INVESTIGATING NOVEL THERAPEUTIC APPROACHES FOR SPORADIC INCLUSION BODY MYOSITIS (sIBM)

Mhoriam Ahmed Bsc (Hons)

University College London, Institute of Neurology

&

MRC Centre for Neuromuscular Diseases

PhD Supervisor:

Professor Linda Greensmith

A Thesis submitted for the degree of Doctor of Philosophy

University College London 2012

DECLARATION

I, Mhoriam Ahmed confirm that the work presented in this Thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the Thesis.

Signed……

ABSTRACT

Sporadic inclusion body myositis (sIBM) is the most common acquired muscle disease affecting adults over the age of 50. Although the aetiology of this disease remains unclear, there is evidence for both inflammatory and myodegenerative processes in sIBM muscle pathology. In particular, abnormal protein aggregation is characteristic of affected muscle, with inclusion bodies incorporating amyloid-beta precursor protein (β-APP) among many others.

Therapeutic interventions tested to date for sIBM have targeted the immune system; but none have been beneficial and sIBM currently remains untreatable. In this study, an *in vitro* model of the degenerative pathology seen in sIBM was established by over-expressing β-APP in primary muscle cultures. This resulted in the formation of inclusion bodies immuno-reactive for β-APP and other sIBM-relevant proteins, as well as increased cytotoxicity, proteasome dysfunction, mitochondrial abnormalities and TDP-43 mis-localisation; all observed in sIBM patient muscle.

The heat shock response (HSR) is an acute endogenous cytoprotective mechanism that responds to misfolded proteins. Up-regulation of the HSR was examined by treatment with Arimoclomol, a co-inducer of the HSR, which showed beneficial effects in this *in vitro* model of IBM by significantly improving cell survival and attenuating cellular pathology.

Since proteasome dysfunction has been implicated in sIBM pathology, I also examined the effects of pharmacological inhibition of the proteasome on muscle cells in culture. Proteasome inhibition did not result in the appearance of several key features of sIBM, suggesting that this is not a suitable approach to modeling sIBM. However, treatment with Arimoclomol was seen to significantly improve proteasome function and cell survival in these experiments.

Using the β-APP model, eight novel pharmacological agents, with known anti-aggregation properties, were subsequently screened and one agent was found to significantly ameliorate the disease outcomes established in this model.

The results of this Thesis show that β-APP over-expression *in vitro* recapitulates many of the characteristic features of sIBM and can be used successfully to screen potential therapies. In particular, Arimoclomol and one novel agent have been identified as potential therapeutic agents for IBM.

ACKNOWLEDGEMENTS

It seems the hardest part of writing this thesis is being able to sufficiently express my gratitude to all those who made it possible. Firstly, I would like to thank my primary supervisor Professor Linda Greensmith for shaping my scientific mind though her support, constructive criticism and encouragement. Many thanks also to Adrian Miller for all your help and advice over the years that we have been 'Team-IBM'. Special thanks to Dr Jenny Morgan, my secondary supervisor, Professor Michael Hanna and Professor Mary Reilly for your advice and guidance, and giving me the opportunity to work in this prestigious institution.

Also thank you to Ralph Andre for teaching me new lab techniques, Alec Smith for help with the muscle cultures and Janice Holton for the muscle sections.

This PhD would not have been half as enjoyable or successful if it were not for the wonderful people in the Greensmith Lab. Thank you all for your help both in the lab, and at times, in my personal life. In particular this thank you goes to Amy Innes for her constant friendship during the ups and downs of the whole PhD process.

Lastly, I would like to thank my amazing family, especially my parents, Sulaman and Shahida Ahmed and my husband Aahsan, for their unyielding love and support, and for putting up with my long hours. I am finally finished and hope that I have made you proud. Jazakallah Khair.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

LIST OF TABLES

PAGE

LIST OF ABBREVIATIONS

° C	**Degrees Celsius**
Aβ	**Amyloid-beta**
AD	**Alzheimer's disease**
ADP	**Adenosine diphosphate**
ALS	**Amyotrophic lateral sclerosis**
ANOVA	**Analysis of variance**
ATP	**Adenosine triphosphate**
β-APP	**Beta amyloid precursor protein**
BACE 1	**Beta-site APP cleaving enzyme 1**
CEE	**Chick embryo extract**
CFTCR	**Cystic fibrosis transmembrane conductance regulator**
CK	**Creatine kinase**
COX	**Cytochrome oxidase**
DAPI	**4, 6-diamidino-2-phenylindole**
DIV	**Days in vitro**
DM	**Dermatomyositis**
DMEM	**Dulbecco's modified Eagle's medium**
DNA	**Deoxyribonucleic acid**
DPX	**Di-n-butyl Phthalate in Xylene**
EDL	**Extensor digitorum Longus**
EDTA	**Ethylenediaminetetraacetic acid**
EMG	**Electromyograph**

ER	**Endoplasmic reticulum**
ERAD	**Endoplasmic reticulum associated degradation**
EV	**Empty vector**
FACS	**Florescent activated cell sorting**
FCS	**Foetal calf serum**
GFP	**Green fluorescent protein**
HD	**Huntingdon's disease**
hIBM	**Hereditary inclusion body myositis**
HLA	**Human leukocyte antigen**
HSE	**Heat shock element**
HSF	**Heat shock factor**
HSP	**Heat shock protein**
HSR	**Heat shock response**
IBM	**Inclusion body myositis**
IBMPFD	**Inclusion body myositis with Paget's disease and Frontotemporal Dementia**
IFN-γ	**Interferon-gamma**
IkB	**Inhibitor of NFkB**
IL-1β	**Interleukin-1β**
IVIG	**Intravenous immunoglobulins**
kDa	**Kilo Dalton**
LDH	**Lactate dehydrogenase**
MACS	**Magnetic associated cell sorting**
MHC	**Major histocompatibility complex**
mRNA	**Messenger RNA**

MTOC	**Microtubule organising centre**
NCAM	**Neural cell adhesion molecule**
NFkB	**Nuclear factor kappa-B**
NHS	**Normal horse serum**
NMJ	**Neuromuscular junction**
PE	**Phycoerythrin**
PBS	**Phosphate buffer saline**
PD	**Parkinson's disease**
PM	**Polymyositis**
RNA	**Ribonucleic acid**
SDH	**Succinate dehydrogenase**
SDS-PAGE	**Sodium dodecyl sulphate polyacrylamide gel electrophoresis**
SEM	**Standard error of the mean**
sIBM	**Sporadic inclusion body myositis**
SOD1	**Superoxide dismutase**
TDP-43	**TAR DNA-binding protein 43**
TNF-α	**Tumour necrosis factor-alpha**
UPR	**Unfolded protein response**
UPS	**Ubiquitin proteasome system**
VCP	**Valosin containing protein**
WT	**Wild type**

CHAPTER 1.

GENERAL INTRODUCTION

1.1. INTRODUCTION: SPORADIC INCLUSION BODY MYOSITIS (SIBM)

Sporadic Inclusion Body Myositis (sIBM) is the most common acquired inflammatory myopathy predominantly affecting men over the age of 50 (Askanas & Engel 2001; Askanas et al. 2009). The prevalence of this condition has been estimated to be between 4.9-10.7 per million people (Phillips et al. 2000; Badrising et al. 2000; Needham & Mastaglia 2007); however there is variation in occurrence between different ethnic groups. The highest frequency has been found among North American, white Australian and northern European Caucasian populations (Needham et al. 2008).

The first account of sporadic inclusion body myositis (sIBM) was presented in 1967 when Chou described the presence of 'intracytoplasmic aggregates' in the muscle biopsy of a patient with a chronic inflammatory myopathy called polymyositis (PM) (Chou 1967; Needham & Mastaglia 2007). Although these aggregates were originally considered to be virus-like structures, they were later defined as proteinaceous inclusion bodies (Sato et al. 1969). Shortly after Yunis and Samaha (1971) coined the term 'Inclusion Body Myositis', after discovering the presence of sarcoplasmic inclusion bodies in light microscopic studies of patient muscle (Yunis & Samaha 1971). IBM became formally established as a separate disease to the other inflammatory myopathies after Carpenter et al (1978) reported on 14 patients with the condition.

This idiopathic condition is slowly progressive yet highly debilitating. The disease affects both proximal and distal muscles with a predilection for atrophy and weakness in finger and wrist flexors, as well as the quadriceps (Engel & Askanas 2006; Greenberg 2010) (see **Figure 1.1**). Other muscles which may be involved, albeit to a lesser degree, include the gluteus maximus, biceps and triceps (Engel & Askanas 2006). Lower limb involvement often causes foot-drop, which increases the propensity of trips and falls (Engel & Askanas 2006; Needham & Mastaglia 2007).

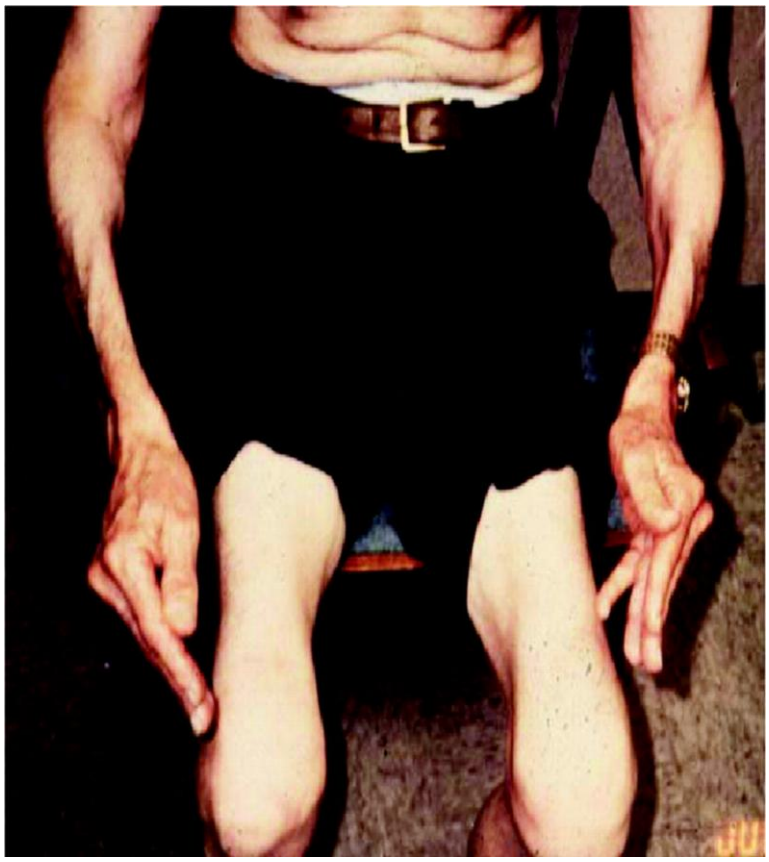


Figure 1.1 Image of a sIBM patient

A patient with sIBM presenting with severe atrophy of the forearm flexors and quadriceps. Taken from Engel W K and Askanas V, Neurology 2006

In addition, up to 40% of patients also experience dysphagia as pharyngeal muscles can become affected, making swallowing food difficult and potentially dangerous due to the increased risk of aspiration (Dabby et al. 2001; Cox et al. 2009; Dalakas 2010a). Although not fatal, sIBM severely affects manual dexterity and significantly impairs mobility, leading to walking aid requirement, usually within 5 years, and often wheelchair confinement within 10 years of disease onset (Needham & Mastaglia 2007; Weihl & Pestronk 2010). To date however, a universally accepted, effective treatment for sIBM has not been identified. **Figure 1.1** shows an image of a sIBM patient with clear atrophy of the forearm flexors and quadriceps (image from Engel and Askanas, Neurology 2006).

1.2. Genetic considerations

A clear genetic component to sIBM has not been defined, making it difficult to ascertain the pathology and to model the disease *in vivo*. However, it is possible that a particular combination of genes and environmental exposure may make an individual more vulnerable to the disease. Genetic susceptibility to sIBM has been very strongly linked to certain haplotypes, namely Human Leukocyte Antigen (HLA) -DR3 and the ancestral haplotype Major Histocompatibility Complex (MHC) 8.1 in Caucasian populations (Mastaglia 2005; Machado et al. 2009a). The HLA-DR3 haplotype has been found present in approximately 75% of sIBM patients (Needham & Mastaglia 2007; Needham et al. 2007). Other ancestral haplotypes have also been associated with sIBM in certain ethnic groups such as HLA-B and HLA-DRB1 in a Japanese study (Scott et al. 2006). These strong HLA and MHC associations make a case for sIBM being primarily more inflammatory than degenerative in nature (Dalakas 2006a), although an antigen that would trigger such an inflammatory response has not been resolved.

Hereditary forms of inclusion body myopathy (hIBM) do however exist, and are a mixture of autosomal dominant and recessive conditions (Needham & Mastaglia 2007). These include pathogenic mutations in the UDP-N-acetylglucosamine-2-epimerase/ N-acetylmannosamine kinase (GNE) gene for a bifunctional enzyme involved in the synthesis

of sialic acid (Malicdan et al. 2007). Hereditary IBM (hIBM) affects both proximal and distal muscles but is 'quadriceps sparing' (Needham et al. 2007), in contrast to sIBM which has high specificity for this muscle. The clinical manifestation of weakness in hIBM progresses over 10-20 years. This is similar to sIBM, but onset is significantly earlier, occurring from 15-40 years of age (Huizing & Krasnewich 2009). Histology of hIBM muscle shows signs of myofibre degeneration with the presence of vacuolated and necrotic fibres, occasional amyloid deposits and some nuclear and sarcoplasmic inclusion formation (Malicdan et al. 2007). However, unlike sIBM there is no evidence of inflammation in the histology of hIBM patients (Ranque-Francois et al. 2005), with no up-regulation of MHC class I expression and absence of lymphocytes. sIBM and hIBM are therefore considered to be distinct disorders.

Another presentation of hIBM is observed in patients with mutations in the gene for valosin containing protein (VCP [p97 in mouse]), a ubiquitously expressed protein of the AAA+ (ATPase) protein family (Ju et al. 2009). Mutations in this gene give rise to a multisystem disorder called "Inclusion Body Myopathy with Paget's Disease of the bone, and Frontotemporal Dementia (IBMPFD)" (Custer et al. 2010). In this disorder some of the characteristic features of sIBM are recapitulated in patient muscle such as the presence of rimmed vacuoles and sarcoplasmic inclusions, but muscle weakness and atrophy is more generalised (Badadani et al. 2010). Paget's Disease affects 49% of IBMPFD patients with bone deformities caused by excessive osteoclastic activity and Frontotemporal Dementia is seen in 27% of IBMPFD patients (Badadani et al. 2010).

1.3. Other inflammatory myopathies

sIBM belongs to a group of muscle disorders called inflammatory myopathies which also include dermatomyositis (DM) and polymyositis (PM). Dermatomyositis is distinguished primarily by the heliotrope rash found in various parts of the body and manifests in patients of all age groups (Dalakas 2010a). Polymyositis is a very rare inflammatory myopathy which has unclear clinical features and remains, to some extent, a diagnosis of

exclusion (Dalakas 2010a). Apart from the inflammation in muscle, the histology of dermatomyositis and polymyositis is quite different to sIBM, with no rimmed vacuoles, no amyloid deposits and no increase in the number of cytochrome oxidase (COX) negative fibres (Dalakas 2010a). Furthermore, both of these myopathies show an improvement with immuno-therapy unlike sIBM (Dalakas 2010a) where conventional immuno-therapies such as intravenous immunoglobulins (IVIG) and steroid treatment have not shown evidence of attenuating the pathology (Dalakas 2010b).

1.3.1. Diagnosis of sIBM

Inflammatory myopathies have increasingly been found to have overlapping features with other neurological disorders such as the fatal neurodegenerative disease Amyotrophic Lateral Sclerosis (ALS), which affects both upper and lower motor neurons, resulting in muscle atrophy and paralysis. This can sometimes lead to diagnostic confusion (Ludolph & Knirsch 1999; Dabby et al. 2001; Ryan et al. 2003). The diagnostic criteria for sIBM takes into account clinical presentation, histological findings and laboratory data. Characteristically patients present with a typical pattern of muscle weakness and atrophy and have a muscle biopsy demonstrating inflammation and degeneration. In addition serum creatine kinase (CK) levels are moderately elevated (Needham & Mastaglia 2007), generally at less than 12 times normal (Tawil & Griggs 2002). Electrophysiologic investigations such as electromyographs (EMG) are often additional diagnostic measures used which show readings of general myopathy (Dabby et al. 2001; Blijham et al. 2006; Hatanaka & Oh 2007). EMG is not sensitive enough to differentiate between inflammatory and other types of myopathies, however is useful in eliminating neurogenic disorders (Dalakas 2010a).

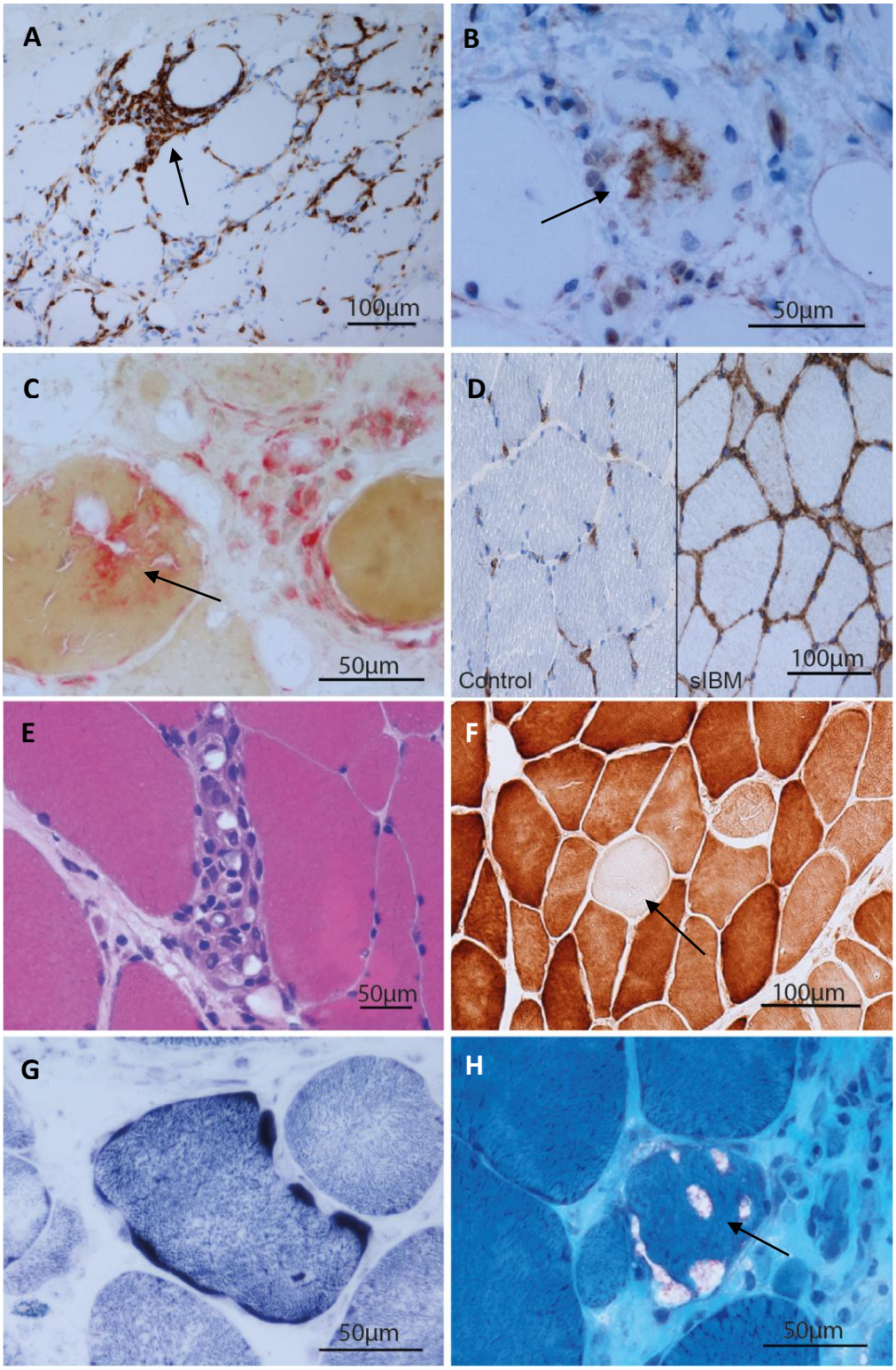
1.4. Histopathology of sIBM

Histopathological analysis of muscle from sIBM patients is a crucial diagnostic tool to differentiate sIBM from the other two inflammatory myopathies. All three myopathies show an influx of inflammatory cells including CD8+ T-cells and macrophages in affected muscle, as well as the up-regulation of MHC Class I (Dalakas 2010a). In addition, general features of muscle disease such as central nuclei and myofibre atrophy are present. However, sIBM is unique in that it also features intracellular protein aggregation, vacuolation within myofibres and increased cytochrome oxidase (COX) negativity compared to aged matched controls. COX negativity and the presence of 'ragged red' fibres in sIBM fibres following SDH staining suggest mitochondrial dysfunction. The presence of a nuclear protein called TDP-43 in the sarcoplasm has recently become an additional marker of sIBM (Verma & Tandan 2009). **Figure 1.2** gives examples of some typical histological features found in sIBM muscle.

Morphological changes are often more obvious in sIBM muscle biopsy than internal cellular abnormalities such as rimmed vacuoles and inclusion bodies, which are sparse and may be missed. sIBM affected muscles contain muscle fibres of varied sizes instead of more uniform dimensions. This is a combination of both atrophied fibres, generally seen as those with a smaller caliber than normal, and hypertrophic fibres, which possibly arise from the development of compensatory work by the muscle (Verma et al. 1992). Additionally angulated fibres may also be present as well as an increase in endomysial connective tissue (Griggs et al. 1995a). Necrotic fibres are also generally easy to identify in histological slides with cells looking highly deformed with oedema and swollen organelles.

Figure 1.2 Histopathological features of sIBM muscle

Images of the typical histopathological features of sIBM muscle. [A] CD8+ T-cell infiltration. [B] Cytoplasmic accumulation of TDP-43. [C] Macrophage infiltration into myofibres. [D] Upregulation of MHC Class I in sIBM muscle compared to healthy control. [E] Necrotic fibre seen with Hematoxylin and Eosin stain (H&E). [F] COX negative fibre. [G] Enhanced mitochondrial accumulation in a myofibre seen with SDH stain. [H] Cytoplasmic rimmed vacuoles seen with Trichrome-Gomori stain. (Sections courtesy of Dr J Holton UCL Institute of Neurology)


A
100µm
B
50µm
C
50µm
D
Control
sIBM
100µm
E
50µm
F
100µm
G
50µm
H
50µm

1.5. Inflammatory changes in sIBM

Typical sIBM muscle biopsies show an infiltration of macrophages and cytotoxic (CD8+) T-cells which surround myofibres at multiple foci within the endomysial parenchyma (Dalakas 2010a). Interestingly it has been noted that inflammatory cells encircle myofibres which do not show presence of sarcoplasmic inclusions, vacuoles or signs of necrosis (Askanas & Engel 2007).

Macrophages and lymphocytes are more commonly found near myofibres which look relatively 'healthy' on a biopsy. In addition, a key feature of the inflammatory changes is the up-regulation of MHC Class I. In muscle this antigen is normally present at low levels, particularly in myofibres targeted by inflammation (Karpati & O'Ferrall 2009).

These inflammatory cells have also been shown to express proinflammatory cytokines and chemokines such as interferon-γ, tumor necrosis factor-α and interleukin-1β as they also do in polymyositis (Karpati & O'Ferrall 2009; Greenberg 2009a).

1.6. Degenerative changes in sIBM

Immunohistochemical analysis of sIBM muscle reveals the abnormal accumulation of proteins in the form of aggregates of known proteins or less defined inclusion bodies within muscle fibres (Askanas & Engel 2007; Karpati & O'Ferrall 2009). This characteristic feature of sIBM muscle is strikingly similar to that observed in Alzheimer's Disease (AD), where protein aggregates containing amyloid form plaques in the brain (Askanas & Engel 2007). Both of these progressive diseases show evidence of cellular oxidative stress and predominantly affect individuals over the age of 50, suggesting that aging may be a factor. However, sIBM and AD remain highly specific for their respective tissue such that sIBM patients, unlike IBMPFD patients, do not have dementia, and AD patients do not show evidence of a muscle disorder (Askanas & Engel 2007).

A wide range of proteins are abnormally present in sIBM muscle fibres including: beta amyloid precursor protein (β-APP), amyloid beta (Aβ); phosphorylated tau (pTau); often in the form of paired helical filaments (Askanas et al. 2009), α-synuclein; α-B crystallin; presenilin-1; apolipoprotein E; α-chymotrypsin; ubiquitin; HSP70; HSP40; TDP-43 and prion protein (Askanas & Engel 2006; Askanas & Engel 2007; Karpati & O'Ferrall 2009; Verma & Tandan 2009; Machado et al. 2009a). In a large study up to 70-80% of vacuolated fibres were found to contain sarcoplasmic inclusions containing a combination of these proteins (Askanas & Engel 2006). Some of these aggregates were also seen in several non-vacuolated fibres.

Most frequently the presence of Aβ and its precursor protein β-APP have been reported and it has been postulated that over-expression of these aggregation-prone proteins or their abnormal proteolysis may be a key feature of the pathology of sIBM (Askanas & Engel 2006; Askanas & Engel 2007). This possibility however, remains controversial.

Intracellular amyloid deposits were first described in muscle in 1991 after biopsy specimens from IBM patients were found to be positive for amyloid filaments as determined by Congo red staining (Mendell et al. 1991). This was later confirmed using Thioflavin-S and crystal violet staining (Askanas et al. 1993; Griggs et al. 1995b). The term 'amyloid' generally refers to congophilic β-pleated sheets of aggregating proteins in an insoluble structure, and should not be confused with Aβ or β-APP which are found as part of multi-protein inclusion bodies (Askanas et al. 2009; Greenberg 2010).

In addition to sarcoplasmic inclusions, sIBM muscle may also contain sarcoplasmic and/or myonuclear tubular filaments, 15-18nm in size, as visualized by electron microscopy (Figarella-Branger et al. 1992; Griggs et al. 1995a), which may be used as a diagnostic feature even in the absence of vacuolated fibres and amyloid deposits.

The presence of sarcoplasmic vacuoles are commonly used as part of the diagnosis of sIBM, however these may not be present in all biopsy specimens and are not specific for

sIBM. Vacuoles are also present in hIBM and other inherited myopathies. The vacuoles in sIBM are often 'rimmed' by a reddish material (indicating lipoproteins at the membrane), however no single protein has yet been defined to be present at the perimeter of these vacuoles (Weihl & Pestronk 2010). Furthermore, the vacuoles do not contain any specific proteins or the characteristic sIBM inclusion bodies (Askanas et al. 2009), but may be observed with or without eosinophilic masses (Karpati & O'Ferrall 2009; Weihl & Pestronk 2010) in biopsy sections. It has been suggested that some of these vacuoles may be lysosomal, since autophagy has been shown to be impaired in sIBM and an up-regulation of the autophagosome marker LC3-II has been found (Nogalska et al. 2010b). Furthermore, lysosomal enzymes such as clathrin are present inside some vacuoles and increased in the sarcoplasm of atrophied fibres (Kumamoto et al. 2004; Askanas et al. 2009).

Recent research into the identification of a biomarker for sIBM has found that other molecules are abnormally present in diseased muscle tissue. For example, the nuclear protein TAR DNA Binding Protein (TDP-43) - a protein known to be involved in post-translational regulation of RNA, has been demonstrated to be abnormally present in the sarcoplasm of sIBM myofibres, either dispersed throughout the cell or as part of an inclusion body (Salajegheh et al. 2009; Verma & Tandan 2009) . Indeed this protein has been identified as one of the most common biomarkers in sIBM with one group finding that 23% of sIBM muscle cells expressing TDP-43 in the sarcoplasm (Salajegheh et al. 2009) and another reporting up to 78% of fibres with TDP-43 inclusions (Weihl et al. 2008).

Another protein which has been shown to be up-regulated in sIBM muscle is p62 (sequestosome 1). This shuttle protein is involved in proteasomal and lysosomal degradation and has been found to be present abnormally in approximately 80% of vacuolated sIBM fibres and 20-25% of non-vacuolated fibres (Nogalska et al. 2009a).

1.7. Pathogenesis of sIBM

The aetiology of sIBM remains elusive. Although the cause of this muscle disease has not been resolved, it is generally accepted that the pathology of sIBM is complex, with possible genetic, environmental and aging factors all playing a role and interacting to bring about the disease (Needham & Mastaglia 2007).

There is much debate as to whether sIBM pathology is primarily caused by an inflammatory response leading to myofibre degeneration or if myofibre degeneration induces an inflammatory response. sIBM is widely considered to be an inflammatory disorder due to the presence of mononuclear cell inflammation (Askanas & Engel 2007). However, many studies have provided evidence for at least a partly myodegenerative process in sIBM muscle (Sugarman et al. 2006).

1.7.1. Inflammation as the primary trigger of sIBM

Muscle inflammation is a key feature of sIBM and is the focal area of research for many groups investigating this disease (Mastaglia et al. 1998; Greenberg 2009a; Dalakas 2010b). However, whether inflammation has any correlation to the muscle degeneration in this myopathy is unclear.

One theory for inflammation being the primary causative factor of sIBM suggests that it is the result of a latent reaction to a viral infection or another pathogen, which triggers an immune reaction (Dalakas 2006a). Such an immune response would begin by inducing clonal expansion of CD8+ cytotoxic T-cells and release of cytokines and chemokines. The inflammatory factors can then instigate the up-regulation of MHC Class I which presents 'self' antigens to the T-cells. As mentioned above, both T-cell expansion and MHC class I up-regulation is observed in sIBM muscle tissue. However, with the increased processing of MHC class I, endoplasmic reticulum (ER) stress can be induced, leading to accumulation of misfolded proteins (Nogalska et al. 2007a). Cytokine release and ER stress both lead to

the activation of nuclear factor kappa-B (NFκB), a transcription factor which regulates T-cell development, maturation and proliferation (Dalakas 2010a). T-cells target the MHC Class I presenting cells and mediate a cytotoxic process using the perforin pathway. This cell would then become necrotic (Dalakas 2006a).

NFκB normally resides in its inactive form in the cytoplasm bound to a protein called Inhibitor of NFκB (IkB) (Bakkar & Guttridge 2010). When a cell signal is transduced, IκB becomes phosphorylated, thereby releasing an active form of the transcription factor which translocates to the nucleus. NFκB has been shown to be present in up to 70% of vacuolated sIBM muscle fibres from a study of 8 sIBM patients (Yang et al. 1997). These experiments, using immunocytochemistry and immunoelectronmicroscopy, showed cytoplasmic localisation of different subunits of the transcription factor in necrotic fibres and normal neuromuscular junctions (Yang et al. 1998). This suggests that each subunit may play a number of roles in physiology and possibly sIBM pathology.

A viral link with sIBM was suggested after publication of case reports of patients with human immunodeficiency viral (HIV) infections who had developed sIBM (Cupler et al. 1996). This increased pathology did not appear to be the result of anti-retroviral therapy, but was rather due to the clonal expansion of specific T-cells which are detrimental to the muscle (Cupler et al. 1996; Dalakas et al. 2007). However, not all patients with HIV develop sIBM and no specific pathogen has yet been established as common among sIBM patients.

Dalakas et al. (2010b) have shown that exposure of muscle cells to inflammatory factors such as INF-γ and IL-β can lead to the over-expression of β-APP (Schmidt et al. 2008), a protein commonly found to be abnormally present in sIBM muscle. This group proposes that a longstanding exposure to inflammatory factors may increase the basal levels of β-APP, which can in turn trigger the release of more inflammatory mediators, creating a self-perpetuating cycle leading to myofibre degeneration.

Despite the evidence suggesting a primary role of inflammation in sIBM, therapies targeted towards the immune system are not as beneficial in sIBM as they are in PM or DM. However, together with the HLA haplotypic associations and associations with other autoimmune disorders, as well as the inflammatory response seen in patients, there is strong evidence that sIBM may well primarily be an inflammatory disorder (Dalakas 2006a).

1.7.2. Degeneration as the primary trigger of sIBM

The presence of several degenerative features in sIBM muscle such as necrotic fibres, TDP-43 mislocalisation, vacuoles and protein aggregates, suggests that a non-immune mechanism may be the primary cause of this disease. Furthermore, it is also possible that degeneration of muscle fibres may subsequently result in the stimulation of an immune response, which may act as described above to cause some of the key inflammatory characteristics of sIBM.

Although the agent or event which may trigger a degenerative cascade remains unknown, it has been proposed that aging may be a significant contributing factor, especially given that sIBM parallels AD in many pathological features.

In cells, accumulations of macromolecules within the sarcoplasm often become toxic and consequently put pressure on ER function, the ubiquitin-proteasome system (UPS) and autophagy (Askanas & Engel 2005a; Nogalska et al. 2007a). Disruption of these three key cellular functions may lead to the pathological changes seen in sIBM muscle. For example, ER stress triggers activation of NFκB which in turn can initiate an immune response via MHC Class I up-regulation (Loell & Lundberg 2010). Dysfunctional UPS increases protein accumulation which can lead to muscle degeneration and atrophy (Kopito 2000). Furthermore abnormal autophagy may result in the formation of large vacuoles that are observed within the sarcoplasm of sIBM muscle fibres together with an accumulation of misfolded proteins (Nogalska et al. 2010a; Nogalska et al. 2010b).

Experiments conducted in our laboratory have also shown that up-regulation of β-APP in primary muscle cultures leads to nuclear translocation of NFkB (Unpublished data, Adrian Miller, Greensmith Lab). Therefore NFkB may be a central component linking degeneration to inflammation.

sIBM has also increasingly been shown to have similarities to other non-immunological disorders such as ALS and hIBM. Both show cytoplasmic protein aggregates and more recently, abnormal expression of TDP-43 (Neumann et al. 2006; Barmada et al. 2010) (Askanas & Engel 1998c). However, there is no T-cell mediated inflammatory response in either ALS or in hereditary IBM (Huizing & Krasnewich 2009), even given the similar degenerative pathologies. This finding indicates that the degenerative features of sIBM can exist in the absence of inflammation and so are not necessarily caused by an immune response.

The most convincing argument that degeneration is the primary cause of sIBM is the fact that as yet disease progression has not been proven to be attenuated by any immunotherapy (Dalakas 2010a). This implies that degenerative changes are not a consequence of inflammation as they continue to progress even with strong immuno-suppression (Karpati & O'Ferrall 2009).

1.8. Protein mishandling in sIBM

Protein aggregation is a key degenerative feature of sIBM and is caused by a disturbance in the protein handling mechanisms within cells. This disturbance may be due to a genetic mutation in a protein involved in protein synthesis, regulation or degradation or may be brought about by internal or external stress on the cells. If the correct mechanisms to handle protein disorder are not in place, or are themselves dysfunctional, this then can become toxic to the cell. Like sIBM, many other diseases such as AD and Huntingdon's disease (HD) show similar signs of protein abnormalities. It is therefore very important to

understand how protein disturbances occur, how they are managed by the cell under normal conditions and how protein mishandling can lead to disease.

Although it is not clear to what extent the dysregulation of one or a few proteins contributes to sIBM pathology, there does appear to be clear evidence of generalised protein mishandling within the sIBM muscle cell. The accumulation of diverse proteins ranging from ER chaperones, heat shock proteins and proteasomal proteins to aggregation-prone molecules and signal transduction components, suggests that the protein equilibrium has been altered in sIBM muscle (Greenberg 2010).

1.8.1 Mechanisms of normal protein folding

With the human genome containing approximately 30,000 protein-coding genes, many of which have multiple functions, a cell's proteome is vast and requires precise regulation. Maintenance of protein homeostasis (proteostasis) is therefore essential for even the most basic cellular functions.

Normal protein folding occurs in stages beginning with translation of the relevant genes. Once translated all proteins are processed until they adopt their native three-dimensional structure which is essential for the macromolecule to be functional. The precise folding into the correct tertiary structure is dependant firstly on the sequence of amino acids synthesised during translation (Anfinsen 1973). This sequence must be chemically exact for the necessary intermolecular bonds to form in adopting the secondary and tertiary structures (Lodish et al. 2005). As the sequence of amino acids is dictated by the genetic code for the protein, genetic mutations are potentially hazardous to exact protein folding if the correct sequence is altered (Hunt et al. 2009).

Protein folding must also occur in an environment suitable for the process to take place. This includes maintaining the optimum temperature, pH, ionic strength and the presence of appropriate catalyst proteins with their co-factors (Anfinsen 1972). Anfinsen et al.

(1961) conducted some important experiments to investigate protein folding, denaturation and renaturation. They demonstrated the ability of a ribonuclease, once denatured with mercaptoethanol in 8M urea, to be able to reform into its native structure when exposed to the optimal protein conditions *in vitro* (Anfinsen et al. 1961). This verifies that the primary structure of a protein is fundamental to its 3D conformation.

Intermediate structures form en route to the final folded state, and are part of the protein folding process for approximately 90% of cellular proteins (Hartl & Hayer-Hartl 2009). Some proteins with partially misfolded conformations or incorrect primary structures may become 'trapped' in an intermediate structure which is kinetically stable but incorrect for the molecule to be functional.

Nascent polypeptide chains can only assume their native states with the assistance of molecular chaperones (Imai et al. 2003a). These mainly ATP-dependent molecules associate with the unfolded/partially-folded protein chain, sheltering it from the high concentration of other non-native proteins and stabilising it under high temperature, (Walter & Buchner 2002) such that folding can occur within a biologically relevant timescale. The group of chaperone proteins which participate in protein biogenesis are the heat shock protein (HSP) 70s and HSP60s (Hartl & Hayer-Hartl 2009). By recognizing exposed hydrophobic amino acids, the chaperones associate with the polypeptide chain and sequester these regions to prevent non-specific interactions with nearby nascent polypeptides (Hartl & Hayer-Hartl 2009).

The final stage in the formation of a complete protein is the quaternary structure. Multimeric proteins require additional organisation of partially folded polypeptide chains, which must converge to form subunits for one functional protein. Between each subunit sufficient intermolecular bonds must be established for the protein to remain stable.

1.8.2 Protein misfolding

The stability of a protein in its native state is relatively low; making disruption to the structure readily possible upon environmental stress (Tyedmers et al. 2010a). The Gibb's free energy separating the folded and unfolded states of a typical protein is marginal, and lies between 20-65kJ/mol (Nelson & Cox 2005), meaning proteins can easily unfold or become misfolded.

Genetic mutation, transcriptional or translational error, faulty mRNA processing and environmental stresses, such as high temperature, are all factors which can result in protein misfolding (Tyedmers et al. 2010a). The consequence of this *in vivo* is loss of a functional protein which can cause disruption in specific cellular pathways or mechanisms. In living organisms such occurrences can be pathological and give rise to disease. Furthermore protein damage or mishandling can alter protein homeostasis, which is detrimental to a cell's viability. Both of these conditions can result in accumulation of impaired or unwanted proteins which are prone to aggregation.

1.8.3 Protein aggregation

Protein aggregation is an inevitable consequence of cellular existence due to the complexity of protein folding (Kopito 2000). Aggregation is directly linked to cell toxicity in neurodegenerative disease such as poly-glutamine-based disorders and Alzheimer's disease (Truant et al. 2008; Gundersen 2010; Crews & Masliah 2010). Misfolded proteins and early aggregates have been suggested as having a detrimental effect on cells through interactions with cell membranes, altering cellular structure and affecting internal biochemistry (Stefani 2007). Furthermore the presence of a large globular structure in the cytoplasm is obstructive especially for cell division, motility and for contraction in myogenic cells.

A protein species which aggregates does not necessarily have to be pathogenic itself (Ben-Zvi & Goloubinoff 2002), but its presence as an aggregate influences the cellular

proteome. Ben-Zvi and Goloubinoff (2002) have demonstrated that the aggregation of one protein strongly accelerates the aggregation of others into insoluble bodies (Ben-Zvi & Goloubinoff 2002). These experiments were conducted *in vitro* using thermal stress to induce the aggregation of glucose-6-phosphate dehydrogenase, Malate dehydrogenase and bovine serum albumin. The addition of chaperone proteins to these aggregated proteins revealed the chaperones' ability to re-solubilise the aggregates.

Aggregates arise from hydrophobic interactions between intermediate-state proteins or those with structural deformity due to mutation or external stress (Ben-Zvi & Goloubinoff 2002). These insoluble structures can consist of oligomers of the same kind (homo-aggregates) or of different varieties (hetero-aggregates) and are energetically more stable than individual hydrophobic oligomers are in the aqueous cellular environment (Tyedmers et al. 2010b). When aggregates of undetermined protein content accumulate in a cell they are referred to as 'inclusion bodies' (Kopito 2000).

For many years it has been assumed that inclusion bodies were a consequence of diffusion-limited accumulation of proteins in the cytoplasm (Kopito 2000), and their benefits as a cytoprotective mechanism has been debated. Johnston et al (1998) investigated the mechanism of inclusion body formation by studying the fate of cystic fibrosis transmembrane conductance regulator (CFTR) in cultured cells where proteasome function was inhibited. They demonstrated that inclusion body formation was controlled by a dynein dependent mechanism which led to the formation of large perinuclear protein structures. These types of inclusion bodies were termed 'aggresomes' and the perinuclear localisation was established to be at the microtubule organising centre (MTOC) (Johnston et al. 1998a). This shows that inclusion body formation is an actual cellular response mediated by the microtubule network to sequester aberrant proteins. When microtubule formation was disrupted in these cells by treatment with nocodazole, which causes microtubule depolymerisation, the formation of aggresomes was prevented (Saunders & Limbird 1997). Instead small protein accumulations were seen dispersed in the cytoplasm.

1.9. Protein Degradation

Protein aggregation is normally prevented by mechanisms which regulate the proteome at two crucial points. Firstly the cell ensures the fidelity of transcription and translation by tightly regulating these processes (Kopito 2000). Secondly, chaperone proteins are utilised at the point of protein synthesis to ensure nascent polypeptides are not exposed to the cellular environment for longer than necessary (Imai et al. 2003b). By immediately stabilising the polypeptide chain, chaperones and co-chaperones prevent it from binding to other polypeptides, but also help fold it into its functional state.

Thirdly, all proteins which are either mutated, damaged, no longer required or are in excess, undergo efficient proteolytic degradation by either the ubiquitin-proteasome system (UPS) (Bukau et al. 2006), the major degradative pathway, or by the lysosome-autophagy pathway. Even with the presence of chaperone proteins, over 30% of newly synthesised proteins are degraded in this way soon after synthesis (Goldberg 2003), which may be due to mistaken ribosomal function or unsuccessful folding (Schubert et al. 2000a).

1.9.1. The Ubiquitin-Proteasome System (UPS)

The ubiquitin-proteasome system is a highly conserved mechanism of protein degradation (Bukau et al. 2006). The 26S proteasome is a large barrel-shaped complex consisting of two 19S regulatory particles and a 20S core particle (Nelson & Cox 2005). Apart from degrading misfolded proteins, it is also involved in general cellular housekeeping by maintaining the turnover of 'aged' proteins (Goldberg 2003) and also degrades foreign proteins which are potentially harmful to the cell. Proteins to be degraded in this manner are tagged by ubiquitin, a small 76-amino acid protein, which is recognised by the proteasome (Rubinsztein 2006). The polypeptide chain is passed through the lumen of the proteasome where it becomes hydrolysed at the core particle into small peptides.

In the case of foreign protein degradation, these small peptides of between 8-10 amino acids are subsequently transported to the endoplasmic reticulum (ER) where they become antigens for MHC Class I (Schubert et al. 2000b). When presented on the cell surface, MHC Class I elicits a T-cell response which triggers an inflammatory response *in vivo* (Nelson & Cox 2005).

1.9.2. The Unfolded Protein Response (UPR) and Endoplasmic Reticulum-Associated Degradation (ERAD)

Proteins within the ER which become aberrant are retro-translocated to the cytosol to be degraded by the proteasome in a process called ERAD (Bukau et al. 2006). In parallel with this, a signalling cascade takes place which recognises the presence of misfolded proteins and induces the up-regulation of ER chaperone proteins as well as other secretory proteins (Bukau et al. 2006). This ER action constitutes the unfolded-protein response (UPR), which acts as a cytoprotective system to handle ER stress (Vattemi et al. 2004).

Askanas *et al* (2004a) investigated whether the UPR is activated in sIBM muscle and found that 5 ER chaperone proteins were present in inclusions in sIBM muscle. This suggests that protein mishandling is widespread throughout the cell, and is sufficient to cause ER stress and is therefore likely to increase the cell's vulnerability to stress and cell death.

1.9.3. The lysosomal autophagy System

Autophagy is a process whereby cytosolic bodies such as aggregates and organelles, which cannot be degraded by the proteasome, are digested in a double-membrane vesicle in the cytoplasm (Tyedmers et al. 2010b). These structures, called autophagosomes, engulf insoluble macromolecules and large structures sequestering them from the cytosol (Clague & Urbe 2010). They then fuse with lysosomes containing proteolytic enzymes which rapidly hydrolyse the captured proteins (Clague & Urbe 2010).

Rideout et al (2004) investigated the effects of inhibition of the proteasome and autophagy in embroyonic rat cortical neuron cultures. They reported an increase in the number of cytoplasmic inclusions in proteasome inhibited cultures following treatment with 3-methyladenine which inhibits autophagic activity. When autophagy was induced, the opposite effect was seen. These experiments demonstrate that blockade of autophagy affects the cell's ability to regulate protein homeostasis and proteasomal inhibition activates the lysosomal pathway (Rideout et al. 2004). Hara et al (2006) have demonstrated that mice deficient for an autophagy related gene called Atg5 develop neurodegeneration with cytoplasmic inclusion bodies in neurons (Hara et al. 2006). Blocking autophagy also increases the risk of apoptosis (Rubinsztein 2006).

Evidence of proteasomal dysfunction, induction of the unfolded protein response and impaired autophagy have all been reported in sIBM tissue (Nogalska et al. 2010b) . Therefore, the altered expression of key proteins and changes in protein handling are interesting areas to focus on in this investigation and may provide clues to potential triggers of sIBM pathology.

1.10. The aging cellular milieu

Alzheimer's disease and sIBM occur in distinctly different tissue types, however have many similarities in terms of pathology (Askanas & Engel 1998a). Although the pathomechanisms of the two conditions are yet to be established, both generally occur in people aged 50 years and over. This suggests that the aging process may be a factor which contributes to, or exacerbates the pathology seen in these debilitating diseases. Muscle weakness and atrophy as a consequence of aging is termed sarcopenia (Shigemoto et al. 2010), the molecular basis of which is still unknown. In aged muscle, the size and often the number of myofibres decreases, as well as the cross-sectional area of fibres, thereby reducing muscle mass (Meng & Yu 2010). These features are very often prematurely observed in sIBM patients and lead to the symptoms of weakness and atrophy (Tawil &

Griggs 2002). As muscle mass is lost, it becomes replaced by an increase in connective tissue and fat (Machado et al. 2009a).

1.10.1. Satellite cells and regeneration

Aged muscle differs from young muscle in many ways, some of which makes it more vulnerable to disease. Most notably, the regenerative capacity of muscle decreases with age and often becomes impaired (Biressi & Rando 2010a).

Muscle development and regeneration is maintained by a population of cells called satellite cells which are found beneath the basal lamina of myofibres (Gopinath & Rando 2008). These muscle-specific stem cells differentiate into myoblasts during development and fuse together to form a multinucleated syncytium which becomes the myofibre (Zammit et al. 2004). In addition, satellite cells remain quiescent in their niche under the basal lamina of mature myofibres until muscle growth, repair or regeneration is required, at which point they become activated (Collins et al. 2005). Activated satellite cells then proliferate and differentiate into myoblasts to form new muscle or can become incorporated into an existing myofibre to add to its myonuclei number (Collins et al. 2005). A proportion of satellite cells will always remain undifferentiated to maintain a precursor population, thereby fulfilling their role as stem cells (Collins et al. 2005). This ability to regulate muscle mass and to repair damage is essential for the neuromuscular system. However, with age, the number of satellite cells becomes reduced (Collins et al. 2007). Furthermore, it has been suggested that satellite cells may lose some of their functionality or it may be altered in aged muscle as their niche becomes modified both locally and systemically (Biressi & Rando 2010b). Shultz and Lipton (1982) demonstrated that the number of progeny cells produced from cultured satellite cells of rats was inversely proportional to the age of the animal (Schultz & Lipton 1982). This shows that the proliferation potential of satellite cells decreases with age.

In sIBM it is still uncertain whether there is an inherent difference in the satellite cells themselves which gives rise to the multiple pathological features. However, Morosetti et al (2008) have found that there is a marked decrease in the proliferative rate of cultured satellite cells obtained from sIBM patients compared to age-matched controls (Morosetti et al. 2008). This early exhaustion of the proliferation rate may be due to genetic susceptibility, accumulation of mutations, change in the local environment or a systemic occurrence which alters satellite cell function.

Morosetti et al (2008) also reported telomere shortening in sIBM satellite cells, indicating that premature senescence may occur in diseased muscle (Morosetti et al. 2008). Senescence is the permanent and irreversible withdrawal of cells from the cell cycle (Young & Smith 2000). This process occurs by the shortening of tandem repeats of DNA found at chromosome terminals called telomeres. With each replicative cell cycle a portion of the telomere is lost until a state is reached where no further cell cycles can take place and growth is arrested (Young & Smith 2000). Senescence can be brought about in this way naturally, as a consequence of aging. Therefore, in aged patients with sIBM, muscle satellite cell senescence would further reduce the regenerative capacity of an already small cell population.

Apart from the difference in satellite cell number and function, aged muscle has various other features which make the environment vulnerable and prone to damage. These changes in muscle cells may result in pathology such as those seen in sIBM. For example, changes in the basal lamina which encloses myofibres have been observed in aged muscle (Gopinath & Rando 2008). The basal lamina not only provides a scaffold for satellite cells to remain adhered to their myofibre, but also acts a reservoir of growth factors which maintain the satellite cell in its quiescent and active forms. In older muscle, the basal lamina has been shown to thicken, accumulate and completely envelope satellite cells, thereby reducing their interactions with the myofibre (Snow 1977) .The extra collagen deposited in this process also alters the exchange of growth factors and nutrients to the satellite cell (Gopinath & Rando 2008).

Aging cells have been shown to have less efficient degradation pathways compared to younger cells, and are therefore more likely to accumulate abnormal proteins (Askanas & Engel 1998b). The reason why this may occur remains unclear, but damage to cellular components, for example, through oxidative stress, is a possible cause (Chung et al. 2011; Aiken et al. 2011).

1.10.2. Oxidative stress

Cellular oxidative stress is caused by an imbalance in oxidants and antioxidant levels (Meng & Yu 2010). In healthy young muscle, oxidative stress is tolerated due to the cell's efficient degradation and re-synthesis of cellular components. However in aged muscle regulatory processes are not as effective and cellular damage cannot be efficiently repaired (Finkel & Holbrook 2000).

Oxidative stress can be caused by both exogenous and endogenous factors, and leads to an increase in the number of reactive oxygen species (ROS) present in the cell (Meng & Yu 2010). These highly toxic molecules are able to cause modulation of key regulatory proteins in the cell, such as transcription factors and protein kinases. This disruption in protein function triggers the up-regulation of the proteolytic pathways which begin protein degradation. Most notably however, oxidative stress leads to the activation of NFkB, triggering a proinflammatory response (Meng & Yu 2010). In aged muscle with the increased levels of oxidative stress, low-level inflammation becomes chronic and as such can be detrimental to cell survival (Meng & Yu 2010).

Maintenance of DNA integrity is essential for cell survival. However, reactive oxygen species are able to damage DNA, causing genetic mutations in both nuclear and mitochondrial DNA (Miura & Endo 2010; Costa et al. 2011). Oxidative stress can also trigger premature cellular senescence by damaging DNA (Finkel & Holbrook 2000). In sIBM where muscle damage is chronic, the pool of satellite cells with a regenerative capacity is

likely to be exhausted earlier through high levels of regeneration. Oxidative stressed-induced premature senescence would further hinder the ability for muscle to regenerate.

Other common changes to DNA induced by oxidative stress include double and single stranded breaks which trigger a cascade of protein and transcription factor activity to repair the damage (Miura & Endo 2010). In response to genotoxic agents and double stranded breaks, a protein kinase called ataxia telangiectasia mutated (ATM) is activated to control the damage. In aged cells however, ATM can be reduced (Miura & Endo 2010). The consequence of DNA damage and impaired repair is the translation of abnormal proteins which are likely to be either immediately targeted for degradation or have altered functionality which does not benefit the cell. Also, the cell would have a reduction in the levels of proteins encoded by the effected genes, which may result in deficits in the pathways they are involved in.

1.10.3. Mitochondrial abnormalities with aging

Research into sarcopenia suggests that 'mitochondrial theory of aging' may be a key component (Loeb et al. 2005). This theory proposes that the aging process is partly brought about by reactive oxygen species causing mutations and deletions in mitochondrial DNA, protein oxidation and electron transport chain dysfunction (Loeb et al. 2005). Indeed, in aged muscle there is evidence of an increase in abnormalities in the electron transport chain, markers of oxidative stress and mutations in mitochondrial DNA (Safdar et al. 2010). Accumulation of mitochondrial abnormalities can be seen in aged muscle biopsy sections with cytochrome oxidase (COX) staining, where a greater proportion of cells are negative for this important electron transport chain protein, compared to younger individuals (Safdar et al. 2010). Mitochondrial abnormalities are commonly observed in sIBM patient muscle and indeed form part of the histological diagnostic criteria (Oldfors et al. 2006a). sIBM patients have a greater number of COX negative fibres compared to age-matched controls (Dahlbom et al. 2002). Also, ragged red fibres are seen in sIBM muscle upon staining for another key mitochondrial protein

succinate dehydrogenase (SDH) (Oldfors et al. 2006b). Diseased cells show an accumulation of abnormal mitochondria around the inner edge of the plasma membrane (see **Figure 1.2**).

The cellular changes described above all contribute to the aging milieu of muscle cells. In sIBM patients it is not known whether the pathogenesis is caused by susceptibility to these general changes or whether one change such as damage to a particular protein or region of DNA is the key trigger. Askanas et al (2007) postulate that the aging muscle environment may facilitate lymphocytic inflammation by making aberrant proteins appear 'foreign' to the immune system (Askanas & Engel 2007). Although the involvement of the aging process in sIBM has not been fully elucidated, there is clear evidence that the aged cell environment is less effective at recovering from the damage caused by the disease.

1.10.4. Neurological changes in sIBM

Although sIBM is clearly a myogenic disorder, the pathogenic events that occur in sIBM muscle results in some neurological deficits. The peripheral nervous system is an essential component for healthy muscle *in vivo*. Axons have an indirect influence on satellite cells. By maintaining myofibre resting membrane potential, ion channel conductance and acetylcholine receptor distribution, axons regulate the homeostasis at the satellite cell niche (Gopinath & Rando 2008). Under normal, healthy conditions, there are a greater number of satellite cells around the neuromuscular junction compared to the rest of the myofibre (Zammit et al. 2006). Shultz et al (1987) demonstrated that following denervation, satellite cells become activated as a result of the physiological changes to their environment, in order to promote muscle regeneration. However, in the absence of re-innervation, new muscle fibres are unable to survive and therefore degenerate (Schultz 1978).

The aging process also has an effect on the peripheral nervous system. In aged muscle there is evidence of partial denervation and remodelling of the neuromuscular junction (Larsson & Ansved 1995). This leads to a decrease in the production of myotrophic factors

from the nerve such as ciliary neurotrophic factor, which are important for muscle homeostasis, and thus contributes to the decline in muscle strength with age (Gopinath & Rando 2008).

Though most of the features of sIBM are myopathic, interestingly, neurogenic findings have also been reported in sIBM patients assessed by histology and by electrophysiology (Arnardottir et al. 2003b). However the neuropathy may not always manifest clinically. Abnormalities are often found with electromyographic (EMG), which suggest the presence of a mild polyneuropathy, with some patients even being initially diagnosed with motor neuron disease (Lotz et al. 1989). It is unclear whether these abnormalities are a consequence of aging, as sIBM occurs in older patients or, as is more likely, whether it is a pathological feature of the disease.

Hermanns et al (2000) reported peripheral neuropathy in 14 sIBM patients using both morphometry and electron microscopy. In this study the sural nerves of patients were examined and results showed predominantly axonal neuropathy with non-specific changes in myelin, Schwann cells, axons and other components of the peripheral nerve (Hermanns et al. 2000). An age-dependant decline in the percentage of myelin area in sIBM patients was observed compared to control cases. However, in comparison, the severity of this observed in sIBM cases was not greater than that of patients with mitochondrial myopathy. The majority of patients in this study also demonstrated neurogenic muscle involvement on electromyography, with a third of patients showing abnormal nerve conduction velocities. Neurogenic features were also present in two patients in this study who had hereditary IBM.

Arnardottir et al (2003) studied the sensory nervous system in sIBM patients and revealed sensory dysfunction in the 9 patients investigated. Examination included a clinical neurological examination, sensibility screening and quantitative determination of somatosensory thresholds such as vibratory and thermal pain thresholds. Results from these studies showed that vibratory thresholds were most commonly abnormal, implying

that large diameter myelinated nerve fibres were affected in sIBM patients (Arnardottir et al. 2003b). As in the previous investigation by Hermanns et al (2002), abnormal conduction velocities were also seen in this study. Cold thresholds were found to be normal in all cases; however, heat pain thresholds were abnormal in sIBM patients compared to controls, possibly reflecting abnormalities in the small, unmyelinated fibres (Hermanns et al. 2000).

Neurogenic abnormalities in sIBM appear to be relatively common in cases where they have been investigated, although these abnormalities have not yet been clearly defined in the literature. Further investigation into the nervous system involvement in sIBM would be beneficial if a universal pattern of abnormalities could be identified to be used as part of diagnosis.

1.11. Therapeutic approaches to sIBM

sIBM is a highly debilitating condition affecting patients over a period of many years. Patients progressively lose their independence and ability to undertake routine tasks without the assistance of others. In view of its debilitating effects, ideally a prompt diagnosis would be made to allow early and effective therapeutic intervention. However, as yet, despite a number of trials, no therapy has yet been proven to have significant benefits for sIBM patients (Askanas & Engel 2007; Christopher-Stine & Plotz 2004). Some of the therapeutic approaches that have been examined in sIBM patients are discussed below.

1.11.1. Steroid and steroid-sparing agents

Two other idiopathic inflammatory myopathies, polymyositis (PM) and dermatomyositis (DM), have both been shown to respond well to immunomodulating therapies (Dalakas 2008). The majority of PM and DM patients respond well to steroid treatment and therefore are given agents such as prednisolone as a first-line therapeutic approach

(Hilton-Jones 2003). Although the precise treatment regimen may vary between patients and between clinicians, it has been found that higher doses early on in the disease, followed by a lower dosage is associated with a faster response to treatment (Hilton-Jones 2003). Those that do not respond well to corticosteroids usually fair better with other immunosuppressants (Christopher-Stine & Plotz 2004).

Treatment of sIBM however, has proven more difficult. Corticosteroids are the main standard treatment used in clinic to treat inflammatory myositis due to their positive effects of on improving clinical weakness in patients and reducing inflammation (Cordeiro & Isenberg 2006). However this effect is not clearly seen in sIBM patients and any benefits are not sustainable (Christopher-Stine & Plotz 2004). To date no controlled efficacy trials have been conducted using corticosteroids for sIBM (Hilton-Jones 2003; Christopher-Stine & Plotz 2004; Wiendl 2008) and their use is generally based on empirical evidence (Dalakas 2001; De Bleecker et al. 2006). The side effects of steroid use can be significant and, importantly for sIBM patients, include muscle wasting. Therefore prolonged use of such drugs is not recommended (De Bleecker et al. 2006). Currently, as second-line therapies, other 'steroid-sparing' immunosuppressants are prescribed in parallel to steroid treatment (Distad et al. 2011). These include agents such as azathioprine, methotrexate, mycophenolate mofetil and cyclosporine (Distad et al. 2011; Hilton-Jones 2003).

Several possible immunomodulating strategies for the treatment of sIBM have been examined but all have been found to benefit only a small number of patients. For example, Mycophenolate mofetil is a new generation immunosuppressant typically used to prevent organ rejection in transplant patients (Mowzoon et al. 2001) and acts by inhibiting B and T-cell proliferation. Mowzoon et al (2001) examined the effect of mycophenolate mofetil treatment in 7 patients with autoimmune neuromuscular diseases, 1 of which was diagnosed with classical sIBM. These authors report that the patients showed a moderate to excellent response to treatment with this drug across a wide range of diseases, which include polymyositis and myasthenia gravis. An additional

small study by this group also showed an improvement in 6 sIBM patients with mycophenolate treatment (Mowzoon et al. 2001). Another small study using this drug was conducted by Pisoni et al (2006) for the treatment of resistant myositis. Six patients refractory to treatment were given mycophenolate and were subsequently found to have increased muscle strength (as measured using the Medical Research Council (MRC) scale) and reduced serum CK levels (Pisoni et al. 2007). Although the results with this drug are encouraging, the studies conducted were extremely small, and so a firm conclusion of the effectiveness of mycophenolate in sIBM cannot be made until a large-scale trial is conducted.

1.11.2. Intravenous immunoglobulin (IVIG) treatment

As an alternative to steroid treatment, modulation of the immune response in sIBM has been trialled using IVIG (Dalakas et al. 2001). IVIG has several modes of action which include activation of Fc receptors on dendritic cells (Siragam et al. 2006), macrophages and B-lymphocytes, inhibition of cytokines and complement molecules and competition with autoantibodies (Dalakas 2008).

IVIG has been shown to be effective in clinical trials for both PM and DM patients (Dalakas et al. 1993). However, as yet trials using IVIG to treat sIBM have not shown a statistically significant improvement in muscle strength, the main outcome measure (Walter et al. 2000). A third of patients however, had some transient benefits with some patients reporting an improvement in dysphagia.

Since previous trials with IVIG reported mild improvements in muscle strength, at least in some sIBM cases, Dalakas et al (2001) undertook another study to investigate the potential benefits of a combination therapy of IVIG with prednisone treatment. Thirty six patients were recruited in this double-blind, placebo controlled study and patients treated with IVIG + prednisone were compared to those on prednisone alone (Dalakas et al. 2001). Treatment was maintained for a three month period. Muscle strength was assessed by

quantitative muscle strength testing and modified MRC scores. The results from this study showed that although there was a reduction in the number of necrotic fibres in the IVIG treated group, there was no significant improvement in muscle strength up to 4 months after treatment. An effect of the steroid treatment was seen in the significant reduction of the number of cells positive for CD2, a surface protein of natural killer cells and T-lymphocytes. This study concluded that treatment with IVIG and prednisone for 3 months was not an effective treatment for sIBM.

As with steroids, IVIG treatment can also have side effects which can range from flu-like symptoms and myalgia to more serious effects such as renal failure and thrombosis (Distad et al. 2011). In addition this IVIG treatment is extremely expensive at approximately £6,000 per treatment (Distad et al. 2011).

1.11.3. Monoclonal antibody based treatment

More recently, new approaches to the treatment of sIBM have been investigated that involve the use of monoclonal antibodies. One drug which has shown some promise is Alemtuzumab (CAMPATH 1-H), a monoclonal antibody against CD52, an antigen present on B cells, T cells and dendritic cells (Wiendl 2008).

Dalakas et al (2009b) conducted a proof-of-principle study to examine the effects of Alemtuzumab infusions on peripheral blood lymphocytes and endomysial T-cells, and to assess whether the natural course of the disease was altered by modulating the immune system in this way (Dalakas et al. 2009b). In this investigation, 13 patients diagnosed with sIBM were recruited and the natural history of the disease monitored in each individual over a 12 month period. The trial was designed to detect at least a 10% increase in muscle strength 6 months after treatment, using Quantitative Muscle Strength Testing and MRC strength measurements. The results showed that following treatment with Alemtuzumab muscle strength did not improve significantly, although short-term stabilisation of disease progression was apparent with a proportion of patients reporting improvements in

performance of daily activities. A significant difference was however seen in the number of CD3 positive lymphocytes in the patients showing improvement, while other peripheral blood lymphocytes also were reduced, including CD8+ cells. The mRNA expression of desmin was increased while the mRNA of stressor molecules such as αB-crystallin were decreased.

Although this study presents preliminary results with Alemtuzumab, the trial was not run as a placebo controlled trial. Together with the small sample size and results showing only 4 out of 13 patients with detectable improvement in muscle strength, this study is not powerful enough to confirm that Alemtuzumab is beneficial to sIBM patients. The authors recognise a need to conduct a larger, controlled trial of this drug (Dalakas et al. 2009a).

Other antibody based treatments have also been tested in sIBM and include a pilot trial for Etanercept, a tumour necrosis factor alpha (TNF-α) blocker (Singh et al. 2001). This investigation showed no effect of the drug compared to control patients in a small study of 9 sIBM patients (Barohn et al. 2006). However, sIBM patients are currently being recruited for a double-blind, randomised, placebo controlled trial of Etanercept to investigate the effects of the drug further (www.clinicaltrial.gov; No. NCT00802815).

Another TNF-α blocker tested in a pilot study is Infliximab. This monoclonal antibody was used in a study of a total of 13 patients with treatment-resistant inflammatory myopathies which included PM and sIBM (Dastmalchi et al. 2008). Of the 13 patients, 9 completed the trial and only 3 patients showed signs of improvement as assessed by histology. The remaining patients had no change or felt the condition worsen. No patients showed improved muscle strength, but type 1 interferon activity was increased after treatment as part of an immune response. This study concluded that Infliximab was not effective in treating resistant inflammatory myopathies.

A phase II clinical trial for Rituximab, a further antibody-based treatment is currently recruiting patients with inflammatory myopathies and myasthenia gravis who are refractory to treatment (www.clinicaltrial.gov; No. NCT00774462).

1.11.4. Exercise as a therapy for sIBM

Physical exercise has historically been discouraged by clinicians for patients with inflammatory myopathies for fear of aggravating the condition (Alexanderson 2009a). Although there is evidence that physical training is beneficial to patients with other neuromuscular diseases (McCartney et al. 1988), the inflammatory pathology observed in inflammatory myopathies makes the potential benefits of exercise doubtful. This is because high intensity exercise characteristically increases the number of natural killer cells, cytokines and cytotoxic T-lymphocytes in muscles, while lengthening exercises can increase the levels of serum creatine kinase (Spector et al. 1997). Both of these increase endomysial inflammation which could exacerbate the myopathy. Fielding et al (1993) found an increase in IL-1β levels and accumulation of neurophils in an exercise study conducted on 9 healthy, untrained men (Fielding et al. 1993). Physiological changes were measured from needle biopsies taken before, 45 minutes after and 5 days after exercise. The results also showed a positive correlation in the number of accumulated neutrophils to the percentage of damaged Z-bands seen by electron microscopy. These and other exercise studies have shown that intense exercise can lead to some muscle damage (Spector et al. 1997), however low intensity exercise may not be effective in increasing muscle hypertrophy and strength (Gualano et al. 2010).

sIBM patients see a progressive decline in muscle strength and functional exercise capacity with time, which is not improved by pharmacological interventions that have been so far trialled (Johnson et al. 2009). With some success in exercise therapy seen in patients with PM and DM, it has become increasingly encouraging that training may be beneficial to sIBM patients (Alexanderson 2009b).

Spector et al (1997) conducted a safety and efficacy study of resistance strength training in sIBM patients over a course of 12 weeks (Spector et al. 1997). Five sIBM patients were followed up and results demonstrated no increase in serum CK, B-cell or T-cell levels upon muscle biopsy. The extent of inflammation and number of degenerating fibres remained unchanged. It was concluded that resistance training in sIBM patients is not detrimental to affected muscle and does not appear to exacerbate the disease. Therefore exercise was considered a safe activity for sIBM patients.

Another 12-week study combining aerobic training with functional muscle strengthening exercises found aerobic exercise to be tolerated by sIBM patients (Johnson et al. 2009). Muscle strength was improved in some muscles post exercise including knee and hip flexors.

Furthermore, Arnardottir et al (2003) conducted an open study comprising of a 12 week home exercise program on sIBM patients (Arnardottir et al. 2003a). Although exercise was well tolerated, muscle strength did not improve. Results from muscle biopsies showed no change in the histology of these patients post exercise.

Thus, from the exercise studies conducted to date, it can be concluded that physical training is safe and well tolerated in sIBM patients. Although exercise may not always increase muscle strength, in sIBM patients, it may help to prevent the loss of muscle strength through inactivity or as a consequence of the disease (Arnardottir et al. 2003a). It has therefore been suggested that an exercise program may be beneficial to sIBM patients, at least in order to supplement any pharmacological treatment (Johnson et al. 2009; Distad et al. 2011). In addition, occupational therapy is recommended for patients, to help them adjust to their muscle weakness and remain mobile using aids such as walking sticks, and to teach patients how to perform tasks that become increasingly difficult with disease progression, for example, those involving fine motor movements (Dalakas 2001).

1.11.5. sIBM therapies based on anecdotal evidence

Other therapies which have been prescribed to patients with sIBM have been largely based on anecdotal evidence of success. Patients are made aware of the lack of proven therapies on offer for their condition before being offered drugs which may help to enhance endurance of the disease (Dalakas 2008). An example of this is prescription of co-enzyme Q_{10}, which is given to patients in the hope that it may help with the effects of mitochondrial abnormalities (Dalakas 2008), despite the absence of any evidence base for such an approach.

1.12. Emerging therapies for sIBM

At present there is no evidence-based, effective treatment for sIBM. The majority of therapeutic agents tested to date have been directed at the inflammatory component of the disease. However, none of these approaches have proved to be beneficial to sIBM patients, and therefore alternative therapeutic strategies need to be considered. One of the major consistent problems with sIBM trials so far is the very low number of patients enrolled and a lack of rigor i.e. not randomised or placebo controlled. Also, many trials showing changes in outcome measures are conducted over only a few months when the disease is very slowly progressive. These limitations therefore mean it is not possible to draw a firm conclusion from these studies.

Other potential therapies currently under investigation include matrix metalloproteinases (MMP) inhibitors. MMP-2 and MMP-9 have been found to be up-regulated in sIBM and PM (Choi & Dalakas 2000). These endopeptidases are secreted by inflammatory cells and play a role in T-cell migration and attachment to the cell wall of muscle fibres and endothelial cells by digesting components of the extracellular matrix (Choi & Dalakas 2000; Dalakas 2001). Another group of molecules involved in adjusting the extracellular matrix to facilitate T-cell movement are integrins and their associated receptors (Dalakas 2001). Both of these may be considered as targets for therapy in future.

Since muscle atrophy is the predominant feature of sIBM (Wojcik et al. 2005) approaches to increase muscle mass have been suggested. Although exercise is the usual method to increase muscle mass and strength in healthy individuals, the benefits to sIBM patients are not yet clear, and so alternate approaches to increase muscle mass have been proposed. Muscle mass *in vivo* is regulated by a protein called myostatin, which is a member of the transforming growth factor-β superfamily (Wojcik et al. 2005). Myostatin negatively regulates the growth of muscle both during development and in adulthood (Nogalska et al. 2007b) and has been found to be increased in sIBM muscle fibres (Wojcik et al. 2005). In humans, depletion of this protein due to mutation causes significant increases in muscle fibre bulk leading to abnormal strength (Wojcik et al. 2005). Myostatin null mice show significant hyperplasia and hypertrophy in skeletal muscle (Elashry et al. 2011). The increased level of this protein in sIBM may be detrimental to the aged and diseased muscle fibres. Therefore by moderating the expression of myostatin precursor protein which is cleaved into the active molecule or by inhibiting the protein itself, muscle atrophy in sIBM may be ameliorated. Myostatin has therefore been proposed as a potential target for therapy (Nogalska et al. 2007c).

1.12.1. Modulating protein handling as a therapeutic approach to sIBM

Whether the degenerative aspect of sIBM is primary to the pathogenesis or not, it clearly plays a role in the deleterious effects of the disease in muscle fibres. Therefore modulating the protein handling mechanisms which may be disrupted in sIBM muscle cells may be a particularly effective disease-modulating strategy for sIBM.

Protein homeostasis (proteostasis) is essential for normal cellular functions and therefore any disruption to this is highly detrimental (Douglas & Cyr 2010). In the event of cell stress, proteins can become unfolded or misfolded which leads to oligomerisation and aggregation (Kopito 2000). Protein misfolding in cells is a usually managed by endogenous chaperone proteins which act to prevent aggregation by bind to polypeptide chains during cell stress (Brown 2007). Either by preventing the protein from interacting with others or

by encouraging folding into it native structure, chaperone proteins ensure the proteostasis is maintained. The main family of chaperone proteins are the heat shock proteins (HSPs) which are up-regulated following activation of the heat shock response (HSR) (Kalmar & Greensmith 2009a). The HSR is an endogenous, ubiquitous, cytoprotective mechanism which ensures proteins are kept in the right place, in the right shape, at the right time. Therefore up-regulating the HSR in disorders with protein mishandling features may be an effective strategy to decrease or delay the degenerative features of sIBM. The HSR and HSPs are discussed in greater detail in Chapter 4.

In this Thesis, the possibility that targeting protein mishandling may be an effective approach to prevent pathological features of sIBM is examined. In order to test this hypothesis, an *in vitro* model of the degenerative features of sIBM was established and characterised. This model was then used to test a number of pharmacological agents that target protein mishandling in cells.

1.13. AIMS OF THIS THESIS

The aims of this study were to:

1. Establish a robust primary muscle culture system which could be manipulated to model the protein mishandling features of sIBM.

2. Characterise the model such that outcome measures can be established to assess the effects of protein mishandling *in vitro*.

3. Test the hypothesis that up-regulation of the HSR would decrease the pathological features of sIBM in this model

4. Examine the role of the proteasome and establish whether it can be a therapeutic target

5. Screen a number of novel agents that target aggregation of β-APP for their ability to reduce the pathological features of sIBM

CHAPTER 2.

MATERIALS AND METHODS

2.1. Primary neonatal mouse and rat cultures

Wild-type Sprague Dawley rats at age postnatal day 0-2 (P0-2) or C57-B6/SJLF1 mice at postnatal day 1-6 were humanely culled in accordance to Schedule 1 of the Animals (Scientific Procedures) Act 1986, and after ethical approval from the Institute of Neurology Ethical Review Panel. Hind limbs were removed after blunt dissection to remove skin and washed in Phosphate Buffer Saline (PBS; (Sigma-Aldrich, Dorset UK)) with 4% PenStrep (GIBCO/ Invitrogen, Paisley UK). Muscle was removed from the bone using forceps and cut into smaller fragments on a dry Petri dish. Muscle fragments were then placed on a shaking incubator at 37°C with 0.1% collagenase II for 40 minutes, triturating every 20 minutes. The resulting muscle solution was then filtered through a 100µm and 40µm cell strainer to remove large pieces of tissue. PBS was added to the tube and the suspension was centrifuged at 480g. The supernatant was then removed and the pellet re-suspended in 1ml of muscle growth media. The cells were then seeded on to appropriate plates for the specific experiment to be undertaken. Muscle media was changed after 48 hours to muscle differentiation media and refreshed using the same media every 2 days *in vitro* (DIV).

The material and methods used for primary neonatal mouse and rat cultures are discussed in greater detail in Chapter 3, which includes the optimization steps taken to obtain the most appropriate cultures.

2.2. Cell fixation

Muscle media was removed from cultured cells and each well was washed once with PBS. Cells were fixed, where appropriate, using ice-cold acetone-methanol solution (1:1 ratio) at 4°C or paraformaldehyde (PFA) at room temperature for 10 minutes. Next, the fixative was removed and the cells were stored at 4°C in PBS until use.

2.3. Transfections

For transfection of β-APP into primary muscle cultures, two DNA constructs were used which contained the full length β-APP gene for the 695 amino acid isoform. One construct contained the murine β-APP gene of 2087bp in a pYX-Asc plasmid backbone (Open Biosystems, AL, USA, See **Figure 2.1** for vector map of this plasmid). The other was a human β-APP gene of 2312bp which had been cloned into the plasmid pcDNA3.1+ (a kind gift from Dr E. Eckman, Mayo Clinic, Jacksonville, USA). pcDNA3.1+ without the β-APP gene was used as an 'empty vector' control for all transfections (see **Figure 2.2** for vector map of this plasmid).

Cell cultures were grown for 2 days *in vitro* in order to reach 80-90% confluency before transfection. A range of DNA and Lipofectamine concentrations were initially tested in 24-well plates in order to determine the optimal ratio for transfection of primary muscle cultures. 1µg of DNA to 2µl of Lipofectamine 2000™ was found to be the optimal ratio. First, differentiation medium was removed and replaced with warm Opti-mem™ (Invitrogen, Paisley, UK). A transfection master mix was prepared consisting of Opti-mem™, DNA and Lipofectamine 2000™ (Invitrogen, Paisley, UK) to a total volume of 50µl for each well, and vortexed to mix. The mixture was left to incubate at room temperature for 20 minutes. 200µl per well of Opti-mem was then added to the transfection mix and this was used to replace the Opti-mem™ already in the wells. The cells were incubated at 37°C for 5 hours after which the transfection mix was replaced with differentiating muscle medium. The cells were cultured for a minimum of 24 hours post transfection to allow for the effects of gene expression to manifest. After this time cells were fixed or processed for further experiments.

2.4. Immunocytochemistry

Following fixation, cultured cells were incubated for 1 hour at room temperature with 10% normal goat/donkey serum (Vector Laboratories inc, Peterborough UK) in 0.1% PBS-Triton-X100 (Sigma-Aldrich, Dorset UK) to block non-specific binding sites.

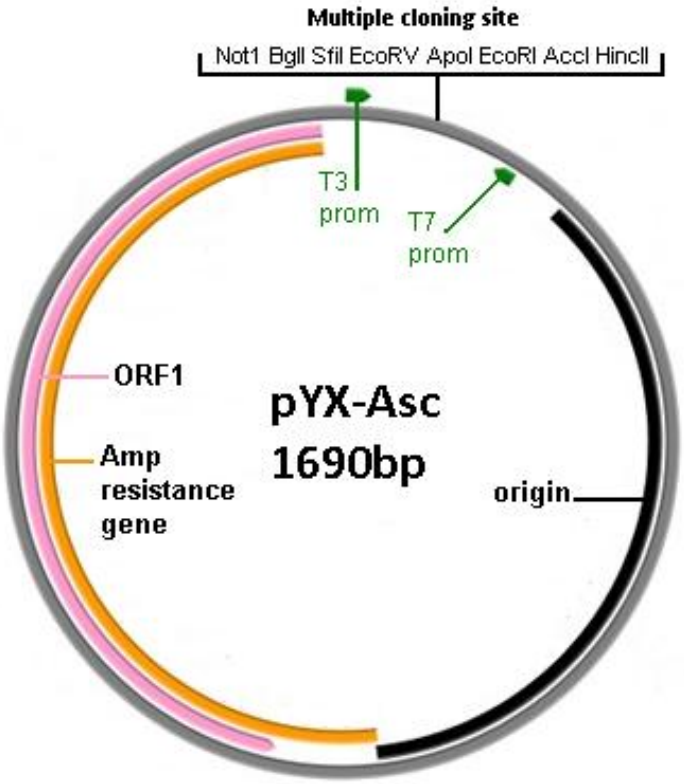


Figure 2.1 Vector map of plasmid pYX-Asc

A Schematic diagram of the plasmid into which the mouse β-APP gene was cloned. The T3 promoter is used to drive transcription of the β-APP gene. The T7 promoter is used to drive transcription of the Open Reading Frame (ORF) and the Ampicillin resistance gene. The plasmid contains Ampicillin resistance genes for selective growth of bacterial clones in plasmid production. The β-APP gene was cloned into multiple-cloning-site. Image adapted from Source Bioscience.

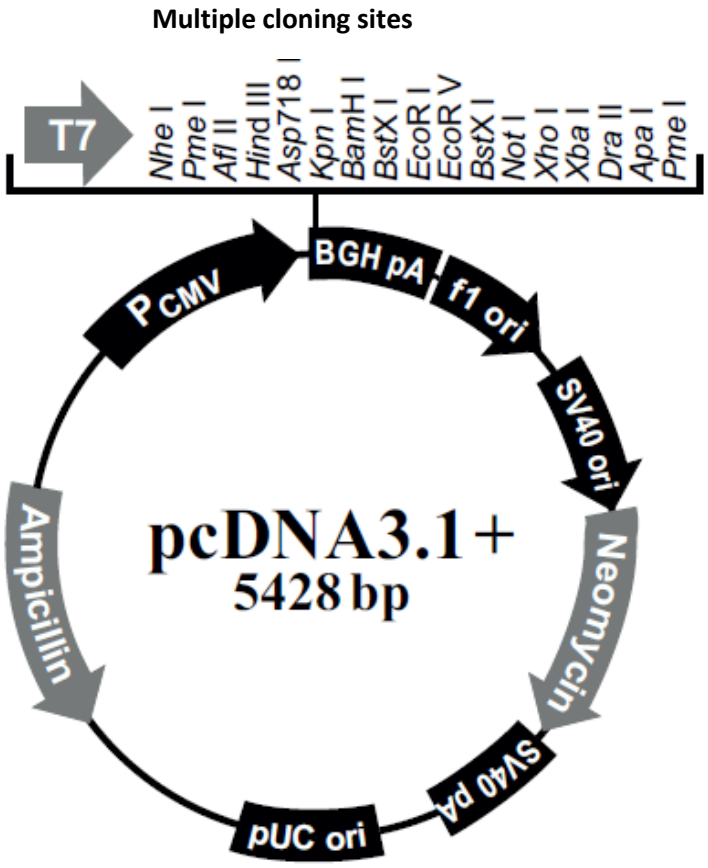


Figure 2.2 Vector map of plasmid pcDNA3.1 (+)

Schematic diagram of the plasmid into which human β-APP was cloned. The T7 Promoter is used to drive transcription of the β-APP gene. The plasmid contains both Ampicillin and Neomycin resistance genes for selective growth of bacterial clones in plasmid production. β-APP gene was cloned into multiple cloning site. Image adapted from Invitrogen Life Sciences.

Blocking solution was then removed and the cells washed three times in PBS with 5 minutes between washes. Next primary antibody was added and the cells incubated for a further 1 hour at room temperature with their respective primary antibodies. The primary antibodies used in the experiments described in this Thesis are presented in the **Table 1**.

Following incubation in the primary antibody, the cells were washed three times in PBS for 5 minutes each. Next the cultures were incubated in the appropriate secondary antibody either goat/donkey anti-mouse Alexa568 (Invitrogen, used 1:1000) or goat/donkey anti-rabbit Alexa488 (Invitrogen, used 1:500) for 1 hour at room temperature. Diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Dorset UK, diluted 1:2000) was applied for 5 minutes at the end of secondary antibody incubation to label nuclei. Negative controls minus the primary or minus the secondary antibodies were prepared in parallel for all experiments. Immuno-labelled cells were visualised with a Leica Fluorescent Microscope at magnifications of x20, x40, x63 or x100.

2.4.1. Cell counts

Cell counts were conducted by taking 5 random images per cover-slip of cultured cells at x20 magnification using a Nikon camera attached to a Leica Microscope. Next, the relevant antibody-labelled cells were counted by eye. The total cell number was calculated by counting all DAPI positive nuclei using image analysis software called MetaMorph®. Labeled cells were then expressed as a percentage of the total cell number. For assessment of inclusion body formation the number of inclusion bodies positive for a particular antibody were counted and recorded as a percentage of the total number of nuclei in each image. For all cell counts, each condition was repeated 2 times from at least three different muscle cultures and the data for each experiment averaged.

Table 1 . Primary antibodies used in this study and respective concentrations

Antibody	Species	Source	Dilution
Desmin (D33 clone)	Mouse monoclonal	Dako	1:100
β-APP	Rabbit polyclonal	Invitrogen	1:250
Myosin (MF20)	Mouse monoclonal	Developmental Studies Hybridoma Bank (DSHB)	1:10
Caspase-3	Rabbit polyclonal	Sigma-Aldrich	1:200
Ubiquitin	Mouse monoclonal	Genetex	1:500
TDP-43	Rabbit polyclonal	Proteintech	1:200
HSP70	Mouse monoclonal	Santa Cruz Biotech	1:200
NCAM/CD56	Mouse monoclonal	Developmental Studies Hybridoma Bank (DSHB)	1:20
p-tau (AT8)	Mouse monoclonal	Pierce Endogen	1:1000
p62	Mouse monoclonal	BD Biosciences	1:100
Aβ 1-42	Rabbit polyclonal	Biosource	1:200
CD34	Mouse monoclonal	Santa Cruz Biotechnology	5µl/10^6 cells
γ-tubulin	Mouse monoclonal	Sigma-Aldrich	1:1000

2.5. LDH assays

Cells were grown in 96-well plates at a density of 1.25 x 10^4 with 200µl of muscle media. High and low toxicity controls were created by treating 3 wells with 2% Triton-X100 and retaining 3 wells which just contained muscle media. On the day of assay, the plates were centrifuged at 250*g* for 5 minutes. 100µl of the supernatant was removed and placed into a new 96-well plate in corresponding wells. An LDH Assay was carried out using the Roche Applied Science Cytotoxicity Detection Kit, Cat No. 11 644 793 001. Following the manufacturers recommended protocol; the two reagent solutions were mixed and added at 100µl to each well. The plates were kept in the dark at room temperature for 20 minutes after which the absorbance at 490nm was measured spectrophotometrically, using a plate reader.

Once LDH data was measured, the remaining cell pellet was used for a protein assay to normalize the LDH dataset to the relative protein content in each well. See Section 2.6 below for protein assays for LDH data). LDH results for each experimental condition were normalized to that of the empty vector control wells to eliminate the effects of transfection on cell survival.

2.6. Protein assays for normalising LDH assays

Initially protein standards were produced by diluting 2mg of BSA (Sigma-Aldrich, Dorset, UK) in 1ml of PBS in a 1ml Eppendorf tube. Serial dilutions of this solution were carried out to obtain further BSA standards of 1, 0.5, 0.25 and 0.125mg/ml. The final Eppendorf contained PBS only.

Protein Assays were carried out using The Bio-Rad Laboratories Protein Assay DC Reagents A and B, Cat No. 500-0114 and 500-0113 to normalize protein levels against LDH Assay data. After all supernatant was removed, the pellet of cells which remained after centrifugation for the LDH assays was homogenized using a buffer containing 5mM Tris

Base and 5mM Tris HCL (at pH6.8) plus 2% sodium dodecyl sulfate (SDS) 2mM EDTA and 2mM EGTA. 10µl of homogenate from each well was placed into a corresponding well of a new plate. 10µl of each of the protein standards was placed into wells along the top of the 96-well plate.

Next, the Bio-Rad reagents A and B were added at 25µl and 200µl respectively. The plate was kept in the dark for 15 minutes at room temperature after which the absorbance at 750nm was measured spectrophotometrically.

To analyse the data, the spectrophotometric measurements for the protein standards were used to produce a scatter graph of known protein concentrations against absorbance readouts. The equation of this line was used to calculate the protein concentration of the unknown samples from the absorbance number.

2.7. Proteasome assays

Cells were harvested from T75 (culture flasks using a cell scraper and centrifuged at 350*g* for 5 minutes. After the supernatant was discarded, the pellet was resuspended in lysis buffer (50mM Tris-HCL, 5mM $MgCl_2$, 250mM sucrose, 1mM Dithiothreitol, 2mM ATP). Cells were lysed using glass beads for 1 hour on a rotator at 4˚C and 10µg of extracted protein was placed per well in a 96-well plate. Epoxomycin was added to control wells to inhibit proteasome activity. Total volume per well was normalized with lysis buffer without sucrose (reaction buffer). Next, 1µl of fluorogenic substrate (Suc-Leu-Leu-Val-Tyr-AMC, Enzo Life sciences, Exeter UK) was added to each well and fluorescence over time measured using a plate reader at 30 second intervals.

2.8. Succinate dehydrogenase (SDH) live cell staining

SDH working solution contained 0.1M phosphate buffer (pH7.6), 1M sodium succinate, 15mM nitroblue-tetrazolium, 0.1M potassium cyanide and 10mM phenazine methosulphate. Mixed solution was stored at 4˚C in a dark bottle.

A working aliquot of SDH stain solution was warmed up to 37˚C. Cultured cells were removed from the incubator and washed once in PBS to remove debris. 2-3 drops of SDH stain solution were placed directly into wells containing coverslips of cells. The plate was then incubated in the dark for 3 minutes at 37˚C. Next, each well was washed with 0.9% saline, followed by 70% acetone, 90% Acetone and 100% ethanol for 1 minute each. The coverslips were then removed from the wells and mounted onto glass slides using DPX mounting medium (BDH laboratory supplies, Poole, UK). Slides were left overnight to dry before bright-field visualisation using a Leica Microscope.

2.9. Data analysis and statistics

All quantitative data were collected on Microsoft Excel 2010 and analysed using this program, SPSS and VassarStats Stastistical Computation program. Statistical tests were performed on SPSS Statistics version 19, using Mann-Whitney U or unpaired *t*-tests where appropriate. Also, 'n' represents the number of individual cultures carried out on different days. In all graphical representations of data * denotes statistical significance where $p<0.05$.

CHAPTER 3.

DEVELOPMENT OF AN *IN VITRO* MODEL OF SIBM PATHOLOGY

3.1. INTRODUCTION: MODELLING SIBM

To date, no effective therapy for sIBM has been developed and any treatments given to patients are based on anecdotal evidence or are only symptom directed. This is mainly due to the lack of understanding of the pathology of this condition. With no clear genetic linkage to sIBM, alternative methods to model the disease *in vitro* and *in vivo* have been investigated by various researchers (Sugarman et al. 2002; Wojcik et al. 2006; Malicdan et al. 2007; Wojcik et al. 2007; Badadani et al. 2010). The aim is to identify therapeutic targets for the development of effective agents. Although many licensed drugs have been tested *in vivo* and in sIBM patients (Dalakas 2008; Mastaglia et al. 1997), there is currently no preclinical setup to screen novel agents specifically for sIBM.

3.1.1. Modelling sIBM *in vivo*

As with any human disease, replicating sIBM in animal models provides the most valuable tool to study the disease. Kim et al (2011) recently published data from experiments on transgenic Drosophila melanogaster which express wild-type human β-APP in muscle. Results from this study show no aggregation of proteins in the muscle of the transgenic flies nor any structural abnormalities (Kim et al. 2011). However, the β-APP transgenic flies displayed age-dependant defects in both flying and climbing. The onset of these defects could be influenced by maintaining the flies in vials coated with different substances. This suggests that environmental conditions alter the onset of muscle abnormalities. Electrophysiological experiments were conducted where intracellular recordings of muscle responses were measured following electrical stimulation of the thoracic dorsal longitudinal flight muscle (DLFM) and tergotrochanteral motor muscle (TTM) of the transgenic flies. The results showed no significant abnormalities compared to control flies, which suggests that there is no motoneuron abnormality as motoneuron activation resulted reliably in action potentials in the muscle. Although this model gives important insight into possible environmental effects in human β-APP transgenic flies, these

experiments suggest that this transgenic fly is not a particularly good model to study mammalian sIBM.

The most widely used mammalian model of human disease is now genetically modified mice in which the human gene is modified and expressed to induce a disease phenotype that resembles the human condition. To date however, a suitable mouse model of sIBM that closely mimics the human disease has proved difficult to develop for sIBM research. This is likely to be due to the unknown aetiology of sIBM and the lack of a known genetic cause.

One proposed mouse model for sIBM was produced by Sugarman et al (2002) who generated a transgenic mouse over-expressing β-APP specifically in skeletal muscle. This group reported that aged mice (>10 months old) exhibit immuno-reactivity for β-APP and its cleaved products in the muscle. These mice also presented other features of myopathy including inflammation and central nuclei as well as calcium dyshomeostasis (Sugarman et al. 2002; Moussa et al. 2006). On the other hand, these mice were not reported to have cytoplasmic inclusion bodies or show TDP-43 pathology in muscle, both of which are key features of the disease. Moreover, as the prevalence of β-APP in sIBM muscle is controversial, this mouse is not an ideal model of sIBM.

In another proposed mouse model of IBM produced by Weihl et al (Weihl et al. 2007), a mutated version of a protein known to facilitate protein degradation called valosin-containing-protein (VCP) was over-expressed. Although an inflammatory response was not observed, histopathology showed myofibre atrophy, formation of ubiquitinated inclusions, vacuolation and rimmed 'cracks'.

In humans, mutations in the VCP gene cause a hereditary form of IBM associated with Paget's disease of the bone and frontotemporal dementia (IBMPFD) and this gene is highly conserved between species (Custer et al. 2010). Custer et al (2010) also generated transgenic mice expressing wild-type and disease-causing versions of human VCP, which

recapitulate the full spectrum of IBMPFD. These mice express either the most common disease-causing human mutation in VCP (R155H) or the mutation which produces the most clinically severe phenotype (A232E). Both mutations showed significant muscle weakness assessed by hanging-wire performance experiments. In addition histological analysis of muscle tissue revealed evidence of myopathy including centralised nuclei, irregular fibre sizes and inflammatory infiltrates. Further staining revealed the presence of rimmed vacuoles and cytoplasmic accumulations of TDP-43, which were immuno-reactive for ubiquitin. All of these histological features are also present in sIBM patient muscle. Although these transgenic mice model a genetic disease which affects a number of tissues, the muscle pathology observed resembles that of sIBM. Therefore this mouse may be a good model for testing therapy for sIBM.

Other mouse models have similarly shown an incomplete phenotype for sIBM and as such cannot be defined as a true model of the disease (Sugarman et al. 2002). However, these models may help to increase our understanding of the pathogenic processes that underlie sIBM.

3.1.2. Modelling sIBM *in vitro*

The advantages of using *in vitro* models of a disease are that they can be easily manipulated and are technically less demanding. Such systems provide a valuable tool for research into fundamental scientific questions. Furthermore, experiments can be completed in a shorter space of time compared to animal models or human trials. Although it can be argued that an *in vitro* system is physiologically far from representing disease in humans, disease pathology on a cellular level can be investigated in real-time in cultured cells without the influence of other systemic components which can be both a benefit and limitation.

One *in vitro* sIBM study on myoblasts cultured from patient muscle suggests inherent abnormalities in the satellite cells from diseased muscle (Morosetti et al. 2008). Morosetti

et al (2008) demonstrate that sIBM patient-derived cells have a lower rate of proliferation and longer doubling time compared to cells from normal age-matched controls. In addition, telomere shortening, the formation of congophilic inclusion bodies and Aβ deposits were detected only in the sIBM cells after several rounds of muscle cell growth. In another *in vitro* study, Muth et al (2009) demonstrated that exposure of primary muscle cells to proinflammatory cytokines interleukin (IL)-1beta and interferon (IFN)-gamma led to an over-expression of the sIBM-relevant proteins β-APP and alphaB-crystallin (Schmidt et al. 2008; Muth et al. 2009).

3.1.3. Beta-Amyloid Precursor Protein (β-APP) in sIBM

Much of the focus in modelling sIBM *in vitro* has been on replicating the degenerative features that have been prominent in patient muscle. A large part of this is the formation of intracellular inclusion bodies which are characteristic of the disease. These inclusion bodies contain a large variety of proteins such as ubiquitin, phosphorylated tau and HSPs, but most commonly, Aβ and its precursor protein β-APP (Askanas et al. 1992a; Askanas et al. 1992b; Bilak et al. 1993; McFerrin et al. 1998; Sugarman et al. 2002; Vattemi et al. 2009). Due to the known pathological effects of β-APP and Aβ in AD neurons, a toxic effect of these proteins in muscle has been proposed (Crews & Masliah 2010). For these reasons, β-APP and Aβ have been widely investigated in sIBM research. However, it must be noted that observation of β-APP and its proteolytic products have not been universal (Sherriff et al. 1995) and thus the role of β-APP as a fundamental component of sIBM pathogenesis remains controversial (Greenberg 2009b).

β-APP is a 240kDa trans-membrane precursor protein with a large N-terminal cell surface domain and short cytoplasmic domain. The largest of the isoforms are the 770, 751 and 695 amino acid proteins which have all also been identified in AD brain. In humans, once translated, β-APP is proteolysed into Aβ (1-40/1-42) by γ-secretase. In mice, cleavage occurs further downstream to produce Aβ (11-40/11-42). For the experiments in this

Thesis, full length β-APP-695 was over-expressed to assess its ability to produce sIBM-like features *in vitro*.

The human *β-APP* gene is located on chromosome 21, has 96% homology between humans, monkeys and rodents and is alternatively spliced into transcripts encoding different isoforms (Thinakaran & Koo 2008). Two other genes in the same family as *β-APP* are *APLP1* and *APLP2* and both, like the *β-APP* gene, encode transmembrane proteins which consist of a large extracellular domain and a smaller cytoplasmic region (Thinakaran & Koo 2008). However, only the β-APP protein is proteolytically cleaved by β-secretase, at the extracellular region and γ-secretase (also known as beta-site APP cleaving enzyme 1 (BACE1)) at the transmembrane domain to produce the Aβ protein (Thinakaran & Koo 2008). **Figure 3.1** presents a schematic diagram of APP processing.

Isoforms of β-APP are expressed in a variety of cell types (Mattson 1997). In neuronal tissue the 695-amino acid isoform is expressed at higher levels than the 751-amino acid isoforms of β-APP. In non-neuronal tissue such as muscle, kidney, liver and the spleen, the opposite is true and β-APP 751 is expressed at a higher level (Mattson 1997; Domingues et al. 2007). Many functions for β-APP have been suggested (reviewed by Mattson 1997); however, the actual physiological role of the protein remains unclear. With interest in β-APP initiating from research into AD, many of the functions of this protein have been investigated in neuronal tissue. One function which has been attributed to β-APP and its proteolytic products is that they act as trophic factors in neuronal cells promoting long-term neuronal survival (Mattson 1997). Other studies into the functions of these proteins have demonstrated an effect on neurite outgrowth, synaptogenesis and cell adhesion (Schubert et al. 1989; Milward et al. 1992; Moya et al. 1994).

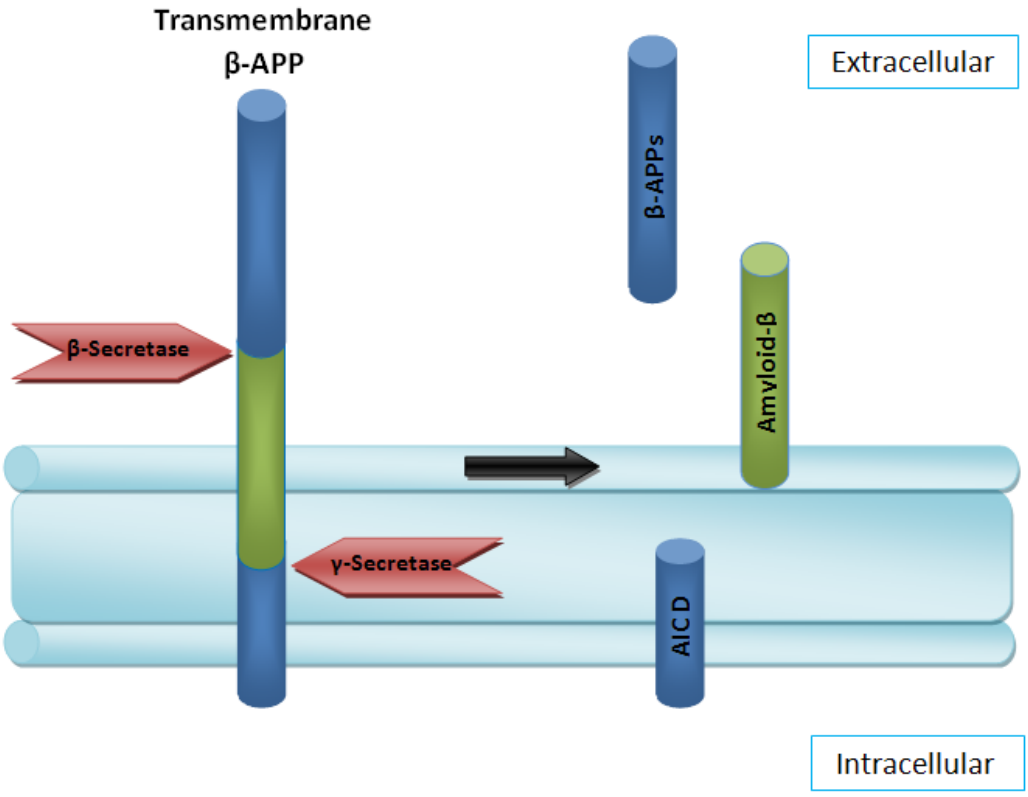


Figure 3.1 APP processing by secretase enzymes

A schematic diagram of the transmembrane β-APP molecule. β-APP is cleaved by β-secretase (BACE 1) at the extracellular region which liberates soluble β-APP (β-APPs). γ-secretase subsequently cleaves at the intramembrane region to release amyloid-β (Aβ40 or 42). The β-APP intracellular domain (AICD) remains at the membrane.

Currently, very little is known about the physiological functions of β-APP and Aβ in muscle. Wang et al (2007) have shown that the β-APP family of proteins are essential in regulating the development of neuromuscular junctions (NMJs) by studying synapse formation in β-APP/APLP2 null mice. These studies have shown that β-APP mediates the presynaptic expression and activity of the high-affinity choline transporter (CHT). CHT is the protein which recycles choline in the synaptic cleft after acetylcholine has been hydrolysed by acetylcholinesterase, returning it to the presynaptic terminal (Wang et al. 2007). In β-APP/APLP2 null mice, CHT fails to localise at the presynaptic terminal causing NMJ defects. Subsequently this group have investigated the effects of inactivating *β-APP* in either the presynaptic motor neuron or post synaptic muscle and report that inactivation in either compartment leads to defective NMJ (Wang et al. 2007). It is suggested that β-APP in postsynaptic muscle is necessary for presynaptic targeting of CHT and therefore synaptic transmission (Wang et al. 2009). Sarkozi et al (1993) previously reported that an increase in mRNA for β-APP was found only in the NMJ of normal human muscle fibres, while sIBM affected muscle had an increase in the mRNA in vacuolated fibres (Sarkozi et al. 1993).

Manipulating the expression of β-APP in cells in culture has been shown to be an effective method for examining potential pathological pathways which may contribute to the cellular degeneration seen in sIBM. In one study an *in vitro* model over-expressing the β-APP protein (751-amino acid isoform) in human cells has shown the formation of congophilic inclusions, vacuolation and mitochondrial dysfunction (Askanas et al. 1996; Askanas & Engel 1998b). In another *in vitro* investigation, Schmidt et al (2008) report that in sIBM, mRNA of β-APP consistently correlates to the levels of mRNA of chemokines and IFN-gamma, suggesting a link between inflammation and protein over-expression (Schmidt et al. 2008).

Regardless of whether β-APP plays a fundamental role in sIBM pathogenesis it is evident that the degenerative features in sIBM patient muscle are related to protein mishandling with aggregation of a large variety of proteins and perturbations in proteasomal function (Askanas & Engel 2006). Furthermore, reports suggest up-regulation of ER stress proteins

in sIBM during the UPR and disturbances in autophagy which are both protein handling mechanisms (Vattemi et al. 2004; Askanas et al. 2009; Nogalska et al. 2010b). In this thesis, over-expression of β-APP in cultured muscle cells was used to model features of sIBM *in vitro*, in order to examine the underlying mechanisms of the disease and to test some potential therapeutic agents.

This Chapter therefore describes the development and characterisation of an *in vitro* model of sIBM pathology.

3.1.4. Growing muscles *in vitro*

In order to remain as close to physiological conditions as possible in this study, primary muscle cultures were used to model sIBM instead of a muscle cell line. Primary muscle cultures can be grown from fresh muscle tissue by extracting satellite cells, the small muscle precursor cells that lie under the basal lamina of myofibres. During muscle regeneration, these satellite cells become activated, enter the myofibre itself and add to its nuclei, thus extending the fibre (Collins et al. 2005). During embryonic development, satellite cells also have the ability to form myofibres themselves by differentiating into single-nucleated myocytes and fusing with neighbouring myocytes until a large, highly multinucleated syncytium forms (Zammit 2008).

In vivo, these myotubes converge to form bundles of myofibres. Satellite cells *in vitro* can be grown under suitable conditions which allow them to differentiate into myocytes and fuse to form myotubes. A major difference is that *in vivo*, different cell types assemble in well-defined niches and myofibres are not contaminated by other cell types. However, due to the lack of structure *in vitro* and the use of generic enzymatic dissociation, different cell types contaminate the muscle culture. These cells include those derived from connective tissue, such as fibroblasts as well as osteocytes, adipose cells and epithelial cells (Sinanan et al. 2004). In this Chapter a variety of methods to isolate highly pure

primary muscle cultures from rodent muscle tissue were tested in order to reduce the number of contaminating cells in the muscle cultures examined.

3.1.4.1. Establishing optimal primary muscle cultures

The first step in modeling features of sIBM *in vitro* was to establish an optimal primary muscle culture which was defined by three criteria:

I. High myogenic potential

Myogenicity was established by immuno-staining cells for the muscle specific proteins desmin and myosin and by the general cellular morphology. Muscle *in vivo* forms as bundles of pure myofibres intersected by other tissue such as vasculature and nerve terminals. Therefore in order to obtain an *in vitro* system as close to the *in vivo* condition as possible, it is necessary for the myogenic potential of cultures to be high with minimal contamination from other cells. The most prevalent contaminating cells in muscle cultures are fibroblasts. Although the presence of fibroblasts in culture is essential for enrichment of muscle media with fibroblast derived growth factors, it is necessary to keep their numbers low to allow for more myogenic cell growth and differentiation.

II. Longevity of cultures in vitro

One aim of this project was to manipulate muscle cells *in vitro* so that they model aspects of sIBM. The muscle cultures used for these experiments must therefore be stable in culture for long enough to undertake the experimental procedures- which may last for up to 10 days. It is important to adjust the nutrient components of muscle medium so that muscle cultures remain viable and healthy throughout this period. A common difficulty faced with primary muscle cultures is their tendency to detach from the culture plate when they become highly confluent and well differentiated. General detachment of cells in culture is seen with many cell types, however, this is exacerbated in muscle cultures by the contractile ability of differentiated myotubes and the network of fibroblasts covering

the culture plate. Therefore the most optimal seeding density of cells must be established with reproducible proportions of fibroblasts in culture.

III. High yield

The nature of the experiments that these muscle cultures were used for required relatively high yields of cells to be isolated from the tissue. Cell counts using a haemocytometer were conducted prior to plating cells at all times to compare the final yields from different isolation and purification techniques.

A number of approaches were tested to identify the cultures which adhered to the criteria set out above. These included the use of different animal species, ages and purification techniques.

3.2. AIMS OF THIS CHAPTER

The Aims of this Chapter were:-

I. To establish a reliable and reproducible method to culture primary muscle cells

II. To induce muscle cultures to express pathological features of sIBM using protein over-expression

III. To characterise the model and establish reliable outcome measures of the effects of protein over-expression for subsequent use in the assessment of potential therapeutic agents

3.3. MATERIALS AND METHODS

3.3.1. Adult mouse primary muscle culture

Wild type C57-B6/SJLF1 hybrid adult mice were culled in accordance to Schedule 1 of the Animals (Scientific Procedures) Act 1986 to obtain muscles from their hind limbs. The two hind limb muscles chosen for dissection were the extensor digitorum longus (EDL) and soleus muscles due to their position deeper within the limb thereby reducing the risk of infection due to exposure to skin and fur. Animals were pinned onto a corkboard in a prone position (face down) to remove the soleus and supine position (face up) to remove the EDL. The hind limbs were sprayed with 70% ethanol to sterilise and all the skin from the hind limbs was removed using dissecting iris scissors and forceps. The EDL and soleus muscles were then identified and removed from both hind limbs.

All subsequent steps were carried out in a Class 1 microbiological flow hood. The EDL and soleus from both hind limbs were then washed in Phosphate Buffered Saline (PBS; (Sigma-Aldrich, Dorset UK)) with 4% Penicillin 100 units/ml and Streptomycin 100µg/ml (PenStrep; (GIBCO/ Invitrogen, Paisley UK)) and dissected into small pieces using fine watchmakers. The muscles were then digested in 1 ml of 0.1% collagenase II (solute PBS, GIBCO/ Invitrogen, Paisley UK) for 20 minutes at 37°C on a shaking incubator. The digesting muscle was next triturated for 5 minutes and centrifuged at 20*g* for 5 minutes and the supernatant discarded. A further 1 ml of 0.1% collagenase plus 1 ml of 0.2% trypsin (Sigma-Aldrich, Ayrshire UK) was added and the incubation repeated for 20 minutes. After repeated trituration and centrifugation, the supernatant was saved. The enzymatic step was repeated with collagenase and trypsin twice more and the supernatants saved. Next all the saved supernatant was combined and centrifuged at 480*g* for 10 minutes. The pellet was washed in PBS and re-suspended in muscle media (High glucose Dulbecco's Modified Essential Medium (DMEM) containing glutamine (GIBCO/ Invitrogen, Paisley UK), 20% fetal calf serum (FCS) (PAA, Somerset UK) and 4%

PenStrep) to be plated at a cell density of 2-5 x 10^4 cells/cm^2. Plated cells were incubated at 37°C/ 5% CO_2 until fixed in 50% Acetone-50% Methanol.

3.3.2. Neonatal mouse and rat primary muscle culture

Wild-type Sprague Dawley rats at age postnatal day 0-2 (P0-2) or C57-B6/SJLF1 mice at postnatal day 1-6 were culled in accordance to Schedule 1 of the Animals (Scientific Procedures) Act 1986. Animals were pinned onto a corkboard prone position and the hind limbs sprayed with 70% ethanol for sterility. Both hind limbs were removed after blunt dissection to remove skin and washed in PBS with 2% PenStrep. Muscle was removed from the bone using watchmaker forceps and cut into smaller fragments in suspension in PBS and then centrifuged. The supernatant was removed and the cells were then placed on a shaking incubator at 37°C with 3ml 0.1% collagenase II per 0.05g of muscle for 40 minutes, triturating every 20 minutes. The muscle solution was then filtered through a 100µm cell strainer (BD Biosciences, Oxford, UK) to remove large pieces of tissue and then filtered through a 40µm strainer. 5ml of PBS per 5ml of cell suspension was added and the suspension centrifuged at 480*g* for 10 minutes. The supernatant was removed and the pellet was re-suspended in 1ml of muscle growth media (High glucose DMEM containing glutamine, 20% FCS and 2% PenStrep).

For immunocytochemistry, cells were plated onto gelatinized coverslips in a 24-well plate with 500µl of muscle media. Plated cells were incubated at 37°C/ 5% CO_2 until fixed in ice-cold acetone-methanol (1:1 solution ratio). For LDH, protein and proteasome assays, the wells were not gelatinized and 96-well and T75 plates were used.

3.3.3. Optimised neonatal primary rat culture

Neonatal rat muscle cultures were prepared as described above but with the following alternations:

- Muscles were removed from bone on a dry Petri dish to eliminate the centrifugation step for removing PBS

- 0.5% CEE added to growth media.

- After 48 hours, muscle medium was replaced with 500µl of muscle differentiation medium (10% Horse Serum, 2% PenStrep and 0.5% Chick Embryo Extract (CEE) in 1x DMEM).

3.3.4. Purification techniques for muscle cultures

Several methods for improving the purity of muscle cultures were tested and are described below.

3.3.4.1. Immuno-panning

Muscle from the hind limbs of neonatal Sprague Dawley rats was removed as described above. After enzymatic digestion and filtration of the digested muscle, cells were plated at 5 x 10^4 per cm^2 in gelatinized wells with muscle media and incubated for 2 days at 37°C/ 5% CO_2. The cells were then trypsinized with 150µl per well of 0.25% trypsin EDTA (Sigma-Aldrich, Ayrshire UK) in DMEM to remove cells from the 24-well plate. The cells were collected in four 15ml Falcon tubes (BD Falcon, Oxford, UK), 2ml of muscle media was added and the suspension centrifuged at 350*g* for 10 minutes at 4°C. The supernatant was removed and the pellet was re-suspended in one tube with 2ml of muscle media to be plated as a non-panned control. To two tubes, 150µl of the primary mouse antibody against neuronal cell adhesion molecule (NCAM/CD56; ββDevelopmental Studies Hybridoma Bank, Iowa USA) was added and the cells incubated on ice for 1 hour. To the fourth tube 150µl of PBS was added instead of the primary antibody as a control. After the incubation, cells were washed with 5% FCS/PBS, centrifuged (350*g* for 10 minutes at 4°C) and the pellet re-suspended in 2ml of 5% FCS/PBS. During the antibody incubation, two

3cm diameter bacteriological grade Petri dishes were coated with 10µg/ml anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, USA) in 0.05M Tris-HCL buffer (pH 9.5). A further plate was left uncoated as a control for the secondary antibody. The plates were incubated for 40 minutes at room temperature after which each dish was washed three times with PBS and twice with 5% FCS in PBS. Using a pipette, cells were carefully layered onto the prepared Petri dishes and incubated for at 4°C. After 40 minutes the dishes were gently swirled then incubated for a further 40 minutes. Next, all the solution from each dish was removed and saved and the surface of the dishes were washed twice with FCS/PBS which was also saved. The collated saved solutions were labelled as the 'non-adherent' fraction. Petri dishes were triturated with FCS/PBS using a sterile Pasteur pipette (glass) to remove cells attached to the dishes. The cell suspension was poured off and saved. Next, the dishes were washed twice with FCS/PBS and all the washes were saved. The saved solutions were pooled as the 'adherent fraction'. Both 'adherent' and 'non-adherent' fractions were subsequently centrifuged at 4°C for 10 minutes at 350*g* to obtain pellets which were re-suspended in 1ml of muscle medium to be plated at 2.5 x 10^4 cells per centimetre squared onto gelatinized culture plates.

3.3.4.2. Density gradient centrifugation

This protocol was adapted from Bischoff (1997) which describes the isolation of muscle satellite cells from rats. Muscle from the hind limbs of neonatal Sprague Dawley rats was removed as described above (Section 3.3.2). After enzymatic digestion the cell suspension was filtered through a 40µm cell strainer and centrifuged at 480*g*. The resulting pellet was re-suspended in muscle media. A homogenous silica-particle solution called Percoll (GE Healthcare Bio-Sciences, Sweden) was diluted in DMEM to three concentrations of 35%, 50% and 70%. A step density gradient was subsequently produced by placing 3.5ml of 70% Percoll into a 50ml Falcon tube, followed carefully by the same volumes of 50% and 35% Percoll. The cell suspension was then carefully placed on top of the Percoll gradient. The Falcon tube was then centrifuged with the brake off at 1250*g* for 20 minutes in a

horizontal rotor at room temperature. The resulting bands of cells and/or debris were recorded and analysed individually for their content.

3.3.4.3. Fluorescent Activated Cell Sorting (FACS)

Wild-type Sprague Dawley rats (n=4-6) at age postnatal day 0-2 were humanely culled and the muscle from both hind limbs removed. The muscle was then either dissociated enzymatically with either collagenase II or trypsin, or manually using iris scissors and rapid trituration in a 1ml Eppendorf tube. After manual dissociation, the muscle fragments were placed in a 15ml Falcon tube with 2ml of PBS to suspend the released satellite cells in solution. Both dissociated muscle solutions were then filtered through a 100µm and a 40µm cell strainer and centrifuged at 480*g* for 10 minutes at room temperature. The pellets were re-suspended in muscle media and a cell count was carried out using a haemocytometer to record the starting number of cells.

Next anti-human NCAM/CD56 conjugated to Phycoerythrin (PE) (BD Biosciences Pharmingen, CA, USA) was added at 10µl/million cells or anti-mouse CD34 conjugated to Fluorescein (FITC) (Santa Cruz Biotech Inc-Insight Biotech, UK) at 5µl/million cells was added to the cells and an aliquot of cells were kept as a negative control for the antibody. The cells were incubated at room temperature for 30 minutes with the antibody. The antibody was then washed off by adding 5ml of PBS to the tube and centrifuging at 480*g* for 5 minutes. The resulting cell pellet was re-suspended in 0.5ml 2% fetal calf serum in PBS as a low serum solution is required for FACS sorting and because preliminary FACS sorting had suggested that the presence of DMEM may interfere with the fluorescence signal. The cell suspensions were then transferred to 5ml polystyrene round bottom tubes (BD Falcon, Oxford, UK) and 1 µg/ml of propidium iodide (Invitrogen, paisley, UK) was added to assess cell viability. The MoFLo-XPD^{TM} cell sorter (Beckman Coulter, USA) was calibrated for the fluorescence and light scatter channels following standard procedures and after analysis of the fluorescently labelled cells, a sterile sort of the NCAM/CD56-

positive or CD34-positive cells was undertaken. Antibody negative cells were also sorted as a control. Sorted cells were collected in sterile 1ml Eppendorf tubes.

3.3.4.4. Magnetic Affinity Cell Sorting

Wild-type Sprague Dawley rats (n=4-6) at age postnatal day 0-2 were culled and the muscles from both hind limbs removed. Following dissociation with collagenase II and centrifugation to remove the enzyme, the cell pellet was re-suspended in muscle media and plated in a T75 culture vessel (BD Falcon, Oxford, UK) and incubated for 24 hours *in vitro*. The cells were then removed from the plate using a cell scraper and centrifuged to remove all media. Two protocols for labelling of the myogenic cells were tested.

In initial experiments, the pellet was re-suspended in a mouse-monoclonal antibody against NCAM/CD56 for 10 minutes after which the primary antibody was removed by adding 1-2ml of MACS labelling buffer (Miltenyi Biotec, Gladbach, Germany) and centrifuging at 300*g* for 10 minutes. Next an anti-mouse Biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) was added to the pellet and the cells incubated for a further 10 minutes. The antibody was removed as before and the cells were re-suspended in 90µl of labelling buffer. To this 10µl of Streptavidin-labelled microbeads (Miltenyi Biotec, Gladbach, Germany) per 10^7 cells were added and the cells were incubated for 15 minutes at 4˚C.

An alternative labelling protocol was then tested where cells were incubated directly with 10µl per 10^7 cells of anti-human NCAM/CD56 microbeads (Miltenyi Biotec, Gladbach, Germany) for 15 minutes at 4˚C. Unlabelled aliquots were removed from both methods as a control and the number of cells before MACS sorting was counted using a haemocytometer. MACS sorting was conducted using the MACS cell separation kit (Miltenyi Biotec, Gladbach, Germany) following the manufacturers recommended protocol. The sorted 'myogenic' fraction, the eluted 'non-myogenic fraction' and the unlabelled control cells were plated onto 24-well plates with gelatin-coated coverslips.

The following methods employed in this Chapter are described in detail in the General Materials and Methods Chapter (Chapter 2).

- Transfection of β-APP plasmid
- Immunocytochemistry
- LDH Assay
- Protein Assay
- Proteasome Assay
- Cell counts
- Data analysis and significance calculations

3.4. RESULTS

The results in this Chapter are presented in three sections. In Part 1, the establishment of an optimal muscle culture is described; in Part 2, the development of a muscle culture that models sIBM is described and in Part 3, the characterisation of this *in vitro* model of sIBM is undertaken.

3.4.1. Results Part 1: Development of an *in vitro* model of sIBM

In order to model features of sIBM *in vitro* robust and reproducible primary muscle cultures were established after testing the use of different source animals, various purification techniques and different feeding media.

3.4.1.1. Identification of muscle cells in culture

To be able to assess the myogenicity and integrity of muscle cultures, it was first necessary to determine what can be classified as a muscle cell. The decisive factors used for these experiments were morphology and immuno-reactivity for myogenic markers. A myogenic cell can be identified at different stages of growth and differentiation by morphology. However, when first plated, satellite cells cannot be distinguished from other cell types by morphology alone, as all cells are uniformly shaped and of similar size (Blanton, Jr. et al. 1999). However, once the cells begin to differentiate, myogenic cells become thin and elongated into spindle-like structures, while other contaminating cells, mainly fibroblasts, form flat, irregular structures. **Figure 3.2** [A and B] shows muscle cultures at different stages of growth. Following immuno-staining with the muscle specific antibodies myosin or desmin, myogenic cells were clearly immuno-reactive and as such could be easily distinguished. **Figure 3.2** [C] shows an image of typical desmin positive myogenic cells in culture.

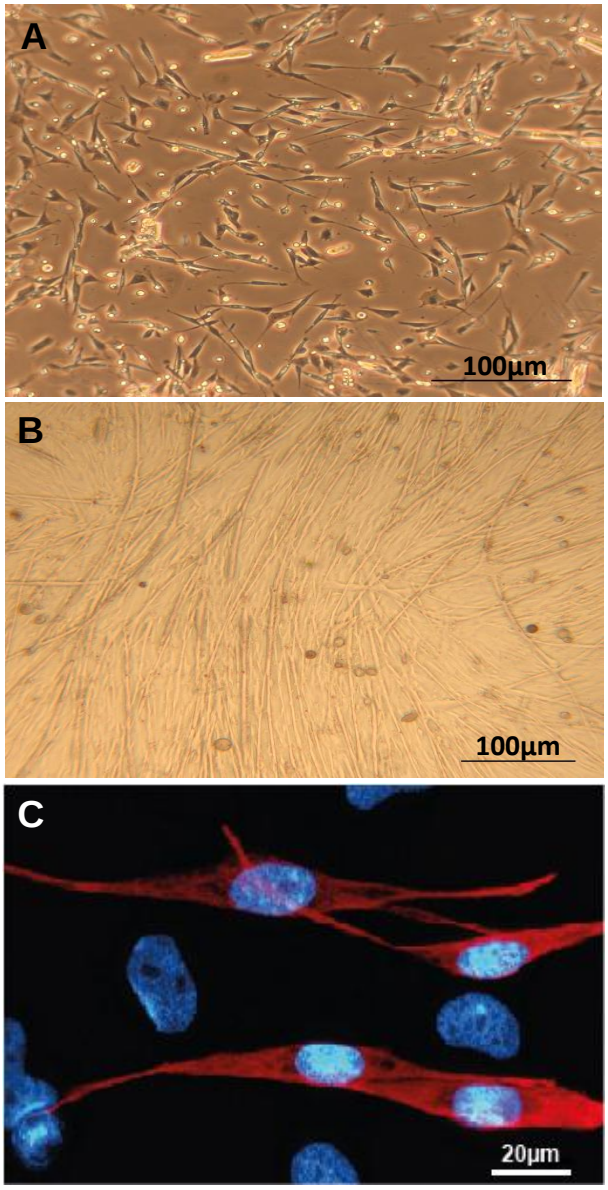


Figure 3.2 Muscle cells *in vitro*

[A and B] Bright-field images of optimised muscle cultures derived from rat muscle. [A] Image of cells at 2 DIV shows myogenic cells are indistinguishable from non-myogenic cells by morphology alone before the formation of myotubes. [B] Image of a typical muscle culture at 7 DIV shows easily identifiable myogenic cells following myotube formation. [C] Myogenic cells in culture immuno-stained for desmin (red) show elongated, spindle-like structure which differentiates them from fibroblasts. DAPI labels nuclei in blue.

3.4.1.2. Assessment of adult mouse satellite cell cultures

Initial primary muscle cultures were set up using satellite cells isolated from adult mice. These cultures were found to be inadequate as they did not meet the three criteria set out in Section 3.1.4.1, i.e. i) myogenicity ii) longevity iii) yield. Thus, the myogenic potential was found to be relatively low and <20% of cells were immuno-reactive for myosin. The cultures were also highly variable in terms of myogenicity, with the satellite cells having a low rate of proliferation such that they were not able to maintain the myogenic potential of the culture for long and were outgrown by fibroblasts. The adult muscle cultures were also prone to bacterial and fungal infection, possibly due to contamination from hair during the dissection, resulting in infections usually within 2 days *in vitro* (DIV). The population of muscle stem cells in healthy adult muscle is also relatively low compared to juvenile muscle, due to lower demand for development or regeneration of muscle cells. As the satellite cells for these cultures were derived from specific adult muscles, the yield of cells after isolation from EDL and soleus muscles was low, with an average of 2.81×10^5 per animal/ml of medium (n=4). Furthermore, immunocytochemistry using antibodies against myogenic markers showed a high level of background staining in mouse cell cultures, making specific antibody staining hard to distinguish. Due to these technical issues this protocol was determined to be inadequate for producing reliable cultures.

3.4.1.3. Assessment of neonatal mouse satellite cell cultures

Muscle has the greatest number of precursor cells during development compared to other stages of life due to the mass muscle generation ongoing during this period. For this reason, muscle cultures from neonatal mice were produced and assessed according to the three criteria of myogenicity, longevity and yield. For neonatal animals all the muscles from both hind limbs were removed as it was not possible to reliably identify different muscle types, or obtain a high enough yield of specific muscle types at such a young age. Compared to adult cultures, neonatal muscle cultures showed an improved proportion of myogenic cells of between 30-40%, although the myogenicity was still highly variable between individual cultures. Due to the lack of fur on the neonatal animals, there was a

reduced incidence of infection, which improved the longevity of cells to up to 5 DIV. These cultures also showed a dramatic increase in the yield of cells obtained from the digestion compared to the adult muscle cultures, with an average of 2.22 x 10^6 per animal/ml (n=3).

3.4.1.4. Assessment of neonatal rat satellite cell cultures

In order to improve the myogenicity and yield of muscle cultures further, satellite cells isolated from neonatal rats were cultured as described in Section 3.3.2. Neonatal rats are significantly larger in size compared to age-matched mice and therefore dissection of the muscles was easier and cleaner, with less contamination. Furthermore, the satellite cells themselves differentiated into larger myocytes and then myotubes allowing the different stages of the differentiation process to be easily observed and distinguished. Although the percentage myogenicity remained similar to the neonatal mouse muscle cultures at an average of 34.3% ± 2.9%, (SEM) there was a large improvement in the yield of cells with an average of 6.42 x 10^6per animal/ml (n=4). At the optimal seeding density the cultures were healthy and viable for up to 14 DIV, after which they would begin to detach from the culture plate. The added benefit of using primary rat cells was that it eliminated the high background staining seen with immunocytochemistry where the antibodies used were often raised in mice. **Figure 3.3** [B, C and D] presents typical images from neonatal rat cultures immuno-stained for desmin at different stages of differentiation.

From the assessment of muscle cultures obtained from adult and neonatal mice and neonatal rats, the cultures that adhered most to the criteria set out of high myogenicity, longevity *in vitro* and high yield were those harvested from neonatal rats. For this reason further *in vitro* work was carried out only using neonatal rat primary muscle cultures.

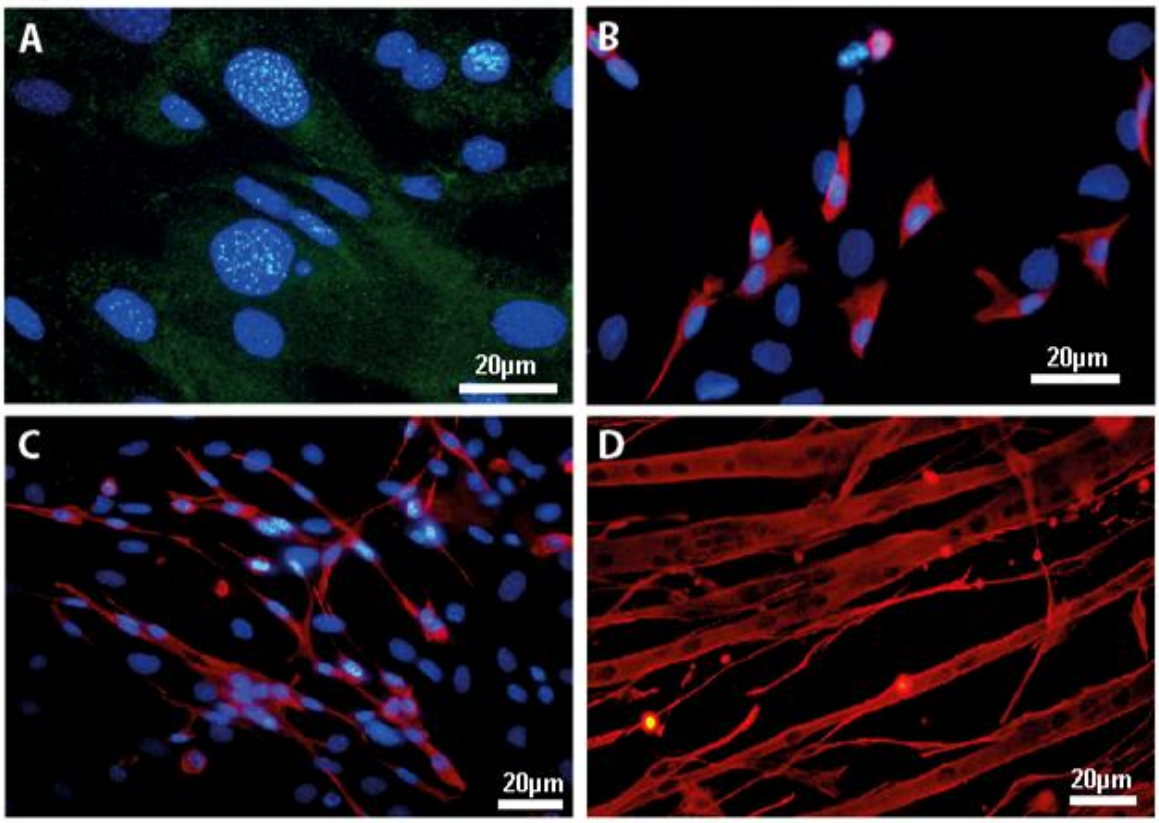


Figure 3.3 Growth of rodent muscle cells *in vitro*

Fluorescent images of cultured primary muscle cells immunostained for desmin (red) or myosin (green). DAPI labels nuclei (blue). [A] Muscle cells cultured from adult mouse satellite cells at 3 DIV [B-D] Muscle cells cultured from rat satellite cells. [B] at 2 DIV, [C] at 3 DIV, [D] at 5 DIV.

3.4.1.5. Purification of neonatal muscle cultures

Although primary cultures from neonatal rats were determined to be optimal, the percentage myogenicity was relatively low with 34.3% ± 2.9% (SEM) desmin- positive cells. To increase the myogenicity of the cultures a number of protocols were tested to improve their purity, while aiming to maintain the yield of cells and longevity of cultures *in vitro.* The results from the techniques tested are described next.

3.4.1.5.1. Immuno-panning

Jones et al. (1990) used a process termed 'panning' to isolate a purer population of myogenic cells from neonatal murine muscle (see Section 3.3.4.1 for protocol). The principle of this procedure is based on differential adhesion of cell types according to the expression of specific surface proteins. Cells labelled with a primary antibody against a muscle specific surface marker were incubated on Petri dishes coated separately with the corresponding secondary antibody. The non-adherent suspension in the Petri dish was taken off as the 'non-myogenic' fraction and the adherent cells were removed as the 'myogenic' fraction. Attempts to replicate these results in this study were unsuccessful. Analysis of the adherent fraction highlighted a substantial population of non-myogenic cells in the myogenic fraction, and the presence of a sizeable population of myogenic cells in the non-myogenic fraction. The problem was caused by the high affinity that all cells types had for the polystyrene of the Petri dish to which they readily adhered. Although some myogenic cells did adhere to the dish, whether this was antibody-specific was difficult to determine. The technique was therefore considered to not be sensitive enough for isolating a myogenic fraction.

3.4.1.5.2. Density gradient centrifugation

Bischoff (1997) used a purification technique based on density gradient centrifugation to isolate a purer myogenic population of cells from adult rat muscle. In this protocol a density gradient is generated using Percoll; a product made up of varying sized silica

particles, though which the mixed cell suspension is passed (see Section 3.3.4.2 for protocol) (Bischoff 1997). When this technique was tested in this study a consistent pattern of bands in the centrifuged Percoll tubes could not be established. Following analysis of the myogenicity of cells from each band, no significant improvement compared to the unsorted control cultures was detected after sorting. Although it was successful in removing debris and contracted fibres from the mixed cell suspension, due to the unreliability of this technique and its inability to improve myogenicity, density gradient centrifugation was not continued as a method for isolating myogenic cells.

3.4.1.5.3. Fluorescent Activated Cell Sorting (FACS)

After investigating relatively low-tech methods of isolating myogenic cells from a mixed population, Fluorescence Activated Cell Sorting (FACS), a more specialised method was tested for its ability to establish a purer muscle culture. Some representative results are displayed in **Figure 3.4**. Cells harvested from neonatal rats after collagenase digestion were labelled with antibodies against the stem cell surface antigens CD34 or neuronal cell adhesion molecule (NCAM/CD56), which is a satellite cell specific protein not expressed by fibroblasts. A secondary antibody conjugated to the fluorophore Phycoerythrin (PE) was used to distinguish the labelled myogenic cells. FACS analysis, indicating the fluorescent distribution of cells labelled with the antibodies, showed no significant positivity for either of the markers from these harvested cells. Repeating the procedure using trypsin instead of collagenase resulted in the same outcome. Since it was possible that enzymatic digestion may interfere with the integrity of the cell surface markers, additional experiments were conducted in which muscle tissue was dissociated manually using iris scissors and trituration. Disruption of cell surface markers by enzymatic dissociation has previously been reported (Abuzakouk et al. 1996). Fluorescent analysis of the manually dissociated cells showed a cell distribution shifted to the right on the PE-fluorescence axis into a gated region when labelled with CD34 or NCAM/CD56.

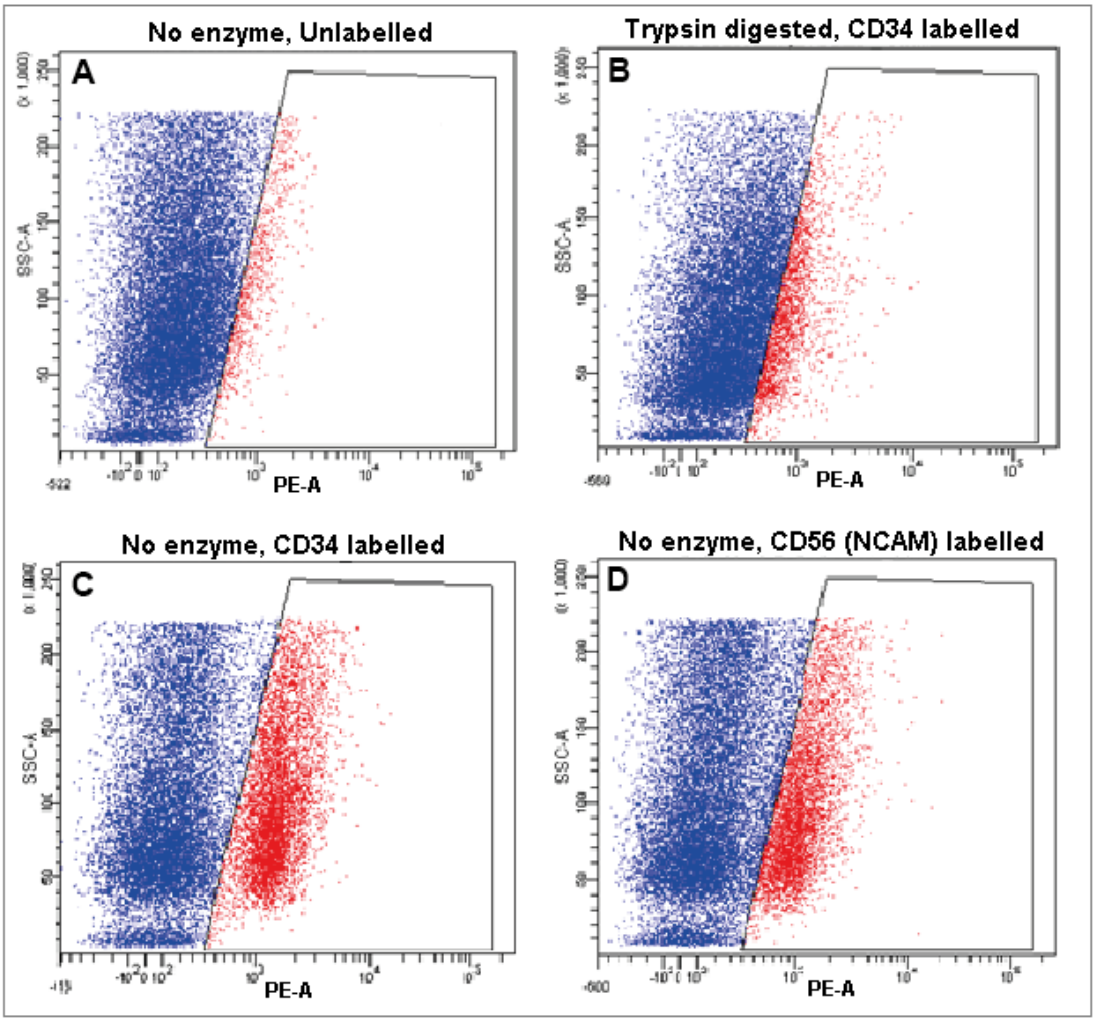


Figure 3.4 FACS sorted satellite cells

FACS analysis showing cell distribution by fluorescence. Cells in the gated region represent labeled cells with high PE fluorescence (red). Unlabelled cells are blue. [A] Unlabelled control showing minimal distribution of cells in the gated region (red). [B] Trypsin digested cells labeled with CD34 show an increased population in the gated region compared to unlabelled control. [C and D] Non-enzymatically digested cells labelled with CD34 and CD56 (NCAM) respectively, show clear isolation of surface-antibody labeled populations.

This corresponds to positive staining for the muscle cell markers. From this result it was deduced that enzymatic digestion either removed the surface markers or altered their affinity for the secondary antibody. **Figure 3.4** illustrates the difference in cell distribution seen from the FACS analysis of labelled cells harvested with or without enzymatic digestion.

The change in cell surface marker expression was confirmed by immunocytochemistry after enzymatic dissociation, where cells were incubated with the anti-NCAM/CD56 antibody after 30 minutes or 24 hours *in vitro*. The results showed no positive immuno-reactivity for NCAM/CD56 after 30 minutes *in vitro* but there was a notable increase in labelled cells after 24 hours (see **Figure 3.5** for images illustrating this occurrence).

Although subsequent FACS procedures were successful in isolating the myogenic proportion from the mixed cell suspension, once plated *in vitro*, the cells were unable to differentiate. This remained the case even after 7 days *in vitro* and after the differentiation medium was altered to 5% horse serum in an attempt to induce differentiation by serum deprivation.

These results suggest that exposure to the internal environment of the FACS machine or the prolonged time in suspension prior to FACS sorting may have reduced the differentiation potential of the myogenic cells. Therefore, FACS sorting to obtain purer muscle cultures was regarded unsuitable and was not continued.

3.4.1.5.4. Magnetic Affinity Cell Sorting (MACS)

Since FACS sorting was able to distinguish myogenic cells using specific antibodies, MACS sorting of immuno-labelled cells was tested next using an alternative method (See section 3.3.4.4 for protocol). MACS sorting makes use of magnetic microbeads targeted towards antibody-labelled cells.

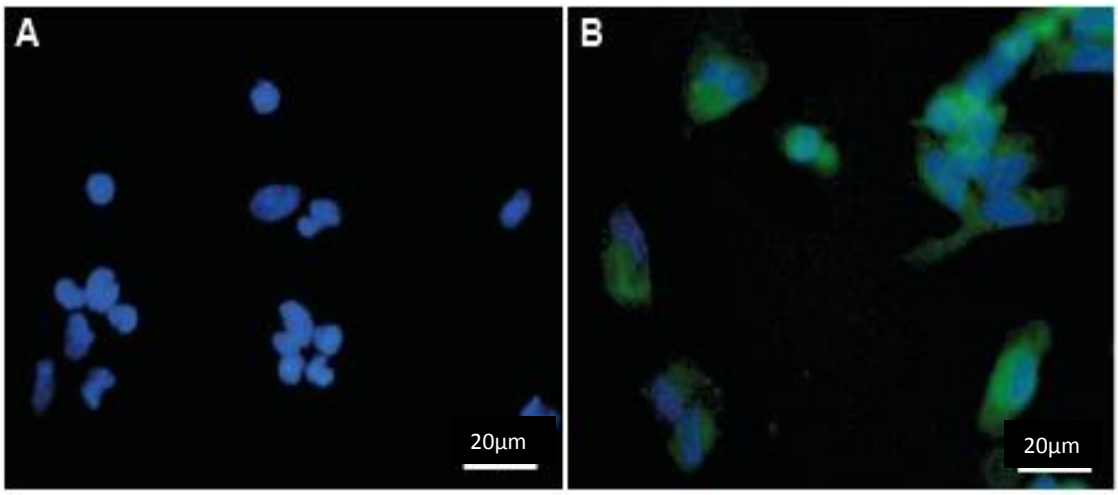


Figure 3.5 Assessment of NCAM labeled cells in culture

Cells were examined for the expression of CD56 (NCAM) (green) by immunocytochemistry. Nuclei are labeled with DAPI (blue). [A] After 30 minutes *in vitro* CD56 expression was not detected. [B] After 24 hours *in vitro,* cells express CD56 on the surface.

These cells, when passed through a magnetised column, are retained and are subsequently eluted as the myogenic fraction. To overcome the problem of reduced surface antigen binding after enzymatic dissociation, cells were grown *in vitro* for 24 hours and then removed from the plate using a cell scraper prior to MACS sorting. As demonstrated in **Figure 3.5**, this restores the NCAM/CD56 expression on the cell surface. In this investigation two different types of magnetic beads were tested for their ability to retain myogenic cells. In both cases the mixed cell suspension harvested from neonatal rats was incubated with a mouse monoclonal antibody against NCAM/CD56.

In the first method tested, magnetic microbeads conjugated to Avidin were used to attach to the anti-CD56 labelled cells via a Biotinylated anti-mouse secondary antibody. This protocol proved successful in significantly improving the percentage of myogenic cells in culture, raising the myogenicity to up to 60% ± 8.8%, (SEM, n=6, p=0.006). However the yield was greatly compromised, with only 10% ± 3.7% (SEM, n=3, p=0.03) of cells being retained in the MACS column. When grown *in vitro* and immuno-stained for the myogenic cell marker desmin, the eluted fraction showed that a very large population of myogenic cells had run through the column and not been retained. It may have been that the magnetic threshold needed for cells to be retained in the column had not been reached because of an insufficient number of microbeads adhering to the myogenic cells. To overcome this problem of poor yield, magnetic microbeads directly conjugated to an anti-NCAM/CD56 antibody were tested next. The expectation was that reducing the number of antibody binding steps in the protocol would improve the magnetic bead adherence to the cells, thereby increasing the quantity of cells reaching the magnetic threshold.

The results from trials with the NCAM/CD56-conjugated beads showed that percentage myogenicity was significantly increased with 86% ± 2.3% (SEM, n=4 $p<0.01$) of the cells being desmin positive. However, the difference was not statistically significant when CD56-conjugated beads were compared to the Avidin conjugated beads (p=0.08).

MACS using NCAM/CD56 was therefore found to be the most effective in isolating myogenic cells and producing pure muscle cultures. Unfortunately, the disadvantage of this technique was that it significantly reduced the yield of cells obtained compared to the protocol using Avidin-conjugated microbeads, to only 1.8% ± 0.25% (SEM, n=3). This suggests that the protocol using Avidin-conjugated beads has an inferior level of specificity for myogenic cells compared to the CD56-conjugated beads, and that perhaps improvement in reaching the magnetic threshold is not achieved even with primary antibody conjugated microbeads. **Figure 3.6** graphically represents this data and shows images of immuno-stained muscle cultures before and after MACS sorting. While MACS sorting resulted in highly myogenic cultures, the very low yield of cells could not be over-looked. Therefore, MACS sorting was not continued as a method of improving the myogenic potential of primary muscle cultures.

In summary, after testing four purification techniques of varying technical complexity, it was concluded that none could be used to produce optimal muscle cultures according to the criteria of myogenicity, longevity and yield (See Section 3.1.4.1). Although myogenicity of cultures was improved by FACS and MACS methods, both had major consequences which could not be disregarded. Therefore, an alternative method of improving the myogenic potential of muscle cultures was assessed, which involved manipulation of the culture conditions for optimal myogenic cell proliferation and differentiation.

3.4.1.5.5. Optimising neonatal rat cultures

Optimisation of the tissue culture conditions was undertaken to improve the survival, proliferation and differentiation of myogenic cells in the mixed cell population while reducing this for the contaminating cells.

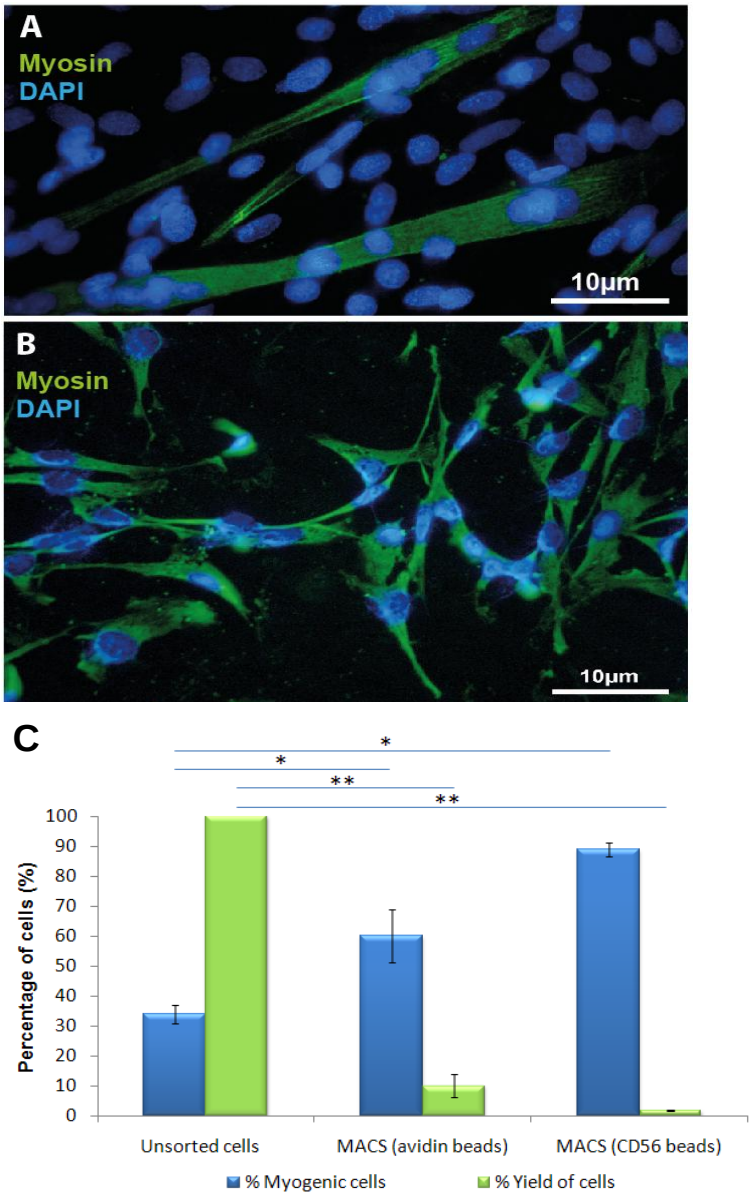


Figure 3.6 Analysis of MACS sorted cells

[A] Images of cells in an unsorted muscle culture immuno-stained for myosin, showing a highly mixed population of cells. DAPI labels nuclei in blue [B] Cells in culture after MACS sorting shows a highly myogenic muscle population. [C] Graph showing an increase in the percentage of myogenic cells after MACS sorting compared to unsorted cultures and the change in yield after MACS. MACS sorting using CD56 conjugated magnetic beads shows the greatest increase in percentage myogenicity but the lowest yield of cells. Error bars= SEM, * denotes statistical significance where $p<0.01$ and ** where $p<0.05$.

The three criteria of high myogenicity, longevity *in vitro* and high yield were used again to assess the optimisation of these muscle cultures.

Changes to the dissection technique

The dissection of muscle from the hind limbs of neonatal rats was altered. During previous dissections the hind limbs were submerged in PBS buffer in a Petri dish in which the bone was removed and the muscle dissociation by forceps was carried out. As satellite cells in neonatal animals are very loosely adhered to the muscle fibres compared to their presence under the basal lamina in adult fibres, there is generally a quick release of these cells when muscle tissue is dissociated. In the previous dissection protocol dissected muscle pieces were centrifuged to remove the PBS; however this allows some loss of cells with the PBS removal. To prevent this loss, the dissection was carried out on a dry Petri dish and the dissociated muscle pieces were immediately placed in 0.1% collagenase II for digestion. This was found to be a more efficient and time-saving modification to the protocol and was used for all subsequent cultures.

Changes to the muscle media

The previous protocol dictated that cells were plated in muscle media containing 15% FCS and 2% PenStrep in high glucose DMEM containing l-glutamine. However, this media also favoured proliferation of contaminating fibroblasts, which outgrew the myogenic cells within the short time *in vitro*. In this study, satellite cells were required to proliferate, then differentiate into myocytes and fuse into myotubes. Muscle differentiation is known to be promoted by serum deprivation and this is a commonly used method (Allen et al. 1991; Machida et al. 2004). Therefore a second media was developed to contain only 10% serum. As adult precursor cells are more geared towards differentiation than proliferation compared to neonatal cells (Gopinath & Rando 2008), to further induce differentiation the serum source was changed from fetal calf to adult horse as horse serum may be better 'conditioned' to differentiation. The protocol was thus changed so that the original media

was removed after the cells were grown 48 hours *in vitro* and replaced with the new 'differentiation' media containing horse serum. This change in media proved highly successful as all cells were allowed to proliferate for 24 hours after which myogenic cell differentiation was induced. Consequently, this appeared to have a negative impact on fibroblast growth, thereby improving the overall myogenic potential of the muscle cultures.

The changes in media also allowed greater control over the longevity of the cultures as maintaining the correct levels of proliferation was necessary to prevent over-confluency of cells in the plate, which leads to nutrient deprivation and detachment of cells.

Addition of supplements

All cells *in vitro* require a specific selection of nutrients and growth factors for healthy development. One substance which is commonly used in tissue culture as part of feeding media is chick embryo extract (CEE). The true components of CEE remains unclear, however some of the active compounds include glutamic acid, serine, taurine, xanthine, uracil, fructose, iron and vitamins (Rosenberg & Kirk 1953). Slater (1976) described the addition of chick embryo extract as beneficial to the differentiation of myogenic cells *in vitro*. In addition, unpublished data from Alec Smith in this lab also shows a significant increase in the number of myotubes which form in culture with the addition of CEE to media (Mann-Whitney U test, see **Figure 3.7**). Therefore to improve the muscle cultures in the present study, 0.5% CEE was added to both the muscle growth media and differentiation media.

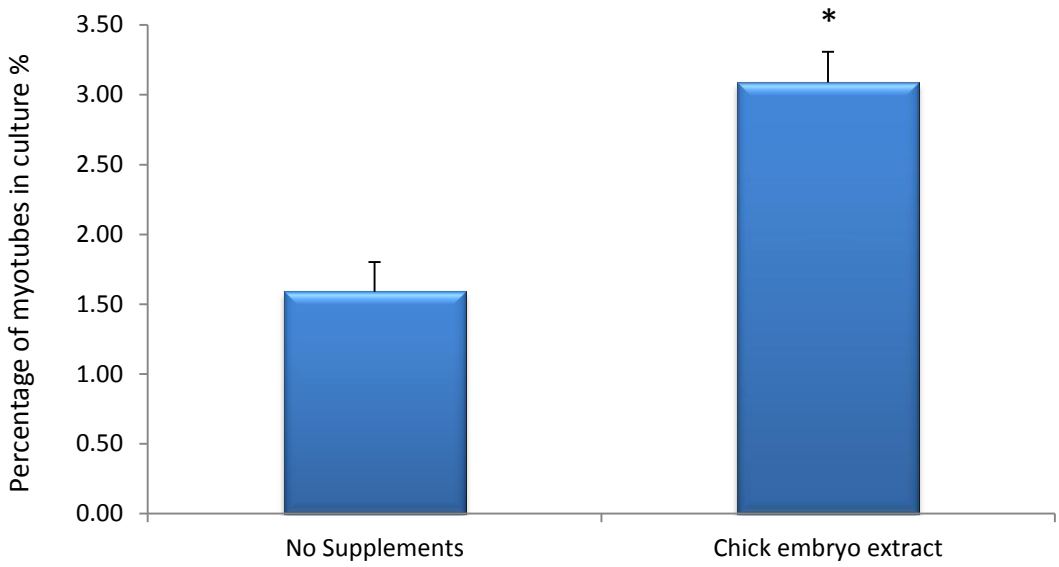


Figure 3.7 The effects of chick embryo extract on muscle cultures

Graph showing a significant increase in the percentage of myotubes formed after the addition of chick embryo extract to muscle media. Culture compared to muscle media without any supplements. $P<0.001$ ($n=3$). Error bars= SEM, * denotes significance. Data courtesy of Alec Smith, UCL institute of Neurology.

Optimisation of the neonatal rat cultures as described above resulted in the most appropriate cultures for this study according to the three criteria of myogenicity, longevity, and yield:-

a) Percentage myogenicity was improved to an average of 60.5% ± 1.9% (SEM, n= 5).

b) Satellite cells were allowed to proliferate over the first 48 hours *in vitro*. Differentiation began after 2 days *in vitro* and large myotubes forming began after 4 days. Cultures were stable and healthy for up to 14 days in culture with regular changing of the differentiation media.

c) This optimised protocol ensured the greatest yield of cells was obtained as no purification steps were required and all the cells harvested could be directly used in culture.

Figure 3.3 shows images of the optimised neonatal rat muscle cultures at three different time points *in vitro* to demonstrate their growth and differentiation. The Figure also shows an image of an adult mouse culture for comparison (**Figure 3.3** [A]).

3.4.1.6. Plating density

Cells derived from neonatal rat muscle were plated onto gelatinized glass coverslips in 24-well plates at different concentrations to assess the optimal plating density for growth and fusion. Results showed that the higher the plating density the greater the number of myotubes which formed. This is due to the increased availability of differentiated myocytes in the culture well, which are also in closer vicinity to one another for fusion to occur. **Figure 3.8** shows images of muscle cultures at increasing plating density. Although greater density of cells promotes myotube formation, it was discovered that once a well becomes over-confluent, due to the contractile nature of these cultures, the entire layer of cells detaches from the coverslip and forms a floating bundle of cells in suspension. To prevent this from occurring, the optimal cell density had to be established where fusion readily occurred but cells did not become over-confluent. Repeated plating density experiments and experience with muscle cultures established that 5×10^4 cells/well was the optimal density for use in 24-well plates. This density could be scaled up or down depending on the size of the plate used for each experiment.

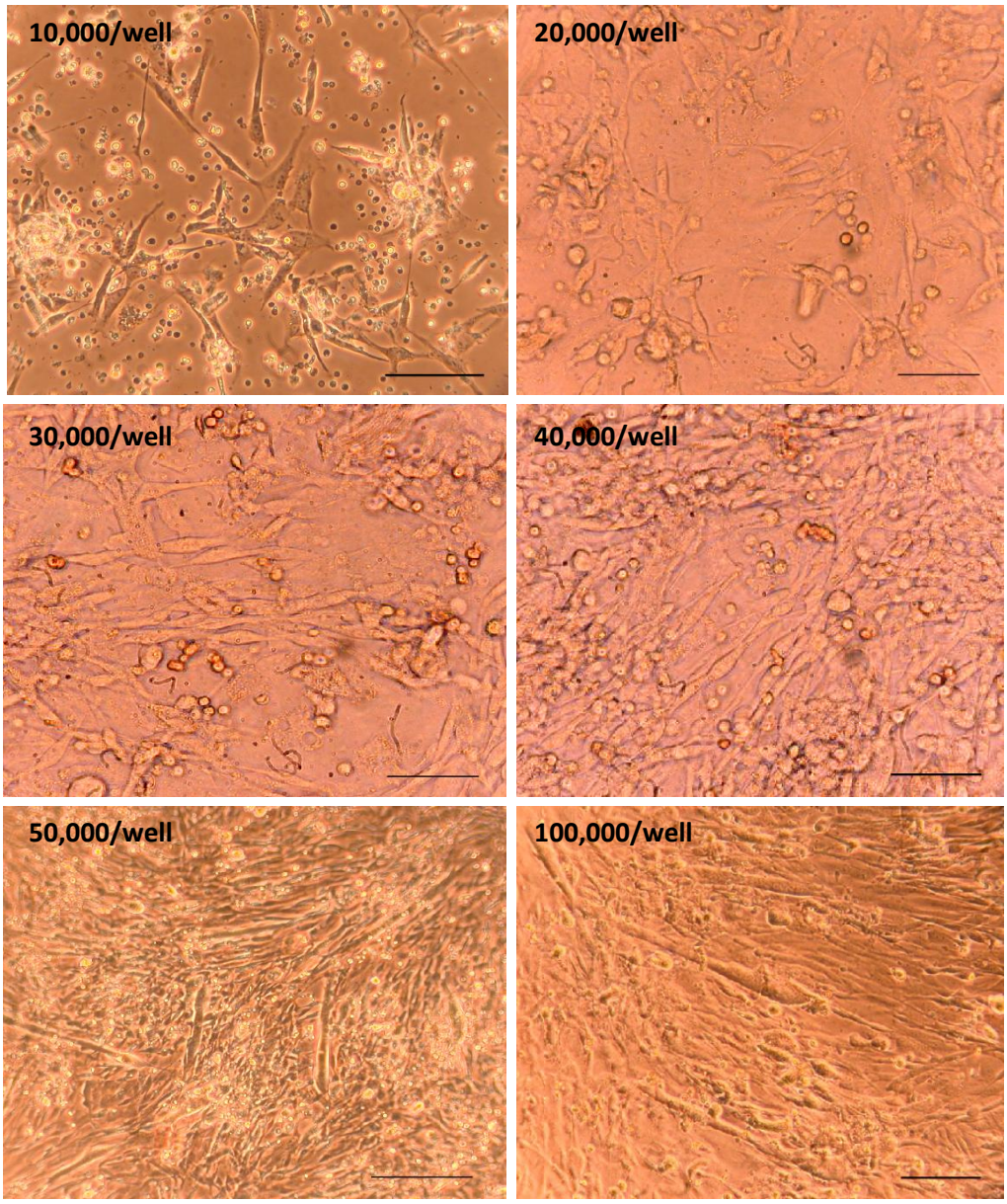


Figure 3.8 Assessing optimal plating density

Cells derived from neonatal rat muscle plated at different densities in 24-well plates. Bright-field images taken at 4 DIV show increased level of fusion and myotube formation with higher plating densities. Scale bar = 100µm.

3.4.2. Results Part 2: Modelling features of sIBM

Sporadic inclusion body myositis is characterised by a combination of its very particular clinical phenotype and histology. Typical sIBM histology shows presence of rimmed vacuoles, central nuclei, atrophied fibres, cytochrome oxidase (COX) negative fibres, cytoplasmic TDP-43 and protein accumulation into inclusion bodies (Askanas & Engel 2007; Verma & Tandan 2009). For the experiments described in this thesis, the protein dysregulation observed in sIBM was chosen as a feature to model *in vitro*. While the other histological features, such as inflammation, would require *in vivo* investigations, genetic modification of cultured cells is commonly carried out to examine the effects of altered protein expression and function.

3.4.2.1. Inducing protein over-expression

β-APP is among the most common proteins found in intracellular protein deposits in sIBM. The gene for the full length human protein is 2312bp whereas the mouse gene is 2087bp. For this investigation, two DNA constructs were used to test their effects on protein over-expression *in vitro*. One construct was a pcDNA3.1 plasmid containing the full length human gene while the other was a full length mouse gene in a pYX-Asc plasmid backbone. These constructs were used to transfect the muscle cultures using Lipofectamine 2000™, resulting in over-expression of β-APP. In the first instance, an optimisation step was carried to determine the optimal ratio of DNA to Lipofectamine to use. 1µg of plasmid DNA was tested with 0.5µl-2µl of Lipofectamine 2000 on cells cultured in 24 well plates which had reached 80-90% confluency.

Next, in order to determine the efficiency of transfection, a method to visualise the translated protein was necessary. A common method for establishing the transfection efficiency is by tagging the protein or plasmid with GFP which can then be easily visualised under a fluorescent microscope. Several attempts to produce a β-APP GFP-tagged pcDNA3.1 plasmid by sub-cloning β-APP proved unsuccessful. This difficulty may have

been due to the large size of the β-APP gene, making ligation more complicated with GFP, a protein over a third of the size of β-APP. GFP-tagging can also alter the function of a protein as previous investigations in this lab have shown when attempting to tag superoxide dismutase 1 (SOD1) with GFP (Stevens et al. 2010). The possibility of altered β-APP function caused by GFP-tagging would make interpretation of the results difficult. Transfection efficiency was therefore determined by counting cells after immuno-staining the cultures with an anti-β-APP antibody to look for cells over-expressing the protein. Using the optimisation step and immunocytochemistry, the most efficient conditions for transfection were determined to be 1µg DNA: 2µl Lipofectamine. Cells were found to reach confluency prior to myocyte fusion after 2 DIV. The transfection efficiency using human β-APP was 13.7% ± 0.8% (SEM, n=6) while the efficiency using the mouse β-APP construct was 17.8% ± 1.4% (SEM, n=6). **Figure 3.9** shows myogenic cells expressing β-APP after 5 days *in vitro*. This protocol for transfection was therefore used in all further experiments.

3.4.2.2. Effects of transfection with the human β-APP plasmid

Cultured cells were transfected after 2 DIV using the human β-APP plasmid. See **Figure 3.9** for images of myocytes over-expressing human β-APP. The same plasmid without the β-APP gene (empty vector) was used as a control to determine the effects of transfection and to ensure any cellular changes were due to the effects of β-APP over-expression itself. The cells were fixed at three time points to observe the changes in β-APP expression over time using immunocytochemistry. **Figure 3.10** shows typical images of the immuno-stained transfected cells at different time points. By 5 DIV β-APP over-expression was evident in transfected cells where immuno-staining with the anti-β-APP antibody showed a 'speckle-like' distribution of the protein throughout the cells. When double-stained with an anti-ubiquitin antibody, there was a clear increase in the expression of ubiquitin compared to empty vector controls, however, the ubiquitin did not appear to co-localise with the β-APP in all transfected cells.

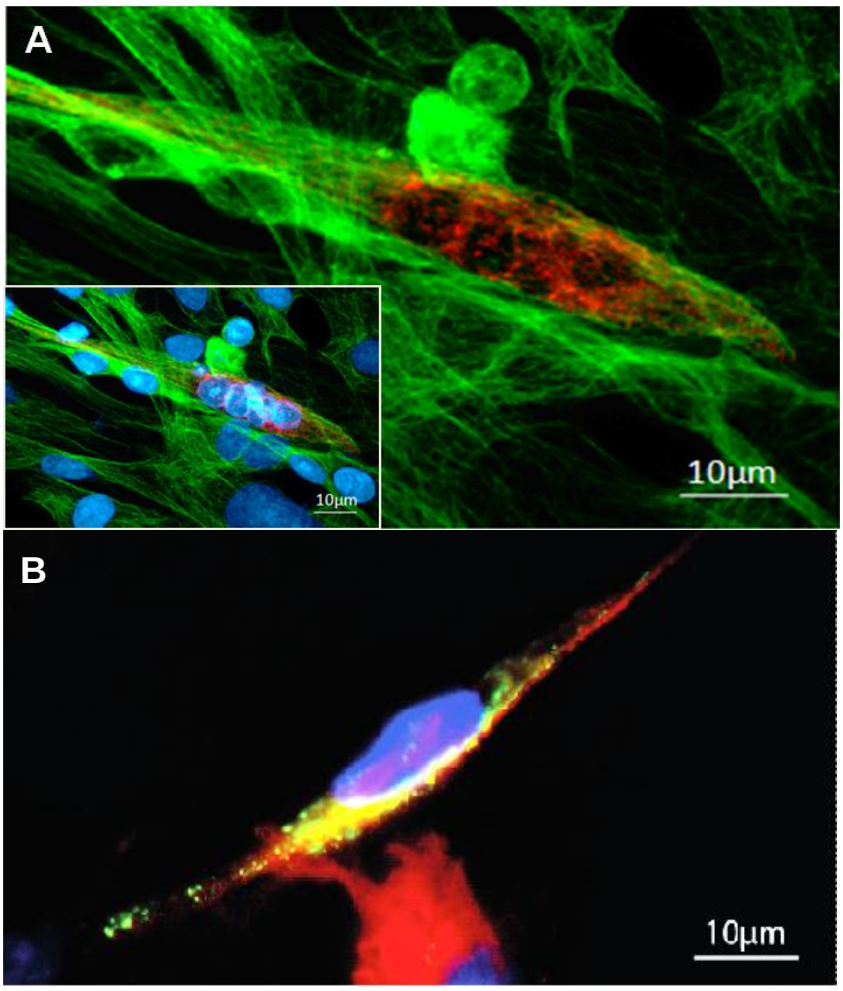


Figure 3.9 Over-expression of human β-APP in muscle cells

Transfection of cultured myocytes with a plasmid containing the β-APP gene induced over-expression of the β-APP protein in cells. [A] A muscle culture immuno-labelled with α-tubulin (green) shows a multinucleated cell over-expressing β-APP (red). Inset shows image in which cells are stained with the nuclear marker DAPI (blue). [B] A desmin antibody was used to identify myocytes (red) in culture that over-expressed human β-APP. The nuclear marker DAPI labels the nucleus (blue).

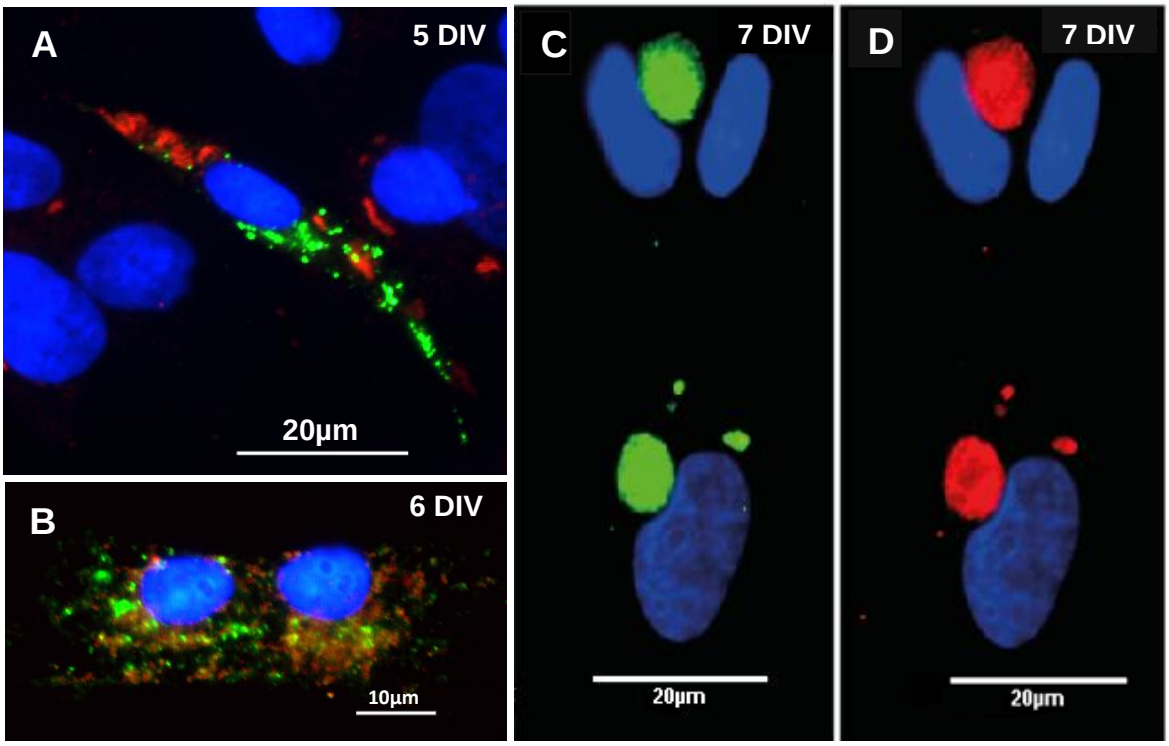


Figure 3.10 Formation of ubiquitinated inclusions in cells transfected with human β-APP

Cells transfected with the human *β-APP* gene, show increased presence of β-APP (green) and ubiquitin (red). DAPI labels the nuclei (blue). [A] At 5 DIV, β-APP is over-expressed in myocytes but is not co-localised with ubiquitin. [B] At 6 DIV, over-expressed β-APP begins to co-localise with ubiquitin [C-D] After 7 DIV, large intracellular inclusions form which co-localise with ubiquitin and often cause nuclear indentations.

By 6 DIV the transfected cells were more varied in their expression of β-APP. By this stage a greater proportion of cells expressed β-APP, which co-localised with ubiquitin, with some cells containing ubiquitinated inclusions. By 7 DIV the majority of transfected cells showed aggregated β-APP protein in large ubiquitinated inclusions which were often found to impinge on the nucleus. At this stage few cells with a speckle-like distribution of β-APP was observed.

3.4.2.3. Effects of transfection with the mouse β-APP plasmid

The protocol used for human β-APP transfection was repeated using the mouse β-APP plasmid. Immuno-staining with antibodies against β-APP and ubiquitin showed that by 5 DIV the majority of transfected cells had already formed large, ubiquitinated, intracellular inclusions. The inclusions were also present in cells fixed at 6 and 7 DIV. As non-aggregated β-APP was rarely observed in the fixed culture at any time point studied, this suggests that the mouse β-APP protein had a greater aggregation potential which allowed it to form inclusions within 48 hours of transfection (5 DIV). **Figure 3.11** shows examples of the ubiquitinated intracellular β-APP inclusions at the three time points studied.

The numbers of desmin-positive cells with β-APP-positive inclusions were quantified to compare the results of empty vector, human β-APP and mouse β-APP transfected cells. The percentage of myogenic cells containing inclusions after transfection with the human gene was found to be 13.5% ± 3.2% (SEM, n=3) and for the mouse β-APP transfected cells 12.6% ± 2.1% (SEM, n=3). This shows that there was a significant increase in the number of inclusions formed after β-APP transfection ($p<0.05$, Mann-Whitney U test) when compared to the empty vector transfected cells (see **Figure 3.12** [A]). Transfection of β-APP therefore resulted in protein over-expression and the formation of inclusion bodies, as required to model this feature of sIBM.

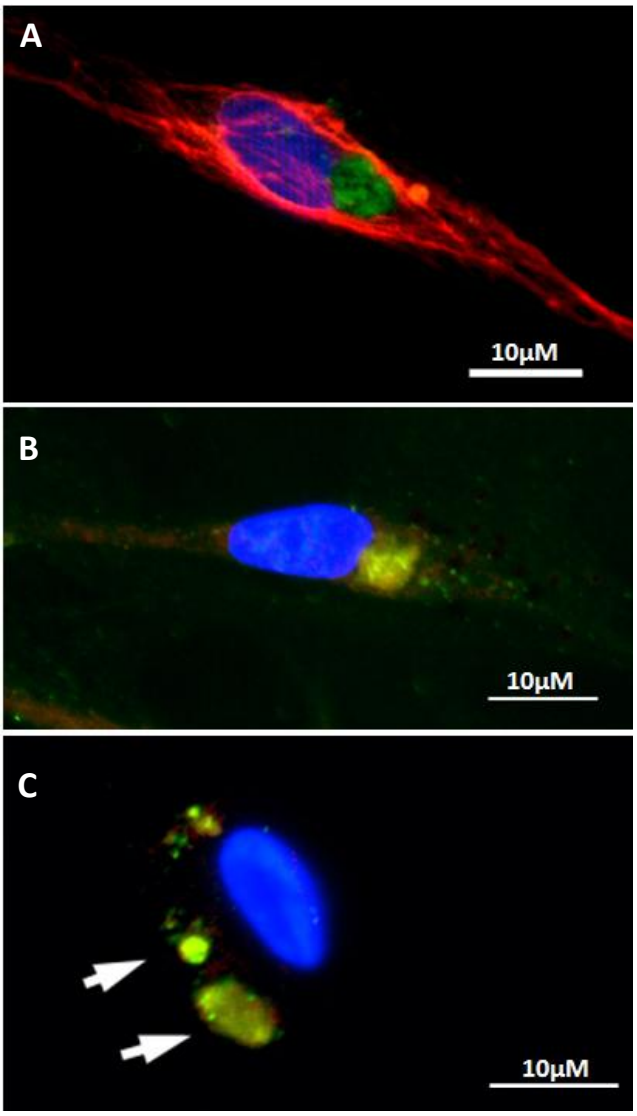


Figure 3.11 Formation of ubiquitinated inclusions in cells transfected with mouse β-APP

Immuno-staining of cells transfected with the mouse β-APP gene shows the formation of intracellular inclusions at 3 different time points. [A] Desmin positive cell (red) with β-APP-positive inclusion body (green). [B and C] β-APP-positive inclusion body (green) co-localises with ubiquitin (red). Co-localised proteins appear orange/yellow. DAPI labels nuclei (blue).

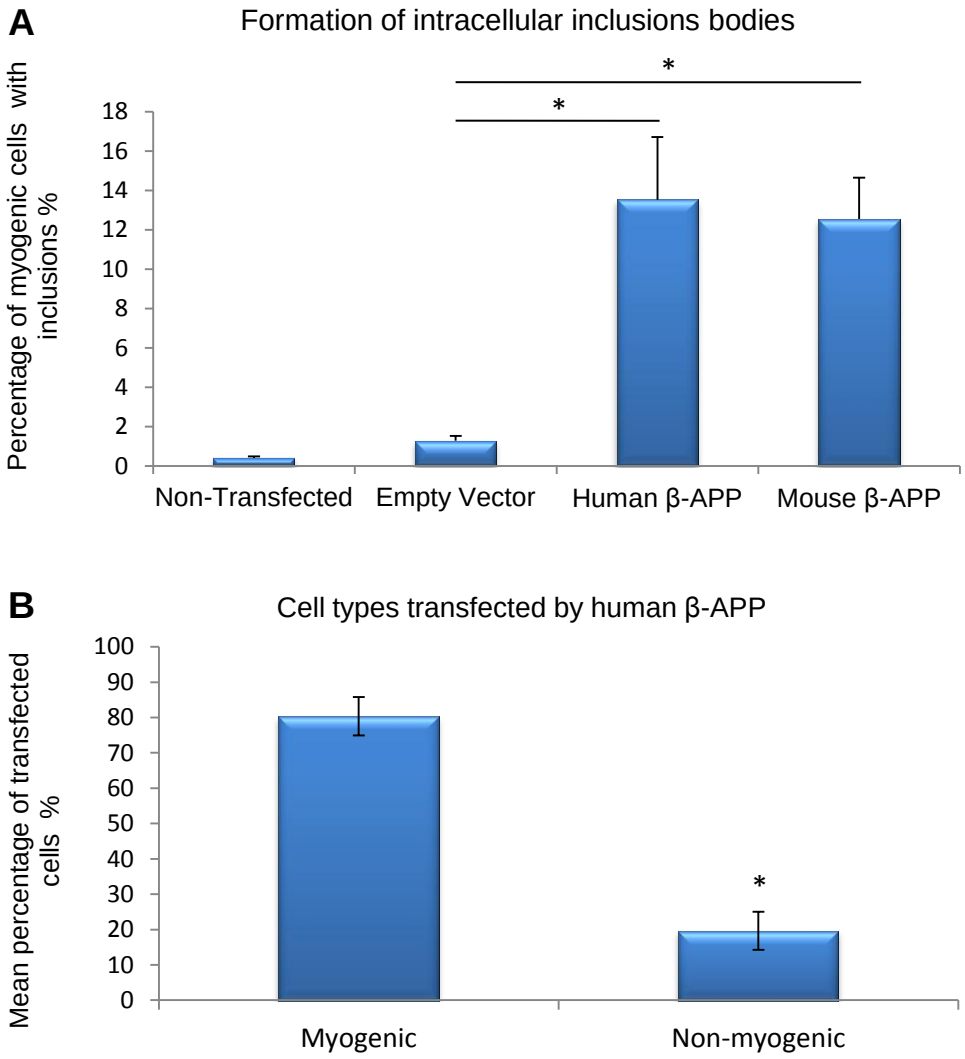


Figure 3.12 Quantification of intracellular inclusion bodies

[A] The bar chart shows the percentage of myogenic cells with inclusion bodies after transfection with human and mouse β-APP. Data shows that transfection with either species of β-APP results in an increase in the formation of inclusions compared to non-transfected cells or those transfected with the empty vector. Error bars= SEM, ($P<0.05$, n=3). [B] The bar chart shows the percentage of human β-APP transfected cells which were found to be myogenic or non-myogenic as determined by desmin staining. The results show that a significantly higher proportion of myogenic cells are transfected compared to non-myogenic ($P<0.01$ n=5). *denotes statistical significance.

The human β-APP transfected model was considered more practical for the current study as progression into inclusion formation was slower and could be visualised and examined at the different stages. Therefore all further transfection procedures were undertaken with the human β-APP plasmid.

Since the muscle cultures used in these experiments are comprised of a mixed population and are not 100% pure, it was interesting to study how the different cell types responded to the transfection process and subsequently overexpressed the β-APP protein. In β-APP transfected cells, the total number of transfected cells was calculated by quantifying those cells which were positive for overexpressed/aggregated β-APP. Next, those β-APP positive cells that were also desmin positive were recorded together with those that were desmin negative. This gives an indication as to how susceptible myogenic and non-myogenic cells are to this type of gene transfer. The results showed that 80.36% ± 5.41% (SEM, n=5) of cells which were transfected were myogenic compared to 20.64% ± 5.41% (SEM, n=5) which were non-myogenic (see **Figure 3.12** [B]).

3.4.2.4. Microtubule mediated inclusion body formation (aggresome)

To explore whether the formation of inclusion bodies in this study was mediated by the inherent protein transport mechanism within the cells, centriole localisation with respect to the cytoplasmic inclusion bodies was investigated by immune-staining of the human β-APP over-expressing cells. The results show that centrioles are located adjacent to the nucleus as expected. However, the β-APP positive inclusion bodies are found associated with the centrioles. **Figure 3.13** shows typical images of the co-localisation of the centrioles and the β-APP positive inclusion bodies. This suggests that the mode of inclusion body formation is not diffusion limited, but mediated by the cell via the microtubule network. These inclusion bodies can therefore be labelled as ‘aggresomes’, a term coined by Kopito (2000) for this type of protein aggregate.

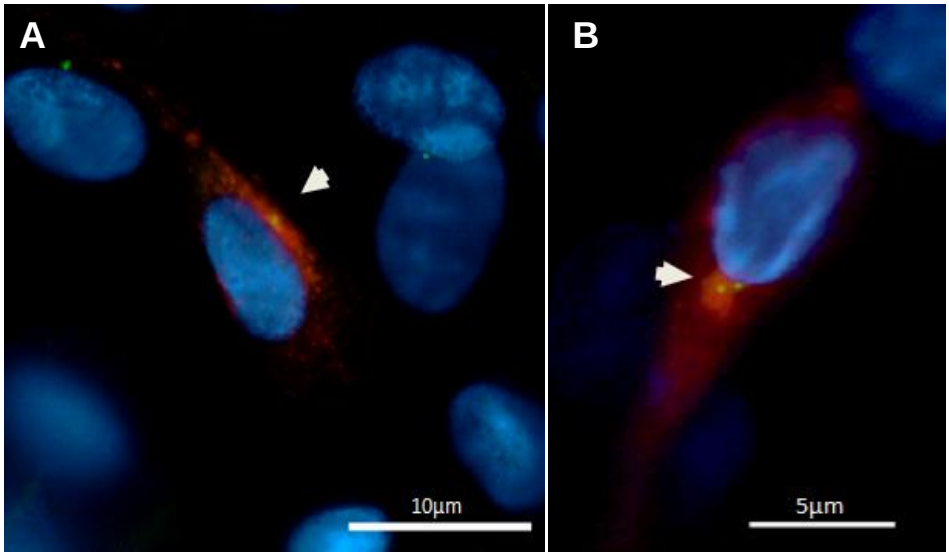


Figure 3.13 Centriole localization in β-APP positive cells

Immuno-staining of cells in culture shows γ-tubulin positive centrioles (in green, indicated by white arrows) in myogenic cells which are positive for β-APP (red). [A] β-APP aggregate forming in a cell with the centriole located at the centre, adjacent to the nucleus. [B] A β-APP positive inclusion body within a cell associated with the centrioles, suggesting microtubule-mediated formation. DAPI labels nuclei (blue).

3.4.3. Results Part 3: Characterisation of the *in vitro* model

To examine the effects of over-expressing β-APP *in vitro*, the cell culture model was further characterised by examining the expression of other proteins relevant to sIBM and assessing the effects of β-APP over-expression on cell survival.

3.4.3.1. Analysis of sIBM-relevant protein expression

The expression pattern of a number of proteins that have been observed to be abnormally present in sIBM were examined in β-APP transfected cultures using immunocytochemistry.

- *HSP70 immuno-reactivity.*

  The β-APP transfected cultures were immuno-stained for HSP70, a well-known stress associated protein chaperone, to determine whether the stress caused by the over-expression of the protein leads to up-regulation of the Heat Shock Response (HSR). Immuno-staining with an anti-HSP70 antibody showed that human β-APP transfected cells up-regulated HSP70 with a uniform distribution in the cytoplasm, 48 hours post transfection (5 DIV) (see **Figure 3.14** [A]). This increased expression of HSP70 however was not maintained over the next 2 days *in vitro* and was seen to decrease as more of the chaperone protein became sequestered within the inclusion bodies.

- *Caspase-3 immuno-reactivity*

  The same pattern of distribution as HSP70 was detected by immuno-staining for activated caspase-3, a protein up-regulated in the pathway of cell apoptosis which indicates the cell is under stress. After day 5 *in vitro*, caspase-3 up-regulation could be seen in cells over-expressing β-APP, however, this distribution was not seen in cells that had formed inclusion bodies. Some inclusion bodies were found to be immuno-reactive for caspase-3 themselves (see **Figure 3.14** [B]).

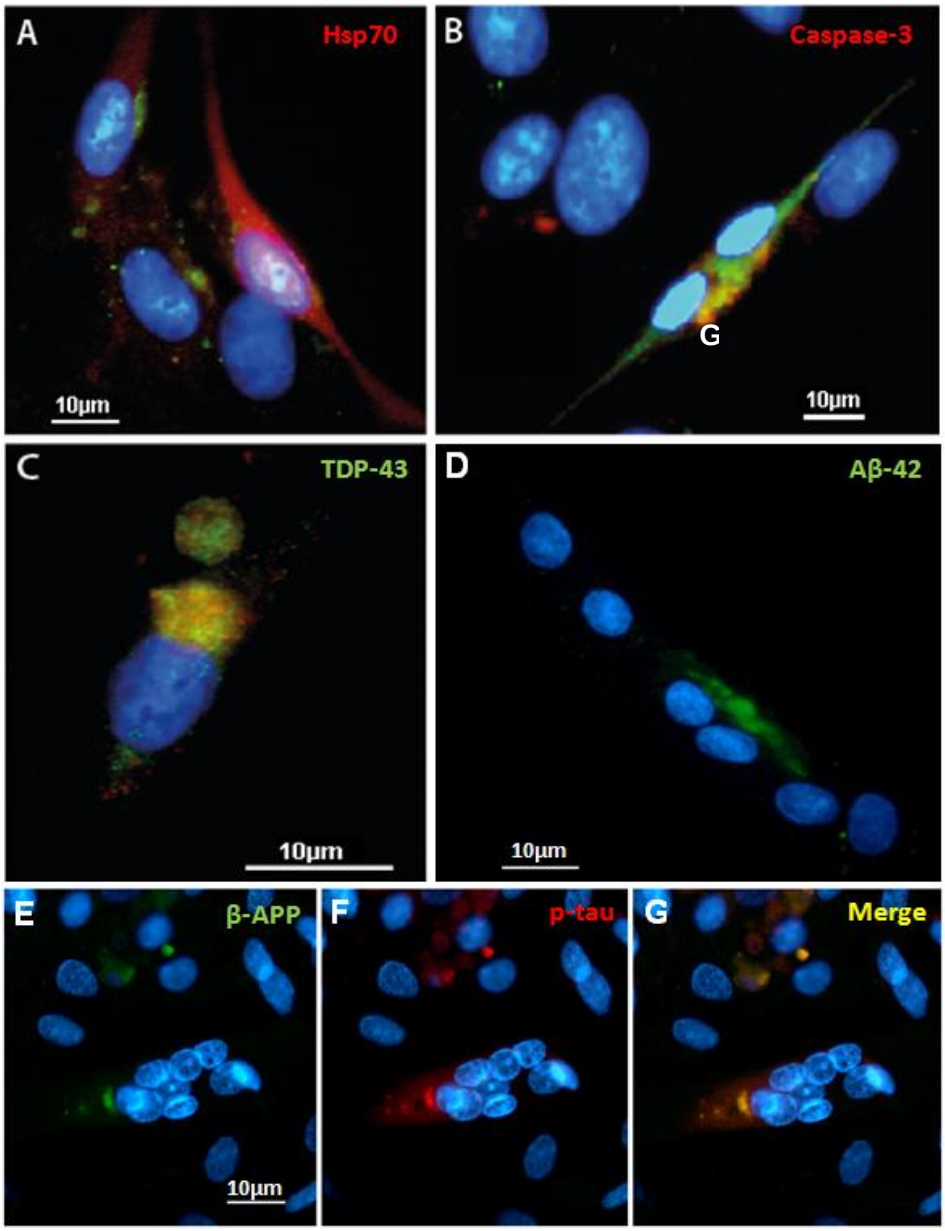


Figure 3.14 β-APP expression induces altered protein expression in muscle cells

[A] Transfection of muscle cells with β-APP (green) leads to the up-regulation of HSP70 (red) and [B] caspase-3 (red). [C] Intracellular inclusion bodies are positive for TDP-43 (green) which co-localises with ubiquitin (red). [D] Up-regulation of the proteolytic product Aβ-42 (green) in β-APP transfected myotubes. [E-G] An example of an inclusion body within a multinucleated cell positive for both β-APP (green) and phosphorylated tau (p-tau) (red). DAPI labels the nuclei (blue).

- *TDP-43 immuno-reactivity*

  TDP-43 is a protein involved in RNA regulation which has recently been implicated in sIBM pathology. TDP-43 has been reported to be present in the cytoplasm of sIBM myofibres. When transfected cells were immuno-stained for TDP-43 it was found to co-localise with ubiquitin in the inclusion bodies (see **Figure 3.14** [C]). Changes in TDP-43 expression in this model are discussed further in section 3.6.3.

- *Aβ-42 immuno-reactivity*

  Along with the up-regulation of β-APP, it was important to assess whether proteolytic cleavage products of this protein were also present at a higher concentration. Results show that Aβ-42 was up-regulated in transfected myogenic cells (see **Figure 3.14** [D]).

- *Phosphorylated tau (p-tau) immuno-reactivity*

  Another protein implicated in sIBM pathology is phosphorylated tau. Immuno-staining revealed that p-tau was also present in the β-APP transfected cells and co-localised with β-APP in aggregates (see **Figure 3.14** [E, F and G]).

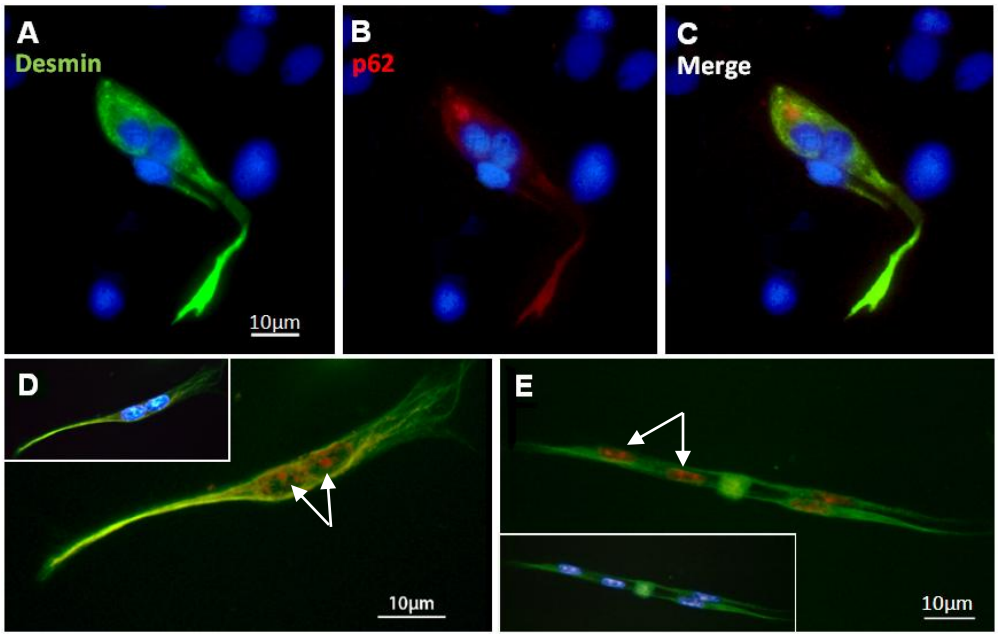
3.4.3.2. p62 localisation in transfected muscle cells

p62 (also known as sequestosome 1) is a multifunctional protein which is known to be involved in nuclear and cytoplasmic shuttling of aberrant proteins (Pankiv et al. 2010). Recent finding have shown p62 to be abnormally expressed in sIBM, and even proposed as a molecular marker for p-tau inclusion bodies (Nogalska et al. 2009b). It may also be a potential marker to distinguish between the inflammatory myopathies (Nogalska et al. 2009b). In this study, p62 positive inclusion bodies were identified in β-APP over-expressing muscle cells, in both nuclear and cytoplasmic inclusions. When quantified, there was a significant increase in the number of cytoplasmic inclusion bodies containing p62 compared to empty vector control cultures ($p<0.001$ Mann-Whitney U, $n=3$) . Nuclear inclusions were not detected in control cells.

Figure 3.15 shows images of these inclusion bodies visualized by immunocytochemistry, and shows the percentage of cells containing p62 positive inclusion bodies.

3.4.3.3. Translocation of TDP-43

TAR DNA-binding Protein 43 (TDP-43) is a nuclear protein which is known to be involved in post transcriptional regulation of RNA and has recently been implicated in sIBM pathology (Verma & Tandan 2009). Cultures transfected with the empty vector and the β-APP plasmid were immuno-stained for the C-terminal and N-terminal of TDP-43. Non-transfected cells were used as a control. The results are summarised in **Figure 3.16** and **Figure 3.17** which show that in non-transfected cultures and those transfected with the empty vector, TDP-43 was localised in the nucleus, as is typical of unstressed cells. However, in cultures transfected with β-APP, in 52.2% ± 5.3% of cells (SEM, n=3 p=0.0001, Mann-Whitney U test) TDP-43 had translocated from the nucleus to the cytoplasm. This translocation was only observed with immuno-stains for the C-terminal of TDP-43. N-terminal TDP-43 remained in the nuclei in β-APP transfected cells. In some cases, C-terminal TDP-43 translocated to the cytoplasm co-localised with ubiquitinated protein aggregates (**Figure 3.14** [C]). These findings suggest that over-expression of the β-APP protein triggers a cellular response which leads to the translocation of a portion of the TDP-43 protein to the cell cytoplasm. **Figure 3.14** shows typical images of the expression of TDP-43 under the different experimental conditions.



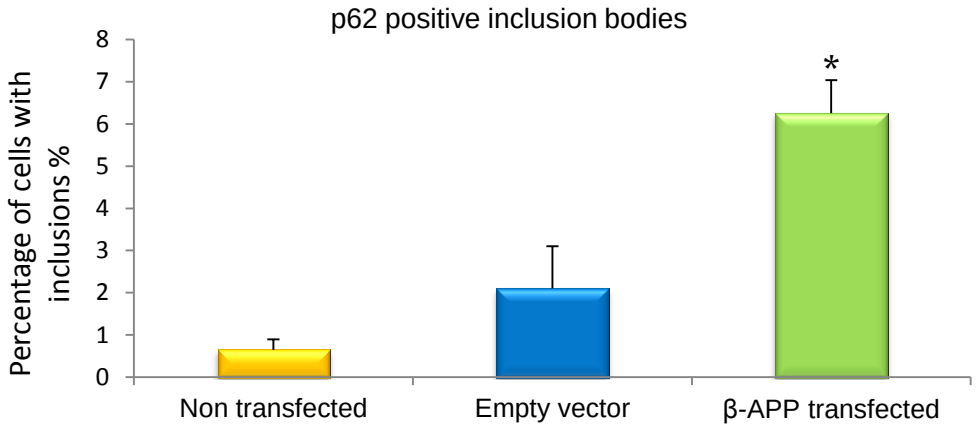


Figure 3.15 β-APP expression induces the formation of p62 positive inclusion bodies in muscle cells.

[A-C] An image of a β-APP over-expressing myogenic cell positive for desmin, containing a large p62 positive inclusion body is shown. [D and E] Myogenic cells containing nuclear inclusions positive for p62. Insets show DAPI stained nuclei. [F] The bar chart shows the percentage of cells containing p62 positive inclusion bodies under different conditions (Non-transfected= 0.65% ± 0.25% SEM; Empty vector= 2.1% ± 1.0% SEM; β-APP= 6.26% ± 0.78% SEM) β-APP over-expressing cells show a significantly greater number of p62 positive inclusions compared to controls. (Error bars =SEM, * denotes $p<0.0001$, n=3)

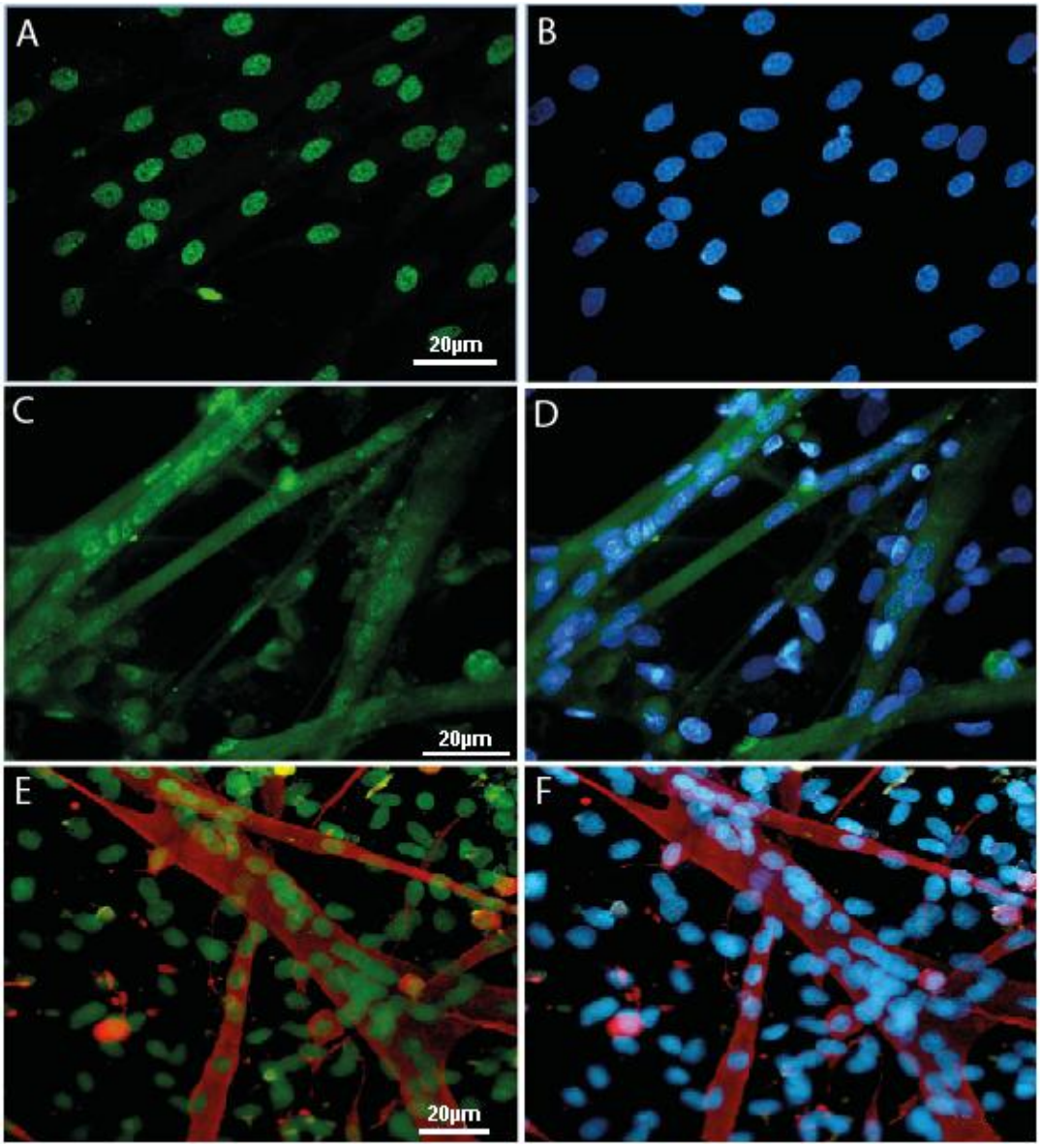


Figure 3.16 β-APP over-expression induces translocation of C-terminal TDP-43 from the nucleus to the cytoplasm

Cultures immuno-stained for TDP-43 are shown. [A-B] Cells transfected with an empty vector show nuclear localisation of TDP-43 (green) using antibodies to both the C and N- terminal TDP-43. DAPI labels the nuclei (blue). [C-D] Cells transfected with β-APP show translocation of C-terminal TDP-43 (green) to the cytoplasm. [E-F] β-APP transfected cells show nuclear localisation of the N-terminal fragment of TDP-43 (green). Myogenic cells are labelled with desmin (red). DAPI labels the nuclei (blue).

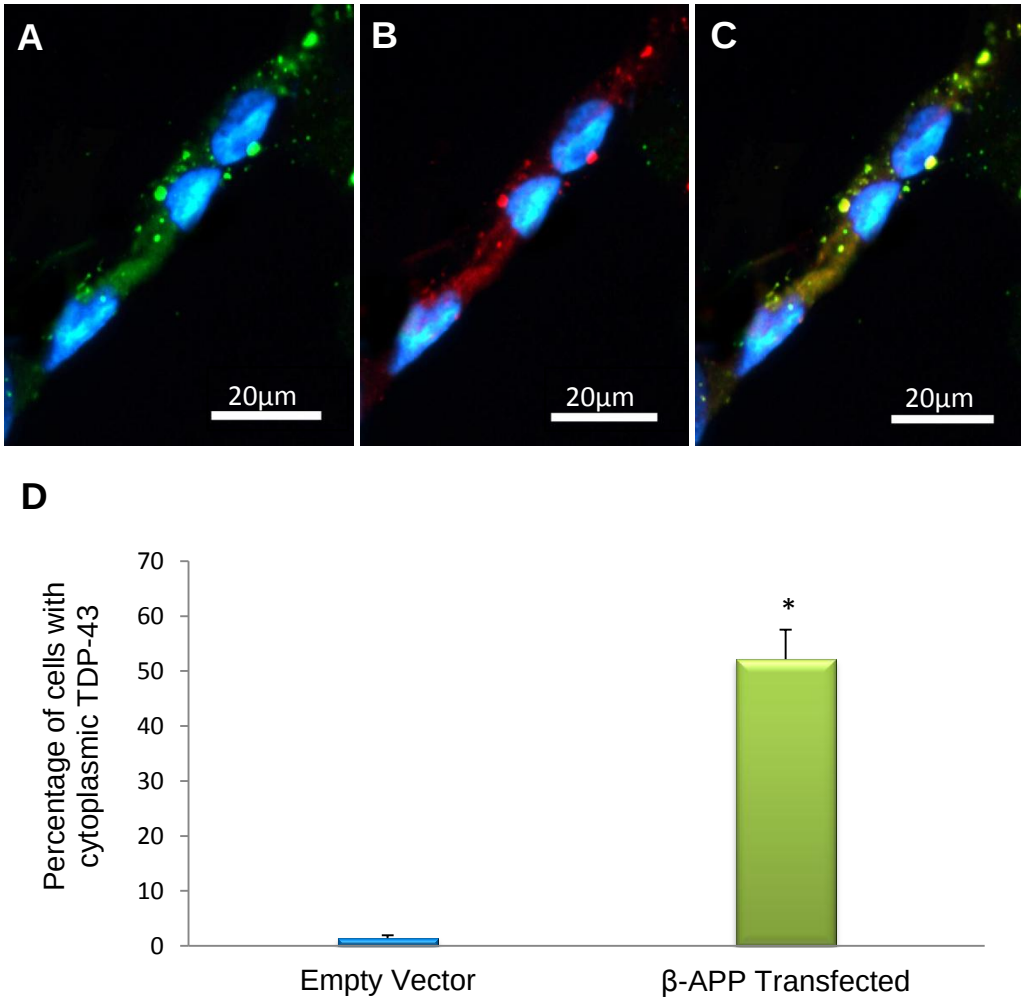


Figure 3.17 The effects of β-APP over-expression on TDP-43 expression

[A-C] Muscle cells transfected with β-APP were immuno-stained for C-terminal TDP-43. Images show co-localisation of TDP-43 and ubiquitin in cytoplasmic inclusions. [D] The bar chart shows that the percentage of cells with cytoplasmic C-terminal TDP-43 increases significantly in β-APP transfected cells after transfection. Error bars= SEM, *denotes P<0.0001 (n=3)

3.4.3.4. The cytotoxic effects of human β-APP over-expression

To investigate the effects of β-APP over-expression in rat primary muscle cultures, cell toxicity was examined using a Lactate Dehydrogenase (LDH) Assay.

The cultured cells were plated into 96-well plates with 6 wells per condition to obtain a reliable mean value. The cells were transfected with the human β-APP plasmid and the empty vector after 3 days *in vitro* and LDH assays were carried out at 5, 6 and 7 days *in vitro* (DIV), the same time points used in the analysis of protein expression above. Each assay was repeated twice, from different cell cultures to confirm the result. Representative data from one assay is shown in **Figure 3.18**. Statistical significance was calculated using a Mann-Whitney U test.

The results show that the percentage of LDH in the media in human β-APP over-expressing cultures was significantly increased by 6 and 7 DIV ($p<0.005$). Thus, at 6 DIV LDH release rose to 134.5% ± 13.1% (SEM, n=6) and 134.8% ± 4.2% (SEM, n=6) at 7 DIV. The percentage LDH was normalised to that released by control cultures transfected with the empty vector, which was set at 100%. Therefore any increase in LDH had to be above that found with the empty vector transfection to be an effect of the β-APP protein over-expression. The LDH data indicates that over-expression of human β-APP is cytotoxic to muscle cells in culture by 6 DIV.

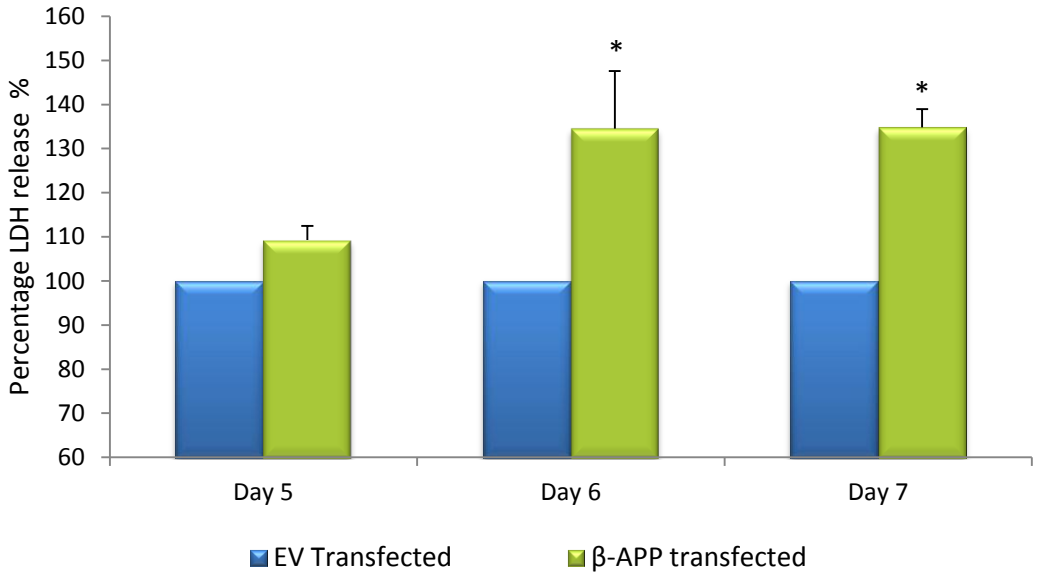


Figure 3.18 The effect of β-APP expression on muscle cell survival

The effect of transfection with β-APP on muscle cells survival was assessed by an LDH Assay. The bar chart summarises the results and shows the percentage of LDH released over three days *in vitro* in cells transfected with either the empty vector or the human β-APP plasmid. Error bars= SEM, * denotes $p<0.05$, representative data from 3 cultures.

3.4.3.5. The effects of β-APP over-expression on mitochondrial function

To investigate the effects of β-APP over-expression on mitochondrial function, the level of succinate dehydrogenase (SDH) was assessed using an assay which causes a reaction between the electron acceptor in the citric acid cycle and nitro-blue tetrazolium. This results in purple/blue colouration of mitochondria within cells which can be visualised and used as a measure of the oxidative potential of the cell.

SDH staining of muscle cultures (**Figure 3.19**) showed firstly that there was a considerable difference in SDH levels between fibroblasts and muscle cells. In non-transfected cultures, myogenic cells were easily distinguishable from non-myogenic cells due to the darker purple/blue colouration and morphology. As muscle cells have a greater oxidative demand, the number of mitochondria they have is significantly higher than most of the other cell types in culture. Therefore a higher level of SDH staining is in line with expectations. **Figure 3.19** [B] demonstrates the differential staining between myogenic and non-myogenic cells in muscle cultures. This observation was found to be very important in assessing the effects on mitochondria in muscle cells after β-APP plasmid transfection.

The results from transfection experiments showed no observable difference in SDH levels between non-transfected and empty vector transfected cultures. However, in β-APP over-expressing cultures, the level of SDH was found to be greatly reduced in transfected muscle cells compared to empty vector and non-transfected control cells. As a positive control for reduced SDH levels, some cells were treated with staurosporine, a widely used cell stressor which affects ATP binding. In the staurosporine treated cells a similar staining pattern was observed to that in the β-APP transfected cultures. These results therefore demonstrate that β-APP over-expression leads to mitochondrial deficits. **Figure 3.19** demonstrates the differences in SDH level in stained muscle cultures with and without β-APP over-expression.

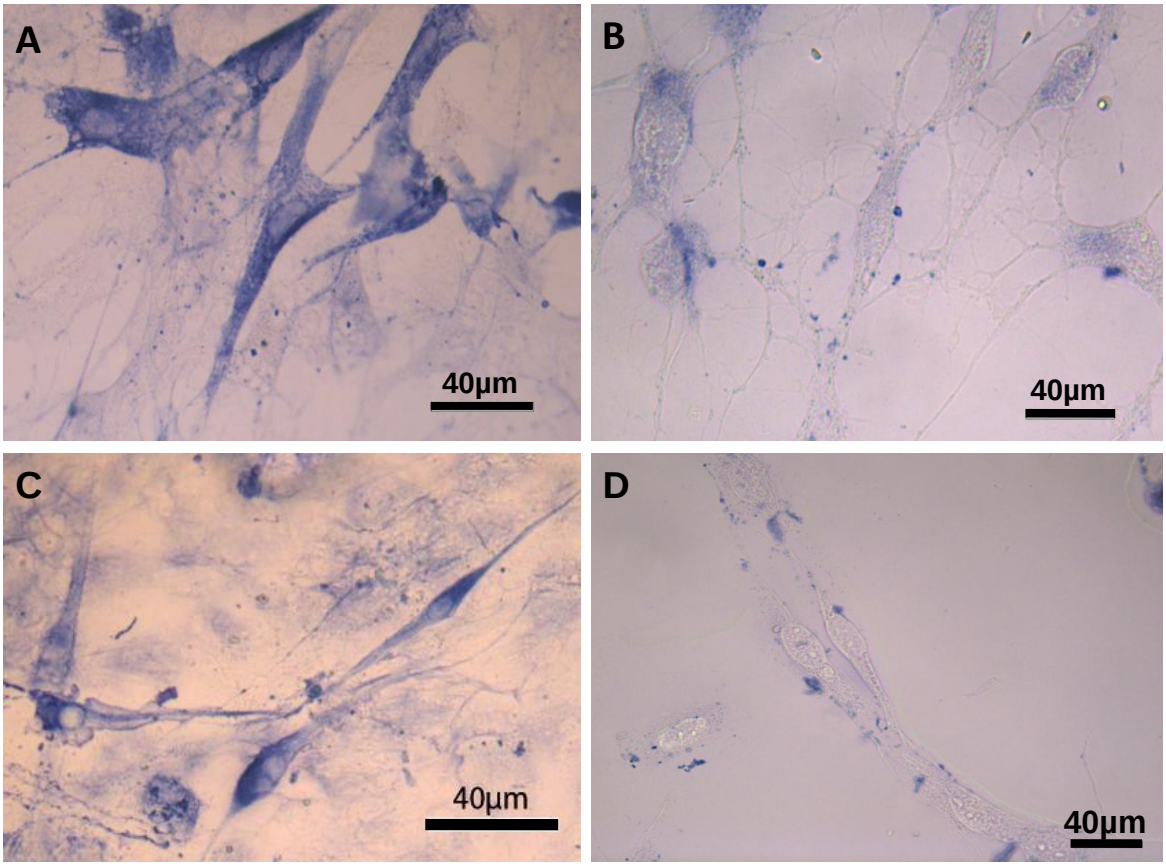


Figure 3.19 β-APP over-expression alters SDH levels in myogenic cells

Myogenic cells were stained for SDH to visualise mitochondrial protein levels. [A] Normal, non-transfected cells. [B] Staurosporine treated cells used as a negative control for SDH levels. [C] Empty vector transfected cells as a control for the transfection process. [D] β-APP transfected cells show a reduction in SDH levels.

3.4.3.6. The effects of β-APP over-expression on proteasome function

The effect of β-APP over-expression on cell viability was also examined by studying proteasomal function. As the proteasome complex is one of the major components of protein handling in cells, instability in protein levels are likely to impact on the function of this complex. In this study proteasome activity was measured biochemically using fluorescent substrates to catalytic sites in the proteasome. Assays were conducted 48 hours after transfection (5 DIV). The results are summarised in **Figure 3.20** which shows that there was a decrease in proteasomal activity in β-APP transfected cells compared to empty vector controls. However the pooled data from 5 individual assays showed the data to be only borderline in statistical significance (Students t-test $p=0.054$, $n=5$). This may be due to the large inherent variations within individual muscle cultures and the relatively low transfection efficiencies.

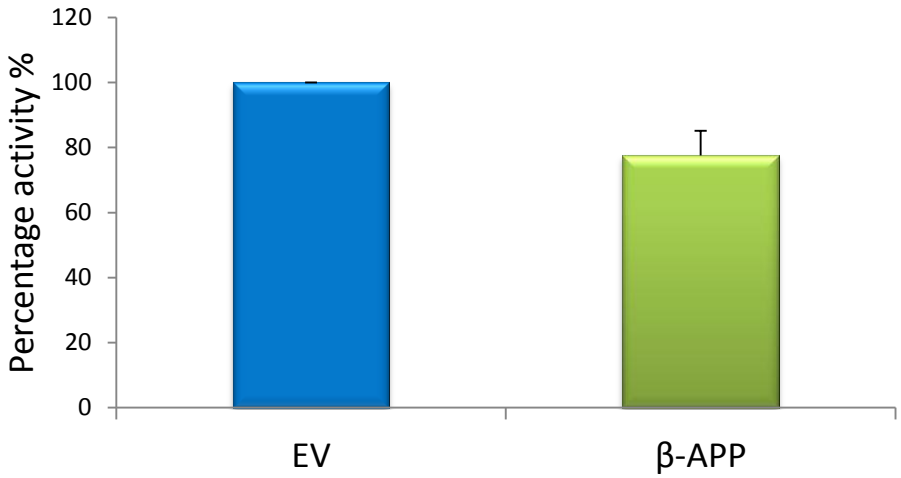


Figure 3.20 The effects of β-APP over-expression on proteasomal function in myogenic cells

The bar chart summarises the results of the percentage proteasomal activity in muscle cultures transfected with either the empty vector (EV) or β-APP. The result from the EV cultures was taken as 100%. The results show that β-APP transfection caused a decrease in proteasome activity. Error bars = SEM

3.5. DISCUSSION

3.5.1. Development of optimal primary muscle cultures

Isolation of muscle precursor cells from biopsy material and animals is a well-established tissue culture technique. However these cultures invariably contain mixed cell types with the proportion of myogenic cells ranging widely from 5-45% (Sinanan et al. 2004). As well as satellite cells, which are known for their myogenic capacity, muscle cultures also contain other Muscle Derived Stem Cells (MDSCs) which may become adipocytic or osteogenic. However, under favourable conditions these MDSCs can also differentiate into muscle cells (Sinanan et al. 2004). As yet, a universal method for obtaining a highly pure population of myogenic cells *in vitro* has not been established. Various methods ranging from FACS sorting (Blanton, Jr. et al. 1999; Sacco et al. 2008) to MyoD transfection of fibroblasts (Qin et al. 2007; Chaouch et al. 2009) have been employed to enrich the myogenic cell population in primary muscle cultures, but each has its disadvantages or has inconsistent reproducibility.

In order to study sIBM *in vitro,* the present investigation required robust and reproducible muscle cultures to be established. Therefore five different techniques were first tested in this study using three criteria: 1) myogenicity, 2) yield of cells and 3) longevity of cultures. Of these, the more technical methods of FACS and MACS sorting gave the most promising results but were found to be unsuitable.

FACS sorting has undoubtedly been a valuable tool for isolating various types of cells from a mixed population. In particular it allows identification of very specific cell types by more than one surface antigen simultaneously, so that FACS sorting is highly precise and sensitive. Leronimakis et al (2008) demonstrated this by the isolation of a particular subtype of endothelial cells which were Sca-1^{+}, $CD31^{+}$, $CD34^{dim}$ and $CD45^{-}$ from skeletal muscle of mice (Leronimakis et al. 2008). It is therefore important that cell surface antigens which are to be used for identifying specific cell populations remain intact for FACS sorting. However, in the present study, FACS analysis revealed that the NCAM/CD56

and CD34 antigens on the cell surface were disrupted by enzymatic digestion using both collagenase and trypsin. This result corroborates the findings of Abuzakouk et al (1996) who investigated the effects of collagenase and dispase on the cell surface proteins of blood lymphocytes. In this study, the authors were unable to see a reappearance of the cleaved surface proteins after incubating cells for 3 hours *in vitro* (Abuzakouk et al. 1996). In the present study NCAM/CD56 antigens on the surface of satellite cells were found to recover when cells were incubated *in vitro* for 24 hours prior to cell sorting. Nonetheless, FACS sorting continued to be problematic as sorted cells would not differentiate *in vitro*.

MACS sorting is used routinely to separate particular cell types from many tissues and is therefore a well-established method in tissue culture. Sinanan et al (2004) first described the use of MACS to isolate purer populations of satellite cells. This group utilised the NCAM/CD56 antigen on the surface of MDSCs to directly label the cells for sorting (Sinanan et al. 2004). In the present investigation the principle of antigen mediated separation by MACS sorting was found to be successful by targeting the same surface protein. MACS sorting produced highly myogenic cultures with approximately 89% desmin-positive cells in culture. On the other hand this technique resulted in a very poor cell yield with the highest myogenic cultures retaining only 1.8% of the original cell number prior to sorting. When incubated *in vitro* and subsequently immuno-stained for desmin, it was apparent that the 'non-myogenic fraction' contained a high proportion of myogenic cells. This loss of cells made the process highly inefficient and this could not be rectified by increasing the concentration of antibodies used or by repeatedly running the 'non-myogenic' fraction through the magnetised column. Although high levels of purity were obtained through MACS sorting, yield could not be compromised on for these experiments. Therefore MACS sorting was also discontinued.

3.5.1.1. Cell culture optimisation

MDSCs are multipotent and can differentiate into adipocytes, osteocytes and other non-myogenic cell types (Sinanan et al. 2004). Even satellite cells have been demonstrated to

be multipotent and undergo 'trans-differentiation' into other lineages (Asakura et al. 2001). This re-enforces the need to grow MDSCs in the most ideal medium to ensure the right environment for differentiation down the myogenic lineage. The final method therefore used to obtain highly myogenic cultures without compromising cell yield or longevity was optimisation of the culture technique, which mainly involved altering the media. The addition of chick embryo extract to the media and the use of a second differentiation media to promote the formation of myocytes from satellite cells produced reliable and reproducible muscle cultures. A maximum myogenicity of 60.5% was achieved when these cells were plated at 5 x 10^4 cells per well in a 24-well plate (or 2.5 x 10^4 per cm^2). These cultures were therefore used in the experiments described in this Thesis.

3.5.2. Over-expression of β-APP as a model of the degenerative features of sIBM

Disruption of proteostasis is a common feature in many neurodegenerative disorders (Stefani 2007). In these disorders, intracellular protein aggregates form which share similar amyloid-like conformations despite different amino acid sequences in the proteins involved (Chiti et al. 1999; Douglas & Cyr 2010). 'Amyloid' refers to aggregated fibrils defined as β-sheet-rich conformations which stain positively with Congo Red or Thioflavin S and are not to be confused with aggregates of A-β pr β-APP. The formation of amyloid structures is not due to the interactions between amino acid side chains, but rather more common interactions such as hydrophobic or hydrogen bonds (Chiti et al. 1999). The formation of amyloid aggregates is therefore not disease or protein specific, but rather a consequence of general disruption to the proteostasis. Amyloid filaments have also been observed in sIBM patient muscle stained with Congo Red (Mendell et al. 1991) and form part of the diagnostic criteria for sIBM. The amyloid structures in sIBM have been reported to contain β-APP, Aβ and phosphorylated tau (Askanas & Engel 2007), indicating that protein mishandling is a key feature of sIBM irrespective of whether or not it is the primary pathological trigger or not.

Protein mishandling is a well-documented feature of sIBM whether in relation to proteasome dysfunction or triggering of the ER unfolded protein response (UPR) (Vattemi et al. 2004; Fratta et al. 2005a). Askanas & Engel (2007) proposed that the intracellular expression of one particular protein, β-APP and its proteolysed products are toxic and play a key upstream role in the pathogenesis of sIBM. This over-expression of β-APP in cultured human muscle cells by viral transduction induced key features of the sIBM cellular phenotype, including the formation of intracellular aggresomes and decrease in proteasome activity (Fratta et al. 2005a). This group also found that β-APP accumulated in cultures of muscle cells from patients with hIBM (with the GNE mutation) (McFerrin et al. 1998).

Furthermore, in contrast to normal human muscle cells, which contract continuously when co-cultured with rat spinal cord explants, muscle cells from hIBM patients or those experimentally over-expressing β-APP do not become readily innervated or show regular contraction of muscle cells (McFerrin et al. 1998). The authors therefore suggest that over-expression of β-APP renders the muscle cells less receptive to innervation.

In the present study, *in vitro* over-expression of β-APP was therefore used to model sIBM. Rat primary muscle cultures were used and over-expression of β-APP was induced with Lipofectamine mediated transfection. The effects of both human and mouse β-APP was initially examined.

3.5.2.1. Comparing the over-expression of human and mouse β-APP

Over-expression of the mouse and human protein was found to result in slightly different outcomes. Although the percentage of cells with inclusions was very similar in both cases, the mouse protein was found to aggregate very rapidly, suggesting that the mouse protein has a greater tendency to aggregate or is processed into inclusion bodies at a faster rate than the human protein when expressed in rat myocytes. This may be due to the 17 amino acid difference between mouse and human β-APP, of which 3 specific amino acid

changes (R5→G, Y10→F and H13→R) are in the N-terminus. This region is known to be important for specificity of interactions between Aβ peptides (Jankowsky et al. 2007). The homology between mouse and rat β-APP protein is 99%, and therefore it is possible that the over-expressed protein is recognized by the rat myocytes swiftly, allowing its endogenous protein handling mechanisms to engage more efficiently. Both the human and the mouse β-APP transfected cells showed significantly more inclusions than empty vector transfected controls, confirming that their formation was not a consequence of the transfection process and was likely to reflect protein mishandling. Therefore the human β-APP plasmid was used in all subsequent experiments.

3.5.2.2. Formation of intracellular aggresomes

The results showed that human β-APP transfection of myogenic cells led to over-expression and aggregation of β-APP throughout the cytoplasm 24 hours after transfection (5 DIV). In parallel, a clear up-regulation of ubiquitin was observed, with ubiquitin appearing in a speckle-like cytoplasmic distribution throughout the cell. At this time point, ubiquitin was not found to co-localise with β-APP in many cells. However, 48 hours post transfection (6 DIV), a greater proportion of β-APP over-expressing cells showed co-localisation of the two proteins. These results suggest that β-APP over-expression leads to disruption of the protein handling mechanism. Ubiquitin is recruited to facilitate the degradation of excess proteins. However, as the rate of excess protein degradation cannot match the rate of transcription and translation of the protein, the surplus protein accumulates in the cytoplasm. The C-terminus of β-APP is hydrophobic and so controls the rate of aggregation (Jankowsky et al. 2007). It is therefore possible that in an area with a high concentration of β-APP, the formation of small aggregates of protein would naturally occur, as we see in these muscle cultures.

The up-regulation of ubiquitin and subsequent co-localisation with β-APP reflects an attempt by the cell to degrade the unwanted protein through the ubiquitin-proteasome system (UPS).

At 7 DIV, very large, ubiquitin and β-APP positive inclusion bodies were present in the cytoplasm of β-APP transfected cells, often seen to cause indentation of the nucleus. A similar finding has been previously reported in HEK cells over-expressing a cystic fibrosis protein (Johnston et al. 1998b) and also cells over-expressing a region of the huntingtin protein (see **Figure 3.21** [b]. These types of inclusion bodies were referred to as 'aggresomes' due to the discovery of their microtubule mediated formation (model described in **Figure 3.21** [A]). The β-APP transfected cells in the present study appear to follow the same pathway to inclusion body formation by first appearing as 'speckle-like' small aggregates all over the cytoplasm then travelling to a larger inclusion near the nucleus by 7 DIV (See **Figure 3.10**).

It has been suggested that the location of large inclusion bodies near the nucleus may be brought about by the perinuclear position of the centrioles, which form the microtubule organising centre in the cell (Johnston et al. 1998b). γ-tubulin, a protein which assists in orientating microtubules and is associated with centrioles has been shown to be present in the inclusion bodies in sIBM muscle (Fratta et al. 2005b). Interestingly, the inclusion bodies formed in the β-APP over-expressing cells also contained γ-tubulin positive centrioles (see **Figure 3.13**). This suggests direct microtubule involvement in the formation of inclusion bodies (aggresomes) *in vivo* and in the *in vitro* experiments described in this Chapter. It is still unknown whether the formation of inclusion bodies in general is entirely a cytoprotective mechanism used by the cell to sequester excessive proteins and allow proteostasis to be re-established, or whether it is a consequence of protein crowding and is, in fact, cytotoxic (Kopito 2000; Stefani 2007).

However, as the formation of aggresomes is mediated by microtubules and is not simply diffusion-limited, this suggests that there is an active mechanism in place which the cell utilises in the presence of unwanted or excessive proteins.

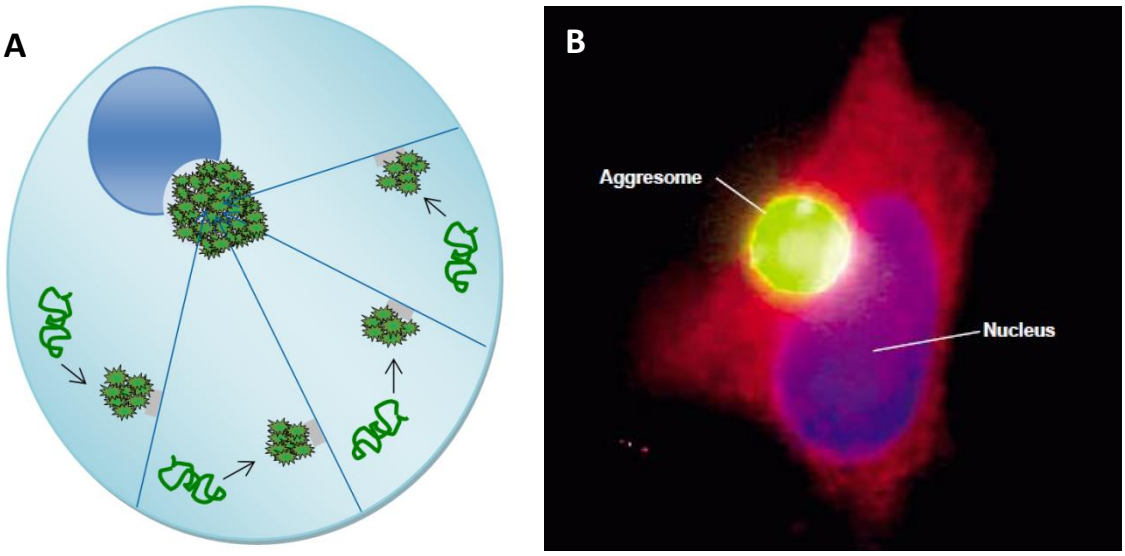


Figure 3.21 Formation of aggresomes

[A] A schematic model for aggresome formation. Small aggregates are retrogradely carried along the microtubule tracks (blue lines) to a juxtanuclear, pericentriolar location, causing indentation of the nucleus. [B] An image of a cytoplasmic aggresome in a HeLa cell formed by over-expression of the polyglutamine region of huntingtin (green). Taken from (Stirling et al. 2003).

3.5.2.3. β-APP over-expression in myogenic and non-myogenic cells

The muscle cultures used in this investigation are a mixed population and therefore manipulating protein expression in muscle cells is likely to also affect the non-myogenic cells in culture also. However, the results presented in this Chapter show that myogenic cells are easier to transfect or over-express β-APP more readily than non-myogenic cells. This suggests that myogenic cells may be more vulnerable to protein mishandling compared to other cell types in culture.

3.5.2.4. Expression of sIBM-relevant proteins in β-APP over-expressing cells

In order to establish whether over-expression of β-APP induced a cellular phenotype that resembled that observed in sIBM patient muscles, the expression of a number of proteins was examined by immunocytochemistry.

Following transfection with human β-APP, a clear up-regulation of HSP70 was observed in cells which contained small, disperse aggregates, but not in cells containing large cytoplasmic aggresomes. This suggests β-APP over-expression is stressful to cells and that aggresome formation is able to stabilise some of the stress brought about by disruption of the proteostasis. Up-regulation of HSP70 has been previously observed in sIBM patient muscle (De et al. 2009), indicating that muscle cells are under stress *in vivo,* possibly due to protein mishandling.

The HSR is a highly acute and sensitive response to protein misfolding and accumulation. Under basal conditions, the levels of HSP70, one of the most prominent components of the HSR, are low but become increased in muscle cells under stress, for example from exercise, ischaemia or protein accumulation (Liu et al. 2006). HSP70 therefore acts as an indicator of cell stress and influences signalling pathways to prevent apoptosis, as well as assisting with protein folding. In its chaperoning capacity, HSP70 is up-regulated to prevent conformational alterations to proteins after translation and shelter their

hydrophobic regions to prevent intermolecular contact, which would lead to aggregation (Kopito 2000).

β-APP transfection also up-regulated the expression of activated caspase-3, a key mediator of the apoptotic cascade (Porter & Janicke 1999). Elevated levels of caspase-3 are also indicative of cell stress, which could lead to apoptosis. Similar to HSP70 expression, caspase-3 up-regulation was only observed when over-expressed β-APP was dispersed around the cell as small aggregates and not in the presence of large aggresomes. It is possible that some cells have already entered the apoptotic pathway, leaving only those cells able to handle the stress remaining in culture. In addition, many of the β-APP transfected cells had inclusion bodies which contained HSP70 and caspase-3, as well as TDP-43. It is conceivable that these stress-proteins became associated with excess β-APP and were transported to the aggresomes by microtubules as part of the small protein aggregates. Alternatively, once the cell initiates aggresome formation, all highly expressed proteins in close proximity may simply aggregate together and be carried to the aggresomes indiscriminately.

TDP-43 and phosphorylated tau (p-tau) have also been observed in sIBM histology (Engel & Askanas 2006; Verma & Tandan 2009) and were also found in β-APP-positive inclusions in this investigation. Phosphorylated and aggregated tau protein is a pathological hallmark for tauopathies such as AD, with the protein being 3-4-fold more hyperphosphorylated in AD brains compared to age-matched controls (Iqbal et al. 2010). Askanas and Engel (2007) discuss the role of tau in sIBM pathology and suggest that although tau-positive aggregates are found in sIBM muscle as congophilic paired helical filaments, there is no direct evidence that this protein is toxic to muscle cells (Askanas & Engel 2007). Nevertheless, p-tau aggregation remains a prominent marker of degeneration in both AD and sIBM and this has been successfully replicated in this *in vitro* model.

3.5.2.5. Translocation of TDP-43

The nuclear protein TDP-43 has recently been proposed to be a potential biomarker for sIBM (Verma & Tandan 2009) and in this study, muscle cultures transfected with β-APP show cytoplasmic translocation of TDP-43 similar to that observed in sIBM muscle. Although some TDP-43 remained exclusively in the nucleus, this was only the N-terminus of the protein. Histology of affected tissue from patients with amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration with ubiquitin positive inclusions (FTLD-U) have also shown pathological TDP-43 in the cytoplasm which is hyper-phosphorylated and cleaved to only the C-terminal fragment (Igaz et al. 2009).

In another study, Zhang et al (2007) investigated the cleavage of TDP-43 by incubating the protein with three different caspase proteins. They found that caspase-3 and caspase-7 both cleaved TDP-43 to produce fragments of ~42, 35 and 25kDa. Using staurosporine treated cells as a positive control for cell stress and induction of the apoptotic cascade, this group demonstrated that TDP-43 cleavage and caspase-3 activity increased in correlation to increasing concentrations of staurosporine (Zhang et al. 2007). This suggests that the apopotic cascade and TDP-43 translocation may be related. Results from the present study shows that the C-terminus of TDP-43 is present in the cytoplasm of β-APP over-expressing cells, while the N-terminal fragment is not. This indicates that TDP-43 is cleaved such that the nuclear localisation and nuclear export signals on the N-terminus are lost and the C-terminus is free to translocate to the cytoplasm.

Figure 3.22 gives a schematic representation of TDP-43, which is highly conserved between species. Moreover, the increased expression of caspase-3 in β-APP over-expressing cells reported in this Chapter endorses the caspase-3 mediated cleavage described by Zhang et al.

Furthermore TDP-43 positive inclusions co-localised with ubiquitin in β-APP over-expressing cells, a finding also observed in sIBM muscle tissue (Salajegheh et al. 2009). As yet, it is not clear whether the translocation of TDP-43 is a cytoprotective mechanism or

one which is detrimental to the cell's viability. Neumann et al (2006) have reported that under pathological conditions TDP-43 is found to translocate to the cytoplasm (Neumann et al. 2006). TDP-43 translocation may therefore be a good outcome measure to study the effects of protein over-expression and test whether therapeutic agents are able to attenuate this response in stressed cells.

3.5.2.6. p62 expression and localisation

The expression of p62 in sIBM muscle has been investigated as a possible biomarker of the disease. p62 is involved in protein degradation via both the UPS and the autophagy system by serving as a delivery shuttle for ubiquitinated proteins (Weihl & Pestronk 2010). This protein also plays a role in signalling pathways such as activation of NFkB (Nogalska et al. 2009b). In sIBM, p62 expression is significantly elevated in muscle (Weihl & Pestronk 2010). p62 has functions in both the cytoplasm and the nucleus and is aggregated in both of these areas under disease conditions (Pankiv et al. 2010). In β-APP over-expressing cells, p62 was also found in the cytoplasm and nucleus as aggregates (see **Figure 3.15**). This suggests that the presence of ubiquitinated proteins is increased in the cell, requiring increased transcription/translation of p62 which then accumulates.

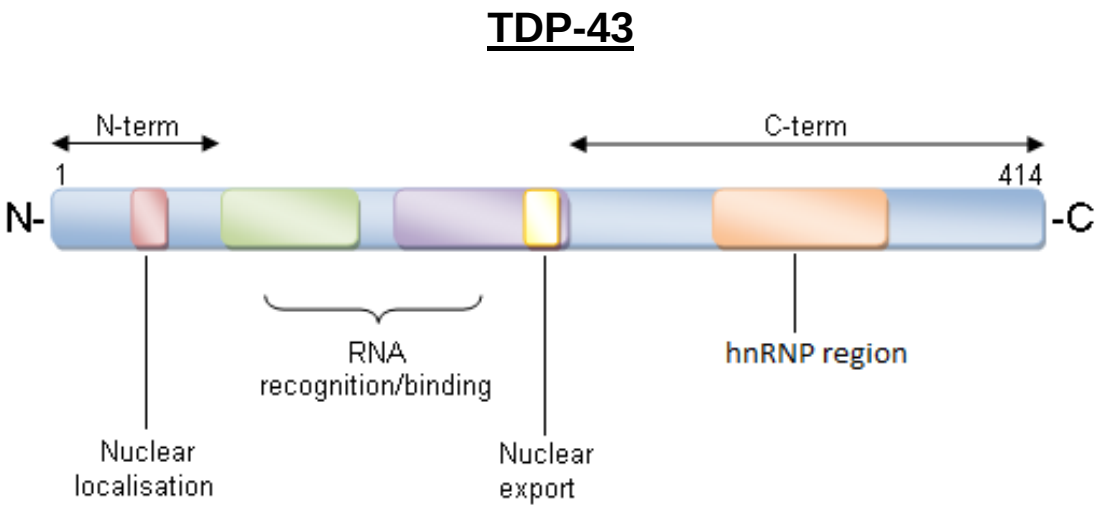


Figure 3.22 Schematic diagram of TDP-43

Schematic representation of the TDP-43 protein shows the nuclear localisation and nuclear export tags on the N-terminus portion of the protein. The C-terminal is released when the protein is cleaved and this is found translocated in the cytoplasm. hnRNP region complexes with RNA polymerase II transcripts and is involved in many cellular regulatory pathways (Krecic & Swanson 1999) .

Alternatively, the UPS and/or the autophagic system may also become dysfunctional so that the ubiquitinated proteins presented to it by the p62 protein cannot be processed. Nevertheless, the accumulation of ubiquitinated proteins and p62 reinforces the effects of disrupted protein homeostasis brought about by the over-expression of β-APP in the experiments described in this Chapter.

3.5.2.7. β-APP over-expression results in cell death

The exact mechanism by which protein misfolding and aggregation leads to toxicity in cells is not yet fully understood. However, proteotoxicity is evident in many protein-related disorders, especially neurodegenerative diseases such as AD and poly-glutamine expansion diseases (Douglas & Cyr 2010). In the present investigation, the effect of β-APP over-expression on cell survival was determined by LDH assays, which showed that over-expression of β-APP resulted in significant cell death by 6 DIV. This time point corresponds well to the increased ubiquitination of β-APP positive 'speckles' or small aggregates and the beginning of inclusion body (aggresomes) formation observed by immunocytochemistry. Furthermore the toxicity level is maintained to 7 DIV when aggresomes have formed.

The mechanism by which disruptions in the proteostasis results in cell death is not clear. Although the formation of aggresomes may form part of the cytoprotective response of the cell, the presence of such a large, globular structure is likely to have a detrimental effect on normal cellular functions, possibly by causing aggregation of other key proteins.

Furthermore, for muscle cells in particular, alterations in the cytoskeleton as a result of the presence of an aggresome and a re-direction of the microtubule network for the aggregation formation would be a major disturbance. Healthy myocytes are required for the formation of the multinucleated myotubes by cytoplasmic fusion. In addition, once myotubes have formed, their primary function is to contract and relax which would be hindered by any large structure in the cytoplasm.

In β-APP over-expressing cultures, some smaller inclusions were observed within myotubes, the formation of which may have resulted from post fusion over-expression of the β-APP gene. Dysregulation of normal muscle cell function may lead to activation of the apoptotic cascade if the cell becomes overwhelmed and damage to the cell becomes irreversible. Up-regulation of activated caspase-3 suggests that over-expression of β-APP does indeed initiate the apoptotic cascade and is therefore toxic to the cell.

Apart from the toxicity inferred from the formation of inclusion bodies, there are many lines of evidence that oligomeric forms of aberrant proteins are highly toxic (Douglas & Cyr 2010). Lajoie and Snapp (2010) studied the effects of expression of soluble polyglutamine oligomers containing a fluorescent GFP-tag in live cells, found that high levels of soluble mutant huntingtin was significantly correlated with increased cytotoxicity, independent of the presence of inclusion bodies (Lajoie & Snapp 2010). Another study where the Aβ peptide was expressed in a muscle cell line (C2C12) found that expression of this peptide was sufficient to cause myotube death (Querfurth et al. 2001). This group found that Aβ peptides induce apoptosis by detecting DNA fragmentation, DNA breaks, nuclear condensation, loss of myotube number and a decrease in redox activity in cultures experimentally expressing Aβ. The pathway by which oligomers cause toxicity remains unclear, although it may involve damage to the cell membrane by peptide binding (Jayaraman et al. 2008), disruption to the UPS (Bence et al. 2001) and/or oxidative stress and mitochondrial dysfunction brought about by an increase in reactive oxygen species (ROS) (Di 2010).

The present study did not demonstrate a precise pathway by which protein mishandling results in cell death. However, together with the current data in the literature, it is possible that disruption of proteostasis by over-expression of a large protein with a tendency to aggregate, leads to cell toxicity and subsequent cell death. This model therefore mimics the reduced cell viability seen in sIBM patient muscle and provides a good outcome measure for assessing cell survival under protein-related cell stress.

3.5.2.8. The effects of β-APP over-expression on mitochondrial function in myocytes

Mitochondrial dysfunction is a known feature of sIBM patient tissue (Oldfors et al. 2006a). The most common mitochondrial abnormalities include a greater number of COX-negative fibres compared to age matched controls and the presence of ragged-red fibres when stained with a modified Gömöri trichrome stain. Ragged red fibres have morphologically abnormal mitochondria which often contain paracrystalline inclusions. In addition, mutations in mitochondrial DNA are also common, with large-scale deletions which become focally expanded and can be the cause of COX-negativity. In the β-APP over-expressing muscle cultures, mitochondrial abnormalities were detectable by staining for SDH, which revealed that a large number of myocytes in the β-APP transfected cultures were deficient in SDH, a key protein in the mitochondrial electron transport chain. This finding suggests that disruption of the protein homeostasis by β-APP over-expression can lead to mitochondrial defects. This may occur through oxidative stress, which is caused by the presence of abnormal protein oligomers (Di 2010). The results therefore demonstrate the ability of this model to replicate another important feature of sIBM *in vitro*.

3.5.2.9. The effects of β-APP over-expression on proteasome function in myocytes

The proteasome is one of the main cellular complexes involved in the removal of damaged or excess proteins. Proteins which become tagged by poly-ubiquitination arrive at the proteasome for lysis into small, manageable peptides (Sorokin et al. 2009). Proteasome function has been previously shown to be decreased in β-APP over-expressing cultures from human tissue (Fratta et al. 2005a) and reduced enzyme activity has been noted in sIBM patient muscle biopsy material. The experiments on proteasome function conducted in this investigation show that there is a trend towards reduced proteasome activity in β-APP over-expressing cultures, although statistical significance was not reached. With the clear aggregation of β-APP in transfected cells and the increased presence of nuclear and cytoplasmic p62, which shuttles proteins to the proteasome, it would be expected that

the proteasomes in the β-APP transfected cells would show increased activity compared to those in the empty vector control cells. However, the results instead show slightly decreased proteasomal activity, suggesting that there is some level of proteasome dysfunction.

3.6. CONCLUSIONS

A reliable *in vitro* model to study the degenerative pathology seen in sIBM was established in this Chapter. Over-expression of β-APP was found to result in the up-regulation of several proteins, characteristic of sIBM pathology.

In addition, β-APP over-expression induced the translocation of TDP-43 and formation of inclusion bodies, both key characteristics of sIBM. Moreover, deleterious changes in mitochondria and the proteasomes were observed β-APP in over-expressing cells, demonstrating that the functional effects of protein mishandling seen in sIBM muscle were recapitulated *in vitro* in this model.

These changes in the phenotype of β-APP transfected myocytes (summarised in Table 2) can therefore be used as outcome measures to assess the potential of pharmacological agents for their ability to modify the pathological features of sIBM.

Table 2. Summary of the proteins examined in this β-APP over-expressing model

Protein examined	Biological function in muscle	sIBM pathology	Observations in this model
β-APP	Unconfirmed. Possible function at the neuromuscular junction	Increased level of β-APP observed in muscle, in particular as part of cytoplasmic inclusion bodies.	Up-regulation of β-APP in the cytoplasm as disperse protein and as part of inclusion bodies
Aβ1-42	Unconfirmed. Possible function at the neuromuscular junction	Increased level of Aβ 1-42 observed in muscle, in particular as part of cytoplasmic inclusion bodies.	Inclusion bodies immuno-reactive for Aβ 1-42
TDP-43	A DNA and RNA binding protein associated with transcriptional and post-translational regulation of RNA and stress response in cells	Abnormal accumulation in the cytoplasm as dispersed protein or as aggregates	C-terminal portion of TDP-43 seen to be translocated to the cytoplasm. Some inclusion bodes are immuno-reactive for TDP-43
p62	A shuttle protein which binds to misfolded proteins in the cytoplasm and nucleus delivering them to proteasomes or lysosomes for degradation	Abnormal accumulation in the cytoplasm and/or nucleus as dispersed protein or as aggregates	Inclusion bodies immuno-reactive for p62 in the cytoplasm and occasionally small p62 positive inclusions observed in the nucleus
HSP70	A chaperone protein up-regulated as part of the HSR. Binds to *de novo* and misfolded proteins to promote correct protein folding and prevent aggregation	Up-regulated in sIBM muscle tissue	Up-regulation of HSP70 following over-expression of β-APP
Ubiquitin	A protein which binds to misfolded proteins targeting them for degradation	Up-regulated and observed as part of cytoplasmic inclusion bodies in the muscle	Observed as part of cytoplasmic inclusion bodies in the cultured myocytes
p-tau	A microtubule associated protein involved in stabilization of the cytoskeleton	Abnormally present in the cytoplasm as clusters of paired helical filaments. Can be found in inclusion bodies	Inclusion bodies immuno-reactive for p-tau
Caspase 3	A protease which mediates apoptosis in stressed cells. Cleaves key cellular proteins	Unexamined to date	Up-regulated in β-APP over-expressing cells. Inclusion bodies are occasionally found to be immuno-reactive for Caspase 3
SDH	A key mitochondrial protein of the electron transport chain. Expressed at high levels in cells with a high oxidative capacity	Abnormal mitochondrial accumulations observed in muscle sections stained for SDH	SDH levels in β-APP over-expressing cultures diminished

CHAPTER 4.

TARGETING THE HEAT SHOCK RESPONSE (HSR) AS A POTENTIAL THERAPY FOR sIBM

4.1. INTRODUCTION: INVESTIGATING POTENTIAL THERAPEUTIC APPROACHES TO sIBM

As sIBM has historically been defined as an inflammatory myopathy, the large majority of therapies examined in clinical trials to date, have largely targeted the inflammatory component of the disease (Dalakas 2010b). However this approach has not proved to be effective in reducing pathology in patients or to significantly improve muscle strength. It is therefore essential that alternative approaches to therapy, which may be more effective, are explored. The degenerative component of sIBM is a major contributor to the pathology and it has been suggested that degeneration may well be upstream to the inflammatory component of sIBM (Askanas et al. 2009). Therefore understanding the mechanisms that underlie the protein mishandling observed in sIBM, which leads to degeneration, is essential to enable the identification of possible drug targets.

In the experiments described in this Thesis, two therapeutic approaches are examined which aim to ameliorate protein mishandling and prevent the aggregation of aberrant proteins in the cell. The first approach, described in this Chapter, involves up-regulating the cell's inherent protein handling machinery to prevent generalised disruption to proteostasis. This was achieved by targeting the endogenous heat shock response (HSR), thereby up-regulating the expression of heat shock proteins. The second approach, described in Chapter 6, is to directly target the formation of amyloid which is a natural consequence of protein deregulation. Amyloid formation leads to cell toxicity through protein oligomerisation and subsequent aggregation (Jayaraman et al. 2008).

4.1.1. The Heat Shock Response (HSR)

The HSR is a highly conserved cytoprotective mechanism which is induced by cell stress. This acute response forms the first-line defence against potentially harmful stresses such as elevations in temperature, oxidative stress, imbalances in protein homeostasis, presence of reactive oxygen species (ROS) and protein aggregation (Westerheide &

Morimoto 2005a). Induction of the HSR results in a large-scale up-regulation of heat shock proteins (HSPs), which are proteins of diverse molecular weight and function (Westerheide & Morimoto 2005a). These HSPs then interact with other proteins and polypeptide chains to ameliorate the toxic effects of cell stress. Elevated levels of heat and other stressors such as toxic chemicals and ROS are detrimental because normal protein handling in a cell becomes disrupted. For example, proteins in their native state are only marginally stable at their optimal physiological temperature. Therefore an increase in temperature leads to rapid unfolding and denaturation. HSPs are thus essential for re-folding polypeptides to their native states and preventing cytotoxic interactions.

4.1.2. Heat shock proteins (HSPs)

HSPs were first discovered by assessing the effects of elevated temperature in cells from Drosophila melanogaster (Tissieres et al. 1974). In these experiments, flies were subjected to temperatures of up to 37.5°C, which is above their physiological temperature. Cells from these flies contained "puffs" in certain chromosomes which were not present in flies that were not heat shocked. These 'puffs' were identified as centres for rapid mRNA synthesis, which was subsequently translated to certain proteins. Using sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE), Tissieres et al (1974) were also able to demonstrate that following heat shock, a number of new protein bands appeared on the SDS-PAGE gels from all tissues examined. These findings led to coining of the term 'heat shock proteins' and introduced a large group of proteins which are essential for normal cellular function in both stressed and unstressed cells.

The HSPs are named according to their molecular weight (Westerheide & Morimoto 2005a). The heat shock gene superfamily can be categorised into six major groups which are the HSP100s, HSP90s, HSP70s, HSP60s (Chaperonins), HSP40 and small HSPs (Westerheide & Morimoto 2005a; Richter et al. 2010). All of these proteins are ubiquitously expressed, well conserved and function as molecular chaperones (Richter). Although all molecular chaperones interact with unfolded proteins, some bind to different

unfolded states and at different times to reduce the effect of cell stress, which mainly causes damage to proteins and an imbalance to proteostasis.

Protein aggregation is a natural consequence of a protein-rich cellular environment, and this occurs in unstressed cells as well as following exposure to a stressor (Kopito 2000). Hydrophobic interactions between unfolded proteins trigger their aggregation. The mode of function of HSPs is therefore through association with exposed hydrophobic regions of unfolded or partially folded polypeptide chains, thereby sequestering them from any neighbouring hydrophobic protein region (Richter et al. 2010).

Apart from acting as molecular chaperones some HSPs are associated with other functions such as DNA/RNA repair, transport and detoxification, metabolism and protein degradation (Richter et al 2010). The major families of HSPs are described next.

4.1.2.1. HSP100

The HSP100 family of chaperones are ubiquitously expressed and all contain AAA-ATPase binding domains (Liberek et al. 2008). This group of proteins are subdivided according to the number of ATP-binding domains they contain into Class I and Class II (Richter et al. 2010). The main function of these HSPs is to disaggregate aggregated proteins in order for them to be subsequently re-folded. By associating with aggregates, HSP100 pulls misfolded proteins through the central pore of its hexameric ring structure resulting in a disaggregation of protein (Liberek et al. 2008; Richter et al. 2010). HSP100 functions in conjunction with HSP70 which subsequently binds the unfolded polypeptide preventing re-aggregation and allowing folding into the correct protein structure.

4.1.2.2. HSP90

HSP90 is present at high concentrations in cells under basal conditions, making up 1-2% of the cytosolic proteins (Krukenberg et al. 2011) and is further up-regulated under conditions of stress (Welch & Feramisco 1982). This chaperone has a smaller spectrum of client proteins than other HSPs such as HSP70, and the majority of its known substrates have been identified as signal transduction proteins (Young et al. 2001). Therefore this HSP is essential in signalling networks. HSP90 binds to polypeptides which are close to their native structure; however its mechanism of action is rather complicated and requires over a dozen co-chaperones forming a multi-protein complex (Richter et al. 2010). Many HSPs co-operate with each other to enable correct handling of misfolded proteins. For example, in some cases, HSP70 delivers substrates to HSP90 which holds the non-native protein in place while its conformation changes. The co-chaperones Hop, Sti1 and p23 have been identified to assist in this mechanism (Eisen et al. 1998). HSP90 is ATP-dependent and hydrolysis of ATP results in release of the substrate protein (Young et al. 2001).

4.1.2.3. HSP70

HSP70s are an essential component of the cellular chaperone network. They are ubiquitously expressed and are present in the cytosol as well as in organelles such as the ER and mitochondria (Richter et al. 2010). This group of HSPs bind to a wide range of short hydrophobic regions of polypeptides, usually seven amino acids in length (Zhu et al. 1996), thereby assisting de novo protein folding under basal conditions as well as preventing protein aggregation in stressed cells (Mayer & Bukau 2005). In addition, HSP70 is also involved in solubilisation of aggregated proteins through interactions with HSP100 (see above). Like many HSPs, HSP70 is also ATP-dependent, with the ATP-bound state having low affinity for substrates, while the ADP-bound state has high affinity. This allows a cycle of substrate binding and release to occur (Mayer & Bukau 2005). The ATPase cycle of HSP70 is regulated by J-domain protein co-chaperones which guide HSP70s to their

substrate. Once bound to a substrate, nucleotide exchange factors determine the length of time for the HSP70-substrate interaction before the substrate it released (Mayer & Bukau 2005).

By binding and releasing substrates, the function of HSP70 in regulating proteins is two-fold. On one hand, bound substrates are sequestered from the cytosol thereby reducing the concentration of free substrates, allowing them to fold into their native structures. This is termed 'kinetic partitioning'. On the other hand, binding and releasing misfolded protein substrates such as β-pleated sheets allows them to detangle, ready for correct folding. This is called 'local unfolding' (Ben-Zvi & Goloubinoff 2001).

In addition to preventing aggregation, HSP70 also has house-keeping functions in the cell such as the control of the activity of regulatory proteins and translocation of proteins (Ryan & Pfanner 2001), which are essential for cell proliferation, differentiation, maintaining homeostasis and apoptosis. These diverse functions have been brought about by the variations in *hsp70* genes, interactions with a range of co-chaperones and co-operation with other HSPs in the cell.

4.1.2.4. HSP60 (Chaperonin)

The HSP60 group of proteins share similar functional properties to HSP70 in that both bind non-native polypeptides and use ATP-dependent substrate binding and release as mechanisms of action. HSP60 however, binds more readily to globular conformations while HSP70 has higher affinity for extended conformations (Bukau & Horwich 1998). The HSP60 equivalent in bacteria, called GroE has been highly characterised and is known to consist of two components which are the GroEL subunits and GroES co-chaperones. Together these form a large protein complex of two heptameric rings which encloses the substrate binding sites (Richter et al. 2010).

4.1.2.5. HSP40

HSP40s, also known as DnaJ proteins, are a highly conserved group of HSPs with 41 family members in humans (Qiu et al. 2006a). Members of the HSP40 family are identified by the J domain, usually at the N-terminal of the protein, which is the site of interaction with HSP70 (Szyperski et al. 1994). These chaperone proteins are highly important for protein translation, degradation, translocation, folding and unfolding, the regulation of which they assist by stimulating the ATPase activity of HSP70 (Qiu et al. 2006b). Therefore by stabilising the interaction of HSP70 with its substrate proteins, different homologs of HSP40 determine the activity of HSP70. In mammals, over 20 homologs of HSP40 have been identified to date, some of which may also regulate other HSPs (Brychzy et al. 2003).

4.1.2.6. Small HSPs (sHSPs)

The most poorly conserved group of HSPs are the sHSPs which vary greatly in sequence and size (Kriehuber et al. 2010), but all have a relatively low molecular weight of between 15-43kD (Liberek et al. 2008). All of these proteins contain a well conserved α-crystallin domain of approximately 100 amino acid residues and function independently of ATP (Horwitz 2003). sHSPs interact with partially folded proteins to prevent aggregation during cell stress, but also act as storage for a large collection of proteins to be sequestered from the cytosol (Richter et al. 2010). Furthermore some sHSPs may even sequester proteins into aggregates to be subsequently remodelled by the larger ATP-dependent HSPs.

Table 3 summaries the general functions of the major HSP families.

4.1.3. Regulation of the HSR

The HSR is transcriptionally regulated by a family of heat shock transcription factors (HSF) which in humans has three members; HSF1, HSF2 and HSF4 (Westerheide & Morimoto 2005a). Of these, HSF1 is the most essential for the HSR and therefore the best

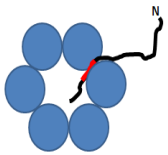
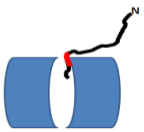
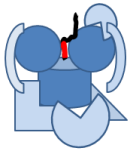
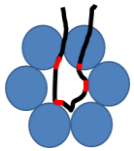
characterised. HSF1 is expressed in almost all cell types (Anckar & Sistonen 2011). Under normal conditions HSF1 exists as an inert monomer in either the cytoplasm or nucleus, shuttling between the two compartments (Pirkkala et al. 2001; Westerheide & Morimoto 2005a). Under basal conditions, the inactive state is maintained by direct association with chaperone proteins, mainly HSP90. However under conditions of stress, cytoplasmic translocation of this protein is inhibited such that it accumulates in the nucleus and HSF1 dissociates from the chaperones proteins modulating it (Westerheide & Morimoto 2005a).

The HSF1 gene consists of a DNA-binding domain, oligomerisation domain, heptad repeat region, regulatory domain and *trans*-activation domain. Once accumulated in the nucleus, HSF1 undergoes a multi-step process of activation through post-translational modifications which includes trimerisation and hyperphosphorylation (Anckar & Sistonen 2011). Trimerisation of HSF1 is regulated by the oligomerisation domain and spontaneous trimerisation in unstressed conditions is inhibited by the heptad repeat region (Anckar & Sistonen 2011). The monomeric state of HSF1 has a low affinity for DNA binding, whereas the trimeric form has significantly higher affinity (Santoro 2000). Therefore trimerisation triggers the DNA-binding activity of HSF1.

The DNA-binding domain of HSF1 recognises a specific repeat sequence of nGAAn located upstream to *hsp* genes which makes up the heat shock element (HSE) (Santoro 2000; Richter et al. 2010). The HSE is a highly conserved promoter sequence of DNA where the transcription complex, which includes, HSF1, RNA polymerase II (RNAPII) and transcription elongation factors, converge (Anckar & Sistonen 2011). In unstressed cells, RNAPII remains associated with *HSP* genes but inactive. Upon stress, mature HSF1 binds to the HSE and RNAPII becomes phosphorylated and therefore activated. At this stage the presence of histones which tightly associate with the DNA inhibit polymerisation (Anckar & Sistonen 2011). Therefore histone displacement and chromatin re-arrangement takes place next to allow *HSP* gene transcription.

The trans-activating and regulatory domains of HSF1 are involved in regulating the magnitude of HSF1 activation as well as activating transcription of *HSP* genes (Anckar & Sistonen 2011) . While activation of the HSF1 is triggered by the presence of misfolding proteins in cells, HSF1 is negatively regulated by the regulatory domain of HSF1, which prevents activation in the absence of protein damage (Anckar & Sistonen 2011).

Table 3. The major HSP families and their general function

Chaperone protein	Substrate binding topology	Functions	References
HSP100	Side  Top 	ATP-dependent disaggregation of misfolded proteins and unfolding for degradation.	(Liberek et al. 2008) (Richter et al. 2010)
HSP90	Multi-protein complex 	Functions in a multi-protein complex to bind near-native proteins, which are mainly signal transduction proteins.	(Young et al. 2001) (Krukenberg et al. 2011)
HSP70		ATP-dependent *de novo* protein folding and aggregation prevention. Also functions as a housekeeping protein.	(Mayer & Bukau 2005)
HSP60 (chaperonin)	Side  Top 	ATP-dependent folding of polypeptides to native conformation using ATP hydrolysis.	(Bukau et al. 2006)
HSP40 (DnaJ)		Interactions with HSP70 and other HSPs to regulate chaperone activity.	(Qiu et al. 2006b)
Small HSPs		Stabilization of aggregating proteins and sequestering proteins into aggregates for remodelling by other HSPs.	(Richter et al. 2010) (Liberek et al. 2008)

In addition the increased presence of the large HSPs such as HSP70 and HSP90, when they are not bound to substrate proteins also acts as a negative control (Richter et al. 2010). This negative feedback cycle ensure the HSR functions efficiently and the correct proteins are expressed in the cell at the correct times.

Several mechanisms for the activation of HSF1 have been proposed. One mechanism suggests that as the presence of damaged proteins in cells trigger HSP translocation to the aberrant protein, this liberates the HSF1 molecule associated with it (Santoro 2000). Un-bound HSF1 can thus trimerise and carry out its transcriptional activity. Another model suggests that HSF1 has an intrinsic ability to sense proteotoxic stress which leads to its trimerisation (Anckar & Sistonen 2011).

4.1.4. Protein chaperones and sIBM

Protein aggregation has been described as 'inevitable' due to the complexity of protein folding in healthy cells (Kopito 2000). Under normal circumstances, the cell has endogenous cytoprotective mechanisms involving chaperone proteins that prevent protein aggregation and toxicity. However, when a cell is unable to control the accumulation of proteins and chaperone levels are insufficient; this results in the formation of inclusion bodies as seen in sIBM. The chaperone system has therefore been widely examined (De et al. 2009) as a target for therapeutic manipulation, particularly in neurodegenerative diseases where protein aggregation is a key feature of disease (Westerheide & Morimoto 2005b; Nagai et al. 2010). However, the possibility that targeting of protein chaperones may be effective in sIBM has not so far been tested. With sIBM being a chronic disease it follows that endogenous levels of HSPs may be unable to cope with the prolonged exposure to stress. Therefore enhancing the HSR in this disease may be beneficial to sIBM patients.

Manipulation of the heat shock response has been explored in several neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) (Kalmar & Greensmith 2009),

Huntington's disease, AD, and Parkinson's disease (Nagai et al. 2010; Hoshino et al. 2011). Although these are neurological disorders, the cytoprotective mechanisms in question are ubiquitous across all cell types. Warrick et al. (1999) explored the effects of directed HSP70 up-regulation in a Drosophila melanogaster model of polyglutamine disease. In these experiments, the internal and external structures of the eyes from flies expressing the mutant polyglutamine gene were compared to wild-type (WT) flies and those co-expressing the human HSP70 gene. The results showed degeneration and reduced pigmentation in the eyes of the mutant polyglutamine flies, which was largely restored in flies in which HSP70 was also induced. The authors suggest that HSP70 suppresses polyglutamine-induced neurodegeneration.

Similarly, Hoshino et al. 2011 investigated the effects of HSP70 over-expression in a transgenic mouse model of Alzheimer's disease which expressed a mutant form of the β-APP protein (APPsw). Wild-type (WT) mice were assessed as controls. The results from these experiments demonstrated that transgenic APPsw/HSP70 mice had a lower level of Aβ40, Aβ42 and Aβ plaque deposits compared to APPsw/WT mice. Furthermore NeuN staining of hippocampal CA2 region showed a significantly higher number of neurons in WT/WT and APPsw/HSP70 mice compared to APPsw/WT mice. The APPsw/HSP70 mice also displayed increased cognitive function compared to the APPsw/WT. This suggests that AD-related neurodegeneration may be suppressed by over-expression of this key chaperone protein. These experiments were the first to investigate the effects of HSP70 over-expression in AD mice *in vivo*. As the cellular phenotype of AD neurons share similarities with sIBM muscle, HSP up-regulation in sIBM is a logical therapeutic strategy to investigate.

4.1.5. Pharmacological manipulation of the HSR

One approach which may be taken to induce increased production of HSPs is using protein synthesis inhibitors, such as puromycin or proteasome inhibitors, such as MG132 (Hightower 1980; Holmberg et al. 2000). These molecules prevent the production and

degradation of polypeptide chains, respectively, and therefore increase the cellular concentration of non-native polypeptides (Hightower 1980; Holmberg et al 2000). The presence of abnormal proteins subsequently triggers the HSR, and HSPs are up-regulated. As these drugs trigger cell stress in order to then induce the HSR, they may lead to detrimental effects in other areas of the cell. For example, proteasome inhibition itself can lead to protein aggregation, even though the endogenous HSR becomes activated (Fratta et al. 2005a).

Sittler et al (2001) conducted *in vitro* experiments with an inhibitor of HSP90 called geldanamycin, in a cell culture model of Huntington's disease, in an attempt to activate the HSR. This compound binds specifically to HSP90 inhibiting its function, thereby triggering HSF1 activation via the negative feedback cycle. The results from these experiments show treatment with geldanamycin suppresses aggregation of the mutant huntingtin protein. A similar cytoprotective effect was observed in a cell culture model of ALS (Turturici et al. 2011). Geldanamycin and related analogues were initially developed as anti-cancer therapies and therefore have since been taken forward into phase I and phase II clinical trials as a cancer drug (Westerheide & Morimoto 2005b).

Labbadia et al (2011) used an alternative method to demonstrate the potential therapeutic effects of HSF1 activation. In these experiments a different inhibitor of HSP90 was used called HSP990 to treat the R6/2 mouse model of Huntington disease (HD). The results showed transient reduction in aggregate load and improvements in the disease phenotype in mice, however, with disease progression, the ability to up-regulate major HSPs was found to be reduced in HD compared to wild-type controls (Labbadia et al. 2011). Although these experiments show proof of principle that HSF-1 activation can be beneficial in the treatment of HD in mammalian models, the authors acknowledge that due to the multitude of functions of HSP90, therapy targeting HSF1 which is independent of HSP90 would be a more promising treatment strategy to use in clinic (Labbadia et al. 2011).

Another agent which has been investigated for its ability to stimulate the HSR is Celastrol. Celastrol is a natural derivative from the *Celastraceae* family of plants and is used in Chinese herbal medicines (Westerheide & Morimoto 2005a). This compound shows striking effects in mammalian cell lines where the HSR is potently activated by induction of HSF-1. Celastrol targets HSF-1, which results in the up-regulation of a number of HSPs rather than individual chaperone molecules. Celastrol triggers the HSR with similar rapid kinetics to those observed following heat shock and is effective in the micromolar range (Westerheide & Morimoto 2005b). Celastrol thus shows promising results for the treatment of neurodegenerative pathology.

However drugs which directly induce the HSR, such as Celastrol, can also increase the risk of oncogenesis (Jaattela 1999). Tumour cells have been found to contain a high level of heat shock proteins compared to normal cells (Jaattela 1999). This is likely to be a mechanism employed by tumour cells to suppress apoptosis caused by the stress of carcinogenic mutations. Therefore systemic up-regulation of the HSR poses a risk to patients and an alternative therapeutic strategy needs investigation.

In addition, activators of the HSR, such as Celastrol, have their effects by inducing the HSR per se i.e. they are cellular stressors and in some cases this can result in direct cellular toxicity and even cell death (Kalmar & Greensmith 2009b). Therefore not all inducers of the HSR will be cytoprotective.

4.1.6. Targeting the HSR as a treatment for sIBM: Arimoclomol

Previous results have identified an agent called Arimoclomol as a pharmacological agent that can up-regulate the HSR (Kieran et al. 2004a). Arimoclomol (/+/-(2R),(Z)-N-[2-hydroxy-3-(piperidin-1-yl)propoxy]-pyridine-1-oxide-3-carbox-imidoylchloride citrate (1:1)) is a member of the hydroxylamine derivatives family and has been shown to **co-induce** the synthesis of heat shock proteins (Hargitai et al. 2003). **Figure 4.1** shows the chemical structure of Arimoclomol.

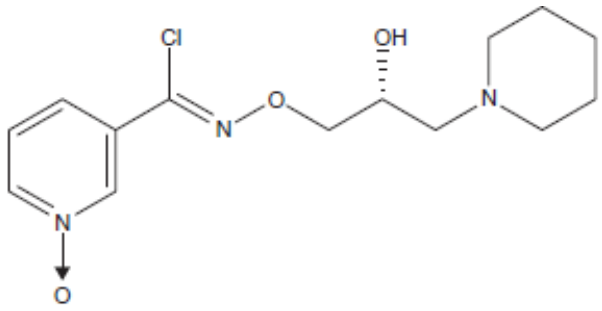


Figure 4.1. The chemical structure of Arimoclomol

(Lanka et al. 2009)

As a co-inducer of the HSR, Arimoclomol and its analogue Bimoclomol function by augmenting the HSR in cells where the endogenous HSR has already been triggered (Vigh et al. 1997). This gives Arimoclomol and Bimoclomol a level of specificity to target cells which are actually under stress, rather than all cells in general. When administered in the absence of a stressor, these agents do not induce the HSR (Vigh et al. 1997), therefore potentially reducing the side effects of activation of the HSR.

These small hydroxylamine molecules bind to HSF1 molecules and increase their level of phosphorylation, thereby prolonging its interaction with the heat shock element (Hargitai et al. 2003). By extending the length of time the transcription factor is associated with the DNA, Arimoclomol and its analogues, thus lead to an increase in the transcription of HSPs (Hargitai et al. 2003).This amplifies the available number of chaperone proteins, thus augmenting the cell's normal response to protein mishandling (see Section 4.1.3 for a more detailed discussion of HSF1 activity). Hargitai et al (2003) demonstrated that Arimoclomol is unable to trigger the HSR in cultured cell from HSF1 -/- mice. This suggests that the presence of the transcription factor is central to the function of this agent.

Arimoclomol is generally non-toxic in animal models (Hargitai et al. 2003; Kieran et al. 2004a) and has been indicated to have a variety of therapeutic benefits in several disorders including diabetic peripheral neuropathy and retinopathy, where up-regulation of HSPs appears to be beneficial (Kurthy et al. 2002). The effects of Arimoclomol has been assessed in adult rats following sciatic nerve injury and showed morphological improvement in sensory neuron markers and restored functional activity of sensory fibres (Kalmar et al. 2003) and in motoneurons (Kalmar et al. 2002).

Arimoclomol has also been found to have major therapeutic effects on mouse models of ALS where up-regulation of HSP in mutant $SOD1^{G93A}$ mice has proved beneficial (Kieran et al. 2004a). These studies found that $SOD1^{G93A}$ mice treatment with Arimoclomol have delayed disease progression and increased lifespan. Thus in Arimoclomol treated SOD1

^{G93A} mice; there was a reduction in muscle atrophy and an improvement in muscle tone, contractile characteristics and motoneuron survival.

4.1.7. Clinical trials of Arimoclomol in ALS

A clinical indication for Arimoclomol is in the treatment of ALS which currently has no effective treatment or cure (Lanka et al. 2009). Research on Arimoclomol demonstrated its potential to enhance cytoprotective mechanisms in stressed cells and pre-clinical studies showed clear improvement in ALS pathology in mice. This drug was therefore approved for clinical testing in ALS patients. Arimoclomol has so far been through seven phase I clinical trials in healthy volunteers to assess safety and tolerability as well as the pharmacokinetic properties of the compound. In addition a small-scale phase II trial on ALS patients has also been completed. The results from these trials have shown Arimoclomol to be safe and well tolerated in both healthy individuals and ALS patients (Lanka et al. 2009).

Further parameters were investigated subsequently in a Phase IIa trial in which dose-ranging was studied in orally administered Arimoclomol (Clinicaltrials.gov ID: NCT00244244). This multi-centre, double-blind, placebo-controlled study assessed safety, tolerability and CSF penetration as well as disease outcome measures using the ALS functional rating scale and physical examination. Up to 100mg 3x daily was given to patients and this dosage was found to be safe and well tolerated.

At present a phase II/III double-blind, randomized, placebo-controlled trial is underway to evaluate the safety and efficacy of Arimoclomol in familial ALS patients with mutations in SOD1 (Clinicaltrials.gov ID: NCT00706147). The hypothesis of this study is that Arimoclomol, at 200mg thrice daily, will reduce disease progression by at least a rate of 30%. In addition, Arimoclomol will be safe and well tolerated in patients. This trial is estimated to be completed in December 2012 and is currently recruiting participants.

4.1.8. Clinical trial using Arimoclomol to treat sIBM

Arimoclomol has shown encouraging results in the treatment of ALS both *in vivo* and in patients. However, as its molecular targets are ubiquitously expressed in all cell types, it follows that up-regulation of the HSR in sIBM patients may be beneficial also. A phase II clinical trial to test the safety and tolerability of Arimoclomol is currently underway jointly at the University of Kansas, USA and at the MRC Centre for Neuromuscular Diseases, London, UK (Clinicaltrials.gov ID:NCT00769860). The aim of this randomized, placebo controlled, double-blinded trial is to primarily look for adverse reactions to the drug. Secondary outcome measures include muscle strength testing, and evaluating muscle biopsies before and after four months of Arimoclomol treatment at 100mg TDI. At present all participants have completed the course of treatment and data from outcome measures are being collected for analysis.

Since Arimoclomol has been found in this lab to be non-toxic and successful in increasing heat shock protein response in nerve and muscle of SOD-1 mice, it follows that the agent may work to reduce protein mishandling and aggregation in sIBM. In the experiments described in this Chapter the effects of Arimoclomol on an *in vitro* model of sIBM was tested as a known anti-aggregation drug.

4.2. AIMS OF THIS CHAPTER

The aims of this Chapter are:

I. To assess the effects of pharmacological up-regulation of the HSR as a therapeutic strategy in β-APP over-expressing cells.

II. To evaluate the suitability of the β-APP over-expressing model as a screen for testing other novel compounds for sIBM.

4.3. MATERIALS AND METHODS

A full description of the materials and methods used in this Chapter are presented in Chapter 2, and include the following:-

- Neonatal rat cell culture
- Transfection of β-APP plasmid
- Immunocytochemistry
- SDH staining
- LDH Assay
- Protein Assay
- Proteasome Assay
- Cell counts
- Data and statistical analysis

4.3.1. Arimoclomol treatment

Muscle cultures were treated with Arimoclomol at various concentrations 24 hours after transfection with β-APP to establish the response to different dosages using LDH assays. Subsequent cell cultures were treated with 10µM Arimoclomol as this was found to be the dose at which a significant reduction in cytotoxicity was observed. Arimoclomol was added to wells containing 500µl of muscle media in 24-well plates or 200µl of media in 96-well plates. Control wells were left untreated.

4.4. RESULTS

The results presented in Chapter 3 have shown that over-expression of β-APP in muscle cells in culture leads to protein mishandling, aggregation and the formation of inclusion bodies. This process has been shown to be cytotoxic and induces the up-regulation of other proteins involved in the handling of aberrant proteins and cell survival such as HSP70 and caspase-3.

In the experiments described in this Chapter, the effect of pharmacologically up-regulating the HRS on pathological features of sIBM was tested by treating β-APP transfected muscle cells with Arimoclomol, a co-inducer of the HSR (Kalmar and Greensmith 2009).

4.4.1. The effects of Arimoclomol on β-APP induced cytotoxicity

In order to establish the optimal dose of Arimoclomol to use in these experiments, β-APP transfected cultures were treated with Arimoclomol at doses ranging from 1-10μM, at 6 DIV. The results are summarised in **Figure 4.2** [A]. it can be seen that treatment with Arimoclomol results in a dose-dependent decrease in LDH release, a measure of cytotoxicity, when analysed 48 hours after treatment. Although a decrease in cytotoxicity can be seen at all concentrations, this was only statistically significant at 10μM Arimoclomol ($p=0.0006$). Therefore this concentration of Arimoclomol was chosen for all subsequent experiments.

The effect of Arimoclomol (10μM) on the survival of cells transfected with β-APP was examined and the results are summarised in **Figure 4.2** [B]. For each experiment, the results were normalised to the values for the empty vector (EV) transfected cells to control for the effects of transfection and this was set as 100% LDH release. Statistical significance was calculated with a One-way ANOVA test. Each experiment was repeated three times and the same trends in significance were observed.

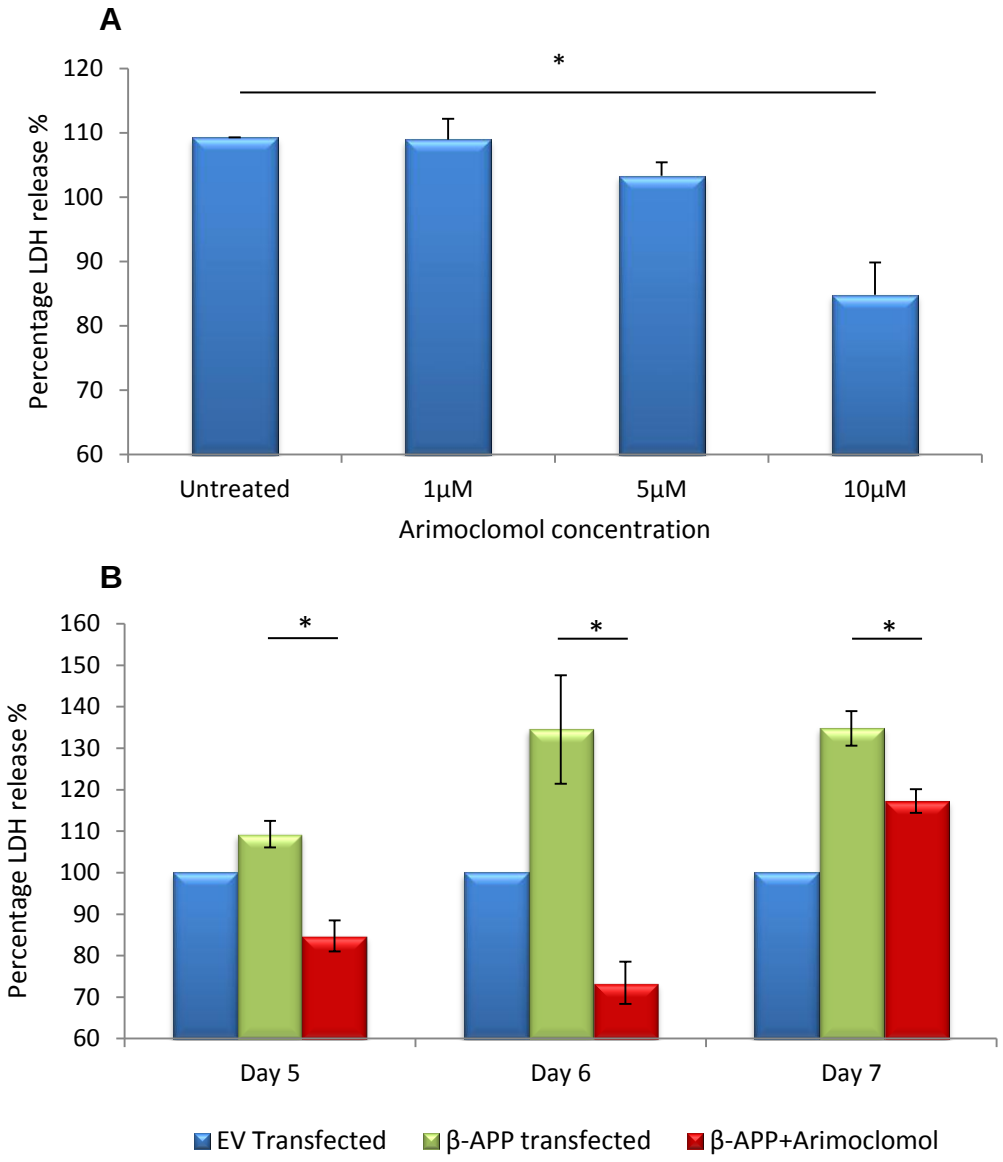


Figure 4.2 Cell survival assays with Arimoclomol treatment

[A] The bar chart shows the effect of increasing concentrations of Arimoclomol on percentage LDH release in myocyte cultures transfected with β-APP at 6 DIV. [B] The bar chart shows percentage LDH release from myocytes cultures transfected with either the empty vector (EV) or β-APP, and the effect of treatment with 10µM Arimoclomol at 5, 6 and 7 DIV. Data is representative of n=3 LDH assays. Error bars = SEM, * denotes $p<0.005$.

The results demonstrate that the treatment of β-APP over-expressing cultures with Arimoclomol significantly reduces LDH released, reflecting an increase in cell survival. These results are consistent at each time *in vitro* investigated. Interestingly, LDH release in Arimoclomol treated cells at 5 and 6 DIV was lower than that in empty vector transfected control cultures, although this did not reach significance. By 7 DIV, LDH release in Arimoclomol treated β-APP over-expressing cultures was no longer lower than that in control cultures, although it remains significantly lower than in untreated β-APP cultures ($p<0.005$).

4.4.2. The effects of Arimoclomol on inclusion body formation

Cultured cells were transfected with either the mouse or human β-APP plasmids at 3 DIV. At 4 DIV the muscle media was removed from wells and 500µl of fresh media containing Arimoclomol was added at a concentration of 10µM. The cells were then fixed at 5, 6 and 7 DIV.

As shown in **Figure 4.3**, immuno-staining for β-APP and ubiquitin revealed that treatment of β-APP over-expressing cells with Arimoclomol results in a significant reduction in inclusion body formation compared to untreated controls. This was consistent over the three time points investigated. The number of desmin-positive cells containing intracellular inclusions was counted over the 3 days investigated. The results showed that in untreated control cultures transfected with human or mouse β-APP, 13.5% ± 3.18% (SEM, n=3) and 12.6% ± 2.11% (SEM, n=3) respectively, of myogenic cells contained inclusion bodies. In contrast, in cultures transfected with human or human β-APP and treated with 10µM Arimoclomol, 1.4% ± 0.5% (SEM, n=6) and 3.5% ± 1.1% (SEM, n=6), respectively contained inclusion bodies. The results summarised in **Figure 4.3** therefore show that the number of myogenic cells containing inclusion bodies is significantly reduced in Arimoclomol treated cultures compared to the untreated cultures (ANOVA, $p<0.01$).

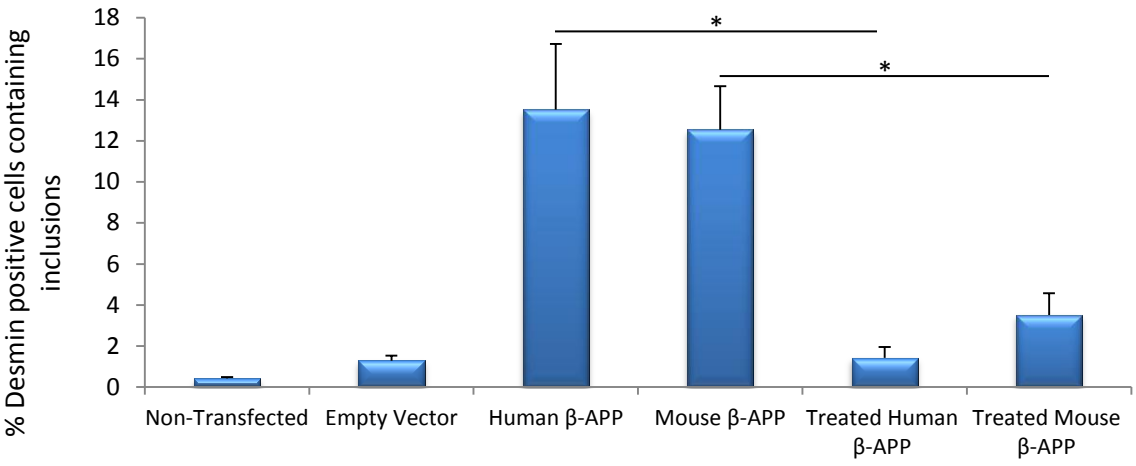


Figure 4.3 The effect of Arimoclomol on inclusion body formation

The bar chart shows the percentage of myogenic cells that contained inclusion bodies in each experimental condition. Error bars = SEM, * denotes $p<0.01$.

This result is consistent for cultures treated with either the human or mouse protein. Further experiments were therefore undertaken using the human β-APP plasmid due to its greater significance to human sIBM.

4.4.3. The effects of Arimoclomol on TDP-43 and p62 expression

Over-expression of β-APP in muscle cells in culture results in the translocation of the C-terminus of TDP-43 from the nucleus to the cytoplasm (See Chapter 3, Section 3.6.3). In the next set of experiments, the effect of treatment with Arimoclomol on these pathological effects of β-APP over-expression was examined. β-APP transfected cultures were treated with 10μM Arimoclomol 24 hours after transfection at 4 DIV, and the cells fixed at 6 DIV, the time when Arimoclomol was observed to have the most significant effect on cell survival (See **Figure 4.2**). Immuno-staining revealed that in contrast to untreated cultures, mislocalisation of TDP-43 to the cytoplasm does not occurin in transfected, Arimoclomol treated cultures. Thus, TDP-43 is only detected in the nucleus in Arimoclomol treated cultures, similar to empty vector transfected cells. **Figure 4.4** shows examples of Arimoclomol treated cultures stained for TDP-43 using both the N and C-terminal TDP-43 antibodies.

This data was quantified by counting the number of cells with cytoplasmic TDP-43 immuno-reactivity in untreated and Arimoclomol treated β-APP transfected cultures. The results are presented in **Figure 4.5** and show that Arimoclomol significantly reduces the number of cells with cytoplasmic TDP-43 to a level similar to that observed in empty vector controls ($p<0.0001$).

Arimoclomol was found to have a similar effect on the expression of p62. In β-APP over-expressing cells, p62 was found to be abnormally present in myocytes, either as cytoplasmic or nuclear inclusions (see Chapter 3, **Figure 3.15**).

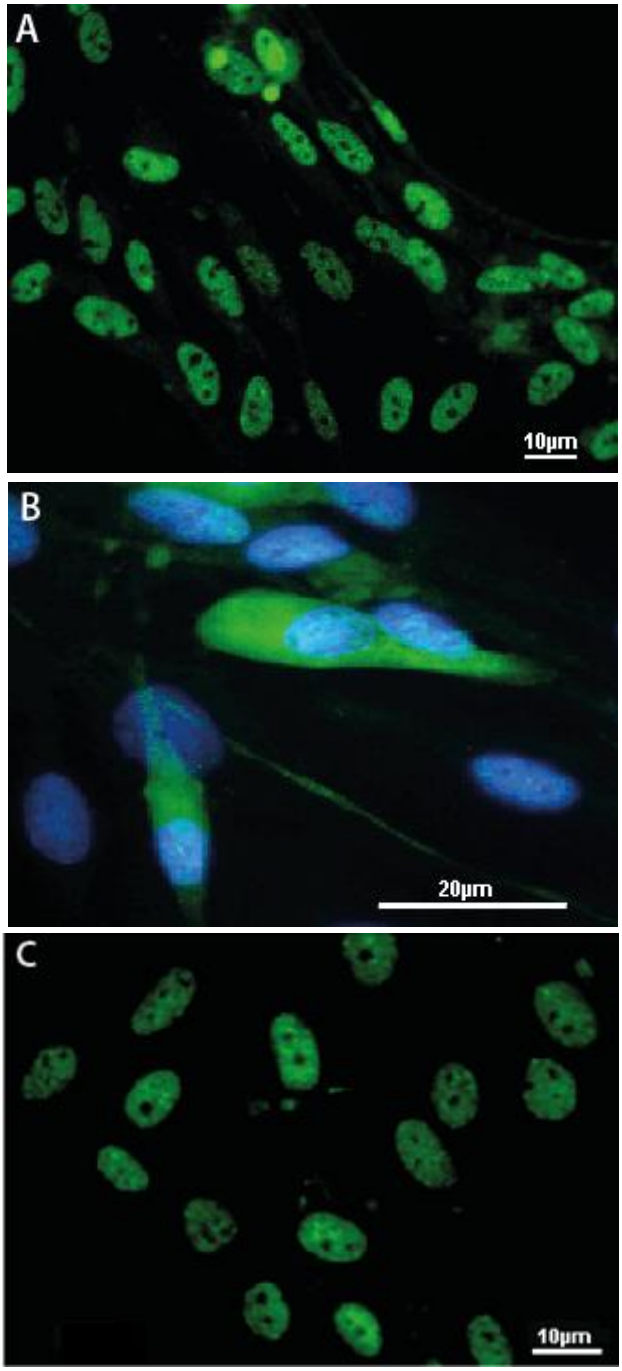


Figure 4.4 The effect of Arimoclomol on TDP-43 expression in β-APP over-expressing cells

Transfected cells were immuno-stained for both the C- and N- terminal TDP-43 (green) and the nuclear marker DAPI (blue). [A] Empty vector transfected cells [B] Untreated β-APP transfected cells [C] β-APP transfected cells treated with Arimoclomol (10µM).

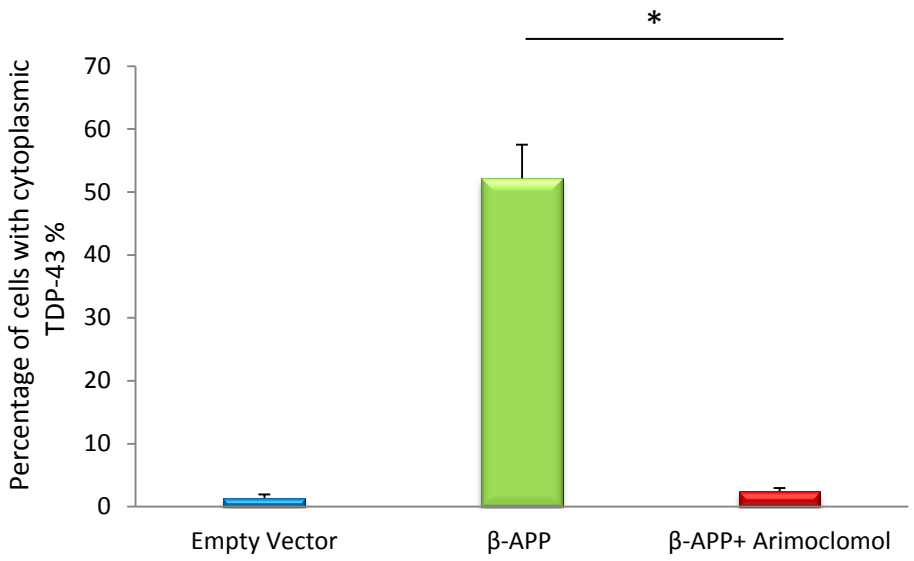


Figure 4.5 The effect of Arimoclomol on TDP-43 expression in β-APP over-expressing cells

Cells were transfected with empty vector or β-APP plasmids in the presence or absence of Arimoclomol (10µM). The bar chart shows the percentage of cells with cytoplasmic TDP-43 immuno-reactivity in each culture condition. The results show that treatment with Arimoclomol significantly decreases the number of β-APP positive cells with cytoplasmic TDP-43. Error bars=SEM, * denotes $p<0.0001$

However, Arimoclomol treatment was found to significantly reduce the number of p62 positive inclusion bodies in β-APP transfected cells to the level seen in empty vector transfected controls. This finding was quantified and the data is presented in **Figure 4.6**.

4.4.4. Arimoclomol increases levels of the mitochondrial protein, SDH in β-APP over-expressing cells

Over-expression of β-APP in myocytes results in a significant reduction in the expression of the mitochondrial protein, succinate dehydrogenase (SDH; Chapter 3, Section 3.6.5). The effect of treatment with Arimoclomol (10μM) on SDH levels in β-APP transfected cells was therefore examined next.

In cultures over-expressing β-APP, treated with Arimoclomol (10μM) 24 hours after transfection, there was a clear increase in SDH staining compared to the untreated controls. As can be seen in **Figure 4.7**, non-transfected cells stain intensely for SDH [A], and this pattern of staining is abolished following treatment with staurosporine, an inducer of apoptosis [B]. Similarly, transfection with β-APP also results in a clear reduction in SDH levels [C]. Treatment of β-APP transfected cells with Arimoclomol (10μM) prevents the loss of SDH expression [D]. These results suggest that Arimoclomol treatment is able to attenuate the mitochondrial abnormality caused by the over-expression of β-APP.

4.4.5. Proteasomal function in β-APP over-expressing cultures with Arimoclomol treatment

Over-expression of β-APP alters proteasomal function in myogenic cells in culture (Chapter 3, Section 3.4.3.6). Therefore, the effect of Arimoclomol on proteasomal function in β-APP transfected cells was also examined.

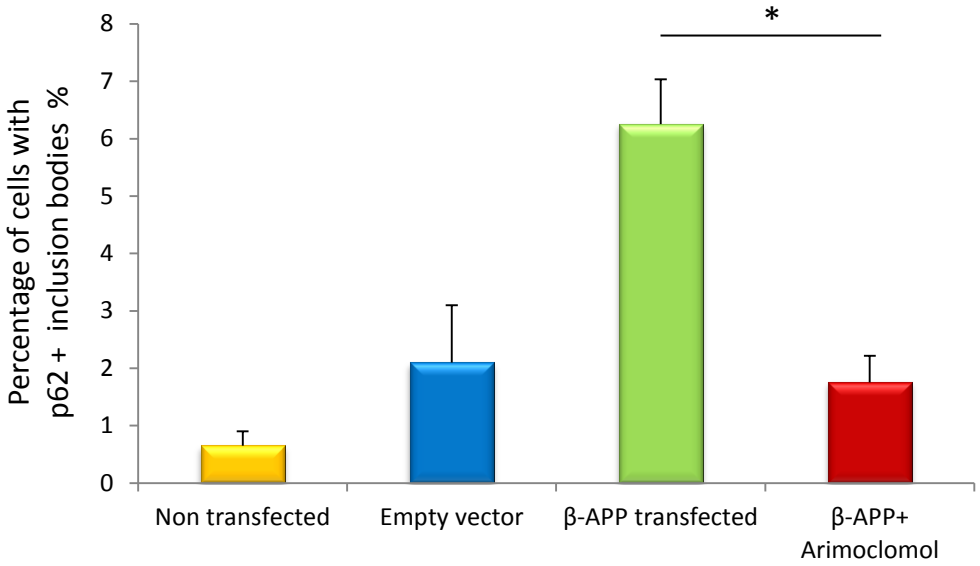


Figure 4.6 The effect of Arimoclomol on p62 expression in β-APP over-expressing cells

Cells were transfected with empty vector or β-APP plasmids in the presence or absence of Arimoclomol (10μM). The bar chart shows the percentage of cells with p62 positive inclusion bodies in each culture condition. The results show that treatment with Arimoclomol significantly reduces the number of p62 positive inclusion bodies in β-APP transfected cultures. Error bars=SEM, * denotes $p<0.0001$

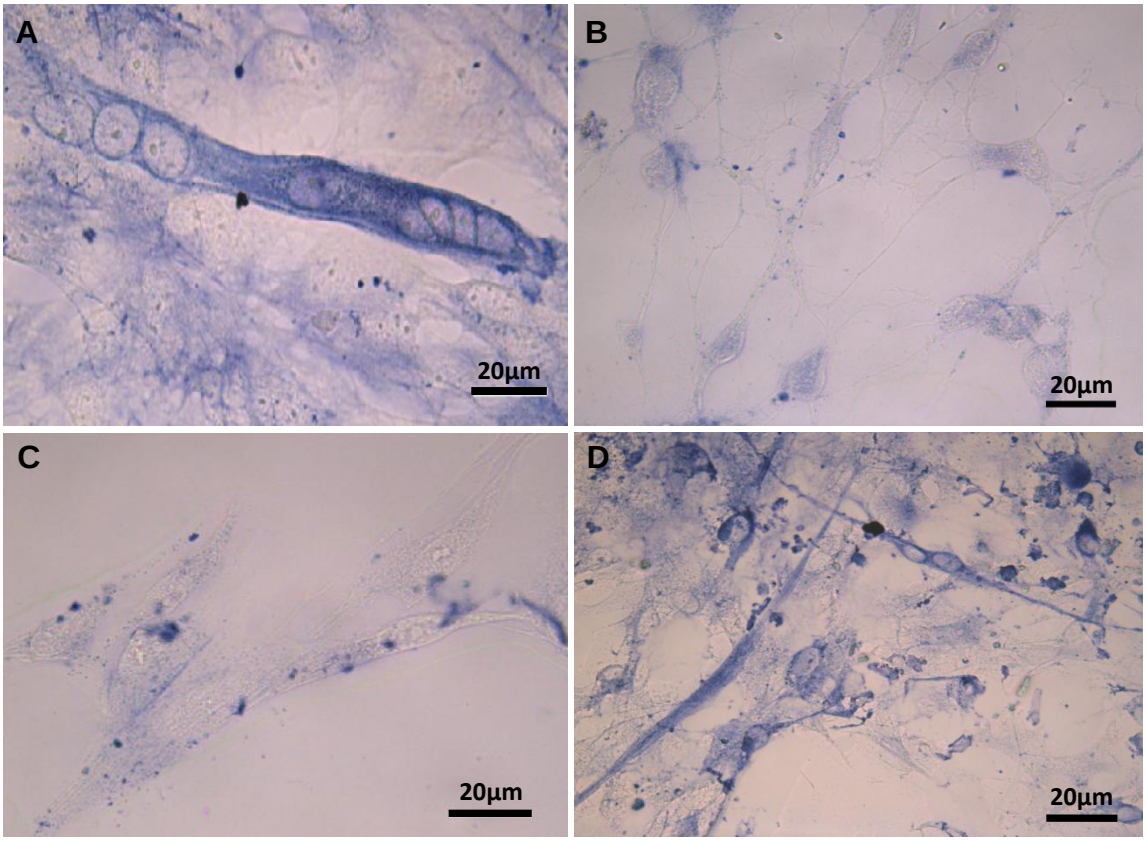


Figure 4.7 The pattern of SDH expression in β-APP transfected cultures with and without Arimoclomol treatment

Cultured muscle cells stained for SDH show the changes in staining intensity under different culture conditions. [A] Cells transfected with the empty vector, [B] Non-transfected cells treated with Staurosporine. [C] β-APP transfected cells. [D] β-APP transfected cells treated with Arimoclomol.

β-APP transfected cultures were treated with 10μM Arimoclomol and assayed for proteasomal activity at 6 DIV. The results are summarised in **Figure 4.8**, which shows that Arimoclomol has no detectable effect on proteasome function in β-APP over-expressing cells, and proteasome activity is not significantly different in untreated and Arimoclomol treated cultures.

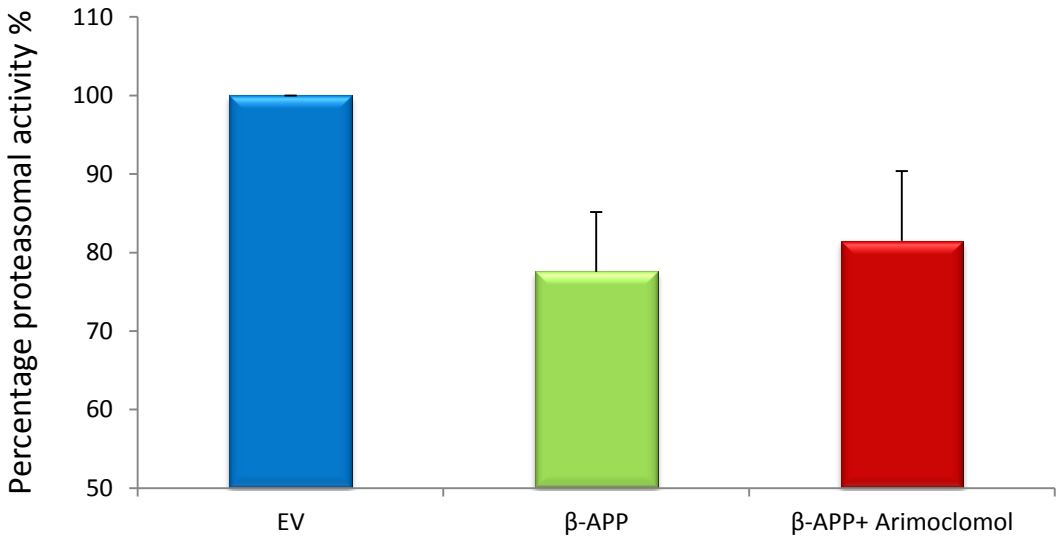


Figure 4.8 Proteasomal function with and without Arimoclomol treatment

The bar chart summarises the results from proteasome activity assays and shows a trend of decreased proteasome activity in β-APP over-expressing cells compared to empty vector (EV) controls (set at 100%). However, no significant change in activity is seen in cells treated with Arimoclomol. Error bars=SEM

4.5. DISCUSSION

There is currently no effective therapy for the treatment sIBM. Although it has been suggested that immunotherapy may have beneficial effects, to date no trials of immuno-suppression in sIBM patients have shown positive effects (Mastaglia et al. 1997). Therefore, there remains a need to investigate alternative drug targets which may reduce the pathological effects of this condition.

In this Chapter, the effects of pharmacological up-regulation of the HSR on the pathological effects of β-APP over-expression shown in Chapter 3 were examined.

Heat shock proteins are essential for the viability of all cells. These proteins not only come into action during intense cellular stress when their expression in the cell rapidly increases, but they are also involved in general housekeeping functions in unstressed cells (Mayer & Bukau 2005). In neurodegenerative diseases such as AD and sIBM, the chronic nature of the condition may result in increased HSP levels, but also the inability to cope with the long-term cell stress (Hoshino et al. 2011). Moreover, such chronic diseases tend to occur in older patients, at a time when HSP genes are poorly induced (Soti & Csermely 2003). Whether this inefficient protein handling, through reduced HSP expression, contributes to the pathogenesis of these diseases remains unclear. However, a poor HSR is likely to contribute to cellular damage and death. Therefore, pharmacologically enhancing the endogenous HSR to improve protein handling in β-APP transfected cells may reduce the pathological effects of β-APP over-expression.

Arimoclomol is a co-inducer of the HSR which prolongs activation of the transcription factor HSF-1 (Kalmar & Greensmith 2009b). However, unlike many other inducers that target HSPs, Arimoclomol co-induces the HSR itself. Arimoclomol works to prolong HSF-1 activation, when HSF-1 is already activated, trimerised and translocated to the nucleus for DNA binding. This mechanism of action of Arimoclomol is important for its function as a co-inducer and sets Arimoclomol aside from other drugs which directly induce the HSR. By only augmenting an already-triggered response, Arimoclomol reduces the risk of

oncogenesis in unstressed cells. Once HSF-1 binds to the HSE, transcription of HSPs takes place. In the presence of Arimoclomol, the expression of HSPs is up-regulated through prolonged HSF-1 binding, leading to a more intense response to stress (Hargitai et al. 2003).

In this Chapter the therapeutic potential of the HSR was examined by testing the effects of Arimoclomol in β-APP over-expressing cells. Muscle cultures were transfected with the human or mouse β-APP plasmid at 4 DIV and the effects were assessed at 5, 6 and 7 DIV. The effects on several pathological features of β-APP over-expression were examined i) cytotoxicity; ii) inclusion body formation; iii) mislocalisation of TDP-43; iv) abnormal p62 expression; v) proteasome activity; and vi) mitochondrial SDH activity.

The effect of Arimoclomol on β-APP induced formation of intracellular inclusion bodies was assessed from immuno-stained images of muscle cultures. The results showed that the number of cells containing inclusion bodies was significantly reduced in β-APP transfected cultures treated with Arimoclomol compared to untreated controls. As the formation of inclusion bodies is highly characteristic and defines this disease, prevention of these protein aggregates is a promising result. Although the mouse and human β-APP proteins are different in their amino acid composition, the same results can be seen following Arimoclomol treatment in cells transfected with either plasmid. These results suggest that the effects of Arimoclomol are not related to the β-APP species or the protein itself, but more are likely to affect generalised protein mishandling.

Over expression of β-APP resulted in a significant cell death. However, in Arimoclomol treated cultures, there was also a significant reduction in β-APP induced cytotoxicity, as determined by LDH assays. Arimoclomol treatment also decreased cell death caused by the process of transfection itself when compared to the empty vector transfected cultures. Thus, the level of cell death in Arimoclomol-treated β-APP cultures at both 5 and 6 DIV was significantly less than both untreated β-APP cultures and cultures transfected with empty vector (EV). However, the beneficial effects of Arimoclomol treatment began

to decline by 7 DIV, when the extent of cell death was still lower in Arimoclomol treated β-APP cultures, but was now significantly greater than detected in EV transfected cultures. The half-life for Arimoclomol in humans is 4 hours (Lanka et al. 2009); however, the half-life *in vitro* has not been established. These experiments therefore suggest that the duration of Arimoclomol's effectiveness may be between 48 and 72 hours, at least *in vitro*.

In addition to the increased cell survival and reduction in inclusion body formation following Arimoclomol treatment of β-APP transfected cultures, cytoplasmic translocation of the C-terminus of TDP-43 induced by β-APP over-expression was also reduced by Arimoclomol treatment. Indeed, Arimoclomol treated cultures were indistinguishable from empty vector transfected cultures, so that TDP-43 immuno-reactivity with both the C- and N- terminal TDP-43 antibodies was restricted to the nucleus. This finding suggests that TDP-43 translocation may well be a detrimental effect of cell stress caused by protein mishandling, as Arimoclomol treatment prevents the translocation and this correlates with an increase in cell survival. As TDP-43 translocation is an increasingly prominent feature of sIBM and other neurodegenerative diseases including ALS and FTD, this data that shows beneficial effects of Arimoclomol on TDP-43 mislocalisation is highly promising.

Arimoclomol was also found to prevent the abnormal pattern of p62 expression observed in β-APP cells. The number of cells with abnormal p62 expression in cytoplasmic or nuclear inclusion bodies was significantly lower in Arimoclomol treated cultures, indicating a reduced level of aberrant protein handling in these cells.

p62 normally functions as a shuttle protein delivering ubiquitinated proteins for degradation via the autophagosomal or proteasomal pathways (Nogalska et al. 2009).

It is possible that improved handling of proteins by a larger number of available chaperone proteins caused by Arimoclomol may make degradation of any unwanted proteins more efficient, thereby preventing the accumulation of p62.

In β-APP over-expressing cultures, levels of the mitochondrial protein, SDH was reduced in β-APP transfected cultures (See Chapter 3, Sections 3.4.3.5 and 3.4.3.6). As SDH is a key protein in the mitochondrial respiratory chain, reduced levels of this protein, especially in highly metabolic muscle cells, is likely to be detrimental to cell viability and indeed, β-APP over-expression does result in an increase in cell death. Treatment with Arimoclomol was found to prevent this reduction in mitochondrial SDH, which correlates with the reduced cell death detected in Arimoclomol treated cultures.

Results presented in this Chapter show that Arimoclomol clearly reduces the effects of protein mishandling, and improvement in the SDH levels in these cultures suggest that the pathogenic consequences of protein mishandling on mitochondrial proteins may also be reduced. It has been suggested that misfolded proteins or protofibrils can become translocated to the mitochondrial membrane and therefore may affect normal mitochondrial functions such as protein import (Hashimoto et al. 2003). A reduction in protein aggregation caused by an increase in chaperone levels in Arimoclomol treated cultures may therefore be reflected by an improvement in expression of mitochondrial proteins in β-APP over-expressing cells.

Up-regulation of the HSR has been shown to have beneficial effects on mitochondrial function in previous studies. Polla et al. (1996), conducted experiments *in vitro* where U937, a human premonocytic cell line was stressed using H_2O_2 treatment. Their results show that increased mitochondrial dysfunction and deformities were present in treated cells compared to untreated cells, which included abnormal mitochondrial membrane potential and disruption to the cristae. However, when cultured cells were heat shocked prior to H_2O_2 treatment, these effects were reduced. The authors suggest that heat shock protein expression, therefore protects against mitochondrial dysfunction. HSP70 expression was found to have a stronger correlation between expression and mitochondrial protection, which may be because specific members of the HSP70 family are expressed in the mitochondria also (Polla et al. 1996).

In view of the positive effects of Arimoclomol on TDP-43 mislocalisation and abnormal p62 expression in β-APP transfected cells, the lack of a positive effect of Arimoclomol on β-APP induced proteasome dysfunction was rather surprising. The deleterious effect of β-APP on proteasome function was relatively small and indeed did not reach significance. It is possible therefore, that β-APP over-expression was insufficient to model *in vitro* the chronic effects of protein aggregation and mishandling that occur *in vivo* in sIBM. Therefore, this model may not be suitable to examine the effects of Arimoclomol on proteasome dysfunction. A more direct method to induce proteasome dysfunction and examine the potential of Arimoclomol to ameliorate proteasome dysfunction is presented in Chapter 5.

Treatment of β-APP over-expressing cells with Arimoclomol has shown to alleviate the pathological effects of β-APP over-expression *in vitro*. It is likely that this is due to the ability of Arimoclomol to co-induce the HSR. Other agents, such as geldanamycin and celastrol, have also been shown to successfully up-regulate HSPs (Sittler et al. 2001) (Westerheide & Morimoto 2005a), however these agents do not have the specificity of Arimoclomol, which augments a cell-triggered HSR. This reduces the possible side effects of triggering the HSR in unstressed cells.

Furthermore, Arimoclomol not only protects against degenerative features caused by protein mishandling, but is also able to reduce the inflammatory effects of β-APP over-expression. Experiments conducted by A. Miller in this lab, have shown that β-APP over-expressing cultures display several inflammatory features that are characteristic of sIBM pathology, such as an increased expression of the major histocompatibility complex class I (MHC Class I) on the cell surface (Miller et al. 2010). MHC Class I presents aberrant cellular antigens at the cell surface to cytotoxic T-cells to instigate an immune response (Sijts & Kloetzel 2011). Treatment of β-APP transfected cells with Arimoclomol was found to significantly reduce MHC Class I expression. As inflammation is a major contributor to the pathology of sIBM, this suggests that Arimoclomol is able to attenuate both the degenerative and inflammatory features of this debilitating condition.

4.6. CONCLUSION

The results presented in this Chapter show that protein mishandling is linked to cell death in this β-APP over-expressing cell culture model of sIBM and that the deleterious effects of β-APP expression can be amerliorated by treatment with Arimoclomol. Since Arimoclomol works, at least in part, by up-regulating the expression of a family of protein chaperones, including HSP70 and HSP90, Arimoclomol is able to prevent the formation of inclusion bodies and promote more efficient protein handling under stressful conditions. Arimoclomol successfully reduces the pathogenic features modelled *in vitro* and therefore is a good candidate to investigate further in the treatment of sIBM.

CHAPTER 5.

INVESTIGATING THE EFFECTS OF PROTEASOME INHIBITION IN PRIMARY MUSCLE CULTURES

5.1. INTRODUCTION

The ubiquitin-proteasome system is not only a pathway for degradation of aberrant proteins that may aggregate in cells if not disposed of, but is also an essential regulator of almost all basic cellular functions (Tanaka 2009). By turning over key transcription factors, chaperones and other enzymes, the proteasome efficiently regulates signal transduction, gene expression, metabolism, the immune response, progression through the cell cycle as well as cell death (Yi & Ehlers 2007; Tanaka 2009; Kwak et al. 2011). Therefore, proteasome dysfunction not only affects degradation of misfolded or damaged proteins but also affects essential biological pathways in the cell.

In the experiments described in this Thesis, the degenerative features of sIBM have been modelled by over-expressing β-APP *in vitro,* which results in disruption to the protein handling mechanisms of the cell. One possible pathway by which this can occur is via disruption of proteasome function, which can lead to an accumulation of misfolded or damaged proteins in the cytoplasm and nucleus and further exacerbate the imbalance in the proteostasis. Bence et al (2001) investigated the transient expression of two aggregation-prone proteins, a fragment of huntingtin containing polyglutamine repeats, and a mutant form of the cystic fibrosis transmembrane conductance regulator (CFTCR). Their results demonstrated that protein aggregates inhibit the UPS and lead to cell cycle arrest (Bence et al. 2001). Therefore, closely examining the effects of proteasome disruption *in vitro* would give insight into protein handling in stressed cells.

The experiments described in Chapter 3 showed that when β-APP was over-expressed in muscle cultures, there was a slight decrease in proteasome activity (Chapter 3, Section 3.4.3.6). In view of this minimal effect of β-APP in proteasome function, it was not surprising that treatment with Arimoclomol had little effect in proteasome activity (Chapter 4, Section 4.4.5).

On the other hand Arimoclomol, treatment reduced cytotoxicity and inclusion body formation, and reduced cytoplasmic translocation of TDP-43, in β-APP over-expressing cultures. These findings therefore suggest that Arimoclomol improves protein handling in

β-APP over-expressing cells which leads to increased cell viability. In order to further investigate the effects of Arimoclomol on proteasome function, the effects of a more direct method of proteasome inhibition was examined in this Chapter.

5.1.1. The proteasome

Proteasomes are large protease complexes composed of 28 subunits arranged into four rings that are ubiquitously expressed in all eukaryotic cells (Meng et al. 1999a). The central role of the proteasome in cells is to carry out degradation of ubiquitin-bound proteins via the ubiquitin-proteasome system (UPS), which accounts for 80-90% of all protein degradation within the cell (Sorokin et al. 2009). All other degradation is carried out by the lysosomal pathway (autophagy) or by smaller proteases (Sorokin et al. 2009).

The enzymatically active 26S proteasome complex consists of a 20S central core particle and either one or two 19S regulatory particles which bind at either end of the core particle (Tanaka 2009). The central core particle contains three catalytic sites known as the chymotrypsin-like, trypsin-like and peptidyl-glutamyl peptide hydrolysing (PGPH) sites which carry out the proteolytic activity (Heinemeyer et al. 1997). Misfolded or damaged proteins ready for degradation by the proteasome are threaded through the regulatory 19S subunit through to the 20S subunit where catalysis commences. However, in order for this to occur, proteins must be tagged by a chain of ubiquitin (containing at least four ubiquitin molecules) (Tanaka 2009). Three key enzymes catalyse the cascade of reaction that lead to ubiquitination of proteins; E1 ubiquitin-activating protein, E2 ubiquitin-conjugating protein and E3 ubiquitin-ligating protein (Sorokin et al. 2009). Ubiquitination of proteins is critical as it ensures the correct proteins are selected for degradation. Shuttle proteins such as p62, then deliver the polyubiquitinated proteins to the proteasome (Pankiv et al. 2010) where the ubiquitin chain must be removed before the protein enters the proteolytic chamber. This reaction is catalysed by deubiquitinating (DUB) enzymes, which are small 20-30kDa proteins (Sorokin et al. 2009). **Figure 5.1** shows a schematic diagram of the ubiquitin-dependant proteasome degradation process.

In addition to the proteolytic function of the proteasome, this complex has other diverse functions such as regulation of the cell cycle and activation of NFκB (Meng et al. 1999b). Most of these functions are regulated by the availability of enzymes, transcription factors and co-factors which are controlled by the activity of the proteasome (Meng et al. 1999b). Therefore this large complex is essential for cell survival.

5.1.2. Proteasome dysfunction and disease

The concentration of proteasomes in cells is relatively high compared to other cellular proteins at 1-20µg/mg reflecting their key role in normal cell activity (Dahlmann & Kuehn 1995). Indeed, mice in which the proteasome has been knocked out are non-viable (Heinemeyer et al. 1991). This may be because up to 90% of intracellular proteins in mammalian cells are degraded by the UPS (Drews et al. 2007). Therefore, through the UPS, the proteasome complex regulates such a large variety of cellular functions that any abnormalities in proteasome function are likely to be deleterious for the cell and may result in disease (Dahlmann 2007).

Proteasome dysfunction has been implicated in many diseases ranging from cancer to neurodegenerative disorders (Dahlmann 2007). As several of these conditions occur in later-life, it is possible that there are age-related elements to these disorders. Indeed several studies have indicated that there may be an age-related decrease in proteasome activity. For example, Bulteau et al (2000) examined proteasome activity and subunit composition in human epidermal cells and showed that a decrease in proteasome activity correlated with increasing age (Friguet et al. 2000; Bulteau et al. 2000). 2D polyacrylamide gel analysis of tissue homogenates showed that the subunit composition of the proteasome was altered in samples from aged volunteers, with some subunits present at higher concentrations and some reduced.

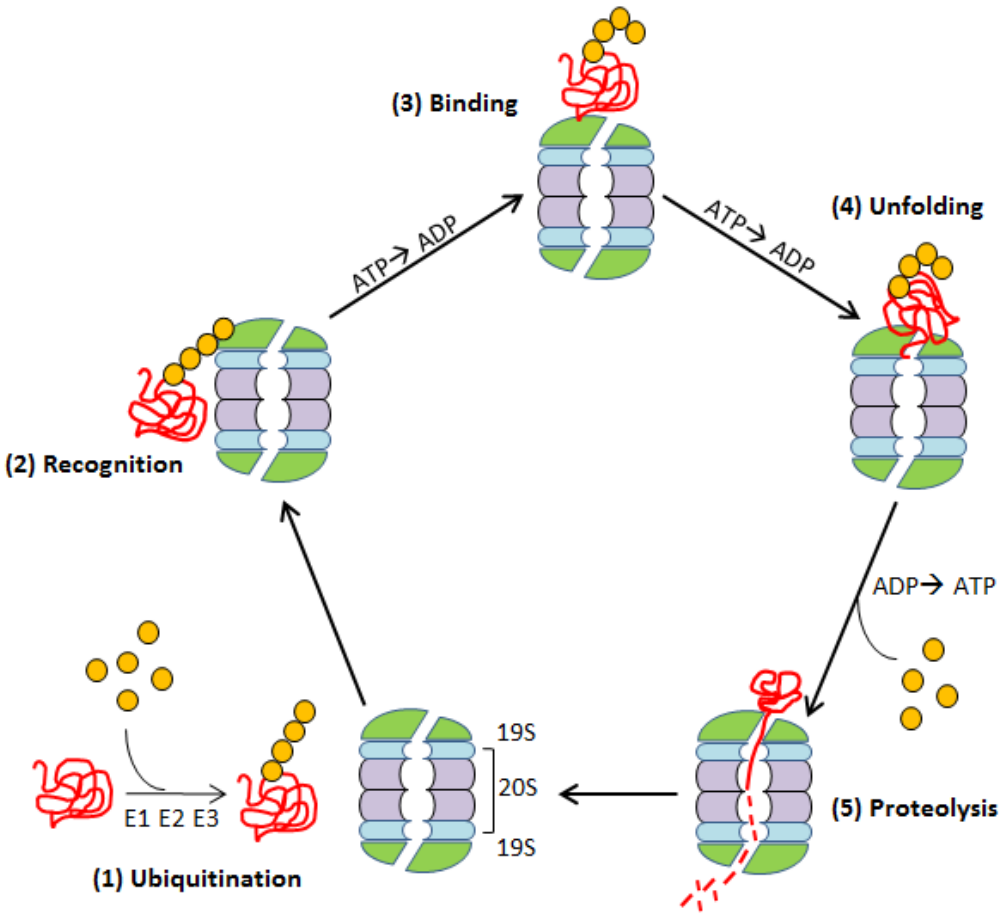


Figure 5.1 Schematic diagram of proteasome function

Proteasome function begins with (1) ubiquitination of misfolded proteins targeted for degradation. (2) The ubiquitinated protein is recognized by the proteasome 19S subunit. (3) Following ATP hydrolysis, the ubiquitinated protein binds to the proteasome. (4) The protein is slowly unfolded and passed through the lumen of the proteasome to the 20S core while ubiquitin is removed by DUB enzymes. (5) The protein is hydrolysed at the 20S core into small peptides and released.

In addition, a correlation between increased levels of oxidised proteins, decreased proteasome activity and increasing age has also been reported in human epidermal cells by (Petropoulos et al. 2000). These results suggest that an age-related decrease in proteasome function may lead to the accumulation of harmful molecules in cells which may become pathogenic.

Proteasome abnormalities have also been identified in AD, Huntington's disease, Parkinson's disease, ALS and sIBM which have all shown decreased activity of the 20S/26S proteasomes (McNaught & Jenner 2001; Keck et al. 2003; Zhou et al. 2003; Kabashi et al. 2004; Oh et al. 2005). In IBM, the induction of immuno-proteasomes in patient tissue has also been observed (Tanaka 2009). Immuno-proteasomes are proteasome complexes with a modified composition of subunits, which form in response to gamma interferon (IFN-γ) induction (Tanaka 2009). Abnormal proteasome activity has also been demonstrated in sIBM biopsy material, and an abnormal association of proteasome subunits with intracellular aggregates has also been reported (Fratta et al. 2005a).

In addition to degenerative diseases, proteasome dysfunction has also been identified in other conditions such as Sjogren's syndrome, cardiac dysfunction and even HIV infections where decreased activity or expression of proteasomes is evident (Keller et al. 2000; Ott et al. 2003; Krause et al. 2006).

5.1.3. Epoxomicin-induced inhibition of the proteasome

Complete or partial inhibition of the proteasome is clearly associated with a wide variety of pathological conditions. Therefore a large number of studies have investigated the effects of proteasome inhibition. For example, Chondrogianni et al. (2004) conducted experiments on human embryonic fibroblasts cultures, where proteasome function was irreversibly or reversibly inhibited with low concentrations of the proteasome inhibitors Epoxomicin or MG132, respectively. Results from these experiments showed an accumulation of oxidized proteins in inhibitor treated cells compared to untreated cells.

These cells also showed evidence of senescence with arrest of the cell cycle although the cells were still viable when the inhibitor was removed from the media (Chondrogianni & Gonos 2004).

Casarejos et al (2011) also tested the effects of Epoxomicin in human neuroblastoma cells and showed that proteasome inhibition led to an increase in the levels of proteins which have been associated with neurodegenerative disorders. These include α-synuclein and hyperphosphorylated tau, which correlated with an increase rate of cell death (Casarejos et al. 2011) .

Although Epoxomicin has been shown to be a potent disruptor of proteostasis in cell culture, this drug was initially developed as an anti-tumour agent (Meng et al. 1999a) (see **Figure 5.2** for chemical structure). Epoxomicin has since been shown to bind covalently to catalytic β-subunits of the proteasome and subsequently inhibit all three catalytic sites, albeit at different rates, thereby eliminating proteasome function. At the optimal concentration of Epoxomicin, proteasome activity in cells may be reduced without shutting down the degradation pathway entirely, which would otherwise lead to cell death in the entire muscle culture.

Inhibition of the proteasome in oncogenic cells is currently under investigation as a possible strategy for cancer treatment, as inhibitors can lower the rate of cell proliferation and induce apoptosis (Wu et al. 2010).

Epoxomicin is therefore a well-studied agent which has been used *in vitro* to successfully induce proteasome dysfunction in cells in culture, and is therefore an ideal agent to use to study the functions of proteasomes under different conditions. In this Chapter, proteasome dysfunction was experimentally induced in cultured primary muscle cells by treatment with Epoxomicin and the effects of Arimoclomol treatment were assessed.

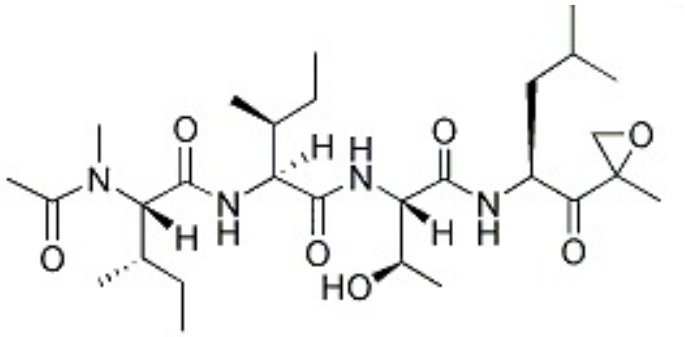


Figure 5.4 Chemical structure of Epoxomicin

5.2. AIMS OF THIS CHAPTER

The aims of this Chapter are:

I. To assess the effects of direct proteasome inhibition in the β-APP over-expressing model of sIBM

II. To examine the effects of Arimoclomol in cultures where the proteasome has been inhibited

5.3. MATERIALS AND METHODS

A full description of the materials and methods used in the experiments described in this Chapter are presented in Chapter 2, and include the following methods:-

- Neonatal rat muscle cell culture
- Immunocytochemistry
- SDH staining
- LDH Assay
- Protein Assay
- Proteasome Assay
- Data and statistical analysis

5.3.1. Epoxomicin treatment

Cultured myocytes were treated with Epoxomicin at 3 DIV and assessed at 6 DIV in order to maintain the timescale of β-APP transfected cultures. The effect of Epoxomicin on cells survival was examined at concentrations of 0.025, 0.05, 0.1 and 0.2µM. In all other cultures Epoxomicin was used at a concentration of 0.1µM.

5.3.2. Arimoclomol treatment

Arimoclomol was added at 10µM to fresh culture media 24 hours after Epoxomicin treatment, i.e. at 4 DIV and the effects examined at 5, 6 and 7 DIV.

5.4. RESULTS

5.4.1. The effects of proteasome inhibition on muscle cell survival in the presence and absence of Arimoclomol

Muscle cultures grown in 96-well assay plates were treated with increasing concentrations of Epoxomicin ranging from 0.025-0.2µM and the effects on cell viability determined by an LDH assay at 3 different time points (5, 6 and 7 DIV). In some cultures, the effect of treatment with Arimoclomol (10µM) for 24 hours on cell survival was also examined. The results are summarised in **Figure 5.3**, which shows that Epoxomicin treatment at concentrations between 0.025-0.1µM results in a similar level of cell death, at approximately 20%. Treatment with Epoxomicin at 0.2µM caused a significantly greater level of cell death, at 31.7% ± 8.7% (SEM, p=0.03). This result was consistent at all 3 time points.

LDH assays further showed that at 5 and 6 DIV, treatment with any concentration of Epoxomicin (0.025-0.2µM) did not result in significantly reduced cytotoxicity following Arimoclomol treatment. However, at 7 DIV, at all concentrations of Epoxomicin, even those cells treated with the highest concentration of the agent (i.e. 0.2µM), Arimoclomol treatment significantly reduced LDH levels to a similar level in all cultures.

In view of the significant cytotoxic effects of 0.2µM Epoxomicin, for all subsequent experiments on proteasome inhibition, Epoxomicin was used at a concentration of 0.1µM, a concentration that produced an LDH level of 20.9% ± 1.53% (SEM). All results are expressed relative to the maximum cell death induced by treatment with Triton X100 (0.1µM), which was set as 100% LDH release.

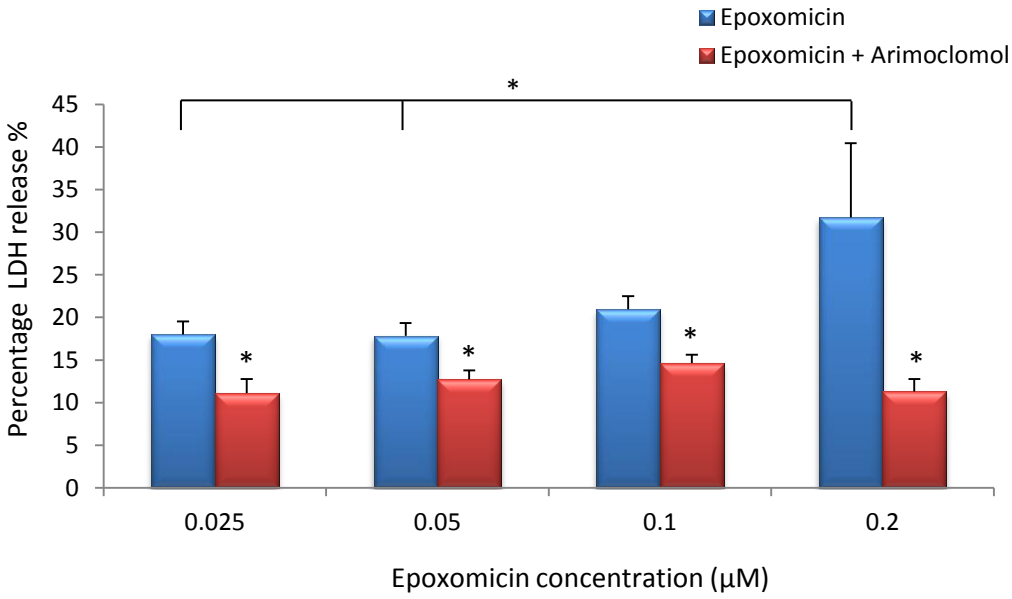


Figure 5.6 Effect of Arimoclomol on Epoxomicin-induced cell death

The effect of Epoxomicin (at various concentrations) on LDH release at 7 DIV is summarised in this bar chart and shows a significant increase in LDH levels in cultures treated with 0.2µM Epoxomicin. The effect of treatment with Arimoclomol is also presented and shows that Arimoclomol reduces LDH release in all cultures. Error bars=SEM, * denotes $p<0.05$

5.4.2. The effect of proteasome inhibition on proteasome activity and the effect of Arimoclomol

The effect of treatment with 0.1µM Epoxomicin on proteasome activity was examined at 7 DIV, when the maximum effect of Epoxomicin on cell death was detected. The results of the proteasome assay are summarised in **Figure 5.4** and show that 0.1µM Epoxomicin dramatically reduced proteasome activity to 16.7% ± 7.0% (SEM, n=3) of control. In contrast, in Epoxomicin treated cultures treated with 10µM Arimoclomol, there was a significant improvement in proteasome activity, with levels reaching 77.6% ± 1.0% (SEM, n=3) of control.

5.4.3. The effect of proteasome inhibition on inclusion body formation

Muscle cultures treated with Epoxomicin were fixed at 7 DIV and immuno-stained for the presence of p62 and β-APP immuno-reactive inclusion bodies (see **Figure 5.5** [A]). A small percentage of cells were found to contain p62 positive inclusion bodies, some of which were also immuno-reactive for β-APP. The total number of inclusion bodies in the Epoxomicin treated cultures was quantified and compared to cultures which were also treated with 10µM Arimoclomol. The results show that there was a significant reduction in the number of inclusion bodies in the Epoxomicin + Arimoclomol treated cultures at 1.6% ± 0.1% (SEM) compared to those only treated with Epoxomicin, which contained 3.4% ± 0.6% (SEM, $p<0.05$). This data is summarised in **Figure 5.5** [B].

5.4.4. The effect of proteasome inhibition on TDP-43 localisation

Since translocation of TDP-43 is a key pathological feature of sIBM that is recapitulated in cells transfected with β-APP, the effect of proteasome inhibition on TDP-43 expression was also examined in Epoxomicin treated cells at 6 and 7 DIV.

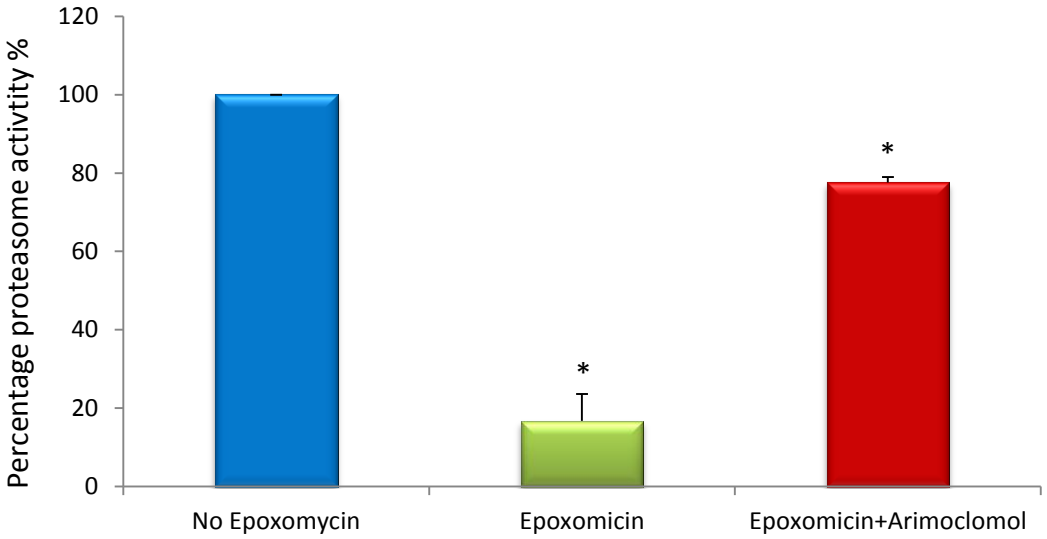


Figure 5.8. The effect of Arimoclomol on Epoxomicin induced proteasome dysfunction

Proteasome activity was examined in untreated muscle cultures and those treated with 0.1µM Epoxomicin in the presence and absence of Arimoclomol (10µM) at 7 DIV. The activity in non-treated cultures was set as 100%. Epoxomicin treatment significantly reduced proteasome activity, and this effect was prevented by treatment with Arimoclomol. Error bars= SEM, ($p<0.001$).

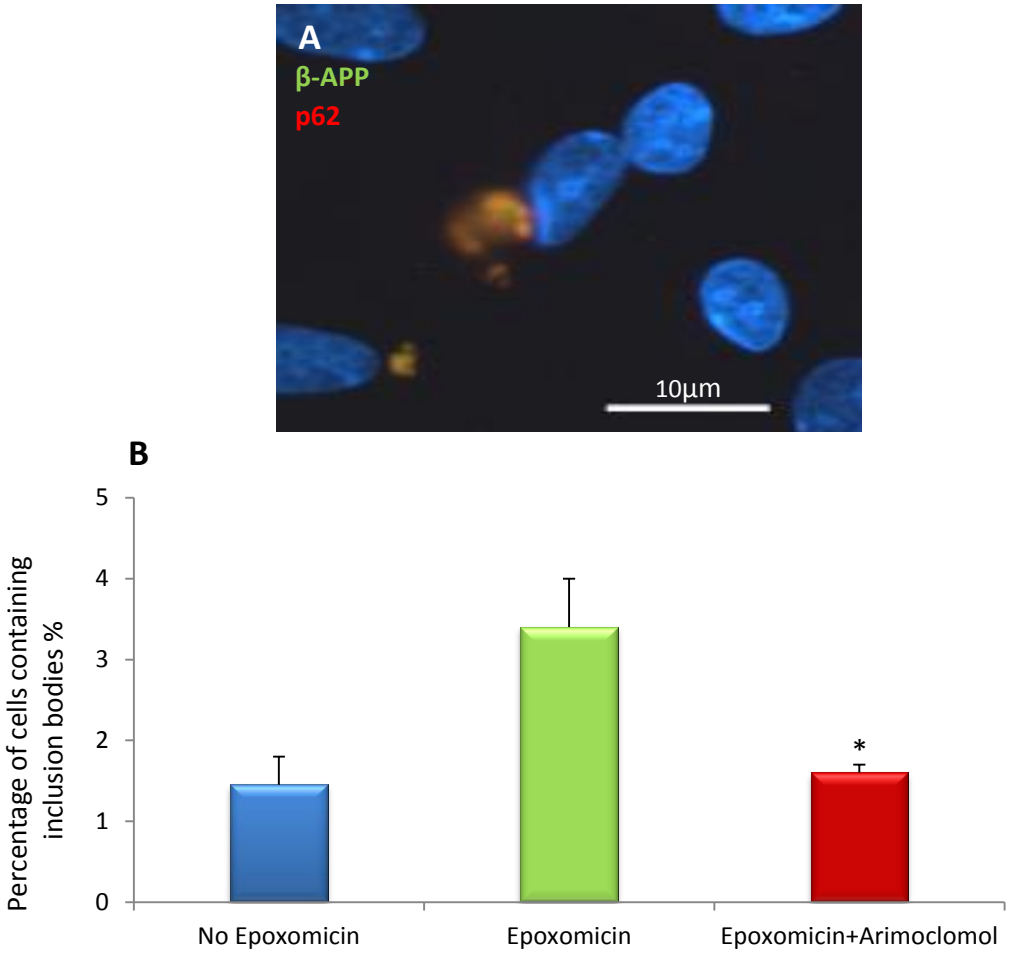


Figure 5.10. Intracellular inclusion body formation following Epoxomicin treatment and the effect of Arimoclomol

Untreated muscle cultures and those treated with Epoxomicin (0.1µM) at 3 DIV were examined for inclusion body formation by immunostaining at 7 DIV in the presence and absence of Arimoclomol. [A] Shows an image of a typical inclusion body immuno-reactive for both β-APP and p62 in an Epoxomicin-treated culture, not treated with Arimoclomol. The bar chart [B] shows the number of cells containing inclusion bodies after 0.1µM Epoxomicin treatment at 7 DIV, expressed as a percentage of the total number of muscle cells. The results also show that treatment with Arimoclomol (10µM) significantly reduces the number of inclusion bodies formed following Epoxomicin treatment. Error bars=SEM, * denotes $p<0.05$.

Figure 5.6 shows examples of Epoxomicin treated and untreated cells immuno-stained for TDP-43. It can be seen that cells treated with 0.1µM Epoxomicin contained only nuclear TDP-43, similar to untreated cells and translocation of TDP-43 to the cytoplasm was not detected with either the N-terminal or C-terminal antibodies. Since Epoxomicin did not have an effect on TDP-43 expression, the effect of Arimoclomol in Epoxomicin-treated cells was not examined.

5.4.5. The effect of proteasome inhibition on mitochondrial function

Mitochondrial function was also assessed in live cells treated with 0.1µM Epoxomicin by examination of SDH levels. The results showed that there was no difference in SDH levels in Epoxomicin treated cultures compared to untreated cultures. **Figure 5.7** shows typical images of cells stained for SDH under both culture conditions. Since Epoxomicin had no effect on SDH expression, the effect of Arimoclomol on SDH expression in Epoxomicin-treated cells was not investigated.

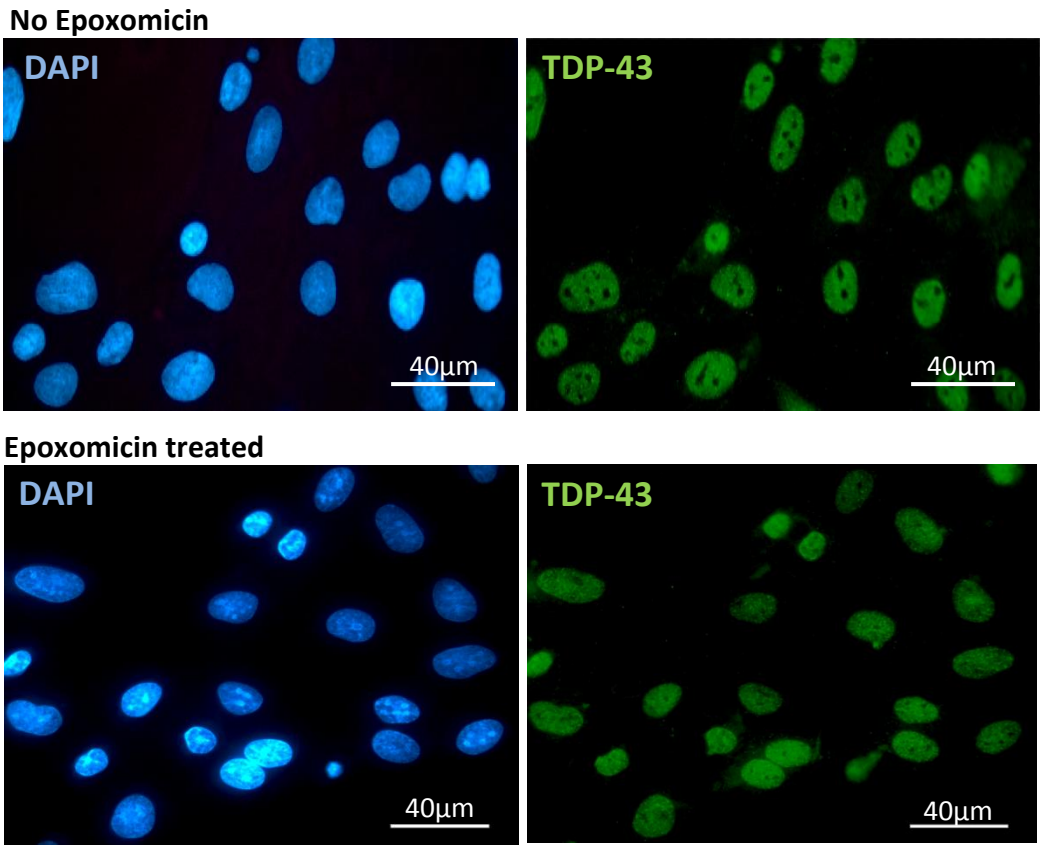
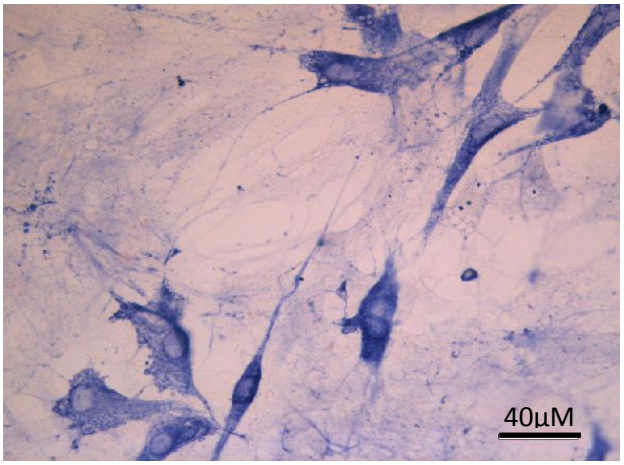
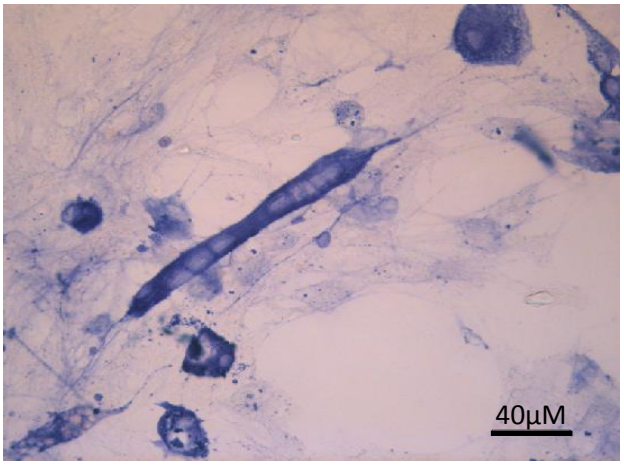


Figure 5.13 Epoxomicin treatment does not alter normal TDP-43 expression in muscle cultures

Following treatment of muscle cells with Epoxomicin (0.1µM) at 3 DIV, the pattern of TDP-43 expression was examined by immuno-staining for TDP-43 (green) and counterstaining the cells with the nuclear marker DAPI (blue). Typical patterns of TDP-43 immuno-reactivity in untreated and Epoxomicin treated cultures are shown here at 7 DIV.


40µM


40µM

5.5. DISCUSSION

A key feature of sIBM is the formation of intracellular protein aggregates which have been reported to contain a large variety of proteins including ubiquitin, β-APP, Aβ, p-tau and α-synuclein among others (Greenberg 2010). The formation of protein inclusions is a reflection of the cell's attempt to sequester an unwanted accumulation of proteins (Kopito 2000). However, in sIBM the mechanism by which protein homeostasis is initially disrupted remains unclear. One possible explanation is that the normal degradation of unwanted proteins is altered, causing a backlog of misfolded proteins to accumulate and thus form aggregates. As the main cellular protein degradation system involves the proteasome, proteasomal dysfunction has been implicated in sIBM pathogenesis.

Askanas et al (2005) found that sIBM patient biopsies demonstrate significantly reduced proteasome activity at all three of the catalytic site of the proteasome. Ferrer et al (2004) conducted an immunohistochemical study on 10 IBM patient muscle biospies, and showed that in all IBM cases examined there was an increase in immuno-reactivity for proteasome subunits which were co-localised with abnormal protein aggregates. The authors suggest their findings indicate a link between protein aggregation and proteasomal expression in IBM (Ferrer et al. 2004a).

5.5.1. The effect of direct inhibition of the proteasome in muscle cells in cultures

Although the experiments described in Chapter 3 showed that β-APP over-expression only resulted in a mild and borderline-significant decrease in proteasome activity in cultured muscle cells, in view of the findings by Askanas et al (2005) in human muscle cells, the effect of direct inhibition of the proteasome on muscle cells was examined in this Chapter. The effects of proteasome inhibition were examined using outcome measures established in Chapter 3.

Abnormal proteins and oligomers have been implicated in inhibiting the proteasome (Gregori et al. 1995; Lindsten et al. 2002; Lindersson et al. 2004). However, in sIBM an

increase in the number of proteasome subunits has also been observed (Ferrer et al. 2004b; Askanas & Engel 2005b). This suggests that inhibition of the proteasome may be counteracted by an up-regulation of proteasome complexes, in order to redress the imbalance in proteostasis.

In Chapter 4, Arimoclomol treatment was not found to significantly improve proteasome activity. However, if the number of proteasomes is increased to compensate for any dysfunctional complexes, then the effects of Arimoclomol treatment on proteasome activity would not be distinguishable. Arimoclomol treatment enhances the number of HSPs, promoting more efficient protein handling under stress. Therefore in treated cells, a reduced amount of misfolded proteins or oligomers would be available for proteasomal degradation. This may be reflected in the lower proteasomal activity.

The results showed that treatment with 0.1µM Epoxomicin induced a significant reduction in proteasome activity, increased cell death and increased the formation of p62 and ubiquitin positive inclusion bodies. However, the normal pattern of TDP-43 and SDH expression were not altered by Epoxomicin treatment. Treatment with Arimoclomol was found to prevent the deleterious changes induced by Epoxomicin treatment.

5.5.2. The effects of Epoxomicin treatment on proteasome activity in the presence and absence of Arimoclomol

Muscle cultures treated with Epoxomicin have significantly decreased proteasomal activity, confirming that this agent effectively inhibits proteasome function *in vitro* (See **Figure 5.4**). In subsequent experiments, cultures treated with Arimoclomol 24 hours after Epoxomicin treatment showed a significant improvement in proteasome activity compared to Epoxomicin-only treated cultures. Although Epoxomicin is an irreversible inhibitor of the proteasome, at the relatively low concentrations used in this study, many proteasome complexes may remain uninhibited, allowing the cells to remain viable. Indeed, cell survival assays showed that increased proteasome inhibition leads to greater

cell death (See **Figure 5.3**). Therefore, by enhancing the HSR, and improving protein handling, Arimoclomol may assist in increasing the activity of uninhibited proteasomes which can then compensate for the disruption caused by the inhibited complexes.

5.5.3. The effects of Epoxomicin on cell survival in the presence and absence of Arimoclomol

The cell survival assays showed a similar level of toxicity across all Epoxomicin concentrations during the first 48 hours after treatment (6 DIV). However, by 7 DIV this level of toxicity was increased, suggesting that there is a cumulative effect of disrupting protein degradation in cells over time, and this causes an imbalance of the proteostasis which is toxic to cells.

The ubiquitin-proteasome system is not only a pathway for degradation of aberrant proteins that may aggregate in cells if not properly disposed of, but is also an essential regulator of almost all basic cellular functions (Tanaka 2009). By turning over key transcription factors, chaperones and other enzymes, the proteasome efficiently regulates signal transduction, gene expression, metabolism, immune response, progression through the cell cycle and cell death (Yi & Ehlers 2007; Tanaka 2009; Kwak et al. 2011). Therefore inhibition of the proteasome may not only effect degradation of misfolded or damaged proteins but also remove healthy proteins from their biological pathways, leading to toxic cell stress.

The cell survival assays further showed that at 7 DIV, Arimoclomol treatment was able to significantly reduce the Epoxomicin induced cytotoxicity, which was not seen at the earlier time points. This indicates that the heat shock response is indeed augmented in cells which already have this pathway triggered by cell stress. Here Arimoclomol is able to attenuate the disruption to the proteostasis leading to improved cell survival.

5.5.4. The effects of Epoxomicin on inclusion body formation in the presence and absence of Arimoclomol

Askanas et al (2005) report that human muscle cultures induced to over-express β-APP +/- Epoxomicin treatment, formed inclusion bodies (as aggresomes) in the cytoplasm of cells. In this Chapter similar results were observed as the formation of intracellular inclusion bodies was evident in Epoxomicin treated cultures, albeit at a lower level compared to the β-APP transfected cells previously examined in Chapter 3. These inclusion bodies were positive for p62 and, on occasion, for β-APP also. This finding implies that the formation of intracellular inclusion bodies is not limited to protein over-expression but is a generic response to protein mishandling in the cell. p62 is a shuttle protein which delivers cytoplasmic and nuclear proteins targeted for degradation, to the proteasome (Moscat & az-Meco 2009). The presence of p62 in the inclusion bodies therefore reflects the cell's attempt to degrade abnormal proteins via the proteasomal pathway. Inhibition of this pathway thus leads to the accumulation of p62-bound proteins. The data also indicates that β-APP is not necessarily a pathogenic protein by itself, as it is not present in all the inclusion bodies, and similar effects on cellular functions can be seen by other methods which disrupt the proteostasis.

In line with the experiments conducted on β-APP over-expressing cultures, Arimoclomol treatment of Epoxomicin treated cultures shows a significant reduction in the percentage of cells containing inclusion bodies. This shows that although the mechanism of proteostasis disruption may be different, up-regulation of the HSR is an effective method to alleviate its disruptive effects.

5.5.5. Comparing the effects of β-APP over-expression and Epoxomicin treatment in muscle cultures

Although Epoxomicin treatment of muscle cultures replicates some of the effects induced by over-expression of β-APP, and therefore some of the features of sIBM, not all of the

features are present when the proteasome is directly inhibited. TDP-43 translocation to the cytoplasm is an increasingly prominent feature of sIBM muscle and can be observed in the β-APP over-expressing cultures (Salajegheh et al. 2009). However, this translocation is not observed in Epoxomicin treated cultures. This indicates that cytoplasmic translocation of TDP-43 is not a direct result of proteasome inhibition, and that disruption to proteostasis may play a more important role. It is possible that β-APP over-expression triggers a separate pathway which causes TDP-43 translocation.

Epoxomicin treated cultures also showed no detectable effect of proteasome inhibition on levels of the mitochondrial protein, SDH, suggesting mitochondrial function is unaffected. This is in contrast to the reduced level of SDH seen in β-APP over-expressing cultures.

Although protein over-expression and treatment with an inhibitor of the proteasome can both alter the normal functions of the proteasome, the mechanism of protein inhibition is likely to differ between the two conditions. Epoxomicin directly and covalently binds to the proteasome inhibiting all 3 catalytic sites (Meng et al. 1999b), while the mechanism by which protein aggregation results in proteasome inhibition remains unconfirmed. This may explain the different results seen in primary muscle cultures treated with the two stressors.

5.6. CONCLUSION

The results from the experiments presented in this Chapter show that direct inhibition of the proteasome is cytotoxic to muscle cells in culture and leads to the formation of inclusion bodies. Both of these effects are attenuated by Arimoclomol treatment. Treatment with Arimoclomol also returns proteasome activity to normal levels in Epoxomicin inhibited cultures. These results provide further information as to how protein mishandling can lead to adverse effects in cells.

A key feature in a model of the degenerative features of sIBM is the number of cells containing inclusion bodies. In Epoxomicin treated cultures, fewer cells contained inclusion bodies than in β-APP over-expressing culture. In addition, Epoxomicin treatment is unable to replicate the translocation of TDP-43 or changes in mitochondrial protein expression which is seen with β-APP over-expression. While it may be argued that culturing Epoxomicin treated cells for longer may lead to a cumulative effect of protein deregulation, which may eventually replicate the same features, this is difficult to examine in these *in vitro* experiments, as longer incubations would lead to over-confluent cultures and detachment of cells from the plate. For these reasons, β-APP over-expression in muscle cultures provides a better model of the degenerative features of sIBM. However, in both β-APP over-expressing models and the model of direct proteasome inhibition examined in this Chapter, treatment with Arimoclomol can ameliorate many of the pathological features that are common between both models as well as with sIBM muscle itself.

CHAPTER 6.

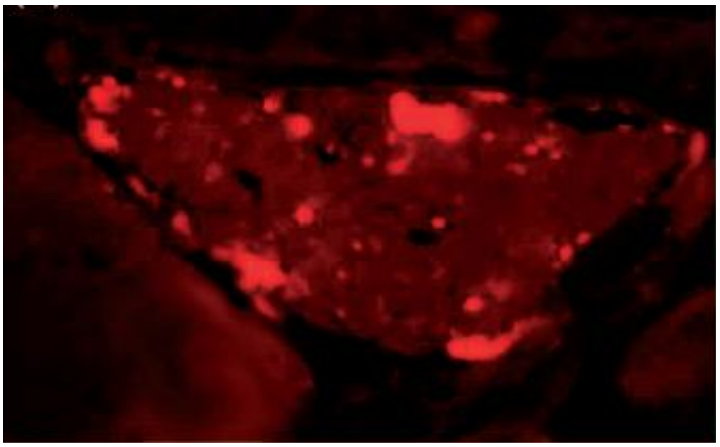
TARGETING AMYLOID FORMATION AS A POTENTIAL THERAPY FOR sIBM

6.1. INTRODUCTION

The formation of amyloid has been well-documented in sIBM muscle and thought to be toxic to the muscle cells (Mendell et al. 1991; Jayaraman et al. 2008). Congo red stained muscle fibres, viewed through Texas-red filters often show the presence of β-pleated amyloid sheets (see **Figure 6.1**) (Askanas & Engel 2007). These amyloid structures are reported to be present in 40-70% of sIBM muscle fibres, suggesting a pathogenic role of protein accumulation in sIBM (Askanas & Engel 2011).

The amyloid hypothesis, first proposed approximately 20 years ago for the pathogenesis of AD, suggests that the neurodegeneration seen in the disease is a result of Aβ deposition (Hardy & Selkoe 2002). The amyloid cascade proposes that accumulation of Aβ in neurons is toxic when Aβ is oligomerised and when in the form of β-sheets (Amijee & Scopes 2009). This toxicity involves activation of microglia and astrocytes, synaptic and neuritic injury, altered ionic homeostasis and oxidative stress as well as altered kinase/phosphatase activity, leading to cell death (Hardy & Selkoe 2002).

Similarly in sIBM, the accumulation of proteins into stable β-pleated amyloid structures has been shown to be cytotoxic both *in vitro* and *in vivo* (Jayaraman et al. 2008; Nogalska et al. 2010a). Over 20 unrelated proteins in general have been identified which can become misfolded and self-aggregate to form β-pleated amyloid, including tau, prion, insulin and Aβ (Ellis & Pinheiro 2002). Therefore therapies that aim to reduce the formation of these amyloid structures are a logical approach for the treatment of sIBM, as well as other diseases where protein mishandling is evident.



6.1 The presence of amyloid in sIBM patient muscle

A typical image of a sIBM patient muscle cell stained with Congo red shows β-amyloid structures appearing as bright red clusters. Image taken from Askanas and Engel 2007.

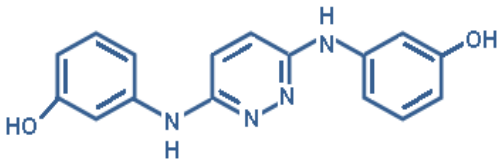
6.1.1. Pharmacologically targeting amyloid formation

Several studies have investigated the self-aggregating nature of key amyloidal proteins such as Aβ. Although protein-protein interactions occur through contact between large surface areas of proteins, there are some amino acid 'hot spots' which are essential for interactions (Amijee & Scopes 2009). These areas have become targets for small compounds which may inhibit the formation of amyloid structures. The difficulty in designing such compounds is that the conformation of Aβ is not well defined. In addition these compounds must have a number of characteristics including, i) a high affinity for the target, ii) must not elicit an immune response, iii) must have a reasonable half-life, iv) be non-toxic and, in the case of AD, v) be able to penetrate the blood-brain barrier. Although most anti-aggregation agents have actually been discovered by serendipity, some have been suitable for chemical optimisation and been taken forward by medicinal chemists.

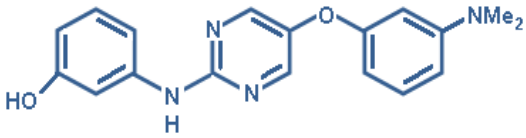
6.1.2. Lead compounds from Senexis

A novel array of compounds has therefore recently been developed by a company called Senexis which have been profiled as 'β-sheet breakers'. These compounds have been specifically designed to target the β-pleated structures which are associated with both AD and sIBM (Amijee & Scopes 2009). The aim of the experiments described in this Chapter was to investigate the potential benefits of a panel of new compounds (provided by Senexis as part of an ongoing collaboration) on the pathological features observed in our β-APP over-expressing *in vitro* model.

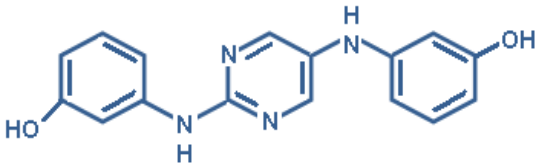
One of these compounds, SEN1000, also known as RS-0406 (N,N'-bis(3hydroxyphenyl)pyridazine-3,6-diamine)) was first discovered by the Japanese pharmaceutical company Sankyo, who identified it as an effective inhibitor of Aβ induced cytotoxicity. (Nakagami et al. 2002; Walsh et al. 2005) (See **Figure 6.2** for the chemical structure).



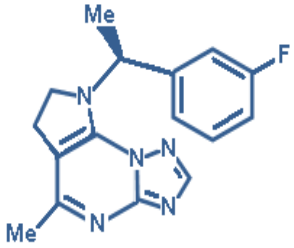
SEN1000



SEN1269



SEN1186



SEN1176

Figure 6.4 Chemical structures of SEN1000, SEN1186, SEN1269 and SEN1176

This group have demonstrated the ability of SEN1000 to significantly inhibit fibrillogenesis of Aβ1-42 as well as disassemble Aβ1-42 fibrils *in vitro* in hippocampal neuron cultures. In addition it has been shown to reverse induced impairment of long-term potentiation in hippocampal slices (Nakagami et al. 2002). It has been suggested that these effects may represent an early manifestation of AD (Amijee & Scopes 2009). Aβ1-42 has been shown by many studies to be more aetiologic in the pathogenesis of AD over other forms of Aβ due to its greater tendency to oligomerise and form aggregates (Amijee & Scopes 2009). This makes SEN1000 a promising compound for further investigation.

Senexis have therefore taken the structure of SEN1000 as a basis for engineering a series of novel analogues which are more potent and specific. Initially, by replacing the central pyridazine structure to a pyrimidine they were able to generate a more potent candidate molecule labelled SEN1186. Later compound SEN1269 was generated which showed similar levels of improved potency but was structurally more desirable. Scopes et al (Scopes 2008, Alzheimer's Association International Conference, Chicago, USA) demonstrated that both SEN1186 and SEN1269 were able to improve cell viability using a colorimetric MTT assay. A thioflavin-T fluorescence assay was also used to measure the aggregation process and both SEN1186 and SEN1269 showed inhibition of this process.

Senexis then investigated another approach to pharmacologically tackling the toxicity brought about by amyloid formation using a compound labelled SEN1176 (see **Figure 6.2** for chemical structure). Instead of directly inhibiting the formation of amyloid structures, this compound targets the inflammatory response to amyloid toxicity. As inflammation is a major contributor to the pathology of both AD and sIBM, attenuation of this effect would greatly benefit the health of the CNS and muscle. In these experiments by O'Hare et al. 2011 bilateral hippocampal injections of Aβ1-42 aggregates were administered in adult male Sprague Dawley rats to induce amyloid-like pathology. These rats then underwent training to press a lever-switching system and were subsequently assessed for dementia using the same technique. Results showed that SEN1176 treatment significantly reduced the number of lever switching errors made by the rats at higher doses of the agent

compared to vehicle treated controls. *In vitro* studies were also conducted by the same authors using this drug where a macrophage cell line (J774.2) was used. The results showed that SEN1176 was able to reduce Aβ-induced nitric oxide production and block Aβ1-42 induced release of cytokines including TNF-α, IL-6 and IL-1β. These findings support the argument that Aβ aggregates can induce an inflammatory response and that SEN1176 is able to function as a novel anti-inflammatory agent to decrease immunological effects of amyloid formation, thereby improving the health of the tissue.

In the present study these novel Senexis compounds were tested using the *in vitro* model of sIBM pathology as part of a screening process. As well as SEN1000, SEN1186, SEN1269 and SEN1176, other lead compounds from Senexis labelled SEN1274, SEN1500, SEN1528 and SEN1576 were also assessed in this investigation. However the chemical structure, biological target and mechanism of function for the latter compounds have not yet been released.

6.2. AIMS OF THIS CHAPTER

The aims of this Chapter are to:

I. Screen a series of novel compounds from Senexis for their ability to reduce sIBM pathology in our *in vitro* model of sIBM pathology

II. Identify the compound(s) which would be most suitable for further preclinical investigation

6.3. MATERIALS AND METHODS

A full description of the materials and methods used in this Chapter are provided in Chapter 2, and include the following:-

- Neonatal rat cell culture
- Immunocytochemistry
- LDH Assay
- Protein Assay
- Proteasome Assay
- Cell counts
- Data analysis and statistical significance

6.3.1. Senexis compound treatment

In the first instance, the optimal dose of each of the 8 compounds provided by Senexis was determined by establishing their effects on cell survival, by means of an LDH assay. The effect of three different doses of each of the Senexis drugs (5, 10 and 50µM) was examined. These results indicated the following doses were optimal for each drug (i.e. resulted in the maximum reduction of cell death) (see **Table 3**). All subsequent cultures were therefore treated with these doses of drugs.

6.3.2. Arimoclomol treatment

In experiments where Arimoclomol and Senexis compounds were tested together, 5µM Arimoclomol was added to the culture media at the same time as the Senexis compounds.

Table 4. Dose of each Senexis compound used to treat muscle cultures

Compound No.	Senexis compound	Optimal dose used (µM)
1	SEN1000	50
2	SEN1186	10
3	SEN1269	50
4	SEN1274	50
5	SEN1500	50
6	SEN1528	50
7	SEN1176	5
8	SEN1576	10

6.4. RESULTS

In order to screen for the most promising Senexis compounds, their effects on a number of key measures of β-APP pathology was examined in this Chapter. In the first instance, the most effective dose of each compound on reducing cell death in β-APP transfected cultures was determined. Based on these results, the most effective compounds were taken forward and their effects on the following parameters were examined: i) inclusion body formation, ii) TDP-43 translocation, iii) proteasome function. In some experiments, Arimoclomol was used as a positive control.

6.4.1. Assessing cell survival

The 8 compounds provided by Senexis were used to treat β-APP transfected muscle cultures in 96-well plates at different concentrations to assess their effect on cytotoxicity using LDH assays. For each compound, 5, 10 and 50μM concentrations were tested to establish the optimum concentrations to use in subsequent experiments. The results are presented in **Figure 6.3**. Although the results do not reach statistical significance, there is a clear trend in the effects of some of the compounds over others. Thus, 10μM Compound 2 and 50μM Compound 6 appear to be more effective in reducing cytotoxicity compared to the other compounds. In addition, Compounds 7 and 8 both also demonstrate a trend towards reduced cytotoxicity at concentrations of 5 and 10μM respectively. This data is presented as representative of three assays conducted per condition where six wells were used for each drug treatment.

Although these assays do not demonstrate a statistically significant difference in cell survival, they indicate the compounds which may be worth further investigation using other methods. Therefore compounds 2, 6, 7 and 8 were chosen as the compounds which showed the lowest level of cytotoxicity at their optimal concentration, and were taken forward to be studied using other key outcome measures relevant to sIBM pathology.

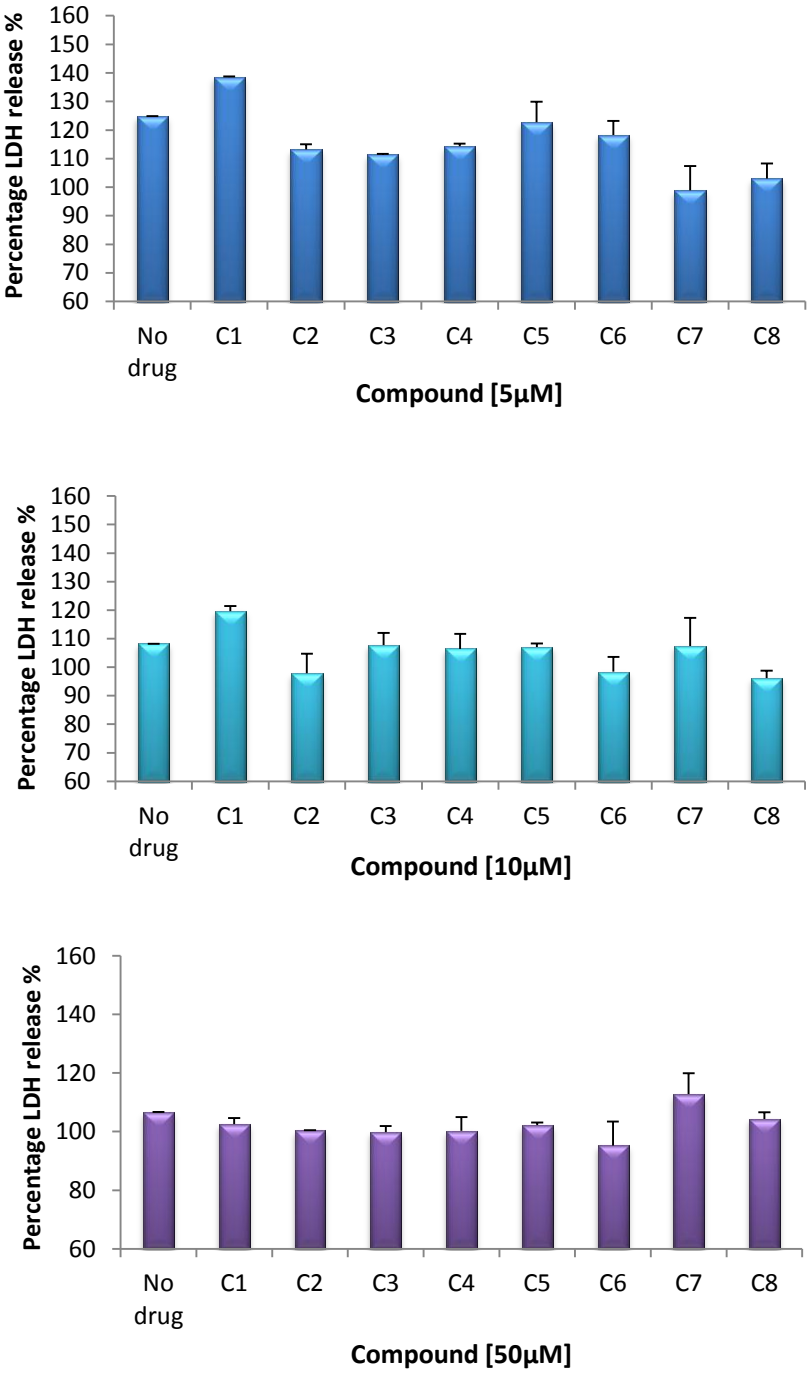


Figure 6.7 Cell survival assay after Senexis drug treatment

The bar charts show the results of the LDH assays after treatment with 8 Senexis compounds at different concentrations: [A] 5µM, [B] 10µM, [C] 50µM. Error bars = SEM.

6.4.2. Assessing the formation of inclusion bodies

Primary muscle cultures transfected with β-APP were treated with Compounds 2, 6, 7 or 8. At 6 DIV the formation of inclusion bodies was assessed by immunocytochemistry. Individual myogenic cells were counted which were desmin-positive and contained intracellular inclusion bodies positive for β-APP. The data is summarised in **Figure 6.4** and shows a reduction in the number of desmin-positive cells which contained inclusion bodies after treatment with all four compounds. However, the data is only statistically significant for Compound 8 which shows the percentage of cells with inclusion bodies to be 2.65% ± 0.69% (SEM, n=3, p=0.04) compared to 13.5% ± 3.18% (SEM) in untreated β-APP transfected cultures. The data thus shows that the four Senexis drugs tested have a positive effect on protein mishandling by reducing the formation of inclusion bodies, with Compound 8 having the greatest effect on this outcome measure.

6.4.3. Assessing the translocation of TDP-43

The effect of the four Senexis compounds on the translocation of TDP-43 from the nucleus to the cytoplasm in β-APP transfected cultures was examined. Cultures were immuno-stained using both the C- and N- terminal TDP-43 antibody and cells which showed any cytoplasmic TDP-43 were counted against the total number of nuclei (i.e. cells). The results are presented in **Figure 6.5**. Arimoclomol treated cultures were used as a positive control (see Chapter 4, section 4.4.3). The data shows that although all four Senexis compounds appear to reduce translocation of TDP-43 to the cytoplasm, only Compounds 2 and 8 resulted in a statistically significant decrease compared to the untreated, β-APP over-expressing cultures. Cultures treated with Compound 2 show the average percentage of cells containing cytoplasmic TDP-43 to be 3.28% ± 0.86% (SEM, n=3, $p<0.02$) and for Compound 8, this was 2.83% ± 0.26% (SEM, n=3, $p<0.02$) compared to 52.17% ± 5.34% (SEM) in untreated cultures. The graph shows the percentage TDP-43 translocation with Compound 8 to be similar to that of Arimoclomol, in which 2.44% ± 0.53% (SEM) of cells showed TDP-43 translocation.

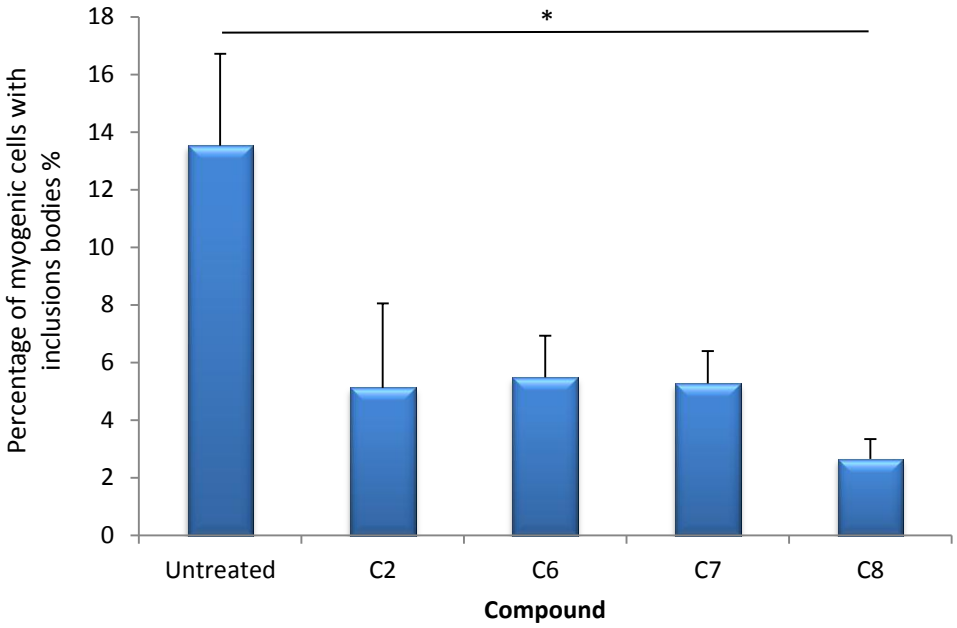


Figure 6.11 Quantification of inclusion bodies after Senexis drug treatment

The bar chart summarises the percentage of desmin-positive cells which contained intracellular inclusion bodies after treatment with the four Senexis compounds. Results show a statistically significant difference in cultures treated with Compound 8 compared to untreated cultures. Error bars= SEM, * denote $p<0.05$.

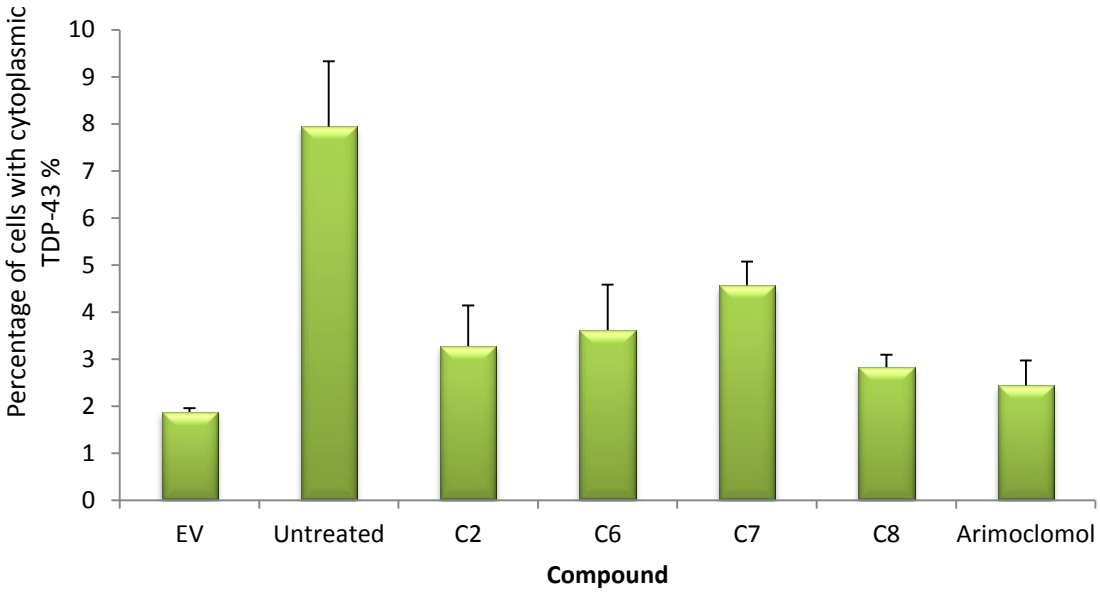


Figure 6.13 Translocation of TDP-43 after treatment with the Senexis drugs

The bar chart presents the percentage of cells with cytoplasmic TDP-43 in muscle cultures treated with the Senexis compounds and Arimoclomol. The data shows a significant reduction in TDP-43 translocation in cultures treated with Compound 2, 8 and Arimoclomol. Error bars= SEM, * denotes $p<0.05$.

6.4.4. Assessing proteasomal function

In order to assess the effects of the Senexis compounds using a functional outcome measure, proteasome activity was investigated in β-APP over-expressing cultures treated with these drugs. The data is summarised in **Figure 6.6** and shows that there is a large, significant increase in proteasome activity with the addition of Compounds 6, 7 and 8 to muscle cultures. This data is relative to empty vector (EV) controls set at 100%. The data shows the greatest effect is seen following treatment with Compound 6 which shows an increase of more than 6 times the activity of untreated, β-APP over-expressing cultures at 375% (SEM 35, $p<0.0001$). Compounds 7 and 8 also show a dramatic improvement in activity. A summary of these results is presented in **Table 4.**

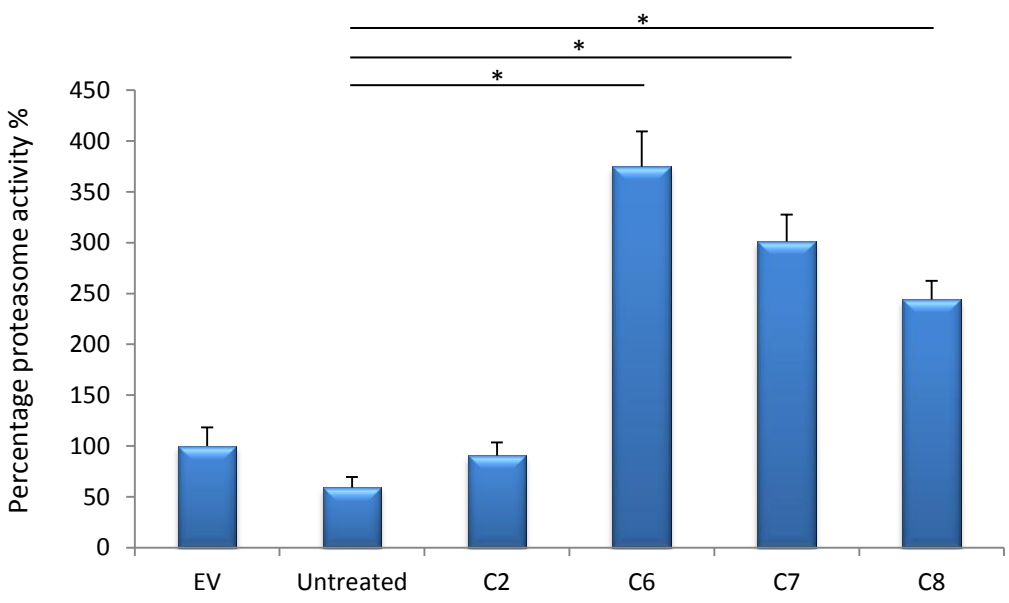


Figure 6.16 Assessing proteasome function after treatment with Senexis drugs

Data from proteasome assays shows improved proteasome activity in cultures treated with Compound 6, 7 and 8 compared to untreated, β-APP over-expressing cultures. Empty vector (EV) transfected control cells are set as 100%.

Table 5. Summary of results from Senexis drugs

Compound No.	Reduced inclusion body formation	Reduced TDP-43 translocation	Improved proteasome activity
(2) SEN1186		+	
(6) SEN1528			+
(7) SEN1176			+
(8) SEN1576	+	+	+

6.5. DISCUSSION

In this Chapter, potential therapies which target the amyloid pathway were tested using lead compounds from the pharmaceutical company Senexis, in our *in vitro* model of sIBM pathology. These small non-peptide molecules have been identified by Senexis as being able to reverse or inhibit the adverse effects of toxicity caused by amyloid *in vitro* and *in vivo*. Although the exact mode of function of these compounds remains undisclosed, they are all associated with the formation of amyloid and/or its effects on the cell, such as amyloid-associated inflammation.

The results presented in this Chapter initially identified 4 out of the 8 compounds that reduced cytotoxicity to a greater extent in muscle cultures transfected with β-APP at their optimal concentrations. These are Compounds 2, 6, 7 and 8. These 4 compounds were also assessed by i) quantifying the formation of inclusion bodies, ii) assessing TDP-43 translocation and iii) measuring proteasome function. The results showed that all 4 compounds attenuated each of the 3 pathological measures, although only Compound 8 was able to act on all three of the features of disease.

Compound 2 (SEN1186) significantly reduced the translocation of TDP-43 from the nucleus to the cytoplasm, but had no effect on any other measure. Previous studies conducted by Scopes et al (2008) showed that Compound 2, as well as the related Compound 3 (SEN1269), were both able to improve cell survival as assessed by MTT assays. However, in the present study, although a slight improvement can be seen at some concentrations when cell survival is measured using LDH assays, this did not reach significance in these experiments. Scopes et al (2008) also found Compound 2 was able to inhibit fibrillogenesis of Aβ1-42 and disassemble fibrils of Aβ1-42 *in vitro*. However, in the present investigation a significant difference in the number of β-APP-positive inclusion bodies was not detected. This inconsistency may be due to the different methods used to measure protein aggregation or the use of different cell types.

The results for Compounds 6 (SEN1528) and 7 (SEN1176) show that although proteasomal activity was significantly increased after treatment with these compounds, there was no effect on the formation of inclusion bodies or the cytoplasmic translocation of TDP-43. As yet, no data on Compound 6 has been published in the literature. However, Compound 7 has been shown to decrease the inflammatory effects brought about in response to amyloid formation (O'Hare et al. 2011). It follows that by increasing proteasome activity in cells over-expressing large quantities of proteins, Compound 7 may reduce the chance that these proteins have to aggregate or to form aberrant oligomers. Therefore fewer misfolded or damaged peptides would exist for antigen presentation by MHC class I, which would normally elicit an inflammatory response. This is consistent with the findings by O'Hare et al (2011) that Compound 7 protects against the effects of aggregated Aβ1-42 injections *in vivo*. Although the pathway which this compound uses to decrease inflammation is not discussed in the literature, the present study suggests that Compound 7 may, at least in part, improve pathology by enhancing proteasome activity.

Of the 4 Senexis compounds examined in detail, Compound 8 (SEN1576) was found to be the most effective at reducing the pathology caused by β-APP over-expression. Compound 8 was found to significantly reduce the formation of inclusion bodies, decrease TDP-43 translocation and improve proteasome activity, as well as demonstrate a trend in increased cell survival. Compound 8 is a second generation molecule, structurally related to Compound 5, which was originally presented by Senexis as a lead candidate for the treatment of Alzheimer's disease. Compound 5 was presented as a non-peptide small molecule which binds to Aβ1-42, reverses long-term potentiation caused by Aβ and protects against cognitive deficit from amyloid toxicity (Senexis LTD, Wellcome Trust European Networking Event 2010). Compound 8 is a related back-up molecule and together with Compound 5, was selected for preclinical development in models of AD. In the present study however, the cell survival data on Compound 5 was unremarkable and therefore this compound was not tested further. On the other hand, Compound 8 showed an improvement in cell survival at both 5 and 10μM concentrations, justifying it to be taken forward for further investigation.

It is possible that the structural differences between Compounds 5 and 8 account for the variation in their ability to reduce toxicity. It would be interesting to investigate Compound 5 with the same outcome measures to compare the results to that of Compound 8. This may identify a potentially useful structural feature and help in designing other compounds with similar targets.

6.6. CONCLUSIONS

This Chapter has demonstrated that that the *in vitro* model established in this report is suitable for successfully screening a number of lead compounds simultaneously using a variety of outcome measures. After screening 8 small molecules from Senexis, the results presented here show that some compounds are more effective than others in reducing pathology induced by β-APP over-expression. The results show that Compound 8 (SEN1576) is the most effective in attenuating pathology by reducing the formation of inclusion bodies, decreasing TDP-43 translocation and improving proteasome activity. Out of the 8 Senexis compounds tested, Compound 8 is the most promising candidate drug for the treatment of amyloid-induced toxicity, and based on the *in vitro* results of this study is a good candidate compound that should be followed up in preclinical studies using *in vivo* models of sIBM.

CHAPTER 7.

GENERAL DISCUSSION

Sporadic inclusion body myositis (sIBM) is a skeletal muscle disease which is poorly understood (Greenberg 2010). Pathologically, although both inflammatory and degenerative features are present in patient muscle, which of these is the primary trigger for the condition remains undetermined and highly debated in recent years (Amato & Barohn 2009). Historically sIBM has been classed as an inflammatory myopathy, grouped together with polymyositis (PM) and dermatomyositis (DM) (Machado et al. 2009b). However, while PM and DM respond to immunotherapy, sIBM remains resistant to the anti-immune therapies tested (Aggarwal & Oddis 2011). This suggests that the degenerative aspect of the disease may play a more prominent role in pathogenesis or at least contribute to the chronic, resistant nature of this disease.

Since a large variety of the anti-inflammatory agents that have been tested have proved negative, failing to improve muscle strength or to prevent disease progression, it is clear that alternative therapeutic strategies for this condition should be investigated. However, in sIBM, there are currently no preclinical outcome measures for use in clinical trials that aim to test the effects of novel agents. Previous trials have focussed on registered agents, with some reports based on evidence from individual patients, detailing the clinician's experience and anecdotal evidence (Aggarwal & Oddis 2011). As this method has not been successful in identifying an effective agent for sIBM, there is a need for a high-throughput screening model to assess potential new compounds before initiating additional human studies.

Although animal models are highly informative and useful for understanding disease pathology and for *in vitro* testing of novel agents, the existing animal models of sIBM are not fully representative of the disease (Sugarman et al. 2002; Kitazawa et al. 2009; Badadani et al. 2010). Furthermore, animal models are complex, time-consuming and expensive and a model without systemic involvement is initially required for more fundamental drug testing. *In vitro* models therefore provide a good system for testing experimental agents, which is fast, relatively uncomplicated and highly reproducible.

The key to any good *in vitro* disease model is that it reflects at least some of the key pathological characteristics of the disease. Therefore, during the course of this project, an *in vitro* system was established which replicates many of the features seen in patient muscle in sIBM. These features are mainly degenerative as a systemic input is required to fully model the inflammatory aspects of sIBM. Once characterised, this model was used to test the effects of novel agents, which showed that the *in vitro* model is suitable for drug screening and produces reliable results. Two novel therapeutic strategies were investigated, one targeting the cell's inherent HSR and therefore protein misfolding and aggregation, and the other targeting amyloid formation.

7.1. Development of reliable primary muscle cultures

In order to produce a reliable *in vitro* system, which would give reproducible results, a robust muscle culture technique was initially developed. Firstly, primary muscle cultures were chosen over a muscle cell line as they would be physiologically closer to cells *in vivo* than immortalised cells, which is particularly important when modelling a disorder that involves cellular degeneration. Secondly, muscle cells from two species, mouse and rat were compared in culture and rat muscle cultures were found to be the most suitable for the experiments described in this Thesis. Primary rat muscle cultures were optimised to fulfil three criteria of longevity, high yield and high purity, which were essential for subsequent experiments.

The most challenging quality to establish in these muscle cultures was the level of purity. Muscle cells were obtained from enzymatic digestion of whole hind limb muscle, which was non-specific and led to the contamination of muscle cultures with non-myogenic cells such as fibroblasts. Previously, various techniques such as FACS sorting and MACS sorting were employed by researchers to obtain high purity muscle cultures, with minimal contamination (Leronimakis 2008, Sinanan et al. 2004). Therefore these techniques, as well as others, were tested to improve the myogenic proportion of muscle cultures. The results from these experiments however showed that each technique had major

disadvantages (Chapter 3). Although MACS sorting was the most successful in isolating a purer population of myogenic cells, this method dramatically reduced the yield of cells, which meant it was highly inefficient. MACS sorting was therefore considered unsuitable.

The most successful method of obtaining highly myogenic cultures was then found by optimising the tissue culture technique itself and altering the feeding media such that myogenic cell growth was favoured over other cell types. Satellite cells, the stem cells of muscle, have been shown to differentiate down alternate pathways under certain environmental conditions such as nutrient content (Asakura et al. 2001). Therefore, a second 'differentiation' media, which was better 'conditioned' for muscle cell differentiation as opposed to proliferation was used to promote growth of satellite cells down the myogenic pathway.

To generate sIBM-like degenerative features in this model, protein mishandling in cells was induced by over-expression of β-APP, a highly aggregation-prone, sIBM-relevant protein. Similar experiments were successfully conducted by Askanas et al (2005; 2010) on human muscle cultures, which showed β-APP over-expression led to the formation of intracellular aggregates, proteasome dysfunction and impaired autophagy. Askanas et al (2005) used human muscle cells obtained from biopsy material and virally transfected the β-APP gene. Although using human cells is physiologically more relevant in modelling a human condition, the yield of cells is low and tissue is not readily available for high-throughput screening *in vitro*. Therefore, as β-APP over-expression was successful in replicating sIBM features, this protein was over-expressed in rat muscle cultures in the present investigation.

7.2. Replicating features of sIBM *in vitro*

7.2.1. Formation of inclusion bodies

Transient over-expression of human β-APP in primary rat muscle cultures resulted in the up-regulation of this protein, initially as disperse 'speckles' throughout the cell, resulting subsequently in the formation of large, intracellular inclusion bodies, which were found to be immuno-reactive for β-APP and ubiquitin. Ubiquitin is a protein tag which becomes associated with misfolded and unwanted proteins and labels them for degradation (Goldberg 2003). This suggests that β-APP over-expression is disruptive to normal cellular functions, instigating the cell's mechanism for removal of these proteins.

The formation of inclusion bodies has been observed in other *in vitro* systems of protein over-expression and mishandling (Kopito 2000), however, whether this is a cytoprotective response to an accumulation of a large volume of proteins is unconfirmed. What has been previously shown, however, is that inclusion body formation is not necessarily a diffusion limited process, where accumulating proteins become associated with each other through intermolecular bonds, to form a growing aggregate (Bence et al. 2001).

On the other hand, Johnston et al (1998) have shown that cells have a specialised mechanism in place which collates small protein aggregates that form at the periphery of cells, and sequesters them to a perinuclear location via the microtubule network. This retrograde transport of aggregates to the minus-end of the microtubule network (the microtubule organising centre (MTOC)) is mediated by dynein binding and ATP hydrolysis (Pierre et al. 2000). Converging of aggregates then leads to the formation of large inclusion bodies, termed 'aggresomes' which are often seen to cause an indentation in the nucleus envelope as they converge to the location of the centrosome (Garcia-Mata et al. 1999).

In Chapter 3 of this Thesis, similar inclusion bodies were evident in β-APP over-expressing cells which were located adjacent to the nucleus, often impinging on its structure. This suggests that in the *in vitro* model, inclusion body formation is a regulated, ATP-dependent mechanism utilised by the cell to sequester unwanted proteins such that the increased protein expression does not interfere with the proteostasis.

Inclusion body formation is a defining feature of the disease in sIBM patients, with protein aggregates forming in affected muscle fibres (Dalakas 2006b). In patient tissue, inclusion bodies have been reported to be immune-reactive for a wide range of proteins such as β-APP, ubiquitin, p-tau, TDP-43, p62, α-sinuclein, HSPs and among many others (Greenberg 2010). It is unknown exactly how these proteins become associated with inclusion bodies, but the protein content is not consistent between biopsies (Greenberg 2010) or even between individual inclusion bodies. It is likely that in the highly protein-dense environment of mammalian cells, disturbances in proteostasis trigger unwanted protein-protein interactions, which result in aggregation of any proteins with exposed hydrophobic surfaces. Some proteins, however, may associate with small aggregates as part of their biological function. For example ubiquitin binds to misfolded protein to label it for degradation, and p62 associates with ubiquitinated proteins to shuttle them to the proteasome or the lysosome (Pankiv et al. 2010). When aggresomes form, it may be that all of these proteins that form the aggregate or are associated with it, become transported to the centrosome indiscriminately, thereby making these inclusion bodies highly variable in their protein content. This would suggest that a particular protein identified in an inclusion body is not necessarily the cause of this pathogenic feature.

In this investigation, all inclusion bodies identified in the β-APP transfected cultures, which were immuno-reactive for ubiquitin, were also immune-reactive for β-APP. Since β-APP was highly over-expressed in these cells, this was an expected result. However, some inclusion bodies were found to be also immuno-reactive for other sIBM-relevant proteins, including, Aβ-42, TDP-43, caspase-3, HSP70, p-tau and p62. Quantification of the number of cells containing β-APP positive and p62-positive inclusion bodies showed β-APP

transfected cultures contained more of these structures compared to empty vector transfected controls. Assessing the number of cells containing inclusion bodies is therefore a good outcome measure for evaluating pathology under different conditions.

This outcome measure was also used to assess the effects of inhibition of the proteasome using Epoxomicin. In this alternate method of disrupting proteostasis in cells, ubiquitin-positive inclusion bodies formed, however, β-APP immuno-reactivity was not observed on most occasions. This supports the possibility that inclusion body formation is not due to the abnormal presence of a particular protein, but rather a disturbance in the protein homeostasis.

7.2.2. Translocation of TDP-43

A diagnosis of sIBM is made using a combination of clinical symptoms and histological features, such as the presence of inclusion bodies or rimmed vacuoles (Amato & Barohn 2009). However, there is no universal set of characteristics which are present in all sIBM patients (Weihl & Pestronk 2010), making diagnosis sometimes difficult and time-consuming. No specific biomarker for sIBM has yet been identified to help make diagnosis easier and faster. One potential biomarker which has recently been proposed is TDP-43. This nucleic acid binding protein has been reported to be abnormally present in approximately 23% of sIBM myofibres (Greenberg 2010). Under normal conditions TDP-43 is present in the nucleus of cells. However, under certain stressful and pathological conditions, this protein becomes cleaved by a caspase protein, and the C-terminal becomes translocated to the cytoplasm (Igaz et al. 2009). This cytoplasmic translocation of TDP-43 may be a diagnostic biomarker for sIBM and a key characteristic of disease pathology.

In the experiments presented in this Thesis, a significant increase in TDP-43 translocation was observed in β-APP over-expressing muscle cultures compared to empty vector controls. Cytoplasmic TDP-43 was found to be the C-terminal portion of the protein with

the N-terminal TDP-43 remaining in the nucleus. On the other hand, when TDP-43 expression was examined in cultures treated with the proteasome inhibitor Epoxomicin, no difference in the pattern of TDP-43 expression was observed compared to untreated cells. This suggests that the stress caused by β-APP over-expression differs from that caused by inhibition of the proteasome, although both methods disrupt normal protein handling in cells. Interestingly, TDP-43 abnormalities are rarely observed in polymyositis or dermatomyositis, indicating that the underlying pathogenesis may differ between these conditions and sIBM (Temiz et al. 2009). The specificity of TDP-43 mislocalisation therefore means it may be a good biomarker for sIBM.

7.2.3. The effect of different stressors on cell survival

In both β-APP over-expressing cultures and cultures treated with Epoxomicin, a significant increase in cell toxicity was observed, which correlated well with the formation of inclusion bodies. Although inclusion body formation may be beneficial to cells by sequestering unwanted proteins to one location, the presence of a large, globular structure containing vital proteins for normal cellular functions, is unlikely to remain beneficial for long. Especially in the case of highly contractile muscle cells which must fuse to form myotubes, this structure would become a hindrance and may thus lead to cell death. Inclusion body formation may therefore be an emergency response to protein aggregation, which requires other mechanisms, perhaps the HSR, to be up-regulated enough for the cell to remain viable.

7.2.4. Replicating functional characteristics of sIBM

An increase in the number of cytochrome oxidase (COX) negative fibres is one of the histological features of sIBM muscle (Oldfors et al. 2006a). This mitochondrial protein plays a central role in the oxidative phosphorylation pathway in all cells, and is particularly important in muscle cells which are highly metabolic (Bratic & Trifunovic 2010). In addition, ragged red fibres are observed in sIBM muscle as well as mutations in

mitochondrial DNA (Oldfors et al. 2006a). Mitochondrial dysfunction is therefore a key feature of sIBM. In order to assess mitochondrial function in this study, the level of another key mitochondrial protein, Succinate dehydrogenase (SDH) was examined in both β-APP over-expressing cultures and Epoxomicin treated cultures. While β-APP transfected cells showed a reduction in the level of SDH compared to empty vector transfected controls, Epoxomicin treatment showed no difference in SDH levels between treated and untreated cells. These results are similar to that seen for the translocation of TDP-43, which again suggests that the pathogenicity of protein over-expression differs to that caused by proteasome inhibition.

When the effects of these two treatments on proteasome activity was examined, β-APP over-expressing cultures did not show a largely significant decrease in activity compared to empty vector transfected controls, although a trend for reduced activity was observed in each individual experiment. On the other hand Epoxomicin treatment did show a reduction in proteasome activity, as expected with the proteasome inhibitor.

Again, these results show a difference between the two stressors on muscle cells. However, it is possible that the relatively mild proteasome dysfunction in β-APP over-expressing cultures is due to reduced activity of inhibited proteasome complexes being masked by highly active proteasomes.

Evidence of proteasome dysfunction has been previously observed in sIBM muscle. Proteasome activity has been found to be decreased in sIBM biopsy material at all three catalytic sites (Fratta et al. 2005b). In addition, subunits of the proteasome have been identified by immunohistochemistry to be associated with protein aggregates in affected muscle. This suggests that proteasome dysfunction may be contributing to the protein mishandling observed in sIBM, although whether it is the initial trigger of the disruption remains unclear.

7.3. The potential therapeutic effects of Arimoclomol in sIBM

The purpose for establishing a cell culture model which replicates the features of sIBM is to understand better the pathology of the disease, but also to act as a screening system to evaluate the effects of novel compounds.

The first agent tested on this model was the heat shock response (HSR) co-inducer Arimoclomol. β-APP transfected cultures and Epoxomicin treated cultures were both treated with Arimoclomol and the same outcome measures were used to assess its effects. Treatment with Arimoclomol showed a significant improvement in cell survival following exposure to either β-APP or Epoxomicin. This reduction in cytotoxicity correlated to a significant decrease in the number of cells containing β-APP and/or p62-positive inclusion bodies. These results suggest that since the formation of inclusion bodies leads to increased cell death, targeting protein aggregation with Arimoclomol prevents this.

Arimoclomol modulates the transcription of HSPs by prolonging the activation of the transcription factor HSF1, so that the time that HSF1 interacts with its DNA target is extended, thereby increasing the expression of HSPs (Hargitai et al. 2003). The increased level of HSPs in the cytosol can assist in protein handling by a) binding to misfolded proteins and sequestering them such that they do not aggregate and/or b) refolding the proteins to their native structure. HSP70 is one of the key chaperone molecules which bind to exposed hydrophobic regions of polypeptide chains (Mayer & Bukau 2005). By its actions of binding and releasing substrate proteins, this chaperone is able to detangle undesirable conformations such as β-pleated sheets (Ben-Zvi & Goloubinoff 2001). The HSP100 family of chaperones are able to assist in the event that large protein aggregates form. By associating with aggregates, HSP100 unravels the polypeptide chains by pulling them through its central core (Liberek et al. 2008). This action generates unfolded polypeptide chains which are immediately targeted by HSP70, preventing its interactions with neighbouring non-native proteins (Richter et al. 2010). HSP70 may then assist in the folding of polypeptide chains to near-native conformations or deliver these to HSP90

which has high affinity for such conformations (Krukenberg et al. 2011). Thus, by a combination of HSPs and their co-chaperones, the HSR prevents protein aggregation.

The mechanism of action of Arimoclomol is therefore highly effective as it increases the expression of a number of HSPs which are essential for effective protein handling, rather than up-regulating individual chaperones. Moreover, an important feature of Arimoclomol is that it is a co-inducer of the HSR and is only effective in cells in which the endogenous HSR has already been instigated, giving it a level of specificity not found with agents which directly induce the HSR and which can indeed be cytotoxic (Kieran et al. 2004b).

When TDP-43 expression was assessed in Arimoclomol treated and untreated, β-APP over-expressing cultures, a significant reduction in cytoplasmic TDP-43 was observed in treated cultures, indicating that an up-regulation on the HSR ameliorates this pathological feature of sIBM. However, in Epoxomicin treated cultures, no difference in TDP-43 translocation was observed. When TDP-43 is translocated, as in the β-APP over-expressing cultures, it becomes cleaved by caspase proteins (Igaz et al. 2009). This indicates the mislocalisation may be linked to apoptosis. Arimoclomol treated cells have improved cell survival rates and therefore apoptosis is unlikely to be triggered in these cells. This may explain why TDP-43 is not translocated to the cytoplasm in unstressed and Arimoclomol treated cultures.

The effect of Arimoclomol on the level of the mitochondrial protein SDH also showed this agent's ability to attenuate pathology, as β-APP over-expressing cultures treated with Arimoclomol were indistinguishable from empty vector transfected cultures. As mitochondria in muscle cells are highly metabolic compared to other cell types, dysfunction in these organelles in muscle is indicative of pathology (Oldfors et al. 2006a). These results therefore show that up-regulation of the HSR prevents mitochondrial dysfunction, perhaps by removing toxic oligomers and aggregates from the cytosol which may have disrupted mitochondrial function.

In Chapters 3 and 4 the effects of modulating proteasome activity was assessed since it is possible that changes in proteasome activity play a role in sIBM. In addition, the effect of Arimoclomol on proteasome dysfunction was also examined. The experiments described in these two Chapters compare the effects of up-regulating the HSR in β-APP over-expressing cells and Epoxomicin treated cells, in which proteasome activity was either mildly or severely affected, respectively. The results demonstrated that proteasome activity was not significantly affected in β-APP over-expressing cultures. In contrast, proteasome activity was significantly reduced in Epoxomicin treated cultures and a dramatic improvement was observed following Arimoclomol treatment. This may be because the mechanisms of proteasome inhibition by the two stressors differ. While protein over-expression can lead to the formation of toxic species which can (perhaps reversibly and mildly) inhibit the proteasome, Epoxomicin covalently binds to the proteasome in an irreversible reaction. Although Epoxomicin halts proteasome function completely, at the low concentration used in these experiments, not all proteasomes would be affected. This means that Arimoclomol treatment was able to restore proteasome activity to a normal level by increasing the activity of uninhibited complexes. A direct function of Arimoclomol on the proteasome has not been previously reported, however, it is possible that by preventing aggregation through the activity of HSPs, degradable polypeptides are made more readily available for proteasomal degradation, thus leading to increased proteasome activity.

The results presented in this Thesis therefore show that up-regulation of the HSR using the co-inducer Arimoclomol is a suitable therapeutic strategy to employ to reduce the effects of protein mishandling and cellular degeneration. As well as degenerative features, the same *in vitro* model shows Arimoclomol to be effective in reducing the inflammatory activation of NFkB and reducing the plasma membrane expression of MHC Class I, the endogenous antigen presenting molecule (Unpublished data from A. Miller in this laboratory). This suggests that Arimoclomol is able to target both of the major components of sIBM, an attribute not observed previously with any other potential sIBM therapy tested to date.

In addition to the success Arimoclomol has shown in reducing both the degenerative and inflammatory features in this *in vitro* model of sIBM pathology, Arimoclomol has been shown to be successful in attenuating pathology in mouse models of ALS (Kalmar et al. 2008), and has been found to be safe and well tolerated in a number of clinical trials (Lanka et al. 2009). Considering these points, Arimoclomol is a suitable candidate to test on proposed *in vivo* models of sIBM. Furthermore, in view of the existing safety and tolerability data of Arimoclomol in humans, if preclinical studies demonstrate a similar efficacy of Arimoclomol to ameliorate sIBM-like pathology *in vivo*, Arimoclomol is well placed to move forward into patient trials.

7.4. The potential therapeutic effects of novel compounds that target amyloid

As part of this Thesis, the *in vitro* model of sIBM pathology was used to screen a set of eight novel compounds from the pharmaceutical company Senexis. These agents were identified as lead compounds which target the formation of amyloid and its associated effects. Although the primary clinical indication for these compounds is in the treatment of Alzheimer's disease (AD), the pathogenic similarities with sIBM muscle means they may also be potentially beneficial in sIBM (Askanas & Engel 2002).

In this study, four outcome measures were used to assess the efficacy of the Senexis compounds in the *in vitro* model of sIBM degeneration. Initially cell survival of β-APP over-expressing cultures treated with the eight compounds was examined, which identified four compounds that were able to reduce cytotoxicity. These compounds were further assessed for their effects on the formation of inclusion bodies, TDP-43 translocation and proteasome activity. Although each compound showed significant beneficial affects with some of the outcome measures, only compound 8 (SEN1576) was successful in attenuating the adverse effects of β-APP as measured by all 4 outcome measures. Compound 8 was therefore identified in the experiments described in this Thesis as a candidate for further testing *in vitro* and to be taken forward for testing in preclinical studies in a proposed mouse model of sIBM.

7.5. Implications of Results for sIBM research

sIBM remains a disease with no effective treatment to offer patients and no widely accepted model to assess the benefits of potential new drugs. The main reason for this is the lack of knowledge about the cause of this condition. Although certain haplotypes are associated with an increased propensity for sIBM, there is no genetic cause (Needham et al. 2007). There is also debate among clinicians and scientists as to whether sIBM is primarily a degenerative condition, similar to AD or whether it is an inflammatory condition, as it has been historically classed. Greater understanding of the cellular pathology of sIBM is therefore needed to allow a targeted therapy to be developed for this condition.

The *in vitro* model developed in this investigation gives some insight into possible pathogenic pathways which may give rise to the histological features observed in sIBM muscle. This model shows that by disrupting the proteostasis, either by manipulating the expression of a protein or by preventing normal protein degradation, the formation of inclusion bodies can be induced which has a deleterious effect on cell viability. The expression of other sIBM-relevant proteins is also altered, for example up-regulation of HSP70. The results presented in this study also show a differential effect of β-APP over-expression compared to proteasome inhibition in terms of proteasome activity, TDP-43 translocation, and SDH levels in the mitochondria, suggesting that how proteostasis is disrupted is also important.

Since no individual protein has yet been identified to be universally present in sIBM muscle (Weihl & Pestronk 2010), it suggests that over-expression of any one protein may not be responsible for triggering this condition. Also, inhibition of the proteasome does not replicate as many degenerative features as shown by protein over-expression. This suggests that sIBM is likely to be caused by a combination of factors which instigate an imbalance of the proteostasis. Askanas et al (2001) suggest that the milieu of aging muscle is likely to lead to pathogenesis, possibly through the cumulative effects of oxidative damage to proteins. As sIBM and AD both occur in an aging population, aging may

certainly contribute to pathology (Askanas & Engel 2007). However, sIBM affects a relatively small population of this age-group and is highly specific for certain muscle groups. In addition, not all the fibres in an affected muscle show signs of disease (Amato & Barohn 2009). This suggests that more specific cellular events must instigate those particular changes to the muscle.

Although the cause of sIBM cannot be deduced from this investigation, it is clear that protein mishandling plays is a significant role, and that pharmacological manipulation to improve the cell's protein handling mechanisms are beneficial. This Thesis identifies two therapeutic strategies which attenuate pathology *in vitro* and which may also be effective *in vivo*. The first strategy utilises the cell's inherent HSR to mount an enhanced response to protein mishandling, using the drug Arimoclomol, while the second strategy targets the prevention of amyloid formation using compound 8 (SEN1576) from Senexis.

In sIBM, the effects of inflammation and degeneration have a circular relationship, with one able to trigger the other in this chronic disease (Askanas & Engel 2007). Therefore, therapeutically targeting only one of these features is unlikely to be effective in altering disease progression. Immunotherapy with agents such as steroids has not been shown to be beneficial to patients (Cordeiro & Isenberg 2006), perhaps because accumulation of misfolded proteins will continue to instigate an immune response. Treating the degenerative features alone is also unlikely to be effective as the chronic immune response would trigger protein mishandling through the actions of cytokines (Nogalska et al. 2007a). Therefore, a combination therapy which targets both degeneration and inflammation simultaneously is more likely to have a beneficial effect in sIBM. In this respect, Arimoclomol has been shown in this study to be able to attenuate many of the degenerative features of sIBM and additionally, has been shown in parallel experiments by A. Miller in this lab, to reduce key inflammatory features of sIBM, namely, NFκB and MHC Class I expression in β-APP over-expressing cells (unpublished data).

The precise mode of function of the Senexis agent Compound 8 (SEN1576) remains undisclosed, but what is known, is that this molecule is a related to Compound 5 (SEN1500) which targets Aβ1-42. It is unknown whether Compound 8 also has an effect on inflammation, although another molecule tested, Compound 7 (SEN1176), does target amyloid-associated inflammation. It is therefore difficult to establish why Compound 8 was the most effective out of all the Senexis compounds in attenuating the pathological measures tested in this investigation.

7.6. Limitations of this investigation

This model of the pathology of sIBM provides insight into possible mechanisms which may be disrupted in sIBM, and provides a model in which to screen potential sIBM-relevant compounds. However, replication of sIBM features *in vitro* is far from the physiological condition of cells *in vivo,* where systemic inputs play important roles. Although expression of some inflammatory markers were investigated by others in the laboratory using this model in parallel to this study, the full implications of protein over-expression on the inflammatory system cannot be examined *in vitro*.

Furthermore, sIBM is a chronic condition which progresses slowly and may be exacerbated by aging. *In vitro* models however, have a short life span which means longitudinal studies cannot be conducted and the aging process cannot be replicated. Protein over-expression in this investigation occurs within days *in vitro*, and due to the use of Lipofectamine-mediated transfection, the effects are transient.

Although many important features of sIBM are replicated in this model, one key characteristic which was not seen in any of the cells is the formation of rimmed vacuoles. Rimmed vacuoles are commonly observed in patient tissue, although they are not universally present in all affected muscle and their cause remains unknown (Amato & Barohn 2009). Conversely, the *in vitro* model presented here demonstrates the formation of inclusion bodies as 'aggresomes' due to their perinuclear localisation and dependence

on the microtubule network, however, such inclusion bodies do not appear to be associated to any nuclei in patient muscle (Fratta et al. 2005b). This dissimilarity may be due to the difference between cells *in vivo* and *in vitro*. Muscle fibres *in vivo* are large, multinucleated syncytia with more complex structure compared to individual myocytes in a culture dish (Zammit et al. 2006). Therefore the cell may use an alternative mechanism to sequester inclusion bodies or the microtubule network may be arranged differently.

The *in vitro* model established in this Thesis has been valuable for identifying potential new therapies for the treatment of sIBM; however, effectiveness *in vitro* does not necessarily mean any agent will also be successful in treating the disease *in vivo*. The relatively simplistic environment of cultured cells does not account for the effects of the host immune system to a new drug or its potential side effects. The half-life and tissue penetrance of a drug also cannot be calculated from *in vitro* experiments. In the case of Arimoclomol, previous experiments on mouse models and human trials have been conducted, which have shown it to be effective and well tolerated (Kieran et al. 2004b; Lanka et al. 2009). However, the novel compounds designed by Senexis have not been tested so thoroughly *in vivo*. Further testing on animal models would therefore be necessary to establish, at least in the first instance, the safety and tolerability of these Compounds.

7.7. Future work

The work presented in this Thesis describes an *in vitro* model which recapitulates many of the features of sIBM, however, further study of the effects of protein over-expression and proteasome inhibition would give insight into other affected mechanisms. This study has shown abnormal localisation of the shuttle protein p62 which delivers misfolded proteins for degradation via either the proteasomal pathway or the lysosomal pathway (Nogalska et al. 2009b). The proteasomal pathway was studied in this investigation; however the lysosomal degradation of proteins was not examined. Dysfunction in autophagy and abnormal p62 expression has been described previously in sIBM muscle (Nogalska et al.

2010b) and therefore more detailed study of this pathway in sIBM would be insightful. The effects of Arimoclomol and Compound 8 (SEN1576) on autophagy would also be interesting to observe.

Other new lead compounds which target protein mishandling may also be investigated using this *in vitro* model which would allow a large range of molecules to be screened relatively quickly. This means a pipeline of successful agents can potentially be taken from *in vitro* to *in vivo* testing to identify an effective treatment for sIBM.

To take the results of this investigation forward, the two drugs identified in this Thesis need to be further investigated *in vitro* but also need to be tested *in vivo* in a mouse model of sIBM. The mutant valosin containing protein (VCP) mouse, which closely mimics Inclusion Body Myositis with Paget's disease and Frontotemporal Dementia (IBMPFD), generated by J.P Taylor's group would be a possible model on which these drugs could be tested (Custer et al. 2010). A mouse model would allow physiological studies to be conducted, giving more representative data about the effects of these agents on mammalian tissue.

7.8. CONCLUDING REMARKS

The results in this Thesis show that over-expression of β-APP in primary rat muscle successfully recapitulates many of the features of sIBM *in vitro*. This includes inclusion body formation, TDP-43 translocation, HSP up-regulation, mitochondrial abnormality and some proteasome dysfunction. These outcome measures were found to be highly reliable, and therefore this model is suitable for undertaking relatively high-throughput, preclinical screens of novel therapeutic agents.

One therapeutic strategy tested in the model presented in this Thesis was up-regulation of the HSR using Arimoclomol. The data presented in this Thesis shows Arimoclomol is able to attenuate the pathogenic features of β-APP over-expression. In addition Arimoclomol also ameliorates the cytotoxic effects of direct proteasome inhibition and restores proteasome activity.

A second therapeutic strategy examined was targeted towards reducing amyloid formation and its associated pathological effects. Testing of eight lead compounds revealed that Compound 8 (SEN1576) significantly improved pathology when tested using four outcome measures established in this Thesis.

This Thesis therefore identifies Arimoclomol and Compound 8 (SEN1576) as potential therapies to be taken forward to *in vivo* assessments using proposed mouse models of sIBM.

REFERENCES

Abuzakouk, M., Feighery, C., & O'Farrelly, C. 1996, "Collagenase and Dispase enzymes disrupt lymphocyte surface molecules", *J.Immunol.Methods*, vol. 194, no. 2, pp. 211-216.

Aggarwal, R. & Oddis, C. V. 2011, "Therapeutic approaches in myositis", *Curr.Rheumatol.Rep.*, vol. 13, no. 3, pp. 182-191.

Aiken, C. T., Kaake, R. M., Wang, X., & Huang, L. 2011, "Oxidative stress-mediated regulation of proteasome complexes", *Mol.Cell Proteomics.*

Alexanderson, H. 2009a, "Exercise effects in patients with adult idiopathic inflammatory myopathies", *Curr.Opin.Rheumatol.*, vol. 21, no. 2, pp. 158-163.

Alexanderson, H. 2009b, "Exercise effects in patients with adult idiopathic inflammatory myopathies", *Curr.Opin.Rheumatol.*, vol. 21, no. 2, pp. 158-163.

Allen, R. E., Rankin, L. L., Greene, E. A., Boxhorn, L. K., Johnson, S. E., Taylor, R. G., & Pierce, P. R. 1991, "Desmin is present in proliferating rat muscle satellite cells but not in bovine muscle satellite cells", *J Cell Physiol*, vol. 149, no. 3, pp. 525-535.

Amato, A. A. & Barohn, R. J. 2009, "Inclusion body myositis: old and new concepts", *J.Neurol.Neurosurg.Psychiatry*, vol. 80, no. 11, pp. 1186-1193.

Amijee, H. & Scopes, D. I. 2009, "The quest for small molecules as amyloid inhibiting therapies for Alzheimer's disease", *J.Alzheimers.Dis.*, vol. 17, no. 1, pp. 33-47.

Anckar, J. & Sistonen, L. 2011, "Regulation of HSF1 function in the heat stress response: implications in aging and disease", *Annu.Rev.Biochem.*, vol. 80, pp. 1089-1115.

Anfinsen, C. B. 1972, "The formation and stabilization of protein structure", *Biochem.J.*, vol. 128, no. 4, pp. 737-749.

Anfinsen, C. B. 1973, "Principles that govern the folding of protein chains", *Science*, vol. 181, no. 96, pp. 223-230.

Anfinsen, C. B., HABER, E., SELA, M., & WHITE, F. H., Jr. 1961, "The kinetics of formation of native ribonuclease during oxidation of the reduced polypeptide chain", *Proc.Natl.Acad.Sci.U.S.A*, vol. 47, pp. 1309-1314.

Arnardottir, S., Alexanderson, H., Lundberg, I. E., & Borg, K. 2003a, "Sporadic inclusion body myositis: pilot study on the effects of a home exercise program on muscle function, histopathology and inflammatory reaction", *J.Rehabil.Med.*, vol. 35, no. 1, pp. 31-35.

Arnardottir, S., Svanborg, E., & Borg, K. 2003b, "Inclusion body myositis--sensory dysfunction revealed with quantitative determination of somatosensory thresholds", *Acta Neurol.Scand.*, vol. 108, no. 1, pp. 22-27.

Asakura, A., Komaki, M., & Rudnicki, M. 2001, "Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation", *Differentiation*, vol. 68, no. 4-5, pp. 245-253.

Askanas, V. & Engel, W. K. 1998a, "Does over-expression of betaAPP in aging muscle have a pathogenic role and a relevance to Alzheimer's disease? Clues from inclusion body myositis, cultured human muscle, and transgenic mice", *Am.J.Pathol.*, vol. 153, no. 6, pp. 1673-1677.

Askanas, V. & Engel, W. K. 1998b, "Does over-expression of betaAPP in aging muscle have a pathogenic role and a relevance to Alzheimer's disease? Clues from inclusion body myositis, cultured human muscle, and transgenic mice", *Am.J Pathol.*, vol. 153, no. 6, pp. 1673-1677.

Askanas, V. & Engel, W. K. 1998c, "Sporadic inclusion-body myositis and hereditary inclusion-body myopathies: current concepts of diagnosis and pathogenesis", *Curr.Opin.Rheumatol.*, vol. 10, no. 6, pp. 530-542.

Askanas, V. & Engel, W. K. 1998d, "Sporadic inclusion-body myositis and its similarities to Alzheimer disease brain. Recent approaches to diagnosis and pathogenesis, and relation to aging", *Scand.J.Rheumatol.*, vol. 27, no. 6, pp. 389-405.

Askanas, V. & Engel, W. K. 2001, "Inclusion-body myositis: newest concepts of pathogenesis and relation to aging and Alzheimer disease", *J.Neuropathol.Exp.Neurol.*, vol. 60, no. 1, pp. 1-14.

Askanas, V. & Engel, W. K. 2002, "Newest pathogenetic considerations in inclusion-body myositis: possible role of amyloid-beta, cholesterol, relation to aging and to Alzheimer's disease", *Curr.Rheumatol.Rep.*, vol. 4, no. 5, pp. 427-433.

Askanas, V. & Engel, W. K. 2005a, "Molecular pathology and pathogenesis of inclusion-body myositis", *Microsc.Res.Tech.*, vol. 67, no. 3-4, pp. 114-120.

Askanas, V. & Engel, W. K. 2005b, "Sporadic inclusion-body myositis: a proposed key pathogenetic role of the abnormalities of the ubiquitin-proteasome system, and protein misfolding and aggregation", *Acta Myol.*, vol. 24, no. 1, pp. 17-24.

Askanas, V. & Engel, W. K. 2006, "Inclusion-body myositis: a myodegenerative conformational disorder associated with Abeta, protein misfolding, and proteasome inhibition", *Neurology*, vol. 66, no. 2 Suppl 1, p. S39-S48.

Askanas, V. & Engel, W. K. 2007, "Inclusion-body myositis, a multifactorial muscle disease associated with aging: current concepts of pathogenesis", *Curr.Opin.Rheumatol.*, vol. 19, no. 6, pp. 550-559.

Askanas, V. & Engel, W. K. 2011, "Sporadic inclusion-body myositis: conformational multifactorial ageing-related degenerative muscle disease associated with proteasomal and lysosomal inhibition, endoplasmic reticulum stress, and accumulation of amyloid-beta42 oligomers and phosphorylated tau", *Presse Med.*, vol. 40, no. 4 Pt 2, p. e219-e235.

Askanas, V., Engel, W. K., & Alvarez, R. B. 1992a, "Light and electron microscopic localization of beta-amyloid protein in muscle biopsies of patients with inclusion-body myositis", *Am.J.Pathol.*, vol. 141, no. 1, pp. 31-36.

Askanas, V., Engel, W. K., & Alvarez, R. B. 1993, "Enhanced detection of congo-red-positive amyloid deposits in muscle fibers of inclusion body myositis and brain of Alzheimer's disease using fluorescence technique", *Neurology*, vol. 43, no. 6, pp. 1265-1267.

Askanas, V., Engel, W. K., Alvarez, R. B., & Glenner, G. G. 1992b, "beta-Amyloid protein immunoreactivity in muscle of patients with inclusion-body myositis", *Lancet*, vol. 339, no. 8792, pp. 560-561.

Askanas, V., Engel, W. K., & Nogalska, A. 2009, "Inclusion body myositis: a degenerative muscle disease associated with intra-muscle fiber multi-protein aggregates, proteasome inhibition, endoplasmic reticulum stress and decreased lysosomal degradation", *Brain Pathol.*, vol. 19, no. 3, pp. 493-506.

Askanas, V., McFerrin, J., Baque, S., Alvarez, R. B., Sarkozi, E., & Engel, W. K. 1996, "Transfer of beta-amyloid precursor protein gene using adenovirus vector causes mitochondrial abnormalities in cultured normal human muscle", *Proc.Natl.Acad.Sci.U.S.A*, vol. 93, no. 3, pp. 1314-1319.

Badadani, M., Nalbandian, A., Watts, G. D., Vesa, J., Kitazawa, M., Su, H., Tanaja, J., Dec, E., Wallace, D. C., Mukherjee, J., Caiozzo, V., Warman, M., & Kimonis, V. E. 2010, "VCP associated inclusion body myopathy and paget disease of bone knock-in mouse model exhibits tissue pathology typical of human disease", *PLoS.ONE.*, vol. 5, no. 10.

Badrising, U. A., Maat-Schieman, M., van Duinen, S. G., Breedveld, F., van, D. P., van, E. B., van den Hoogen, F., Hoogendijk, J., Howeler, C., de, J. A., Jennekens, F., Koehler, P., van der Leeuw, H., de, V. M., Verschuuren, J. J., & Wintzen, A. R. 2000, "Epidemiology of inclusion body myositis in the Netherlands: a nationwide study", *Neurology*, vol. 55, no. 9, pp. 1385-1387.

Bakkar, N. & Guttridge, D. C. 2010, "NF-kappaB signaling: a tale of two pathways in skeletal myogenesis", *Physiol Rev.*, vol. 90, no. 2, pp. 495-511.

Barmada, S. J., Skibinski, G., Korb, E., Rao, E. J., Wu, J. Y., & Finkbeiner, S. 2010, "Cytoplasmic mislocalization of TDP-43 is toxic to neurons and enhanced by a mutation associated with familial amyotrophic lateral sclerosis", *J.Neurosci.*, vol. 30, no. 2, pp. 639-649.

Barohn, R. J., Herbelin, L., Kissel, J. T., King, W., McVey, A. L., Saperstein, D. S., & Mendell, J. R. 2006, "Pilot trial of etanercept in the treatment of inclusion-body myositis", *Neurology*, vol. 66, no. 2 Suppl 1, p. S123-S124.

Ben-Zvi, A. P. & Goloubinoff, P. 2001, "Review: mechanisms of disaggregation and refolding of stable protein aggregates by molecular chaperones", *J.Struct.Biol.*, vol. 135, no. 2, pp. 84-93.

Ben-Zvi, A. P. & Goloubinoff, P. 2002, "Proteinaceous infectious behavior in non-pathogenic proteins is controlled by molecular chaperones", *J.Biol.Chem.*, vol. 277, no. 51, pp. 49422-49427.

Bence, N. F., Sampat, R. M., & Kopito, R. R. 2001, "Impairment of the ubiquitin-proteasome system by protein aggregation", *Science*, vol. 292, no. 5521, pp. 1552-1555.

Bilak, M., Askanas, V., & Engel, W. K. 1993, "Strong immunoreactivity of alpha 1-antichymotrypsin co-localizes with beta-amyloid protein and ubiquitin in vacuolated muscle fibers of inclusion-body myositis", *Acta Neuropathol.*, vol. 85, no. 4, pp. 378-382.

Biressi, S. & Rando, T. A. 2010a, "Heterogeneity in the muscle satellite cell population", *Semin.Cell Dev.Biol.*, vol. 21, no. 8, pp. 845-854.

Biressi, S. & Rando, T. A. 2010b, "Heterogeneity in the muscle satellite cell population", *Semin.Cell Dev.Biol.*, vol. 21, no. 8, pp. 845-854.

Bischoff, R. 1997, "Chemotaxis of skeletal muscle satellite cells", *Dev.Dyn.*, vol. 208, no. 4, pp. 505-515.

Blanton, J. R., Jr., Grant, A. L., McFarland, D. C., Robinson, J. P., & Bidwell, C. A. 1999, "Isolation of two populations of myoblasts from porcine skeletal muscle", *Muscle Nerve*, vol. 22, no. 1, pp. 43-50.

Blijham, P. J., Hengstman, G. J., Hama-Amin, A. D., van Engelen, B. G., & Zwarts, M. J. 2006, "Needle electromyographic findings in 98 patients with myositis", *Eur.Neurol.*, vol. 55, no. 4, pp. 183-188.

Bourgeron, T., Rustin, P., Chretien, D., Birch-Machin, M., Bourgeois, M., Viegas-Pequignot, E., Munnich, A., & Rotig, A. 1995, "Mutation of a nuclear succinate dehydrogenase gene results in mitochondrial respiratory chain deficiency", *Nat.Genet.*, vol. 11, no. 2, pp. 144-149.

Bratic, I. & Trifunovic, A. 2010, "Mitochondrial energy metabolism and ageing", *Biochim.Biophys.Acta*, vol. 1797, no. 6-7, pp. 961-967.

Brown, I. R. 2007, "Heat shock proteins and protection of the nervous system", *Ann.N.Y.Acad.Sci.*, vol. 1113, pp. 147-158.

Brychzy, A., Rein, T., Winklhofer, K. F., Hartl, F. U., Young, J. C., & Obermann, W. M. 2003, "Cofactor Tpr2 combines two TPR domains and a J domain to regulate the HSP70/HSP90 chaperone system", *EMBO J.*, vol. 22, no. 14, pp. 3613-3623.

Bukau, B. & Horwich, A. L. 1998, "The HSP70 and HSP60 chaperone machines", *Cell*, vol. 92, no. 3, pp. 351-366.

Bukau, B., Weissman, J., & Horwich, A. 2006, "Molecular chaperones and protein quality control", *Cell*, vol. 125, no. 3, pp. 443-451.

Bulteau, A. L., Petropoulos, I., & Friguet, B. 2000, "Age-related alterations of proteasome structure and function in aging epidermis", *Exp.Gerontol.*, vol. 35, no. 6-7, pp. 767-777.

Casarejos, M. J., Solano, R. M., Gomez, A., Perucho, J., de Yebenes, J. G., & Mena, M. A. 2011, "The accumulation of neurotoxic proteins, induced by proteasome inhibition, is reverted by trehalose, an enhancer of autophagy, in human neuroblastoma cells", *Neurochem.Int.*, vol. 58, no. 4, pp. 512-520.

Chaouch, S., Mouly, V., Goyenvalle, A., Vulin, A., Mamchaoui, K., Negroni, E., Di, S. J., Butler-Browne, G., Torrente, Y., Garcia, L., & Furling, D. 2009, "Immortalized skin fibroblasts expressing conditional MyoD as a renewable and reliable source of converted human muscle cells to assess therapeutic strategies for muscular dystrophies: validation of an exon-skipping approach to restore dystrophin in Duchenne muscular dystrophy cells", *Hum.Gene Ther.*, vol. 20, no. 7, pp. 784-790.

Chiti, F., Webster, P., Taddei, N., Clark, A., Stefani, M., Ramponi, G., & Dobson, C. M. 1999, "Designing conditions for *in vitro* formation of amyloid protofilaments and fibrils", *Proc.Natl.Acad.Sci.U.S.A*, vol. 96, no. 7, pp. 3590-3594.

Choi, Y. C. & Dalakas, M. C. 2000, "Expression of matrix metalloproteinases in the muscle of patients with inflammatory myopathies", *Neurology*, vol. 54, no. 1, pp. 65-71.

Chondrogianni, N. & Gonos, E. S. 2004, "Proteasome inhibition induces a senescence-like phenotype in primary human fibroblasts cultures", *Biogerontology.*, vol. 5, no. 1, pp. 55-61.

Chou, S. M. 1967, "Myxovirus-like structures in a case of human chronic polymyositis", *Science*, vol. 158, no. 807, pp. 1453-1455.

Christopher-Stine, L. & Plotz, P. H. 2004, "Adult inflammatory myopathies", *Best.Pract.Res.Clin.Rheumatol.*, vol. 18, no. 3, pp. 331-344.

Chung, H. Y., Lee, E. K., Choi, Y. J., Kim, J. M., Kim, D. H., Zou, Y., Kim, C. H., Lee, J., Kim, H. S., Kim, N. D., Jung, J. H., & Yu, B. P. 2011, "Molecular Inflammation as an Underlying Mechanism of the Aging Process and Age-related Diseases", *J.Dent.Res.*

Clague, M. J. & Urbe, S. 2010, "Ubiquitin: same molecule, different degradation pathways", *Cell*, vol. 143, no. 5, pp. 682-685.

Collins, C. A., Olsen, I., Zammit, P. S., Heslop, L., Petrie, A., Partridge, T. A., & Morgan, J. E. 2005, "Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche", *Cell*, vol. 122, no. 2, pp. 289-301.

Collins, C. A., Zammit, P. S., Ruiz, A. P., Morgan, J. E., & Partridge, T. A. 2007, "A population of myogenic stem cells that survives skeletal muscle aging", *Stem Cells*, vol. 25, no. 4, pp. 885-894.

Cordeiro, A. C. & Isenberg, D. A. 2006, "Treatment of inflammatory myopathies", *Postgrad.Med.J.*, vol. 82, no. 969, pp. 417-424.

Costa, R. A., Romagna, C. D., Pereira, J. L., & Souza-Pinto, N. C. 2011, "The role of mitochondrial DNA damage in the citotoxicity of reactive oxygen species", *J.Bioenerg.Biomembr.*

Cox, F. M., Verschuuren, J. J., Verbist, B. M., Niks, E. H., Wintzen, A. R., & Badrising, U. A. 2009, "Detecting dysphagia in inclusion body myositis", *J.Neurol.*, vol. 256, no. 12, pp. 2009-2013.

Crews, L. & Masliah, E. 2010, "Molecular mechanisms of neurodegeneration in Alzheimer's disease", *Hum.Mol.Genet.*, vol. 19, no. R1, p. R12-R20.

Cupler, E. J., Leon-Monzon, M., Miller, J., Semino-Mora, C., Anderson, T. L., & Dalakas, M. C. 1996, "Inclusion body myositis in HIV-1 and HTLV-1 infected patients", *Brain*, vol. 119 (Pt 6), pp. 1887-1893.

Custer, S. K., Neumann, M., Lu, H., Wright, A. C., & Taylor, J. P. 2010, "Transgenic mice expressing mutant forms VCP/p97 recapitulate the full spectrum of IBMPFD including degeneration in muscle, brain and bone", *Hum.Mol.Genet.*

Dabby, R., Lange, D. J., Trojaborg, W., Hays, A. P., Lovelace, R. E., Brannagan, T. H., & Rowland, L. P. 2001, "Inclusion body myositis mimicking motor neuron disease", *Arch.Neurol.*, vol. 58, no. 8, pp. 1253-1256.

Dahlbom, K., Lindberg, C., & Oldfors, A. 2002, "Inclusion body myositis: morphological clues to correct diagnosis", *Neuromuscul.Disord.*, vol. 12, no. 9, pp. 853-857.

Dahlmann, B. 2007, "Role of proteasomes in disease", *BMC.Biochem.*, vol. 8 Suppl 1, p. S3.

Dahlmann, B. & Kuehn, L. 1995, "The 20S/26S proteasomal pathway of protein degradation in muscle tissue", *Mol.Biol.Rep.*, vol. 21, no. 1, pp. 57-62.

Dalakas, M. C. 2001, "Progress in inflammatory myopathies: good but not good enough", *J.Neurol.Neurosurg.Psychiatry*, vol. 70, no. 5, pp. 569-573.

Dalakas, M. C. 2006a, "Inflammatory, immune, and viral aspects of inclusion-body myositis", *Neurology*, vol. 66, no. 2 Suppl 1, p. S33-S38.

Dalakas, M. C. 2006b, "Sporadic inclusion body myositis--diagnosis, pathogenesis and therapeutic strategies", *Nat.Clin.Pract.Neurol.*, vol. 2, no. 8, pp. 437-447.

Dalakas, M. C. 2008, "Therapeutic advances and future prospects in immune-mediated inflammatory myopathies", *Ther.Adv.Neurol.Disord.*, vol. 1, no. 3, pp. 157-166.

Dalakas, M. C. 2010a, "Immunotherapy of myositis: issues, concerns and future prospects", *Nat.Rev.Rheumatol.*, vol. 6, no. 3, pp. 129-137.

Dalakas, M. C. 2010b, "Inflammatory muscle diseases: a critical review on pathogenesis and therapies", *Curr.Opin.Pharmacol.*, vol. 10, no. 3, pp. 346-352.

Dalakas, M. C., Illa, I., Dambrosia, J. M., Soueidan, S. A., Stein, D. P., Otero, C., Dinsmore, S. T., & McCrosky, S. 1993, "A controlled trial of high-dose intravenous immune globulin infusions as treatment for dermatomyositis", *N.Engl.J.Med.*, vol. 329, no. 27, pp. 1993-2000.

Dalakas, M. C., Koffman, B., Fujii, M., Spector, S., Sivakumar, K., & Cupler, E. 2001, "A controlled study of intravenous immunoglobulin combined with prednisone in the treatment of IBM", *Neurology*, vol. 56, no. 3, pp. 323-327.

Dalakas, M. C., Rakocevic, G., Schmidt, J., McElroy, B., Harris-Love, M. O., Shrader, J. A., Levy, E. W., & Dambrosia, J. 2009a, "Reply to: Comment on alemtuzumab and inclusion body myositis", *Brain*.

Dalakas, M. C., Rakocevic, G., Schmidt, J., Salajegheh, M., McElroy, B., Harris-Love, M. O., Shrader, J. A., Levy, E. W., Dambrosia, J., Kampen, R. L., Bruno, D. A., & Kirk, A. D. 2009b, "Effect of Alemtuzumab (CAMPATH 1-H) in patients with inclusion-body myositis", *Brain*, vol. 132, no. Pt 6, pp. 1536-1544.

Dalakas, M. C., Rakocevic, G., Shatunov, A., Goldfarb, L., Raju, R., & Salajegheh, M. 2007, "Inclusion body myositis with human immunodeficiency virus infection: four cases with clonal expansion of viral-specific T cells", *Ann.Neurol.*, vol. 61, no. 5, pp. 466-475.

Dastmalchi, M., Grundtman, C., Alexanderson, H., Mavragani, C. P., Einarsdottir, H., Helmers, S. B., Elvin, K., Crow, M. K., Nennesmo, I., & Lundberg, I. E. 2008, "A high incidence of disease flares in an open pilot study of infliximab in patients with refractory inflammatory myopathies", *Ann.Rheum.Dis.*, vol. 67, no. 12, pp. 1670-1677.

De Bleecker, J. L., Creus, K. K., & De, P. B. 2006, "Potential therapeutic targets for idiopathic inflammatory myopathies", *Drug News Perspect.*, vol. 19, no. 9, pp. 549-557.

De, P. B., Creus, K. K., Martin, J. J., Weis, J., & De Bleecker, J. L. 2009, "A dual role for HSP90 and HSP70 in the inflammatory myopathies: from muscle fiber protection to active invasion by macrophages", *Ann.N.Y.Acad.Sci.*, vol. 1173, pp. 463-469.

Di, C. M. 2010, "Beta amyloid peptide: from different aggregation forms to the activation of different biochemical pathways", *Eur.Biophys.J.*, vol. 39, no. 6, pp. 877-888.

Distad, B. J., Amato, A. A., & Weiss, M. D. 2011, "Inflammatory Myopathies", *Curr.Treat.Options.Neurol.*

Domingues, S. C., Henriques, A. G., Wu, W., Da Cruz e Silva EF, & Da Cruz e Silva OA 2007, "Altered subcellular distribution of the Alzheimer's amyloid precursor protein under stress conditions", *Ann.N.Y.Acad.Sci.*, vol. 1096, pp. 184-195.

Douglas, P. M. & Cyr, D. M. 2010, "Interplay between protein homeostasis networks in protein aggregation and proteotoxicity", *Biopolymers*, vol. 93, no. 3, pp. 229-236.

Drews, O., Wildgruber, R., Zong, C., Sukop, U., Nissum, M., Weber, G., Gomes, A. V., & Ping, P. 2007, "Mammalian proteasome subpopulations with distinct molecular compositions and proteolytic activities", *Mol.Cell Proteomics.*, vol. 6, no. 11, pp. 2021-2031.

Eisen, M. B., Spellman, P. T., Brown, P. O., & Botstein, D. 1998, "Cluster analysis and display of genome-wide expression patterns", *Proc.Natl.Acad.Sci.U.S.A*, vol. 95, no. 25, pp. 14863-14868.

Elashry, M. I., Otto, A., Matsakas, A., El-Morsy, S. E., Jones, L., Anderson, B., & Patel, K. 2011, "Axon and muscle spindle hyperplasia in the myostatin null mouse", *J.Anat.*, vol. 218, no. 2, pp. 173-184.

Ellis, R. J. & Pinheiro, T. J. 2002, "Medicine: danger--misfolding proteins", *Nature*, vol. 416, no. 6880, pp. 483-484.

Engel, W. K. & Askanas, V. 2006, "Inclusion-body myositis: clinical, diagnostic, and pathologic aspects", *Neurology*, vol. 66, no. 2 Suppl 1, p. S20-S29.

Ferrer, I., Martin, B., Castano, J. G., Lucas, J. J., Moreno, D., & Olive, M. 2004a, "Proteasomal expression, induction of immunoproteasome subunits, and local MHC class I presentation in myofibrillar myopathy and inclusion body myositis", *J.Neuropathol.Exp.Neurol.*, vol. 63, no. 5, pp. 484-498.

Ferrer, I., Martin, B., Castano, J. G., Lucas, J. J., Moreno, D., & Olive, M. 2004b, "Proteasomal expression, induction of immunoproteasome subunits, and local MHC class I presentation in myofibrillar myopathy and inclusion body myositis", *J.Neuropathol.Exp.Neurol.*, vol. 63, no. 5, pp. 484-498.

Fielding, R. A., Manfredi, T. J., Ding, W., Fiatarone, M. A., Evans, W. J., & Cannon, J. G. 1993, "Acute phase response in exercise. III. Neutrophil and IL-1 beta accumulation in skeletal muscle", *Am.J.Physiol*, vol. 265, no. 1 Pt 2, p. R166-R172.

Figarella-Branger, D., Pellissier, J. F., Pouget, J., Calore, E. E., Azulay, J. P., Desnuelle, C., & Serratrice, G. 1992, "[Inclusion body myositis and neuromuscular diseases with rimmed vacuoles]", *Rev.Neurol.(Paris)*, vol. 148, no. 4, pp. 281-290.

Finkel, T. & Holbrook, N. J. 2000, "Oxidants, oxidative stress and the biology of ageing", *Nature*, vol. 408, no. 6809, pp. 239-247.

Fratta, P., Engel, W. K., McFerrin, J., Davies, K. J., Lin, S. W., & Askanas, V. 2005a, "Proteasome inhibition and aggresome formation in sporadic inclusion-body myositis and in amyloid-beta precursor protein-over-expressing cultured human muscle fibers", *Am.J.Pathol.*, vol. 167, no. 2, pp. 517-526.

Fratta, P., Engel, W. K., McFerrin, J., Davies, K. J., Lin, S. W., & Askanas, V. 2005b, "Proteasome inhibition and aggresome formation in sporadic inclusion-body myositis and in amyloid-beta precursor protein-over-expressing cultured human muscle fibers", *Am.J Pathol.*, vol. 167, no. 2, pp. 517-526.

Friguet, B., Bulteau, A. L., Chondrogianni, N., Conconi, M., & Petropoulos, I. 2000, "Protein degradation by the proteasome and its implications in aging", *Ann.N.Y.Acad.Sci.*, vol. 908, pp. 143-154.

Galimberti, D. & Scarpini, E. 2011, "Disease-modifying treatments for Alzheimer's disease", *Ther.Adv.Neurol.Disord.*, vol. 4, no. 4, pp. 203-216.

Garcia-Mata, R., Bebok, Z., Sorscher, E. J., & Sztul, E. S. 1999, "Characterization and dynamics of aggresome formation by a cytosolic GFP-chimera", *J.Cell Biol.*, vol. 146, no. 6, pp. 1239-1254.

Goldberg, A. L. 2003, "Protein degradation and protection against misfolded or damaged proteins", *Nature*, vol. 426, no. 6968, pp. 895-899.

Gopinath, S. D. & Rando, T. A. 2008, "Stem cell review series: aging of the skeletal muscle stem cell niche", *Aging Cell*, vol. 7, no. 4, pp. 590-598.

Grammas, P. 2011, "Neurovascular dysfunction, inflammation and endothelial activation: implications for the pathogenesis of Alzheimer's disease", *J.Neuroinflammation.*, vol. 8, p. 26.

Greenberg, S. A. 2009a, "Comment on 'Interrelation of inflammation and APP in sIBM: IL-1beta induces accumulation of beta-amyloid in skeletal muscle'", *Brain*, vol. 132, no. Pt 4, p. e106.

Greenberg, S. A. 2009b, "How citation distortions create unfounded authority: analysis of a citation network", *BMJ*, vol. 339, p. b2680.

Greenberg, S. A. 2010, "Theories of the pathogenesis of inclusion body myositis", *Curr.Rheumatol.Rep.*, vol. 12, no. 3, pp. 221-228.

Gregori, L., Fuchs, C., Figueiredo-Pereira, M. E., Van Nostrand, W. E., & Goldgaber, D. 1995, "Amyloid beta-protein inhibits ubiquitin-dependent protein degradation *in vitro*", *J.Biol.Chem.*, vol. 270, no. 34, pp. 19702-19708.

Griggs, R. C., Askanas, V., DiMauro, S., Engel, A., Karpati, G., Mendell, J. R., & Rowland, L. P. 1995a, "Inclusion body myositis and myopathies", *Ann.Neurol*, vol. 38, no. 5, pp. 705-713.

Griggs, R. C., Askanas, V., DiMauro, S., Engel, A., Karpati, G., Mendell, J. R., & Rowland, L. P. 1995b, "Inclusion body myositis and myopathies", *Ann.Neurol.*, vol. 38, no. 5, pp. 705-713.

Gualano, B., Neves, M., Jr., Lima, F. R., Pinto, A. L., Laurentino, G., Borges, C., Baptista, L., Artioli, G. G., Aoki, M. S., Moriscot, A., Lancha, A. H., Jr., Bonfa, E., & Ugrinowitsch, C. 2010, "Resistance training with vascular occlusion in inclusion body myositis: a case study", *Med.Sci.Sports Exerc.*, vol. 42, no. 2, pp. 250-254.

Gundersen, V. 2010, "Protein aggregation in Parkinson's disease", *Acta Neurol.Scand.Suppl* no. 190, pp. 82-87.

Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H., & Mizushima, N. 2006, "Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice", *Nature*, vol. 441, no. 7095, pp. 885-889.

Hardy, J. & Selkoe, D. J. 2002, "The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics", *Science*, vol. 297, no. 5580, pp. 353-356.

Hargitai, J., Lewis, H., Boros, I., Racz, T., Fiser, A., Kurucz, I., Benjamin, I., Vigh, L., Penzes, Z., Csermely, P., & Latchman, D. S. 2003, "Bimoclomol, a heat shock protein co-inducer, acts by the prolonged activation of heat shock factor-1", *Biochem.Biophys.Res.Commun.*, vol. 307, no. 3, pp. 689-695.

Hartl, F. U. & Hayer-Hartl, M. 2009, "Converging concepts of protein folding *in vitro* and *in vivo*", *Nat.Struct.Mol.Biol.*, vol. 16, no. 6, pp. 574-581.

Hashimoto, M., Rockenstein, E., Crews, L., & Masliah, E. 2003, "Role of protein aggregation in mitochondrial dysfunction and neurodegeneration in Alzheimer's and Parkinson's diseases", *Neuromolecular.Med.*, vol. 4, no. 1-2, pp. 21-36.

Hatanaka, Y. & Oh, S. J. 2007, "Single-fiber electromyography in sporadic inclusion body myopathy", *Clin.Neurophysiol.*, vol. 118, no. 7, pp. 1563-1568.

Heinemeyer, W., Fischer, M., Krimmer, T., Stachon, U., & Wolf, D. H. 1997, "The active sites of the eukaryotic 20 S proteasome and their involvement in subunit precursor processing", *J.Biol.Chem.*, vol. 272, no. 40, pp. 25200-25209.

Heinemeyer, W., Kleinschmidt, J. A., Saidowsky, J., Escher, C., & Wolf, D. H. 1991, "Proteinase yscE, the yeast proteasome/multicatalytic-multifunctional proteinase: mutants unravel its function in stress induced proteolysis and uncover its necessity for cell survival", *EMBO J.*, vol. 10, no. 3, pp. 555-562.

Hermanns, B., Molnar, M., & Schroder, J. M. 2000, "Peripheral neuropathy associated with hereditary and sporadic inclusion body myositis: confirmation by electron microscopy and morphometry", *J.Neurol.Sci.*, vol. 179, no. S 1-2, pp. 92-102.

Hightower, L. E. 1980, "Cultured animal cells exposed to amino acid analogues or puromycin rapidly synthesize several polypeptides", *J.Cell Physiol*, vol. 102, no. 3, pp. 407-427.

Hilton-Jones, D. 2003, "Diagnosis and treatment of inflammatory muscle diseases", *J.Neurol.Neurosurg.Psychiatry*, vol. 74 Suppl 2, p. ii25-ii31.

Holmberg, C. I., Illman, S. A., Kallio, M., Mikhailov, A., & Sistonen, L. 2000, "Formation of nuclear HSF1 granules varies depending on stress stimuli", *Cell Stress.Chaperones.*, vol. 5, no. 3, pp. 219-228.

Horwitz, J. 2003, "Alpha-crystallin", *Exp.Eye Res.*, vol. 76, no. 2, pp. 145-153.

Hoshino, T., Murao, N., Namba, T., Takehara, M., Adachi, H., Katsuno, M., Sobue, G., Matsushima, T., Suzuki, T., & Mizushima, T. 2011, "Suppression of Alzheimer's disease-related phenotypes by expression of heat shock protein 70 in mice", *J.Neurosci.*, vol. 31, no. 14, pp. 5225-5234.

Huizing, M. & Krasnewich, D. M. 2009, "Hereditary inclusion body myopathy: a decade of progress", *Biochim.Biophys.Acta*, vol. 1792, no. 9, pp. 881-887.

Hunt, R., Sauna, Z. E., Ambudkar, S. V., Gottesman, M. M., & Kimchi-Sarfaty, C. 2009, "Silent (synonymous) SNPs: should we care about them?", *Methods Mol.Biol.*, vol. 578, pp. 23-39.

Ieronimakis, N., Balasundaram, G., & Reyes, M. 2008, "Direct isolation, culture and transplant of mouse skeletal muscle derived endothelial cells with angiogenic potential", *PLoS.ONE.*, vol. 3, no. 3, p. e0001753.

Igaz, L. M., Kwong, L. K., Chen-Plotkin, A., Winton, M. J., Unger, T. L., Xu, Y., Neumann, M., Trojanowski, J. Q., & Lee, V. M. 2009, "Expression of TDP-43 C-terminal Fragments *in vitro* Recapitulates Pathological Features of TDP-43 Proteinopathies", *J.Biol.Chem.*, vol. 284, no. 13, pp. 8516-8524.

Imai, J., Yashiroda, H., Maruya, M., Yahara, I., & Tanaka, K. 2003a, "Proteasomes and molecular chaperones: cellular machinery responsible for folding and destruction of unfolded proteins", *Cell Cycle*, vol. 2, no. 6, pp. 585-590.

Imai, J., Yashiroda, H., Maruya, M., Yahara, I., & Tanaka, K. 2003b, "Proteasomes and molecular chaperones: cellular machinery responsible for folding and destruction of unfolded proteins", *Cell Cycle*, vol. 2, no. 6, pp. 585-590.

Iqbal, K., Liu, F., Gong, C. X., & Grundke-Iqbal, I. 2010, "Tau in Alzheimer disease and related tauopathies", *Curr.Alzheimer Res.*, vol. 7, no. 8, pp. 656-664.

Jaattela, M. 1999, "Escaping cell death: survival proteins in cancer", *Exp.Cell Res.*, vol. 248, no. 1, pp. 30-43.

Jankowsky, J. L., Younkin, L. H., Gonzales, V., Fadale, D. J., Slunt, H. H., Lester, H. A., Younkin, S. G., & Borchelt, D. R. 2007, "Rodent A beta modulates the solubility and distribution of amyloid deposits in transgenic mice", *J.Biol.Chem.*, vol. 282, no. 31, pp. 22707-22720.

Jayaraman, M., Kannayiram, G., & Rajadas, J. 2008, "Amyloid toxicity in skeletal myoblasts: Implications for inclusion-body myositis", *Arch.Biochem.Biophys.*, vol. 474, no. 1, pp. 15-21.

Johnson, L. G., Collier, K. E., Edwards, D. J., Philippe, D. L., Eastwood, P. R., Walters, S. E., Thickbroom, G. W., & Mastaglia, F. L. 2009, "Improvement in aerobic capacity after an exercise program in sporadic inclusion body myositis", *J.Clin.Neuromuscul.Dis.*, vol. 10, no. 4, pp. 178-184.

Johnston, J. A., Ward, C. L., & Kopito, R. R. 1998a, "Aggresomes: a cellular response to misfolded proteins", *J.Cell Biol.*, vol. 143, no. 7, pp. 1883-1898.

Johnston, J. A., Ward, C. L., & Kopito, R. R. 1998b, "Aggresomes: a cellular response to misfolded proteins", *J.Cell Biol.*, vol. 143, no. 7, pp. 1883-1898.

Jones, G. E., Murphy, S. J., & Watt, D. J. 1990, "Segregation of the myogenic cell lineage in mouse muscle development", *J Cell Sci.*, vol. 97 (Pt 4), pp. 659-667.

Ju, J. S., Fuentealba, R. A., Miller, S. E., Jackson, E., Piwnica-Worms, D., Baloh, R. H., & Weihl, C. C. 2009, "Valosin-containing protein (VCP) is required for autophagy and is disrupted in VCP disease", *J.Cell Biol.*, vol. 187, no. 6, pp. 875-888.

Kabashi, E., Agar, J. N., Taylor, D. M., Minotti, S., & Durham, H. D. 2004, "Focal dysfunction of the proteasome: a pathogenic factor in a mouse model of amyotrophic lateral sclerosis", *J.Neurochem.*, vol. 89, no. 6, pp. 1325-1335.

Kalmar, B. & Greensmith, L. 2009a, "Activation of the heat shock response in a primary cellular model of motoneuron neurodegeneration-evidence for neuroprotective and neurotoxic effects", *Cell Mol.Biol.Lett.*, vol. 14, no. 2, pp. 319-335.

Kalmar, B. & Greensmith, L. 2009b, "Activation of the heat shock response in a primary cellular model of motoneuron neurodegeneration-evidence for neuroprotective and neurotoxic effects", *Cell Mol.Biol.Lett.*, vol. 14, no. 2, pp. 319-335.

Kalmar, B., Greensmith, L., Malcangio, M., McMahon, S. B., Csermely, P., & Burnstock, G. 2003, "The effect of treatment with BRX-220, a co-inducer of heat shock proteins, on sensory fibers of the rat following peripheral nerve injury", *Exp.Neurol.*, vol. 184, no. 2, pp. 636-647.

Kalmar, B., Novoselov, S., Gray, A., Cheetham, M. E., Margulis, B., & Greensmith, L. 2008, "Late stage treatment with arimoclomol delays disease progression and prevents protein aggregation in the SOD1 mouse model of ALS", *J.Neurochem.*, vol. 107, no. 2, pp. 339-350.

Karpati, G. & O'Ferrall, E. K. 2009, "Sporadic inclusion body myositis: pathogenic considerations", *Ann.Neurol*, vol. 65, no. 1, pp. 7-11.

Keck, S., Nitsch, R., Grune, T., & Ullrich, O. 2003, "Proteasome inhibition by paired helical filament-tau in brains of patients with Alzheimer's disease", *J.Neurochem.*, vol. 85, no. 1, pp. 115-122.

Keller, J. N., Huang, F. F., Zhu, H., Yu, J., Ho, Y. S., & Kindy, T. S. 2000, "Oxidative stress-associated impairment of proteasome activity during ischemia-reperfusion injury", *J.Cereb.Blood Flow Metab*, vol. 20, no. 10, pp. 1467-1473.

Kieran, D., Kalmar, B., Dick, J. R., Riddoch-Contreras, J., Burnstock, G., & Greensmith, L. 2004a, "Treatment with arimoclomol, a coinducer of heat shock proteins, delays disease progression in ALS mice", *Nat.Med.*, vol. 10, no. 4, pp. 402-405.

Kieran, D., Kalmar, B., Dick, J. R., Riddoch-Contreras, J., Burnstock, G., & Greensmith, L. 2004b, "Treatment with arimoclomol, a coinducer of heat shock proteins, delays disease progression in ALS mice", *Nat.Med.*, vol. 10, no. 4, pp. 402-405.

Kim, C., Srivastava, S., Rice, M., Godenschwege, T. A., Bentley, B., Ravi, S., Shao, S., Woodard, C. T., & Schwartz, L. M. 2011, "Expression of human amyloid precursor protein in the skeletal muscles of Drosophila results in age- and activity-dependent muscle weakness", *BMC.Physiol*, vol. 11, no. 1, p. 7.

Kitazawa, M., Vasilevko, V., Cribbs, D. H., & LaFerla, F. M. 2009, "Immunization with amyloid-beta attenuates inclusion body myositis-like myopathology and motor impairment in a transgenic mouse model", *J.Neurosci.*, vol. 29, no. 19, pp. 6132-6141.

Kopito, R. R. 2000, "Aggresomes, inclusion bodies and protein aggregation", *Trends Cell Biol.*, vol. 10, no. 12, pp. 524-530.

Krause, S., Kuckelkorn, U., Dorner, T., Burmester, G. R., Feist, E., & Kloetzel, P. M. 2006, "Immunoproteasome subunit LMP2 expression is deregulated in Sjogren's syndrome but not in other autoimmune disorders", *Ann.Rheum.Dis.*, vol. 65, no. 8, pp. 1021-1027.

Krecic, A. M. & Swanson, M. S. 1999, "hnRNP complexes: composition, structure, and function", *Curr.Opin.Cell Biol.*, vol. 11, no. 3, pp. 363-371.

Kriehuber, T., Rattei, T., Weinmaier, T., Bepperling, A., Haslbeck, M., & Buchner, J. 2010, "Independent evolution of the core domain and its flanking sequences in small heat shock proteins", *FASEB J.*, vol. 24, no. 10, pp. 3633-3642.

Krukenberg, K. A., Street, T. O., Lavery, L. A., & Agard, D. A. 2011, "Conformational dynamics of the molecular chaperone HSP90", *Q.Rev.Biophys.*, vol. 44, no. 2, pp. 229-255.

Kumamoto, T., Ueyama, H., Tsumura, H., Toyoshima, I., & Tsuda, T. 2004, "Expression of lysosome-related proteins and genes in the skeletal muscles of inclusion body myositis", *Acta Neuropathol.*, vol. 107, no. 1, pp. 59-65.

Kürthy M, Mogyorósi T, Nagy K, Kukorelli T, Jednákovits A, Tálosi L, Bíró K. 2002 " Effect of BRX-220 against peripheral neuropathy and insulin resistance in diabetic rat models" *Ann N Y Acad Sci.* vol. 967:482-9

Kwak, J., Workman, J. L., & Lee, D. 2011, "The proteasome and its regulatory roles in gene expression", *Biochim.Biophys.Acta*, vol. 1809, no. 2, pp. 88-96.

Labbadia, J., Cunliffe, H., Weiss, A., Katsyuba, E., Sathasivam, K., Seredenina, T., Woodman, B., Moussaoui, S., Frentzel, S., Luthi-Carter, R., Paganetti, P., & Bates, G. P. 2011, "Altered chromatin architecture underlies progressive impairment of the heat shock response in mouse models of Huntington disease", *J.Clin.Invest*, vol. 121, no. 8, pp. 3306-3319.

Lajoie, P. & Snapp, E. L. 2010, "Formation and toxicity of soluble polyglutamine oligomers in living cells", *PLoS.ONE.*, vol. 5, no. 12, p. e15245.

Lanka, V., Wieland, S., Barber, J., & Cudkowicz, M. 2009, "Arimoclomol: a potential therapy under development for ALS", *Expert.Opin.Investig.Drugs*, vol. 18, no. 12, pp. 1907-1918.

Larsson, L. & Ansved, T. 1995, "Effects of ageing on the motor unit", *Prog.Neurobiol.*, vol. 45, no. 5, pp. 397-458.

Liberek, K., Lewandowska, A., & Zietkiewicz, S. 2008, "Chaperones in control of protein disaggregation", *EMBO J.*, vol. 27, no. 2, pp. 328-335.

Lindersson, E., Beedholm, R., Hojrup, P., Moos, T., Gai, W., Hendil, K. B., & Jensen, P. H. 2004, "Proteasomal inhibition by alpha-synuclein filaments and oligomers", *J.Biol.Chem.*, vol. 279, no. 13, pp. 12924-12934.

Lindsten, K., de Vrij, F. M., Verhoef, L. G., Fischer, D. F., Van Leeuwen, F. W., Hol, E. M., Masucci, M. G., & Dantuma, N. P. 2002, "Mutant ubiquitin found in neurodegenerative disorders is a ubiquitin fusion degradation substrate that blocks proteasomal degradation", *J.Cell Biol.*, vol. 157, no. 3, pp. 417-427.

Liu, Y., Gampert, L., Nething, K., & Steinacker, J. M. 2006, "Response and function of skeletal muscle heat shock protein 70", *Front Biosci.*, vol. 11, pp. 2802-2827.

Lodish, H., Berk, A., Kaiser, C. A., Krieger, M., Scott, M. P., Bretscher, A., Ploegh, H., & Matsudairia, P. 2005, *Molecular Cell Biology*, 5th edn.

Loeb, L. A., Wallace, D. C., & Martin, G. M. 2005, "The mitochondrial theory of aging and its relationship to reactive oxygen species damage and somatic mtDNA mutations", *Proc.Natl.Acad.Sci.U.S.A*, vol. 102, no. 52, pp. 18769-18770.

Loell, I. & Lundberg, I. E. 2010, "Can muscle regeneration fail in chronic inflammation: a weakness in inflammatory myopathies?", *J.Intern.Med.*

Lotz, B. P., Engel, A. G., Nishino, H., Stevens, J. C., & Litchy, W. J. 1989, "Inclusion body myositis. Observations in 40 patients", *Brain*, vol. 112 (Pt 3), pp. 727-747.

Ludolph, A. C. & Knirsch, U. 1999, "Problems and pitfalls in the diagnosis of ALS", *J.Neurol.Sci.*, vol. 165 Suppl 1, p. S14-S20.

Machado, P., Miller, A., Holton, J., & Hanna, M. 2009a, "Sporadic inclusion body myositis: an unsolved mystery", *Acta Reumatol.Port.*, vol. 34, no. 2A, pp. 161-182.

Machado, P., Miller, A., Holton, J., & Hanna, M. 2009b, "Sporadic inclusion body myositis: an unsolved mystery", *Acta Reumatol.Port.*, vol. 34, no. 2A, pp. 161-182.

Machida, S., Spangenburg, E. E., & Booth, F. W. 2004, "Primary rat muscle progenitor cells have decreased proliferation and myotube formation during passages", *Cell Prolif.*, vol. 37, no. 4, pp. 267-277.

Malicdan, M. C., Noguchi, S., Nonaka, I., Hayashi, Y. K., & Nishino, I. 2007, "A Gne knockout mouse expressing human V572L mutation develops features similar to distal myopathy with rimmed vacuoles or hereditary inclusion body myopathy", *Hum.Mol.Genet.*, vol. 16, no. 2, pp. 115-128.

Mastaglia, F. L. 2005, "Neuromuscular disorders: molecular and therapeutic insights", *Lancet Neurol.*, vol. 4, no. 1, pp. 6-7.

Mastaglia, F. L., Phillips, B. A., & Zilko, P. 1997, "Treatment of inflammatory myopathies", *Muscle Nerve*, vol. 20, no. 6, pp. 651-664.

Mastaglia, F. L., Phillips, B. A., & Zilko, P. J. 1998, "Immunoglobulin therapy in inflammatory myopathies", *J.Neurol.Neurosurg.Psychiatry*, vol. 65, no. 1, pp. 107-110.

Mattson, M. P. 1997, "Cellular actions of beta-amyloid precursor protein and its soluble and fibrillogenic derivatives", *Physiol Rev.*, vol. 77, no. 4, pp. 1081-1132.

Mayer, M. P. & Bukau, B. 2005, "HSP70 chaperones: cellular functions and molecular mechanism", *Cell Mol.Life Sci.*, vol. 62, no. 6, pp. 670-684.

McCartney, N., Moroz, D., Garner, S. H., & McComas, A. J. 1988, "The effects of strength training in patients with selected neuromuscular disorders", *Med.Sci.Sports Exerc.*, vol. 20, no. 4, pp. 362-368.

McFerrin, J., Engel, W. K., & Askanas, V. 1998, "Impaired innervation of cultured human muscle over-expressing betaAPP experimentally and genetically: relevance to inclusion-body myopathies", *Neuroreport*, vol. 9, no. 14, pp. 3201-3205.

McNaught, K. S. & Jenner, P. 2001, "Proteasomal function is impaired in substantia nigra in Parkinson's disease", *Neurosci.Lett.*, vol. 297, no. 3, pp. 191-194.

Mendell, J. R., Sahenk, Z., Gales, T., & Paul, L. 1991, "Amyloid filaments in inclusion body myositis. Novel findings provide insight into nature of filaments", *Arch.Neurol.*, vol. 48, no. 12, pp. 1229-1234.

Meng, L., Mohan, R., Kwok, B. H., Elofsson, M., Sin, N., & Crews, C. M. 1999a, "Epoxomicin, a potent and selective proteasome inhibitor, exhibits *in vivo* antiinflammatory activity", *Proc.Natl.Acad.Sci.U.S.A*, vol. 96, no. 18, pp. 10403-10408.

Meng, L., Mohan, R., Kwok, B. H., Elofsson, M., Sin, N., & Crews, C. M. 1999b, "Epoxomicin, a potent and selective proteasome inhibitor, exhibits *in vivo* antiinflammatory activity", *Proc.Natl.Acad.Sci.U.S.A*, vol. 96, no. 18, pp. 10403-10408.

Meng, S. J. & Yu, L. J. 2010, "Oxidative stress, molecular inflammation and sarcopenia", *Int.J.Mol.Sci.*, vol. 11, no. 4, pp. 1509-1526.

Miller, A. Ahmed, M. Hanna, M G. Greensmith. L. 2010 Abstract: "Augmentation of the heat shock response in an in vitro model of sporadic inclusion body myositis" *J Neurol Neurosurg Psychiatry.*

Milward, E. A., Papadopoulos, R., Fuller, S. J., Moir, R. D., Small, D., Beyreuther, K., & Masters, C. L. 1992, "The amyloid protein precursor of Alzheimer's disease is a mediator of the effects of nerve growth factor on neurite outgrowth", *Neuron*, vol. 9, no. 1, pp. 129-137.

Miura, Y. & Endo, T. 2010, "Survival responses to oxidative stress and aging", *Geriatr.Gerontol.Int.*, vol. 10 Suppl 1, p. S1-S9.

Morosetti, R., Broccolini, A., Sancricca, C., Gliubizzi, C., Gidaro, T., Tonali, P. A., Ricci, E., & Mirabella, M. 2008, "Increased aging in primary muscle cultures of sporadic inclusion-body myositis", *Neurobiol.Aging*.

Moscat, J. & az-Meco, M. T. 2009, "p62 at the crossroads of autophagy, apoptosis, and cancer", *Cell*, vol. 137, no. 6, pp. 1001-1004.

Moussa, C. E., Fu, Q., Kumar, P., Shtifman, A., Lopez, J. R., Allen, P. D., LaFerla, F., Weinberg, D., Magrane, J., Aprahamian, T., Walsh, K., Rosen, K. M., & Querfurth, H. W. 2006, "Transgenic expression of beta-APP in fast-twitch skeletal muscle leads to calcium dyshomeostasis and IBM-like pathology", *FASEB J.*, vol. 20, no. 12, pp. 2165-2167.

Mowzoon, N., Sussman, A., & Bradley, W. G. 2001, "Mycophenolate (CellCept) treatment of myasthenia gravis, chronic inflammatory polyneuropathy and inclusion body myositis", *J.Neurol.Sci.*, vol. 185, no. 2, pp. 119-122.

Moya, K. L., Benowitz, L. I., Schneider, G. E., & Allinquant, B. 1994, "The amyloid precursor protein is developmentally regulated and correlated with synaptogenesis", *Dev.Biol.*, vol. 161, no. 2, pp. 597-603.

Muth, I. E., Barthel, K., Bahr, M., Dalakas, M. C., & Schmidt, J. 2009, "Proinflammatory cell stress in sporadic inclusion body myositis muscle: over-expression of alphaB-crystallin is associated with amyloid precursor protein and accumulation of beta-amyloid", *J.Neurol.Neurosurg.Psychiatry*, vol. 80, no. 12, pp. 1344-1349.

Nagai, Y., Fujikake, N., Popiel, H. A., & Wada, K. 2010, "Induction of molecular chaperones as a therapeutic strategy for the polyglutamine diseases", *Curr.Pharm.Biotechnol.*, vol. 11, no. 2, pp. 188-197.

Nakagami, Y., Nishimura, S., Murasugi, T., Kaneko, I., Meguro, M., Marumoto, S., Kogen, H., Koyama, K., & Oda, T. 2002, "A novel beta-sheet breaker, RS-0406, reverses amyloid beta-induced cytotoxicity and impairment of long-term potentiation *in vitro*", *Br.J.Pharmacol.*, vol. 137, no. 5, pp. 676-682.

Needham, M., Corbett, A., Day, T., Christiansen, F., Fabian, V., & Mastaglia, F. L. 2008, "Prevalence of sporadic inclusion body myositis and factors contributing to delayed diagnosis", *J.Clin.Neurosci.*, vol. 15, no. 12, pp. 1350-1353.

Needham, M. & Mastaglia, F. L. 2007, "Inclusion body myositis: current pathogenetic concepts and diagnostic and therapeutic approaches", *Lancet Neurol.*, vol. 6, no. 7, pp. 620-631.

Needham, M., Mastaglia, F. L., & Garlepp, M. J. 2007, "Genetics of inclusion-body myositis", *Muscle Nerve*, vol. 35, no. 5, pp. 549-561.

Nelson, D. L. & Cox, M. M. Lehninger Principles of Biochemisty. 2005.

Neumann, M., Sampathu, D. M., Kwong, L. K., Truax, A. C., Micsenyi, M. C., Chou, T. T., Bruce, J., Schuck, T., Grossman, M., Clark, C. M., McCluskey, L. F., Miller, B. L., Masliah, E., Mackenzie, I. R., Feldman, H., Feiden, W., Kretzschmar, H. A., Trojanowski, J. Q., & Lee, V. M. 2006, "Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis", *Science*, vol. 314, no. 5796, pp. 130-133.

Nogalska, A., D'Agostino, C., Engel, W. K., Klein, W. L., & Askanas, V. 2010a, "Novel demonstration of amyloid-beta oligomers in sporadic inclusion-body myositis muscle fibers", *Acta Neuropathol.*, vol. 120, no. 5, pp. 661-666.

Nogalska, A., D'Agostino, C., Terracciano, C., Engel, W. K., & Askanas, V. 2010b, "Impaired autophagy in sporadic inclusion-body myositis and in endoplasmic reticulum stress-provoked cultured human muscle fibers", *Am.J.Pathol.*, vol. 177, no. 3, pp. 1377-1387.

Nogalska, A., Terracciano, C., D'Agostino, C., King, E. W., & Askanas, V. 2009a, "p62/SQSTM1 is overexpressed and prominently accumulated in inclusions of sporadic inclusion-body myositis muscle fibers, and can help differentiating it from polymyositis and dermatomyositis", *Acta Neuropathol.*, vol. 118, no. 3, pp. 407-413.

Nogalska, A., Terracciano, C., D'Agostino, C., King, E. W., & Askanas, V. 2009b, "p62/SQSTM1 is overexpressed and prominently accumulated in inclusions of sporadic inclusion-body myositis muscle fibers, and can help differentiating it from polymyositis and dermatomyositis", *Acta Neuropathol.*, vol. 118, no. 3, pp. 407-413.

Nogalska, A., Wojcik, S., Engel, W. K., McFerrin, J., & Askanas, V. 2007a, "Endoplasmic reticulum stress induces myostatin precursor protein and NF-kappaB in cultured human muscle fibers: relevance to inclusion body myositis", *Exp.Neurol.*, vol. 204, no. 2, pp. 610-618.

Nogalska, A., Wojcik, S., Engel, W. K., McFerrin, J., & Askanas, V. 2007b, "Endoplasmic reticulum stress induces myostatin precursor protein and NF-kappaB in cultured human muscle fibers: relevance to inclusion body myositis", *Exp.Neurol.*, vol. 204, no. 2, pp. 610-618.

Nogalska, A., Wojcik, S., Engel, W. K., McFerrin, J., & Askanas, V. 2007c, "Endoplasmic reticulum stress induces myostatin precursor protein and NF-kappaB in cultured human muscle fibers: relevance to inclusion body myositis", *Exp.Neurol.*, vol. 204, no. 2, pp. 610-618.

O'Hare, E., Scopes, D. I., Treherne, J. M., Monaghan, J., Palmer, P. M., Amijee, H., & Kim, E. M. 2011, "Novel Anti-Inflammatory Compound SEN1176 Alleviates Behavioral Deficits Induced Following Bilateral Intrahippocampal Injection of Aggregated Amyloid-beta1-42", *J.Alzheimers.Dis.*, vol. 25, no. 2, pp. 219-229.

Oh, S., Hong, H. S., Hwang, E., Sim, H. J., Lee, W., Shin, S. J., & Mook-Jung, I. 2005, "Amyloid peptide attenuates the proteasome activity in neuronal cells", *Mech.Ageing Dev.*, vol. 126, no. 12, pp. 1292-1299.

Oldfors, A., Moslemi, A. R., Jonasson, L., Ohlsson, M., Kollberg, G., & Lindberg, C. 2006a, "Mitochondrial abnormalities in inclusion-body myositis", *Neurology*, vol. 66, no. 2 Suppl 1, p. S49-S55.

Oldfors, A., Moslemi, A. R., Jonasson, L., Ohlsson, M., Kollberg, G., & Lindberg, C. 2006b, "Mitochondrial abnormalities in inclusion-body myositis", *Neurology*, vol. 66, no. 2 Suppl 1, p. S49-S55.

Ott, D. E., Coren, L. V., Sowder, R. C., Adams, J., & Schubert, U. 2003, "Retroviruses have differing requirements for proteasome function in the budding process", *J.Virol.*, vol. 77, no. 6, pp. 3384-3393.

Pankiv, S., Lamark, T., Bruun, J. A., Overvatn, A., Bjorkoy, G., & Johansen, T. 2010, "Nucleocytoplasmic shuttling of p62/SQSTM1 and its role in recruitment of nuclear polyubiquitinated proteins to promyelocytic leukemia bodies", *J.Biol.Chem.*, vol. 285, no. 8, pp. 5941-5953.

Petropoulos, I., Conconi, M., Wang, X., Hoenel, B., Bregegere, F., Milner, Y., & Friguet, B. 2000, "Increase of oxidatively modified protein is associated with a decrease of proteasome activity and content in aging epidermal cells", *J.Gerontol.A Biol.Sci.Med.Sci.*, vol. 55, no. 5, p. B220-B227.

Phillips, B. A., Zilko, P. J., & Mastaglia, F. L. 2000, "Prevalence of sporadic inclusion body myositis in Western Australia", *Muscle Nerve*, vol. 23, no. 6, pp. 970-972.

Pierre, Vernace, Wang, & Figueiredo-Pereira 2000, "Assembly of Protein Aggregates in Neurodegeneration: Mechanisms Linking the Ubiquitin/Proteasome Pathway and Chaperones," in *Madame Curie Bioscience Database* , Landes Bioscience.

Pirkkala, L., Nykanen, P., & Sistonen, L. 2001, "Roles of the heat shock transcription factors in regulation of the heat shock response and beyond", *FASEB J.*, vol. 15, no. 7, pp. 1118-1131.

Pisoni, C. N., Cuadrado, M. J., Khamashta, M. A., Hughes, G. R., & D'Cruz, D. P. 2007, "Mycophenolate mofetil treatment in resistant myositis", *Rheumatology.(Oxford)*, vol. 46, no. 3, pp. 516-518.

Polla, B. S., Kantengwa, S., Francois, D., Salvioli, S., Franceschi, C., Marsac, C., & Cossarizza, A. 1996, "Mitochondria are selective targets for the protective effects of heat shock against oxidative injury", *Proc.Natl.Acad.Sci.U.S.A*, vol. 93, no. 13, pp. 6458-6463.

Porter, A. G. & Janicke, R. U. 1999, "Emerging roles of caspase-3 in apoptosis", *Cell Death.Differ.*, vol. 6, no. 2, pp. 99-104.

Qin, R. F., Mao, T. Q., Gu, X. M., Hu, K. J., Liu, Y. P., Chen, J. W., & Nie, X. 2007, "Regulation of skeletal muscle differentiation in fibroblasts by exogenous MyoD gene *in vitro* and *in vivo*", *Mol.Cell Biochem.*, vol. 302, no. 1-2, pp. 233-239.

Qiu, X. B., Shao, Y. M., Miao, S., & Wang, L. 2006a, "The diversity of the DnaJ/HSP40 family, the crucial partners for HSP70 chaperones", *Cell Mol.Life Sci.*, vol. 63, no. 22, pp. 2560-2570.

Qiu, X. B., Shao, Y. M., Miao, S., & Wang, L. 2006b, "The diversity of the DnaJ/HSP40 family, the crucial partners for HSP70 chaperones", *Cell Mol.Life Sci.*, vol. 63, no. 22, pp. 2560-2570.

Querfurth, H. W., Suhara, T., Rosen, K. M., McPhie, D. L., Fujio, Y., Tejada, G., Neve, R. L., Adelman, L. S., & Walsh, K. 2001, "Beta-amyloid peptide expression is sufficient for myotube death: implications for human inclusion body myopathy", *Mol.Cell Neurosci.*, vol. 17, no. 5, pp. 793-810.

Ranque-Francois, B., Maisonobe, T., Dion, E., Piette, J. C., Chauveheid, M. P., Amoura, Z., & Papo, T. 2005, "Familial inflammatory inclusion body myositis", *Ann.Rheum.Dis.*, vol. 64, no. 4, pp. 634-637.

Richter, K., Haslbeck, M., & Buchner, J. 2010, "The heat shock response: life on the verge of death", *Mol.Cell*, vol. 40, no. 2, pp. 253-266.

Rideout, H. J., Lang-Rollin, I., & Stefanis, L. 2004, "Involvement of macroautophagy in the dissolution of neuronal inclusions", *Int.J.Biochem.Cell Biol.*, vol. 36, no. 12, pp. 2551-2562.

Rosenberg, S. & Kirk, P. L. 1953, "Tissue culture studies; identification of components and synthetic replacements for the active fraction of chick embryo extract ultrafiltrate", *J.Gen.Physiol*, vol. 37, no. 2, pp. 239-248.

Rubinsztein, D. C. 2006, "The roles of intracellular protein-degradation pathways in neurodegeneration", *Nature*, vol. 443, no. 7113, pp. 780-786.

Ryan, A., Nor, A. M., Costigan, D., Foley-Nolan, D., El-Rafie, A., Farrell, M. A., & Hardiman, O. 2003, "Polymyositis masquerading as motor neuron disease", *Arch.Neurol.*, vol. 60, no. 7, pp. 1001-1003.

Ryan, M. T. & Pfanner, N. 2001, "HSP70 proteins in protein translocation", *Adv.Protein Chem.*, vol. 59, pp. 223-242.

Sacco, A., Doyonnas, R., Kraft, P., Vitorovic, S., & Blau, H. M. 2008, "Self-renewal and expansion of single transplanted muscle stem cells", *Nature*.

Safdar, A., Hamadeh, M. J., Kaczor, J. J., Raha, S., deBeer, J., & Tarnopolsky, M. A. 2010, "Aberrant mitochondrial homeostasis in the skeletal muscle of sedentary older adults", *PLoS.ONE.*, vol. 5, no. 5, p. e10778.

Salajegheh, M., Pinkus, J. L., Taylor, J. P., Amato, A. A., Nazareno, R., Baloh, R. H., & Greenberg, S. A. 2009, "Sarcoplasmic redistribution of nuclear TDP-43 in inclusion body myositis", *Muscle Nerve*, vol. 40, no. 1, pp. 19-31.

Santoro, M. G. 2000, "Heat shock factors and the control of the stress response", *Biochem.Pharmacol.*, vol. 59, no. 1, pp. 55-63.

Sarkozi, E., Askanas, V., Johnson, S. A., Engel, W. K., & Alvarez, R. B. 1993, "beta-Amyloid precursor protein mRNA is increased in inclusion-body myositis muscle", *Neuroreport*, vol. 4, no. 6, pp. 815-818.

Sato, T., Walker, D. L., Peters, H. A., Reese, H. H., & Chou, S. M. 1969, "Myxovirus-like inclusion bodies in chronic polymyositis: Electron microscopic and viral studies", *Trans.Am.Neurol.Assoc.*, vol. 94, pp. 339-341.

Saunders, C. & Limbird, L. E. 1997, "Disruption of microtubules reveals two independent apical targeting mechanisms for G-protein-coupled receptors in polarized renal epithelial cells", *J.Biol.Chem.*, vol. 272, no. 30, pp. 19035-19045.

Schmidt, J., Barthel, K., Wrede, A., Salajegheh, M., Bahr, M., & Dalakas, M. C. 2008, "Interrelation of inflammation and APP in sIBM: IL-1 beta induces accumulation of beta-amyloid in skeletal muscle", *Brain*, vol. 131, no. Pt 5, pp. 1228-1240.

Schubert, D., Jin, L. W., Saitoh, T., & Cole, G. 1989, "The regulation of amyloid beta protein precursor secretion and its modulatory role in cell adhesion", *Neuron*, vol. 3, no. 6, pp. 689-694.

Schubert, U., Anton, L. C., Gibbs, J., Norbury, C. C., Yewdell, J. W., & Bennink, J. R. 2000a, "Rapid degradation of a large fraction of newly synthesized proteins by proteasomes", *Nature*, vol. 404, no. 6779, pp. 770-774.

Schubert, U., Anton, L. C., Gibbs, J., Norbury, C. C., Yewdell, J. W., & Bennink, J. R. 2000b, "Rapid degradation of a large fraction of newly synthesized proteins by proteasomes", *Nature*, vol. 404, no. 6779, pp. 770-774.

Schultz, E. 1978, "Changes in the satellite cells of growing muscle following denervation", *Anat.Rec.*, vol. 190, no. 2, pp. 299-311.

Schultz, E. & Lipton, B. H. 1982, "Skeletal muscle satellite cells: changes in proliferation potential as a function of age", *Mech.Ageing Dev.*, vol. 20, no. 4, pp. 377-383.

Scott, A. P., Allcock, R. J., Mastaglia, F., Nishino, I., Nonaka, I., & Laing, N. 2006, "Sporadic inclusion body myositis in Japanese is associated with the MHC ancestral haplotype 52.1", *Neuromuscul.Disord.*, vol. 16, no. 5, pp. 311-315.

Sherriff, F. E., Joachim, C. L., Squier, M. V., & Esiri, M. M. 1995, "Ubiquitinated inclusions in inclusion-body myositis patients are immunoreactive for cathepsin D but not beta-amyloid", *Neurosci.Lett.*, vol. 194, no. 1-2, pp. 37-40.

Shigemoto, K., Kubo, S., Mori, S., Yamada, S., Akiyoshi, T., & Miyazaki, T. 2010, "Muscle weakness and neuromuscular junctions in aging and disease", *Geriatr.Gerontol.Int.*, vol. 10 Suppl 1, p. S137-S147.

Sijts, E. J. & Kloetzel, P. M. 2011, "The role of the proteasome in the generation of MHC class I ligands and immune responses", *Cell Mol.Life Sci.*, vol. 68, no. 9, pp. 1491-1502.

Sinanan, A. C., Hunt, N. P., & Lewis, M. P. 2004, "Human adult craniofacial muscle-derived cells: neural-cell adhesion-molecule (NCAM; CD56)-expressing cells appear to contain multipotential stem cells", *Biotechnol.Appl.Biochem.*, vol. 40, no. Pt 1, pp. 25-34.

Singh, R., Cuchacovich, R., Huang, W., & Espinoza, L. R. 2001, "Inclusion body myositis unresponsive to etanercept", *J.Clin.Rheumatol.*, vol. 7, no. 4, pp. 279-280.

Siragam, V., Crow, A. R., Brinc, D., Song, S., Freedman, J., & Lazarus, A. H. 2006, "Intravenous immunoglobulin ameliorates ITP via activating Fc gamma receptors on dendritic cells", *Nat.Med.*, vol. 12, no. 6, pp. 688-692.

Sittler, A., Lurz, R., Lueder, G., Priller, J., Lehrach, H., Hayer-Hartl, M. K., Hartl, F. U., & Wanker, E. E. 2001, "Geldanamycin activates a heat shock response and inhibits huntingtin aggregation in a cell culture model of Huntington's disease", *Hum.Mol.Genet.*, vol. 10, no. 12, pp. 1307-1315.

Slater, C. R. 1976, "Control of myogenesis *in vitro* by chick embryo extract", *Dev.Biol.*, vol. 50, no. 2, pp. 264-284.

Snow, M. H. 1977, "The effects of aging on satellite cells in skeletal muscles of mice and rats", *Cell Tissue Res.*, vol. 185, no. 3, pp. 399-408.

Sorokin, A. V., Kim, E. R., & Ovchinnikov, L. P. 2009, "Proteasome system of protein degradation and processing", *Biochemistry (Mosc.)*, vol. 74, no. 13, pp. 1411-1442.

Soti, C. & Csermely, P. 2003, "Aging and molecular chaperones", *Exp.Gerontol.*, vol. 38, no. 10, pp. 1037-1040.

Spector, S. A., Lemmer, J. T., Koffman, B. M., Fleisher, T. A., Feuerstein, I. M., Hurley, B. F., & Dalakas, M. C. 1997, "Safety and efficacy of strength training in patients with sporadic inclusion body myositis", *Muscle Nerve*, vol. 20, no. 10, pp. 1242-1248.

Stefani, M. 2007, "Generic cell dysfunction in neurodegenerative disorders: role of surfaces in early protein misfolding, aggregation, and aggregate cytotoxicity", *Neuroscientist.*, vol. 13, no. 5, pp. 519-531.

Stevens, J. C., Chia, R., Hendriks, W. T., Bros-Facer, V., van, M. J., Martin, J. E., Jackson, G. S., Greensmith, L., Schiavo, G., & Fisher, E. M. 2010, "Modification of superoxide dismutase 1 (SOD1) properties by a GFP tag--implications for research into amyotrophic lateral sclerosis (ALS)", *PLoS.ONE.*, vol. 5, no. 3, p. e9541.

Stirling, P. C., Lundin, V. F., & Leroux, M. R. 2003, "Getting a grip on non-native proteins", *EMBO Rep.*, vol. 4, no. 6, pp. 565-570.

Sugarman, M. C., Kitazawa, M., Baker, M., Caiozzo, V. J., Querfurth, H. W., & LaFerla, F. M. 2006, "Pathogenic accumulation of APP in fast twitch muscle of IBM patients and a transgenic model", *Neurobiol.Aging*, vol. 27, no. 3, pp. 423-432.

Sugarman, M. C., Yamasaki, T. R., Oddo, S., Echegoyen, J. C., Murphy, M. P., Golde, T. E., Jannatipour, M., Leissring, M. A., & LaFerla, F. M. 2002, "Inclusion body myositis-like phenotype induced by transgenic over-expression of beta APP in skeletal muscle", *Proc.Natl.Acad.Sci.U.S.A*, vol. 99, no. 9, pp. 6334-6339.

Szyperski, T., Pellecchia, M., Wall, D., Georgopoulos, C., & Wuthrich, K. 1994, "NMR structure determination of the Escherichia coli DnaJ molecular chaperone: secondary structure and backbone fold of the N-terminal region (residues 2-108) containing the highly conserved J domain", *Proc.Natl.Acad.Sci.U.S.A*, vol. 91, no. 24, pp. 11343-11347.

Tanaka, K. 2009, "The proteasome: overview of structure and functions", *Proc.Jpn.Acad.Ser.B Phys.Biol.Sci.*, vol. 85, no. 1, pp. 12-36.

Tawil, R. & Griggs, R. C. 2002, "Inclusion body myositis", *Curr.Opin.Rheumatol.*, vol. 14, no. 6, pp. 653-657.

Temiz, P., Weihl, C. C., & Pestronk, A. 2009, "Inflammatory myopathies with mitochondrial pathology and protein aggregates", *J.Neurol.Sci.*, vol. 278, no. 1-2, pp. 25-29.

Thinakaran, G. & Koo, E. H. 2008, "Amyloid precursor protein trafficking, processing, and function", *J Biol.Chem.*, vol. 283, no. 44, pp. 29615-29619.

Tissieres, A., Mitchell, H. K., & Tracy, U. M. 1974, "Protein synthesis in salivary glands of Drosophila melanogaster: Relation to chromosome puffs", *J.Mol.Biol.*, vol. 85, no. 3, pp. 389-398.

Truant, R., Atwal, R. S., Desmond, C., Munsie, L., & Tran, T. 2008, "Huntington's disease: revisiting the aggregation hypothesis in polyglutamine neurodegenerative diseases", *FEBS J.*, vol. 275, no. 17, pp. 4252-4262.

Turturici, G., Sconzo, G., & Geraci, F. 2011, "HSP70 and its molecular role in nervous system diseases", *Biochem.Res.Int.*, vol. 2011, p. 618127.

Tyedmers, J., Mogk, A., & Bukau, B. 2010a, "Cellular strategies for controlling protein aggregation", *Nat.Rev.Mol.Cell Biol.*, vol. 11, no. 11, pp. 777-788.

Tyedmers, J., Mogk, A., & Bukau, B. 2010b, "Cellular strategies for controlling protein aggregation", *Nat.Rev.Mol.Cell Biol.*, vol. 11, no. 11, pp. 777-788.

Vattemi, G., Engel, W. K., McFerrin, J., & Askanas, V. 2004, "Endoplasmic reticulum stress and unfolded protein response in inclusion body myositis muscle", *Am.J.Pathol.*, vol. 164, no. 1, pp. 1-7.

Vattemi, G., Nogalska, A., King, E. W., D'Agostino, C., Checler, F., & Askanas, V. 2009, "Amyloid-beta42 is preferentially accumulated in muscle fibers of patients with sporadic inclusion-body myositis", *Acta Neuropathol.*, vol. 117, no. 5, pp. 569-574.

Verma, A., Bradley, W. G., Soule, N. W., Pendlebury, W. W., Kelly, J., Adelman, L. S., Chou, S. M., Karpati, G., & Brenner, J. F. 1992, "Quantitative morphometric study of muscle in inclusion body myositis", *J.Neurol.Sci.*, vol. 112, no. 1-2, pp. 192-198.

Verma, A. & Tandan, R. 2009, "TDP-43: a reliable immunohistochemistry marker for inclusion body myositis?", *Muscle Nerve*, vol. 40, no. 1, pp. 8-9.

Vigh, L., Literati, P. N., Horvath, I., Torok, Z., Balogh, G., Glatz, A., Kovacs, E., Boros, I., Ferdinandy, P., Farkas, B., Jaszlits, L., Jednakovits, A., Koranyi, L., & Maresca, B. 1997, "Bimoclomol: a nontoxic, hydroxylamine derivative with stress protein-inducing activity and cytoprotective effects", *Nat.Med.*, vol. 3, no. 10, pp. 1150-1154.

Walsh, D. M., Townsend, M., Podlisny, M. B., Shankar, G. M., Fadeeva, J. V., El, A. O., Hartley, D. M., & Selkoe, D. J. 2005, "Certain inhibitors of synthetic amyloid beta-peptide (Abeta) fibrillogenesis block oligomerization of natural Abeta and thereby rescue long-term potentiation", *J.Neurosci.*, vol. 25, no. 10, pp. 2455-2462.

Walter, M. C., Lochmuller, H., Toepfer, M., Schlotter, B., Reilich, P., Schroder, M., Muller-Felber, W., & Pongratz, D. 2000, "High-dose immunoglobulin therapy in sporadic inclusion body myositis: a double-blind, placebo-controlled study", *J.Neurol.*, vol. 247, no. 1, pp. 22-28.

Walter, S. & Buchner, J. 2002, "Molecular chaperones--cellular machines for protein folding", *Angew.Chem.Int.Ed Engl.*, vol. 41, no. 7, pp. 1098-1113.

Wang, B., Yang, L., Wang, Z., & Zheng, H. 2007, "Amyolid precursor protein mediates presynaptic localization and activity of the high-affinity choline transporter", *Proc.Natl.Acad.Sci.U.S.A*, vol. 104, no. 35, pp. 14140-14145.

Wang, Z., Wang, B., Yang, L., Guo, Q., Aithmitti, N., Songyang, Z., & Zheng, H. 2009, "Presynaptic and postsynaptic interaction of the amyloid precursor protein promotes peripheral and central synaptogenesis", *J.Neurosci.*, vol. 29, no. 35, pp. 10788-10801.

Warrick, J. M., Chan, H. Y., Gray-Board, Chai, Y., Paulson, H. L., & Bonini, N. M. 1999, "Suppression of polyglutamine-mediated neurodegeneration in Drosophila by the molecular chaperone HSP70", *Nat.Genet.*, vol. 23, no. 4, pp. 425-428.

Weihl, C. C., Miller, S. E., Hanson, P. I., & Pestronk, A. 2007, "Transgenic expression of inclusion body myopathy associated mutant p97/VCP causes weakness and ubiquitinated protein inclusions in mice", *Hum.Mol.Genet.*, vol. 16, no. 8, pp. 919-928.

Weihl, C. C. & Pestronk, A. 2010, "Sporadic inclusion body myositis: possible pathogenesis inferred from biomarkers", *Curr.Opin.Neurol.*, vol. 23, no. 5, pp. 482-488.

Weihl, C. C., Temiz, P., Miller, S. E., Watts, G., Smith, C., Forman, M., Hanson, P. I., Kimonis, V., & Pestronk, A. 2008, "TDP-43 accumulation in inclusion body myopathy muscle suggests a common pathogenic mechanism with frontotemporal dementia", *J.Neurol.Neurosurg.Psychiatry*, vol. 79, no. 10, pp. 1186-1189.

Welch, W. J. & Feramisco, J. R. 1982, "Purification of the major mammalian heat shock proteins", *J.Biol.Chem.*, vol. 257, no. 24, pp. 14949-14959.

Westerheide, S. D. & Morimoto, R. I. 2005a, "Heat shock response modulators as therapeutic tools for diseases of protein conformation", *J Biol.Chem.*, vol. 280, no. 39, pp. 33097-33100.

Westerheide, S. D. & Morimoto, R. I. 2005b, "Heat shock response modulators as therapeutic tools for diseases of protein conformation", *J Biol.Chem.*, vol. 280, no. 39, pp. 33097-33100.

Wiendl, H. 2008, "Idiopathic inflammatory myopathies: current and future therapeutic options", *Neurotherapeutics.*, vol. 5, no. 4, pp. 548-557.

Wojcik, S., Engel, W. K., McFerrin, J., & Askanas, V. 2005, "Myostatin is increased and complexes with amyloid-beta within sporadic inclusion-body myositis muscle fibers", *Acta Neuropathol.*, vol. 110, no. 2, pp. 173-177.

Wojcik, S., Engel, W. K., McFerrin, J., Paciello, O., & Askanas, V. 2006, "AbetaPP-over-expression and proteasome inhibition increase alphaB-crystallin in cultured human muscle: relevance to inclusion-body myositis", *Neuromuscul.Disord.*, vol. 16, no. 12, pp. 839-844.

Wojcik, S., Nogalska, A., McFerrin, J., Engel, W. K., Oledzka, G., & Askanas, V. 2007, "Myostatin precursor protein is increased and associates with amyloid-beta precursor protein in inclusion-body myositis culture model", *Neuropathol.Appl.Neurobiol.*, vol. 33, no. 2, pp. 238-242.

Wu, W. K., Cho, C. H., Lee, C. W., Wu, K., Fan, D., Yu, J., & Sung, J. J. 2010, "Proteasome inhibition: a new therapeutic strategy to cancer treatment", *Cancer Lett.*, vol. 293, no. 1, pp. 15-22.

Yang, C. C., Alvarez, R. B., Engel, W. K., Haun, C. K., & Askanas, V. 1997, "Immunolocalization of nitric oxide synthases at the postsynaptic domain of human and rat neuromuscular junctions--light and electron microscopic studies", *Exp.Neurol.*, vol. 148, no. 1, pp. 34-44.

Yang, C. C., Askanas, V., Engel, W. K., & Alvarez, R. B. 1998, "Immunolocalization of transcription factor NF-kappaB in inclusion-body myositis muscle and at normal human neuromuscular junctions", *Neurosci.Lett.*, vol. 254, no. 2, pp. 77-80.

Yi, J. J. & Ehlers, M. D. 2007, "Emerging roles for ubiquitin and protein degradation in neuronal function", *Pharmacol.Rev.*, vol. 59, no. 1, pp. 14-39.

Young, J. & Smith, J. R. 2000, "Epigenetic aspects of cellular senescence", *Exp.Gerontol.*, vol. 35, no. 1, pp. 23-32.

Young, J. C., Moarefi, I., & Hartl, F. U. 2001, "HSP90: a specialized but essential protein-folding tool", *J.Cell Biol.*, vol. 154, no. 2, pp. 267-273.

Yunis, E. J. & Samaha, F. J. 1971, "Inclusion body myositis", *Lab Invest*, vol. 25, no. 3, pp. 240-248.

Zammit, P. S. 2008, "All muscle satellite cells are equal, but are some more equal than others?", *J Cell Sci.*, vol. 121, no. Pt 18, pp. 2975-2982.

Zammit, P. S., Golding, J. P., Nagata, Y., Hudon, V., Partridge, T. A., & Beauchamp, J. R. 2004, "Muscle satellite cells adopt divergent fates: a mechanism for self-renewal?", *J Cell Biol.*, vol. 166, no. 3, pp. 347-357.

Zammit, P. S., Partridge, T. A., & Yablonka-Reuveni, Z. 2006, "The skeletal muscle satellite cell: the stem cell that came in from the cold", *J Histochem.Cytochem.*, vol. 54, no. 11, pp. 1177-1191.

Zhang, Y. J., Xu, Y. F., Dickey, C. A., Buratti, E., Baralle, F., Bailey, R., Pickering-Brown, S., Dickson, D., & Petrucelli, L. 2007, "Progranulin mediates caspase-dependent cleavage of TAR DNA binding protein-43", *J.Neurosci.*, vol. 27, no. 39, pp. 10530-10534.

Zhou, H., Cao, F., Wang, Z., Yu, Z. X., Nguyen, H. P., Evans, J., Li, S. H., & Li, X. J. 2003, "Huntingtin forms toxic NH2-terminal fragment complexes that are promoted by the age-dependent decrease in proteasome activity", *J.Cell Biol.*, vol. 163, no. 1, pp. 109-118.

Zhu, X., Zhao, X., Burkholder, W. F., Gragerov, A., Ogata, C. M., Gottesman, M. E., & Hendrickson, W. A. 1996, "Structural analysis of substrate binding by the molecular chaperone DnaK", *Science*, vol. 272, no. 5268, pp. 1606-1614.