treatment (Howitz et al., 2003; Lakshminarasimhan et al., 2012; Price et al., 2012). However, data from Chapter 4 showed that, following resveratrol treatment, Sirt1 protein content was decreased in primary myotubes from rat and this was associated with increased myotube diameter compared with untreated myotubes. Sirt1 has been shown to inhibit myogenesis (Rathbone et al., 2009) and so the hypothesis was that the decrease in Sirt1 was beneficial to muscle cells as it resulted in initiation of differentiation. Although some studies have shown beneficial effects of Sirt1 in other tissues, Sirt1 may be inhibitory during muscle development (Rathbone et al., 2009). In contrast to our *in vitro* findings in Chapters 3 and 4, resveratrol treatment *in vivo* resulted in an increase in Sirt1 protein content of muscle, suggesting that Sirt1 may be mediating the effects of resveratrol, particularly in the reduction of muscle CSA seen in muscles of adult mice, which is in agreement with published data by Price and colleagues (Price et al., 2012).

#### **7.4.2. Effect of resveratrol on cross-sectional area of EDL muscles from adult and old mice**

A significant decrease in CSA was seen in muscles of resveratrol treated adult mice compared with DMSO vehicle treated adult mice. These data may provide some evidence of the hypothesis that resveratrol may be detrimental to muscle of healthy

individuals (Chapter 5). Previous Chapters have indicated that treatment of healthy muscle with resveratrol may be detrimental. For instance; a pre-treatment of resveratrol followed by a non-detrimental treatment of IP10 led to increases in the muscle specific ubiquitin ligase, Atrogin1 (Figure 5.17). Resveratrol treatment of myotubes also resulted in an increase in some pro inflammatory cytokines in the media of myotubes (Figure 6.3).

In agreement with others (Russell et al., 2015) treatment of mice with resveratrol decreased the CSA of muscles of old mice. This was in contrast to other studies that have shown that treatment with resveratrol resulted in increases the CSA of muscles of old animals (Bennett et al., 2013; Ballak et al., 2016). Discrepancies in these data may also be due to the health state of the animals used for these studies. In the study by Ballak et al (2016), mice were 25-months old and the rats used in the study by Bennett et al (2013) had undergone unloading, further agreeing with previous data in this Chapter suggesting that resveratrol may be more beneficial in older animals with a more severe sarcopenic phenotype.

#### **7.4.3. Effect of treatment of mice with resveratrol on muscle force generation**

The decrease in muscle mass and force generation with age is well characterised in rodents (Brooks et al., 1994; McArdle et al., 2004) and humans (Bemben et al., 1991) and was also shown in this study as well as a significant decrease in gastrocnemius and EDL mass when expressed relative to body mass was seen in old mice when compared with adult mice which is in agreement with published data (Brooks et al., 1994; McArdle et al., 2004).

Published data has previously shown that resveratrol administration resulted in an increased force generation in muscles of adult animals (Dolinsky et al., 2012; Wu et al., 2013), however in the current study, resveratrol treatment caused no significant increase in muscle mass and function in adult mice when compared with data from adult DMSO vehicle treated mice. Despite studies demonstrating that treatment of mice with resveratrol resulted in an increased force generation by muscles of adult animals (Dolinsky et al., 2012; Wu et al., 2013) and showing some beneficial effects on the muscle of sarcopenic animals, such as a decrease in ROS generation (Jackson *et al.*, 2010), this study and others have been unable to show the ability of resveratrol to attenuate the loss of force with age has not been demonstrated (Jackson et al., 2011). Data from this study and others have suggested that resveratrol may need a pre-existing condition to have beneficial effects (Baur et al., 2006), therefore resveratrol treatment may be more beneficial in older mice with a more severe sarcopenic phenotype.

#### **7.4.4. Optimisation of 3D construct**

Additional studies were aimed at optimising the formation of 3D muscle constructs that could be used to measure force generation *in vitro* for future studies which would allow us to examine the direct effects of resveratrol and its metabolites on skeletal muscle in culture and therefore further understand any different effects of interventions reported *in vivo* compared with in culture. In addition, this model provides a novel technique for manipulating the environment of the muscle cells to examine the effects of specific treatments directly on muscle cells.

I was trained in techniques to assess contractile function in 3D muscle constructs by Professor Lisa Larkin (University of Michigan, Ann Arbor, USA) who has developed these functional skeletal muscle constructs *in vitro*. Upon my return from the University of Michigan, I spent a substantial amount of time optimising this technique. 3D muscle constructs are extremely fragile and break easily when they are attached to the force transducer. In addition, delamination of cells from the substrate and rolling into a cylinder can also be problematic, therefore 3D muscle constructs are not always formed properly. Nevertheless, a small number of 3D muscle constructs were successfully produced and force generation from one construct demonstrated. The maximum force measurement from the 3D construct in this study was comparable to published data (Larkin et al., 2006). The construct requires additional standardisation since myotubes were enclosed within a fibroblast surrounding in a similar manner to published data (Dennis et al., 2000). These preliminary data demonstrate the feasibility of this technique and future work will allow the use of this model to measure the effect of resveratrol and other compounds directly on skeletal muscle function.

## **7.5. Conclusions**

Data from this Chapter identified an effective concentration of resveratrol in muscle *in vivo* and these data will be used to power future studies although the functional effects of resveratrol were disappointing. This study was also able to successfully produce 3D muscle constructs *in vitro* and future work will allow use of

this model to measure the effect of resveratrol and other compounds directly on skeletal muscle function.

## **8. DISCUSSION**

## 8.1 Summary of main findings

The initial aims of the work reported in this thesis were to:

1. Establish an *in vitro* model to test the effects of resveratrol on primary muscle cells in culture and identify a concentration of resveratrol that was effective in both undifferentiated (myoblasts) and differentiated (myotubes) primary skeletal muscle cells.
2. Establish an *in vitro* model to study the effect of increased levels of IP10 on muscle cell atrophy and inflammation.
3. Examine whether a treatment of primary myotubes with resveratrol is able to protect against IP10 induced effects on skeletal muscle cell atrophy or inflammation.
4. Identify an effective dose of resveratrol in mice *in vivo* and examine the effect of this concentration on skeletal muscle force generation in adult and old mice.
5. Establish a 3D muscle construct model that could potentially be used to study the direct effects of compounds on skeletal muscle function *in vitro*.

The major findings of the work were:

1. Data have shown that concentrations of resveratrol achievable by diet showed some initial toxicity but were non-toxic to myoblasts in the longer term, conferred some protective effects against cell death, increased cell proliferation and potentially caused early myogenesis. Physiological concentrations of resveratrol resulted in increases in MnSOD and catalase protein contents in myoblasts, indicating that resveratrol is functional of primary rat myoblasts. Data suggested that a potential

mechanism for this was through upregulation of the NAD-dependent deacetylase Sirt1, however results were inconclusive.

**2.** Data from primary rat myotubes demonstrated that treatment of myotubes with concentrations of resveratrol achievable by diet initially caused an increase in cell death, which was associated with an increase in myotube diameter and decreased hydrogen peroxide production of the remaining cells. Data suggest that the increase in myotube diameter was likely through the decrease in the myogenesis inhibitor Sirt1 thus allowing early progression of myogenesis. The decrease in hydrogen peroxide production was likely due to the increase in the antioxidant defence enzymes MnSOD and catalase; however, these data suggest that, in myotubes, this does not occur through the upregulation of Sirt1.

**3.** Data from primary rat myotubes treated with concentrations of IP10 found in old individuals led to decreases in myotube diameter and increases in the muscle levels of specific ubiquitin ligase Atrogin1. Treatment of myotubes with levels of IP10 found in young individuals had no effect on markers of atrophy. Treatment of myotubes with both concentrations of IP10 had some subtle effects on the cytokine profile in the media of myotubes. These data suggest that the age-related changes in serum IP10 concentrations likely contributes to sarcopenia but are unlikely to have a major role in the age-related increase in cytokine production by muscle.

**4.** Data from myotubes treated with resveratrol demonstrated that resveratrol treatment alone had anti-inflammatory effects by inducing IL13, IL2 and reducing levels of IL7. Treatment of myotubes with resveratrol prior to treatment with IP10 was able

to prevent IP10-induced decreases in myotube diameter and was able to inhibit the IP10-induced increases in the muscle specific ubiquitin ligase Atrogin1. Pre-treatment of myotubes with resveratrol was able to partly reduce some of the IP10-induced pro-inflammatory cytokine levels in the media but also prevented some of the anti-inflammatory effects of IP10. Thus resveratrol may be a potential treatment for sarcopenia; however it is unlikely these effects are mediated through anti-inflammatory properties.

5. Data from force measurements demonstrated that treatment of mice with 125mg resveratrol/kg/day resulted in increased muscle content of antioxidant defences but had little effect on age-related changes in force generation. It is possible that that a longer term treatment and a more severe sarcopenic phenotype is required to produce a beneficial effect with resveratrol.

6. The study successfully established a 3D muscle construct which can be used to study the direct effects of compounds such as resveratrol or its metabolites on muscle function *in vitro*.

## **8.2 General discussion of the data presented**

A number of general areas for discussion are highlighted by these data:

During ageing, the function and mass of skeletal muscle declines and this is known as sarcopenia (Rosenberg, 1989). In a large number of cases sarcopenia leads to a loss of mobility resulting in the loss of independence and a poor quality of life as well as an increase in the likelihood of development other diseases and mortality (Landi et al., 2013). The poor prognosis of individuals with sarcopenia coupled with the ever

increasing lifespan means that sarcopenia is becoming a huge economic burden for society (WHO, 2014).

The mechanisms responsible for sarcopenia are unclear, however it is accepted that the increased state of inflammation that occurs with ageing (Fagiolo *et al.*, 1993; Sorby Borge *et al.*, 2009; Caldow *et al.*, 2013) is likely to play a major role. Some other factors thought to contribute to sarcopenia include; changes in the production and effect of ROS (Harman, 1956), mitochondrial dysfunction (Short *et al.*, 2005), changes in autophagy (Cuervo *et al.*, 2000) and ubiquitin-proteasomal pathways (Ponnappan *et al.*, 1999; Chondrogiannia *et al.*, 2000) and anabolic resistance (Welle *et al.*, 1993). Changes to important systems during ageing results in the complete loss of muscle fibres, inability of muscles to regenerate (Gibson *et al.*, 1983) (Shefer *et al.*, 2006; Day *et al.*, 2010), a decrease in type II fibre numbers and cross sectional area (Lee *et al.*, 2006), a decrease in the number of motor neurons (Rowan *et al.*, 2012), denervation of neuromuscular junctions, (Deschenes *et al.*, 2010), infiltration of connective tissue (Brack *et al.*, 2007) and fat (Borkan *et al.*, 1983; Visser *et al.*, 2005) all of which subsequently contribute to the decrease in muscle mass and function that is seen in sarcopenia.

Polyphenols are plant extracts and resveratrol is one of the most studied polyphenols, due to the beneficial effects shown in a number of diseases (Jang *et al.*, 1997; Brasnyó *et al.*, 2011; Harati *et al.*, 2015). Resveratrol has also been proposed to have beneficial effects in sarcopenia (Dolinsky *et al.*, 2012).

The overall aim of this study was to identify a concentration of resveratrol that could be functional in skeletal muscle and identify whether resveratrol could be a useful therapeutic for sarcopenia.

### **8.2.1 Effect of resveratrol on myoblast and myotube viability**

Treatment of myoblasts with 0.1, 1 and 10 $\mu$ M of resveratrol for up to 24H resulted in a decrease in the number of myoblasts, suggesting that a short term treatment of resveratrol may be toxic to myoblasts. Similar results were seen in human lung cells, where treatment with resveratrol resulted in a decrease in metabolic rate suggesting inhibition of oxidative phosphorylation (Robb et al., 2006). Despite this initial decrease in cell number, at 48H following treatment with 0.1, 1 and 10 $\mu$ M of resveratrol, an increase in the proliferation rate of myoblasts, pre-mature elongation and fusion in resveratrol-treated myoblasts could be seen in agreement with other studies and demonstrating that resveratrol can increase proliferation and cause early myogenesis (Kaminskia et al., 2012a; Montesano et al., 2013).

Taken together, these data suggest that resveratrol is not chronically toxic to myoblasts. The initial decrease in myoblast number may be due to the ability of resveratrol to affect the motility of myoblasts which has previously been shown (Bosutti *et al.*, 2015).

Treatment of myotubes with 0.1, 1 and 10 $\mu$ M of resveratrol also resulted in an initial increase in cell death seen at 3 days following treatment and this effect was extended until 5 days following treatment with 10 $\mu$ M of resveratrol. However this cell

death was not evident at later time points. This suggests that myotubes may be more susceptible to death than myoblasts and may be due to myotubes having a higher uptake of resveratrol than myoblasts (Kaminskia et al., 2012a), although this was not determined in this study. However, as myotube specific staining was not carried out it is possible that the cell death that was detected may have occurred in myoblasts that did not differentiate into myotubes or fibroblasts that are also present in primary muscle cultures, therefore, in future, elimination of fibroblasts would be useful.

These data agrees with existing data that resveratrol may initially cause some damage to skeletal muscle however in the long term, resveratrol is relatively non-toxic. Furthermore, data also suggest that resveratrol is able to induce fusion and thus early differentiation to myotubes (Kaminskia et al., 2012a; Montesano et al., 2013) which may be beneficial in certain condition such as muscles of older people recovering from damage.

### **8.2.2 Effect of resveratrol on antioxidant defence enzyme content in myoblasts and myotubes**

Increases in the protein levels and activity of the antioxidant defence enzymes MnSOD and catalase were seen following resveratrol treatment of muscle (Jackson *et al.*, 2010; Ryan *et al.*, 2010; Bennett *et al.*, 2013). Treatment of myoblasts with resveratrol resulted in a transient upregulation of MnSOD protein content in myoblasts; whereby 0.1 $\mu$ M of resveratrol to increased MnSOD content at 3H after treatment; 1 $\mu$ M upregulated MnSOD content for up to 24H after treatment and 10 $\mu$ M for up to 48H. The different concentrations had different temporal effects suggesting

that they may function through different pathways (Centeno-Baez *et al.*, 2011; Bosutti *et al.*, 2015).

Treatment of myoblasts with resveratrol resulted in a dose dependent increase in catalase protein content with 0.1 $\mu$ M of resveratrol increasing catalase content for up to 6H, 1 $\mu$ M for up to 24H and 10 $\mu$ M for up to 48H. These data show that resveratrol is able to have functional effects in myoblasts and the longer maintenance of MnSOD upregulation with 10 $\mu$ M resveratrol treatment, also agrees with data from Chapter 3 where cell viability assays identified that treatment of myoblasts with 10 $\mu$ M of resveratrol provided a longer term protection than the lower concentrations of resveratrol. The increase in MnSOD and catalase protein content in the myoblasts also suggests a potential mechanism by which resveratrol is able to protect myoblasts from cell death (Figure 3.8).

In a similar manner to myoblasts, treatment of myotubes with resveratrol resulted in increases in MnSOD and catalase protein content for up to 7 days following treatment with 1 $\mu$ M and 10 $\mu$ M of resveratrol. Treatment of myotubes with 0.1 $\mu$ M of resveratrol resulted in an increase in MnSOD protein content in myotubes for up to 3 days and catalase for up to 5 days. These data suggested that resveratrol is able to have a functional effect on myotubes.

Some would argue that the increases in antioxidant defence enzymes following a polyphenol treatment are predictable as polyphenols have been shown to react with components in cell culture media to produce hydrogen peroxide (Long *et al.*,

2010). However unpublished data from our lab and other data showed that the addition of resveratrol to the media used in these studies did not result in hydrogen peroxide production. Furthermore, this study demonstrated that treatment of myotubes with resveratrol led to a decrease in hydrogen peroxide production by myotubes (Figure 4.12). This is likely to be due to the increase in the antioxidant MnSOD and catalase protein content and suggests that the increases in MnSOD and catalase is a likely to be direct effect of the resveratrol treatment.

### **8.2.3 Effect of resveratrol on Sirt1 protein content in myoblasts and myotubes.**

Sirt1 is a deacetylase involved in silencing of gene transcription and has been shown to be the direct target of resveratrol in numerous tissues as well as in skeletal muscle (Price et al., 2012). It is through the increase in Sirt1 the activation of the mitochondrial master regulator PGC1- $\alpha$  is thought to occur. Despite the evidence for a role of Sirt1, the activation of Sirt1 following resveratrol treatment is highly debated (Beher et al., 2009; Pacholec et al., 2010). Therefore, the aim was to examine the effect of a treatment of resveratrol on Sirt1 protein content of rat primary skeletal muscle cells.

Treatment of myoblasts with 0.1, 1 and 10 $\mu$ M of resveratrol for up to 48H resulted in temporal increases in Sirt1 protein content that did not correlate with antioxidant defence enzyme protein content and was therefore inconclusive as to whether Sirt1 was involved in resveratrol- mediated effects on myoblasts.

Treatment of myotubes with 0.1, 1 and 10 $\mu$ M of resveratrol resulted in a decrease in Sirt1 protein content following 3 and 5 days of treatment. This was supported by data showing more acetylation of proteins in resveratrol treated myotubes than in untreated myotubes. These data support other studies that suggest Sirt1 is not a direct target for resveratrol (Pacholec et al., 2010). However, as Sirt1 has been shown to inhibit myogenesis (Fulco et al., 2003), the decreases in Sirt1 in resveratrol treated myotubes that were observed in this study also support a role for resveratrol as an initiator of early myogenesis. These data suggests that resveratrol may cause early myogenesis by decreasing Sirt1 activity and thus preventing the Sirt1 mediated inhibition of myogenesis (Rathbone et al., 2009).

#### **8.2.4 Hypertrophic effects of resveratrol on myotubes**

Hypertrophic and atrophic effects are studied in cell culture by measuring the diameter of myotubes, with increases in myotube diameter suggesting hypertrophic effects and decreases showing atrophic effects. Treatment of myotubes with 0.1, 1 and 10 $\mu$ M of resveratrol resulted in an increase myotube diameter following 3 days of treatment suggesting that resveratrol has hypertrophic effects in skeletal muscle. This is supported by other studies that have found hypertrophic effects (Montesano et al., 2013) and increases in force production (Dolinsky et al., 2012) following resveratrol treatment in skeletal muscle. Other studies have also shown that, as well as preventing the increase in proteasomal degradation, resveratrol is able to upregulate the Akt pathway (Wang *et al.*, 2014), which is responsible for hypertrophy in skeletal muscle.

This suggests a potential mechanism for the increase in myotube diameter seen in resveratrol treated myotubes. However this was not considered in the current study.

Treatment of myotubes with 10 $\mu$ M of resveratrol resulted in an increased myotube diameter treatment for up to 3 days and this effect was still seen following a 5 day treatment with 0.1 $\mu$ M and 1 $\mu$ M of resveratrol however any further treatment with resveratrol did not illicit any effects. The absence of any long term increases in myotube diameter following resveratrol treatment suggests that resveratrol does not maintain a hypertrophic response in skeletal muscle myotubes. However, it does further support the theory that resveratrol causes early myogenesis. It is possible therefore that resveratrol treated myotubes reach peak diameter earlier than control myotubes, and thus explains the absence of any differences in myotube diameter at later time points when myogenesis would be complete.

These data provide further evidence for resveratrol increasing myogenesis and also highlights the advantageous effects of the lower concentrations of resveratrol on skeletal muscle.

#### **8.2.5 Effect of IP10 on markers of atrophy in myotubes, role of resveratrol**

The increase in inflammation during ageing is well characterised and recently the chemokine IP10 has been shown to be a major contributor to this age-related increase in inflammation (Miles et al., 2008). Only a sparse amount of work has been carried out examining the effect of IP10 on muscle. Data in this thesis have shown that treatment of myotubes with doses of IP10 found in the old (200pg/ml) resulted in a

decrease in myotube diameter. In contrast, treatment of myotubes with IP10 at levels found in younger individuals (150pg/ml) had no major effect on myotube diameter. Pre-treatment of myotubes with resveratrol prevented this IP10-induced decrease in myotube diameter. These data suggest that the increase in levels of IP10 during ageing may contribute to skeletal muscle atrophy and therefore may play an important role in sarcopenia, particularly as data demonstrated more detrimental effects with more chronic treatment. A role for IP10 in the development of sarcopenia is further supported by the absence of atrophic effects on muscle using levels of IP10 found in the younger individuals.

It should be noted that it is possible that the ability of a resveratrol pre-treatment with IP10 in preventing the decrease in myotube diameter following treatment was due to the ability of resveratrol to increase myotube diameter and thus the myotubes may have a larger diameter at the time of IP10 treatment. However, this is unlikely, as no differences were seen in the diameter of myotubes treated with IP10 at lower concentrations found in the young with or without a pre-treatment with the same concentration of resveratrol.

Treatment of myotubes with IP10 resulted in temporal increases in the mRNA expression of the muscle specific ubiquitin ligase Atrogin1. This suggested an increase in proteasomal degradation as a mechanism responsible for the atrophic effects of IP10. This theory is further supported by the fact that a pre-treatment of myotubes with resveratrol prevented both the decrease in diameter and the increase in Atrogin1 mRNA expression caused by treatment of myotubes with IP10.

Data from this study showed that a pre-treatment of resveratrol followed by a 10 day treatment with IP10 at concentrations found in the young also resulted in an increase in Atrogin1 mRNA expression that was absent in myotubes treated with the lower level of IP10 alone. These data suggest that, at least in skeletal muscle, resveratrol supplementation of a healthy subject could be detrimental. It also highlights the possibility that resveratrol should only be used as a treatment for sarcopenia when there is a pre-existing condition and not as a preventative to retain skeletal muscle. Furthermore, these data support other studies that show the health status of the subject dictates the outcome of the resveratrol treatment (Lagouge et al., 2006) and that if no pre-existing condition is present, a treatment of resveratrol may be detrimental, at least in skeletal muscle.

#### **8.2.6 Effect of IP10 and resveratrol on muscle inflammation**

Treatment of myotubes with resveratrol alone led to anti-inflammatory effects with an increase in IL13 and IL2 and decreasing IL7. Treatment of myotubes with IP10 had only subtle effects on the inflammatory profile in the media of myotubes, treatment of myotubes with levels of 150pg/ml and 200pg/ml of IP10 surprisingly showing changes in both pro and anti-inflammatory cytokines. There was also no major difference in the effects of the two concentrations of IP10 on the cytokine profile in the media of myotubes. Similarly, pre-treatment of myotubes with resveratrol prior to IP10 treatment had both pro and anti-inflammatory effects; treatment with resveratrol was able to prevent some of the increases in pro-inflammatory cytokines however, pre-treatment with resveratrol also prevented some of the IP10-induced anti-inflammatory

effects in the media of myotubes. Although the data were relatively ambiguous, data provided no evidence for a major anti-inflammatory role of resveratrol in skeletal muscle and suggest that the beneficial effects seen in skeletal muscle following resveratrol treatment in Chapters 3-4 were not due to anti-inflammatory effects. Furthermore, the lack of differential effects with a treatment of IP10 at 150pg/ml or 200pg/ml suggest that the increases in IP10 during ageing are not a major regulator of the changes in the cytokine profile of skeletal muscle that occur with ageing.

Clustergram analysis of the cytokine content of the media of myotubes following treatment with IP10, suggested that MCP-1 and IL6 may be major regulators of skeletal muscle inflammation in this situation. Data also provided evidence that resveratrol affects myoblast proliferation by the clustering of IL13 and GRO-KC, as well as affecting IL7 content in the media of myotubes. Similarly to previous data in this study, clustergram data showed temporal effects, suggesting a potential role of the stage of myogenesis in the differences observed in cytokine production.

### **8.2.7 Effect of resveratrol on muscle function *in vivo***

Daily oral treatments of mice with 125mg resveratrol/kg/day for 14 days demonstrated that in contrast to *in vitro* studies, Sirt1 was upregulated *in vivo* following resveratrol treatment. Daily oral treatments of resveratrol for 14 days resulted in no significant effect on force generation in adult or old mice. The lack of statistical differences may be due to the lack of gross sarcopenia in old mice and furthermore, the low number of animals used in this pilot study. Therefore, future studies would utilise these data for a powered study if appropriate.

### 8.2.8 Resveratrol as a therapeutic compound for sarcopenia

Data have shown that resveratrol can be beneficial to skeletal muscle and can result in the upregulation of antioxidant defence enzymes to protect against increases in ROS, as well as a decrease in proteasomal degradation. This leads to an increase in myotube diameter of myotubes in culture as well as preventing the atrophic effects induced by an IP10. Furthermore, data demonstrated that resveratrol was able to increase proliferation of satellite cells and initiate early myogenesis, suggesting that it may be a good treatment for injured muscle or following muscle unloading. Functional measurements of the effect of resveratrol as a supplementation *in vivo* were disappointing in the pilot study and it is likely that a longer treatment is needed possibly with a more severe phenotype of sarcopenia, when inflammation is substantially increased to see more benefits.

*In vitro* data suggested that treatment with resveratrol may be detrimental to 'healthy' muscle; force measurements in young mice showed no suggestion of this. However treatment of adult mice with resveratrol resulted in a decrease in CSA, agreeing with cell culture data that resveratrol can be detrimental to skeletal muscle.

In conclusion this study showed that resveratrol has the potential to be an efficient treatment for the alleviation of some of the molecular changes that occur with sarcopenia. However, cell culture work suggested that further work is needed and it is likely that personalised doses of resveratrol may be required depending on the stage of sarcopenia.

### **8.3 Potential future studies**

These studies showed that resveratrol has the ability to alleviate IP10 induced atrophy and that increases in serum levels of IP10 may contribute to sarcopenia and thus propose resveratrol as a potential treatment for sarcopenia as well as IP10 as a potential therapeutic target. Several further experiments could be carried out to further test these findings.

Chapter 4 provided evidence that treatment of myotubes with resveratrol increases myotube diameter and further prevented the decrease in myotube diameter induced by concentrations of IP10 found in the old. Some studies propose that this is due to induction of hypertrophy whereas these data suggest that resveratrol initiates early myogenesis. Therefore the identification of changes in the Akt pathway and myogenesis regulatory factors following treatment of myotubes with IP10 treatment +/- resveratrol pre-treatment would identify whether resveratrol was able to cause hypertrophy or solely induce pre-mature myogenesis.

This study has suggested that resveratrol may have some beneficial effects in sarcopenic skeletal muscle however; data from Chapter 5 showed that lower levels of IP10 such as those seen in healthy adults together with a pre-treatment with resveratrol resulted in an increase in Atrogin1 in myotubes. Therefore, future experiments identifying the molecular effects of resveratrol on healthy muscle would identify any negative effects of such treatment and whether resveratrol could be used

as a treatment for sarcopenia or whether caution should be used when using resveratrol as a preventative measure.

Data showed that, in skeletal muscle, resveratrol had functional effects through the upregulation of antioxidant defence enzymes, MnSOD and catalase. As there is an age-related increase in the muscle content of these enzymes (Vasilaki et al., 2006), cell culture studies using siRNA to inhibit or the addition to overexpress these enzymes would confirm whether this is the mechanism through which resveratrol was functioning and would further allow identification of pathways that resveratrol may be functioning through. This would also identify whether resveratrol was able to further increase these enzymes when their muscle contents was already at elevated levels (Vasilaki et al., 2006).

Finally, although this thesis has shown that resveratrol can alleviate some of the molecular changes hypothesised to be involved in sarcopenia the study lacks clear measurements of muscle function. Therefore using the optimised 3D construct (myooid) treated with IP10 and resveratrol or its metabolites followed by measurement of force generation would allow identification of the effect of increasing levels of IP10 directly on skeletal muscle and therefore validate a role of IP10 in sarcopenia as well as the ability of resveratrol or other compounds on muscle function.

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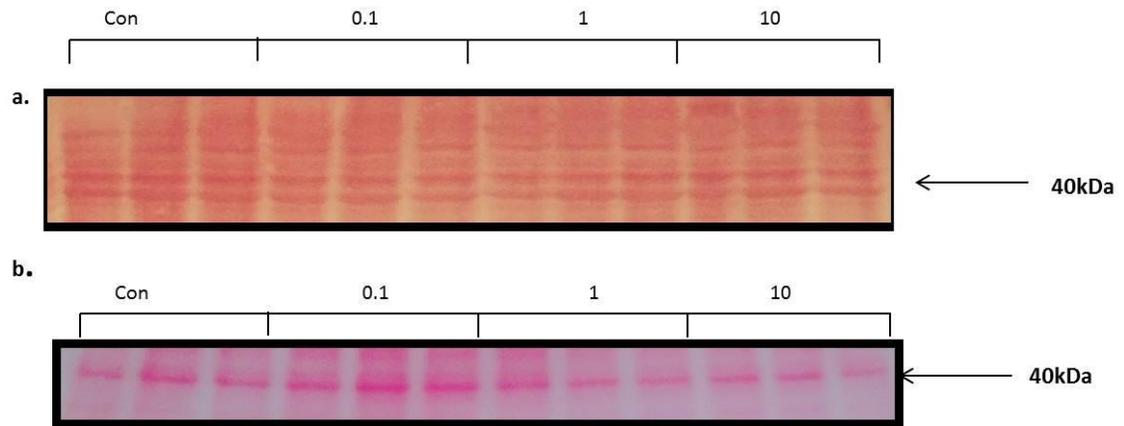
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## **10. APPENDIX**

## 10.1. Ponceau stain for western blotting

### 10.1.1. Three hour resveratrol treated myoblasts Ponceau S

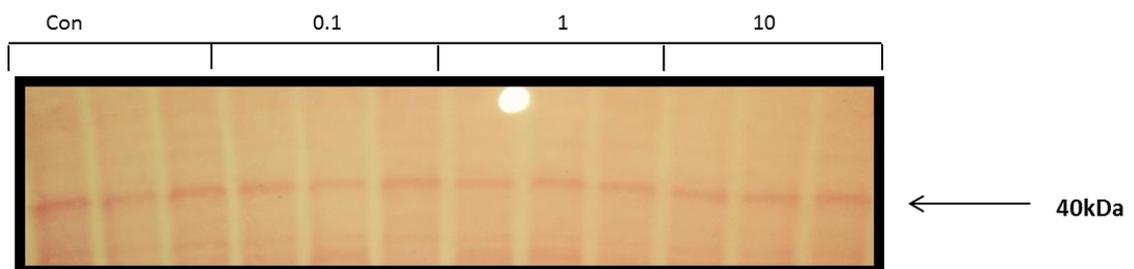
Ponceau S (Sigma Aldrich, Dorset, UK) staining for normalisation for western blot analysis of MnSOD, catalase and Sirt1 protein content at 3H following resveratrol treatment (Figure 3.8, Figure 3.10 and Figure 3.12) is shown in Figure 10.1.



**Figure 10.1** Ponceau S staining for normalisation for (a) MnSOD and catalase and (b) Sirt1 content at following 3H resveratrol treatment.

### 10.1.2. Six hour resveratrol treated myoblasts Ponceau S

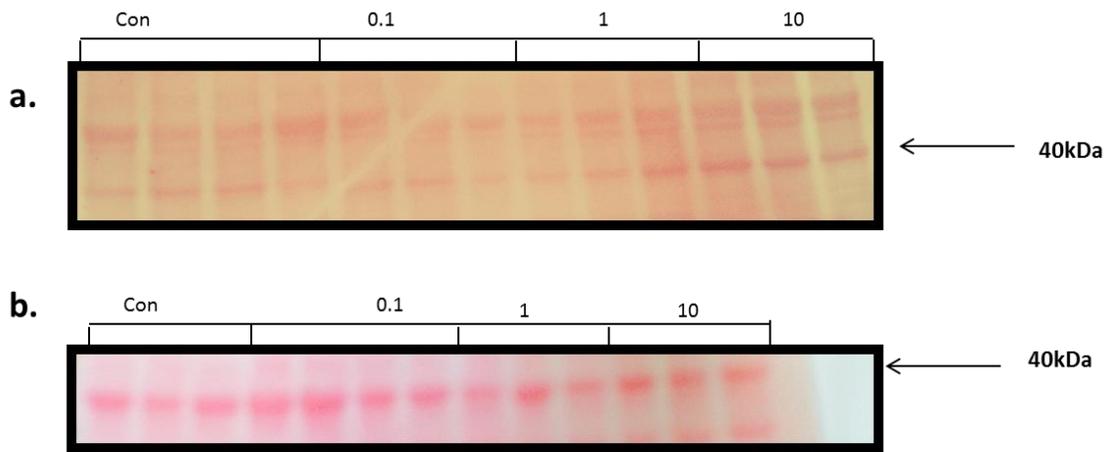
Ponceau S (Sigma Aldrich, Dorset, UK) staining for normalisation for western blot analysis of MnSOD, catalase and Sirt1 protein content at 6H following resveratrol treatment (Figure 3.8, Figure 3.10 and Figure 3.12) is shown in is shown in Figure 10.2



**Figure 10.2** Ponceau S staining for normalisation for MnSOD, catalase and Sirt1 protein content following 6H resveratrol treatment.

### 10.1.3. Twelve hour resveratrol treated myoblasts Ponceau S

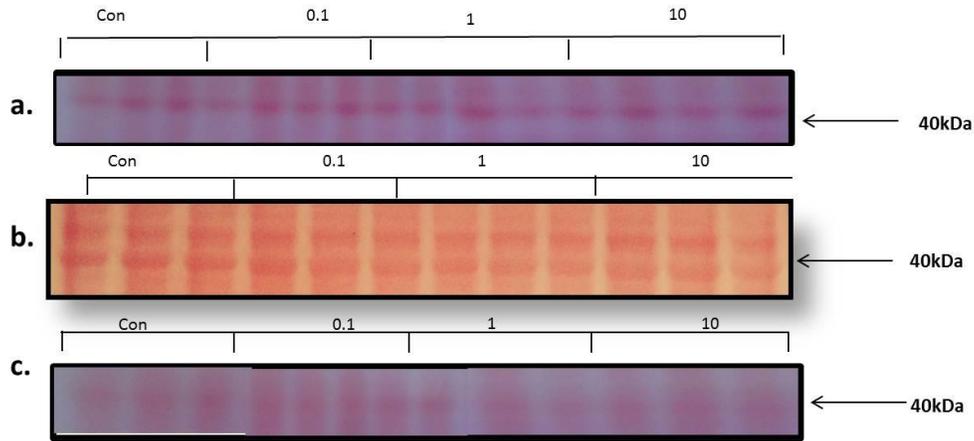
Ponceau S (Sigma Aldrich, Dorset, UK) staining for normalisation for western blot analysis of MnSOD, catalase and Sirt1 protein content following 12H resveratrol treatment (Figure 3.8, Figure 3.10 and Figure 3.12) is shown in is shown in Figure 10.3



**Figure 10.3 Ponceau S staining for normalisation for (a) MnSOD, catalase and (b) Sirt1 protein content following 12H resveratrol treatment.**

### 10.1.4. Twenty four hour resveratrol treated myoblasts Ponceau S

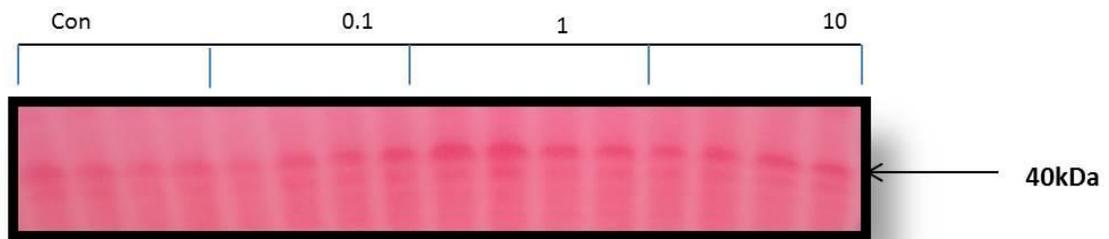
Ponceau S (Sigma Aldrich, Dorset, UK) staining for normalisation for western blot analysis of MnSOD, catalase and Sirt1 protein content following 24H resveratrol treatment (Figure 3.8, Figure 3.10 and Figure 3.12) is shown in is shown in Figure 10.4.



**Figure 10.4 Ponceau S staining for normalisation for (a) MnSOD, (b)catalase and (c) Sirt1 protein content following 24H resveratrol treatment.**

#### **10.1.5. Forty eight hour resveratrol treated myoblasts Ponceau S**

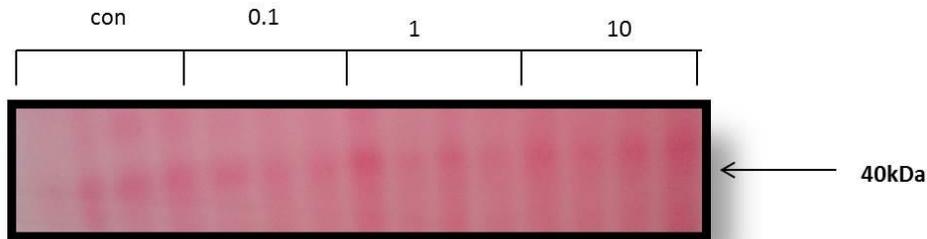
Ponceau S (Sigma Aldrich, Dorset, UK) staining for normalisation for western blot analysis of MnSOD, catalase and Sirt1 protein content following 48H resveratrol treatment (Figure 3.8, Figure 3.10 and Figure 3.12) is shown in is shown in Figure 10.5.



**Figure 10.5 Ponceau S staining for normalisation for MnSOD, catalase and Sirt1 protein content following 48H resveratrol treatment.**

### 10.1.6. Three day resveratrol treated myotube Ponceau S

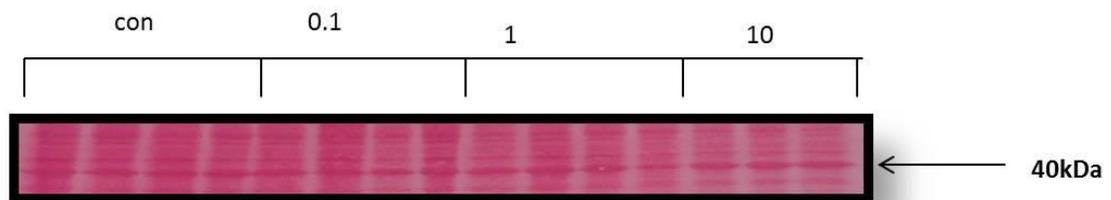
Ponceau S (Sigma Aldrich, Dorset, UK) staining for normalisation for western blot analysis of MnSOD, catalase and Sirt1 protein content following 3 day resveratrol treatment (Figure 4.6, Figure 4.7 and Figure 4.8) is shown in is shown in Figure 10.6.



**Figure 10.6** Ponceau S staining for normalisation for MnSOD, catalase and Sirt1 protein content following 3 day resveratrol treatment.

### 10.1.7. Five day resveratrol treated myotube Ponceau S

Ponceau S (Sigma Aldrich, Dorset, UK) staining for normalisation for western blot analysis of MnSOD, catalase and Sirt1 protein content following 5 day resveratrol treatment (Figure 4.6, Figure 4.7 and Figure 4.8) is shown in is shown in Figure 10.7.



**Figure 10.7** Ponceau S staining for normalisation for MnSOD, catalase and Sirt1 protein content following 5 day resveratrol treatment.

### 10.1.8. Seven day resveratrol treated myotube Ponceau S

Ponceau S (Sigma Aldrich, Dorset, UK) staining for normalisation for western blot analysis of MnSOD, catalase and Sirt1 protein content following 7 day resveratrol treatment (Figure 4.6, Figure 4.7 and Figure 4.8) is shown in is shown in Figure 10.8.

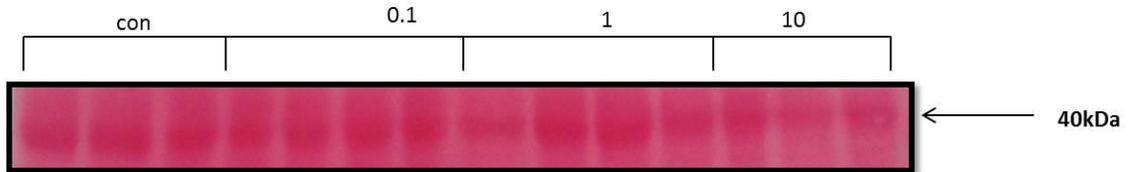


Figure 10.8 Ponceau S staining for normalisation for MnSOD, catalase and Sirt1 protein content following 7 day resveratrol treatment.

### 10.2. Effect of resveratrol and IP10 treatment on Murf1 expression in myotubes

The effect of a 24H 150pg/ml and 200pg/ml of IP10 with and without a resveratrol treatment on Murf1 mRNA levels is shown in Figure 10.9.

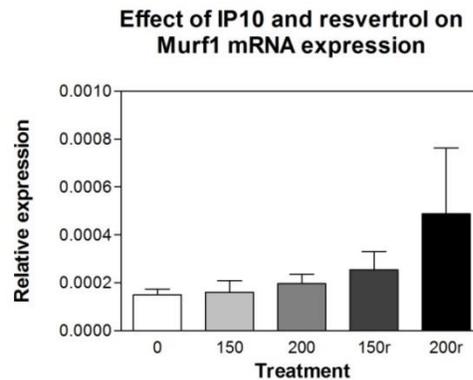


Figure 10.9 Effect of IP10 and resveratrol on Murf1 mRNA levels following a 1 day IP10 treatment +/- a 7 day resveratrol pre-treatment.