Title: Behaviour of human myoblasts in vitro: role of ageing and inflammatory cytokines

Author: Mansour Alsharidah

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BEHAVIOUR OF HUMAN MYOBLASTS IN VITRO: ROLE OF AGEING AND INFLAMMATORY CYTOKINES

MANSOUR SALEH ALSHARIDAH

PhD Thesis

Centre of Human & Aerospace Physiological Sciences
School of Biomedical Sciences
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London
2012
Abstract

Ageing is associated with a loss of muscle mass, a processes known as “sarcopenia”. It has been postulated that one of the reasons underlying this muscle loss is a decreased ability to repair itself in response to damage. Repair of muscle is facilitated by a specific population of progenitor adult stem cells known as satellite cells in situ and myoblasts ex vivo. The work in this thesis has used a cell culture approach to study the behaviour of human myoblasts and compare the inherent effects of age (by studying myoblasts taken from young and old people) and using an in vitro model of ageing, (i.e. proliferative senescence), and investigating the contribution of environmental factors by culturing the cells in human serum from young and old participants and treating them with recombinant cytokines.

Several parameters were studied, but the main focus was on ability of myoblasts to proliferate and differentiate. Markers of proliferating muscle cells were desmin, NCAM and Ki67, and markers of differentiating muscle cells were myogenin and myosin heavy chain. Additional parameters observed were DNA damage and analysis of specific cytokines in the cell secretome. No differences in any parameter measured were found between cells of young and old people. However senescent myoblasts differed significantly in all parameters from early passage cells. Differentiation was studied over a seven day period. There was a delay of two-four days in the onset of markers expression between senescent and early passage cells, as well as a decrease in the expression levels of myogenin (50 ± 3% in young, 49 ± 3% in old and 6 ± 1% in senescent) after three days of differentiation and myosin heavy chain (MHC; 71 ± 2% in young, 70 ± 1.4% in old and 15 ± 1% in senescent) after five days of differentiation. In addition, increased DNA damage (7 ± 1% in young, 8 ± 1% in old and 90 ± 4% in senescent), increased TGF-β secretion (111 ± 13 pg/ml in young, 115 ± 19 pg/ml in old and 268 ± 11 pg/ml in senescent), and decreased myotube area (151574 ± 22968 µm² in young, 132531 ± 25106 µm² in old and 47765 ± 763 µm² in senescent) were observed in senescent cells compared to early passage cells.

For influence of environmental factors, freshly isolated cells were cultured in human sera or medium with or without cytokines. No differences were observed in the myoblasts cultured in sera from young and elderly individuals (Ki67 expression was 85 ± 2% in young and 84 ± 2% in old, and desmin expression was 81 ± 2% in young and 83 ± 3% in old at three days after isolation). There was, however, a significant decrease in desmin and myogenin expression when cells were exposed to TGF-β1 (1 ng/ml), TNF-α (1 ng/ml) or IL-1β (1 ng/ml). Committed myoblasts were also cultured in the presence or absence of TGF-β1, TNF-α, or IL-1β. Myogenin but not desmin expression was significantly inhibited in the presence of cytokines.

These findings support the observation of many studies in vivo in both human and animal models which show that satellite cells from old muscle can contribute to muscle repair and regeneration. This suggests the satellite cells per se may not be critically involved in the mechanism responsible for sarcopenia. However, their sensitivity to inflammatory cytokines suggests that if these were present in vivo they would affect the myogenic behaviour of the cells.
DECLARATION OF ORIGINAL AUTHORSHIP

I declare that this work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge, this thesis has not been presented to any other University for examination either in the United Kingdom or overseas. No portion of the work referred to in this Research Project has previously been published except where due references has been made in this thesis. All the work described in this thesis was undertaken by the author, except the expansion of freshly isolated cells was performed by Tomasz George.

______________________________
Mansour Saleh Alsharidah

2012
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<tr>
<td>Akt</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>ALK</td>
<td>Anaplastic Lymphoma Kinase</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>APAF</td>
<td>Apoptosis protein activating factor 1</td>
</tr>
<tr>
<td>ATF-2</td>
<td>Activating transcription factor 2</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2-associated death promoter</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcl-2 homologous antagonist/killer</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>Bid</td>
<td>Bcl2 interacting protein</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>Caspase-activated DNase</td>
</tr>
<tr>
<td>Cdc37</td>
<td>Co-chaperon of HSP90</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinases</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>DD</td>
<td>Death domain</td>
</tr>
<tr>
<td>DIABLO</td>
<td>Direct IAP binding protein with low pI</td>
</tr>
<tr>
<td>DM</td>
<td>Differentiation medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EndoG</td>
<td>Endonuclease-G</td>
</tr>
<tr>
<td>Erk</td>
<td>Extra cellular signal regulated kinase</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
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<tr>
<td>FOXO</td>
<td>Forkhead Homeobox Type O</td>
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<tr>
<td>GSK3β</td>
<td>Glycogen-synthase kinase 3 beta</td>
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<td>GM</td>
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gp130  glycoprotein 130
H2AX  Gene coding for histone H2A
HSP90  Heat shock protein-90
IAP  Inhibitor of apoptosis protein
IGF-1  Insulin growth factor-one
IGFBP  Insulin growth factor binding protein
IL-1β  Interleukin one
IL-4  Interleukin four
IL-6  Interleukin six
IL-7  Interleukin seven
IL-8  Interleukin eight
IL-10  Interleukin ten
IL-13  Interleukin thirteen
JAK  Janus family tyrosine kinase
Jnk  C-Jun N-terminal kinase
MADD  MAPK activating death domain
MAPK  Mitogen-Activated Protein Kinase
MHC  Myosin heavy chain
MKK  Mitogen-activated protein Kinase Kinase
MNF  Myocyte nuclear factor
MRF  Myogenic regulatory factor
mRNA  Messenger ribonucleic acid
Myf5  Myogenic factor five
MyoD  Myogenic determination
NAN3  Sodium azide
NCAM  Neural cell adhesion molecule
NF-κB  Nuclear factor-kappaB
NO  Nitric oxide
NOS  Nitric Oxide Synthase
<table>
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<tr>
<td>Par6</td>
<td>Partitioning defective 6 homolog</td>
</tr>
<tr>
<td>Pax3</td>
<td>Paired box transcription factor 3</td>
</tr>
<tr>
<td>Pax7</td>
<td>Paired box transcription factor 7</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor-gamma</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinases</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
</tr>
<tr>
<td>Ras</td>
<td>Rat sarcoma</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
</tr>
<tr>
<td>S100B</td>
<td>S100 calcium binding protein B</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Smad</td>
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<tr>
<td>Smurf1</td>
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<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
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<tr>
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<td>TNF converting enzyme</td>
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</table>
Introduction

Ageing is associated with a loss of muscle mass. This process is known as “sarcopaenia”. It negatively affects the quality of life as functional ability is reduced; and there are negative implications given the role of muscle as a source of heat, a dynamic metabolic store and a source of protective padding (Young, 1997). Despite several investigations, the mechanisms responsible for sarcopenia remain unknown. There are numerous factors, both intrinsic and extrinsic to muscle, that may be contributed to sarcopenia. These include denervation (Rowan et al., 2012), decreased protein synthesis (Short et al., 2004), oxidative damage (Jang et al., 2009, Kondo et al., 1992), mitochondrial dysfunction (Romanello et al., 2010) and increase in inflammatory cytokines (Carlson et al., 2009a, Dobbs et al., 1999, Njemini et al., 2011, Paolisso et al., 1998, Wei et al., 1992), and decrease in repair mechanisms (Grounds, 1998).

Skeletal muscle is a post-mitotic tissue which has the ability to repair itself in response to injury. This process is largely dependent on a specific population of progenitor adult stem cells known as satellite cell “myoblasts” (Lepper et al., 2011, Murphy et al., 2011, Sambasivan et al., 2011). There is no agreement as to whether defective regeneration of aged muscles is determined by changes in systemic factors, factors intrinsic to muscle tissue or by impairment of the satellite cells themselves. The work in this thesis uses a cell culture approach to study both the inherent effects of age and environmental factors on behaviour of human myoblasts. The former are assessed by isolating cells from muscle biopsies taken from both young and elderly people and comparing their behaviour in culture, whereas the latter are assessed by analysing the response of satellite cells to treatment with serum from young and elderly people as well as purified cytokines.
In chapter one, a review of literature is presented. First, sarcopaenia and its underlying mechanisms are detailed. Next, satellite cells are described, and the current ways that ageing appears to affect these cells is reviewed. Finally, the effects of cytokines on myoblasts proliferation and differentiation are presented. In chapter two, the general methodology undertaken in the data chapters is described.

The experimental work is reported in chapters three through to chapter six. Chapter three describes how ageing “in vivo” affects the proliferation and differentiation of human myoblasts when compared to a model of “in vitro” ageing, namely proliferative senescence. Chapter four reports the secretory profile of human myoblast during differentiation. Chapters five and six examine the effects of cytokines and human serum on the behaviour of human myoblast, respectively. Finally, chapter seven summarises the work, and discusses how these results contribute to the field of muscle ageing.
Chapter 1: Literature review
1.1 Population ageing

The worldwide population is ageing (Degens, 2007, Dunnell, 2007). There is a growing proportion of older people and decreasing proportion of children in the general population (Tomassini, 2005). Demographic trends estimate that global population of those aged 60 over will increase from 600 million (year 2000 estimate) to 2 billion (year 2050 estimate) (Koopman and van Loon, 2009). Meanwhile, the fertility is declining as average life expectancy continues to increase, and people aged 85 years and over (Campion, 1994) constitute the fastest growing subpopulation in the developed world (Manini and Pahor, 2009, Mazzeo et al., 1998). These changes create significant financial pressures on public health and social services. In the UK approximately 40% of the health and social care costs of the National Health Service (NHS) is currently spent on those over 65 years of age (Dunnell, 2008).

1.2 Sarcopenia

The term was first described by Rosenberg (1989) and derives from the Greek language meaning ‘poverty of flesh’ (Rosenberg, 1989, Roubenoff and Hughes, 2000). Sarcopenia is defined as an appendicular skeletal muscle mass divided by height in meters, two standard deviations below the mean value for healthy young people under 30 years of age (Baumgartner et al., 1998). Using this criterion, it is estimated that 25% of people above the age of 60 years old and 40% of people over the age of 80 suffer from sarcopenia. Similar results are reported by Ianuzzi-Sucich et al. (2002) and Janssen et al. (2002). It is now broadly accepted that sarcopenia is an intrinsic feature of ageing and as such, it cannot be classified as a disease (Roubenoff and Castaneda, 2001). The most severe effects of sarcopenia occur when mobility and activity of daily
living become markedly impaired and eventually lead to loss of independence, a reduced quality of life and an increased risk of falls (Doherty, 2003, Roubenoff, 2000, Sayer et al., 2006).

Although inter-individual variation exists, around 10% of the decrease in muscle size seems to occur between the ages of 25 and 50, as determined by cross-sectional area of whole vastus lateralis muscle obtained from cadavers (Lexell et al., 1988). One noteworthy consideration about this seminal study is that the medical history and physical activity profile of the subjects examined was not reported. After the age of 50 the rate of muscle loss increases dramatically and by 80 years a total decrease of 40% in muscle size is apparent (Lexell et al., 1988). This decline in muscle mass with ageing is associated with both a decrease in the number and muscle fibre size. Lexell et al. (1988) has demonstrated that by the eighth decade a 50% reduction in the total fibre number is present when compared with muscle tissue in young subjects and that cross sectional area of type II fibres is nearly 35% smaller in muscles from older individuals when compared with young individuals (Lexell and Taylor, 1991). A growing body of literature generally agrees that type II fibre atrophy with age whereas type I fibre size remains unchanged (Figure 1.1); (Coggan et al., 1992, Kosek et al., 2006, Petrella et al., 2006, Verdijk et al., 2007). It must be noted that all subjects on these studies were sedentary, suggesting that any differences observed could be either due to age itself or simply due to inactivity. Klitaard and co-workers (1990) showed that elderly strength trained subjects have cross-sectional areas larger than of sedentary subjects and similar to those of young adult control subjects, suggesting that age-related changes in skeletal muscle function may be counteracted by long-term strength training in elderly men. Therefore, the model used to study the effects of age needs to be carefully considered,
in order to confirm whether differences between young and old individuals are indeed due to age or the combination of age and other deleterious factors to health, such as inactivity (Lazarus and Harridge, 2010).

Muscle strength and power also decrease with age (Figure 1.2); (Frontera et al., 2000, Newman et al., 2003, Skelton et al., 1994). Cross sectional studies indicate that every ten years, absolute strength will experience a loss of 8-15% additionally, after the age of 50, the loss increases to 25-40% (Goodpaster et al., 2006, Hughes et al., 2001, Lindle et al., 1997). A loss of muscle power occurs by the age of 40 years in both men and women at a much more rapid rate than strength (Izquierdo et al., 1999, Metter et al., 1997). The effects of loss of muscle power are seen in elite athletes at a similar rate to those of healthy untrained individuals (Pearson et al., 2002). This implies that the loss of muscle power that occurs with advancing age is not simply due to disuse.
Figure 1.1. Cross sectional area of type I and type II fibre in muscle tissue collected from elderly (A) and young (B) males. ATPase histochemistry shows, type I fibres a dark/black, and type IIa fibres as areas pale/white and type IIb/IIx with intermediate staining. Type II fibres are significantly smaller in the elderly vs. the young. adapted from (Verdijk et al., 2007).

Figure 1.2. Age-related decreases in thigh muscle area (based on one-cut computed tomography scan) and, knee extensor strength (180°/s) in 78 health persons who did not regularly exercise. Taken from (Nair, 2005).
1.3 Underlying causes of sarcopaenia

1.3.1 Protein turnover

The maintenance of muscle mass and function relies on a dynamic equilibrium between muscle protein synthesis and muscle protein breakdown (Smith and Rennie, 1996). Changes in the basal rates of muscle protein synthesis have been demonstrated in some (Balagopal et al., 1997, Rooyackers et al., 1996, Short et al., 2004, Welle et al., 1993, Yarasheski et al., 1993) but not all studies (Cuthbertson et al., 2005, Volpi et al., 1998, Volpi et al., 2001). The loss of muscle mass with age is attributed to a phenomenon called ‘anabolic resistance’, where muscle protein synthesis is blunted in response to the main anabolic stimuli, i.e. protein intake and/or physical activity (Cuthbertson et al., 2005, Volpi et al., 2004). For example, older men showed anabolic resistance of muscle protein synthesis to feeding, revealing a reduced sensitivity and responsiveness to the anabolic effects of protein or amino acid ingestion (Cuthbertson et al., 2005, Katsanos et al., 2005, Volpi et al., 2000). Furthermore, older men showed anabolic resistance of signalling and muscle protein synthesis following a single bout of resistance type exercise (Kumar et al., 2009), but Hasten and co-workers showed that older people enrolled in short term resistance exercise study can respond with increased muscle protein synthesis rates in similar manner to young subjects (Hasten et al., 2000), suggesting that there may be a delay in response, which means it is not observed in single bout studies, but is in longer term studies.

1.3.2 Denervation and motor unit loss

An age-related change in motor units is considered an important factor in the development of sarcopaenia (Rowan et al., 2012). A motor unit is defined as the
motoneuron and all the myofibres it innervates. A month post-denervation, the muscle mass of an adult rat hindlimb muscles will decrease by 50% (Gutmann, 1962). This process is related to motor neuron cell death or neuromuscular junction remodelling both of which occur with age (Ansved and Larsson, 1990, Cardasis and LaFontaine, 1987, Hashizume et al., 1988, Larsson, 1995, Pettigrew and Gardiner, 1987, Wokke et al., 1990). Early studies on ageing human muscle reported a reduction in motor unit numbers with an increase in the size of the remaining motor units (Brown, 1972, Campbell et al., 1973, Stalberg and Fawcett, 1982, Sica et al., 1976). In human beings, half of the motor neurons loss occurs in people above the sixth decade of life (Tomlinson and Irving, 1977). Denervation is associated with both fibre type grouping and fibre atrophy grouping, resulting in a reduction in the mosaic composition of a muscle (Grimby et al., 1982, Larsson et al., 1991, Lexell and Downham, 1991). In addition, neuromuscular junction (Lexell, 1997, Luff, 1998) and axon myelination (Grover-Johnson and Spencer, 1981, Kazui and Fujisawa, 1988, Knox et al., 1989) abnormalities, which should impede stimulation for muscle contractions, are observed in aged rodents and humans. These observations are highly suggestive of neural degeneration occurring with age.

1.3.3 Apoptosis

Apoptosis, also known as programmed cell death, is an important process by which the cell actively participates in its own destruction (Baker and Reddy, 1998). Accumulated evidence suggests that an aged-related acceleration of apoptosis might represent one of the major factors to drive age-related muscle atrophy. Microarray experiments report that muscle biopsies of older persons show differences of expression in genes involved
in mediating cellular responses to apoptosis when compared with young subjects (Giresi et al., 2005). Loss of nuclei during muscle atrophy is attributed to DNA degradation, nuclear condensation and cell fragmentation, all of which are characteristics of apoptosis (Allen et al., 1997, Allen et al., 1999, Borisov and Carlson, 2000, Dupont-Versteegden et al., 2006, Leeuwenburgh et al., 2005, McArdle et al., 1999). Apoptosis is classified into two different pathways, caspase-dependent and caspase-independent apoptosis (Marzetti and Leeuwenburgh, 2006). Muscle atrophy is associated with an increased level of caspase-3 (Leeuwenburgh et al., 2005, Libera et al., 1999, Yasuhara et al., 2000). In vitro studies have also shown caspase-3 to be increased during apoptosis in myotubes formed by fusion of C2C12 myoblasts (a mouse cell line); (McArdle et al., 1999). Moreover, Bcl-2, an anti-apoptotic protein, decreases while Bax, a pro-apoptotic protein, increases during muscle atrophy (Jin et al., 2001, Libera et al., 1999, Persinger et al., 2003, Tews and Goebel, 1997).

1.3.4 Mitochondria and oxidative stress

It has been proposed that an increased level of oxidative stress and mitochondrial dysfunction are potential contributors to sarcopaenia (Jang et al., 2009, Kondo et al., 1992, Romanello et al., 2010). Ageing has been suggested to be a cumulative accretion of mutations in mitochondrial DNA (Wallace, 1999). These mutations can lead to an impairment of ATP production and therefore a decline of energy at the tissue level. This is largely due to a cytochrome c oxidase (COX) deficiency and the numbers of cells containing this deficiency is reported to increase with age in human extraocular muscles and in cardiac muscle (Brierley et al., 1998, Muller-Hocker et al., 1992). It has been shown that both protein and mRNA level of genes involved in mitochondrial biogenesis
decrease in age murine human skeletal muscle compared to the young (Chabi et al., 2008, Safdar et al., 2010), which eventually contribute to the decreased function of these cells, and their increased susceptibility to stress. As mentioned previously, it must be noted that all subjects on these studies were sedentary, suggesting that any differences observed could be either due to ageing itself or simply due to inactivity. Safdar et al. (2010) showed that physically active elderly subject had protein content of PGC-1α, a master regulator of mitochondrial biogenesis higher than of the sedentary elderly subjects and similar to those of young adult control subjects, suggesting that an active lifestyle is counteract mitochondrial abnormalities associated with ageing.

Mitochondria are the main cellular sites of generation of reactive oxygen species. As a result of its proximity to the electron transport chain (ETC), the lack of protective histones and the less efficient repair system, mitochondrial DNA is very susceptible to oxidative damage by free radicals (Greenlund and Nair, 2003, Wei and Lee, 2002, Yakes and Van Houten, 1997). An increase in free radical production, along with a decrease in the activity of antioxidative mechanisms, leads to oxidative damage of DNA, proteins and lipids in elderly healthy subjects (Fano et al., 2001).

### 1.3.5 Inflammageing

Ageing has been associated with systemic low-grade inflammation, so-called “inflammageing”. Inflammageing is defined, in part, by increased serum and plasma levels of a variety of pro-inflammatory cytokines and growth factors, including IL-6, TNF-α, and TGF-β1 (Carlson et al., 2009a, Dobbs et al., 1999, Njemini et al., 2011, Paolisso et al., 1998, Wei et al., 1992). Higher cytokine levels are associated with loss
of muscle mass and strength with advancing age (Aleman et al., 2011, Cesari et al., 2005, Pedersen et al., 2003, Schaap et al., 2009, Schaap et al., 2006, Visser et al., 2002) and have a negative effect on satellite cell behaviour that may contribute directly to sarcopenia. This discussed further in section 1.5.

1.3.6 Impairment of satellite cell function in repair and hypertrophy

Skeletal muscles are the most abundant tissue in the human body and comprise approximately 40-45% of the total body mass. Throughout life, skeletal muscles have a remarkable ability to repair after damage (McCroskery et al., 2005). The tremendous capacity for mammalian skeletal muscle regeneration is demonstrated by the histological examination of skeletal muscle specimens in rodents following chemical insult, where large areas of damage are restored to their normal architectural integrity within ten days (Otto et al., 2008, Yan et al., 2003). The remarkable regenerative capacity of postnatal skeletal muscle has been found to be largely dependent on a specific population of progenitor adult stem cells known as satellite cells. Transplantation of freshly isolated myofibres into the radiation-ablated muscles of dystrophic mdx-nude mice, has provided important evidence that the satellite cells are able to give rise to both new myofibres and myofibre-associated cells (Collins et al., 2005).
Recent studies using genetic ablation of the satellite cell pool in adult mice have provided evidence that muscle regeneration is entirely dependent on the presence of the satellite cells (Lepper et al., 2011, Murphy et al., 2011, Sambasivan et al., 2011). Satellite cells also have their capacity to “self renew” during the regeneration process, ensuring that undifferentiated precursors are available for future repair (Collins, 2006, Collins and Partridge, 2005), it is now widely accepted that satellite cells are mononucleated myogenic precursor cells (myoblasts), that adhere to existing muscle fibres under the basal lamina (Bischoff, 1975) and are present throughout the lifespan of all myofibres. They play a major role in the maintenance and repair of postnatal skeletal muscle tissue. Satellite cells are normally present in a quiescent undifferentiated state, but when activated in response to muscle damage by injury or exercise, they have the ability to proliferate, migrate to the site of injury and fuse with existing fibres for regeneration and hypertrophy (Bischoff, 1975, Konigsberg et al., 1975, Morgan and Partridge, 2003, Moss and Leblond, 1971, Snow, 1977b); (Figure 1.4). As this thesis is concerned with the in vitro behaviour of satellite cell derived myoblasts obtained from human biopsies, Section 1.4 details several aspects of satellite cells from their identification in the early 1960s to details of markers used in their identification. The effect of age on satellite cells is addressed in detail in section 1.4.5.

Although satellite cells have been shown to play a central role in muscle regeneration, other cell populations have also been implicated in this process. For example, a pioneering study by Ferrari et al. (1998) showed that bone marrow derived cells can participated to muscle regeneration and are capable of differentiating into myofibres following injury. More recently, genetic ablation of “muscle side population cells” demonstrated that skeletal muscle regeneration is delayed (Doyle et al., 2011). These
cells are distinct from satellite cells by their ability to efflux the dye Hoechst 33342 and show a unique pattern on fluorescence-activated cell sorting analysis (Goodell et al., 1996). Subsequently, several other cell types have been reported to incorporate into muscle during regeneration, including mesoangioblasts (Sampaolesi et al., 2003), pericytes (Dellavalle et al., 2007), and PW1<sup>ev</sup> interstitial cells (Mitchell et al., 2010). The functional interactions between satellite cells and other muscle residing cell types following muscle injury are unclear and the role of the non-muscle derived cells in sarcopenia is even less so.

1.4 Satellite cells

1.4.1 Identification and morphology of satellite cells

Satellite cells were first identified in 1961 based on their anatomical position using electron microscopic examination of the frog tibialis anticus muscle, located between the plasma membrane and the basal lamina of muscle fibres (Mauro, 1961). Human satellite cells are found to be shorter in length than myonuclei (8 µm compared to 11 µm); (Watkins and Cullen, 1986) and display cytoplasm with few organelles (ribosomes, rough endoplasmic reticulum, and Golgi apparatus), and high amounts of nuclear heterochromatin (Schultz, 1976). These features demonstrate that satellite cells are transcriptionally less active than myonuclei, i.e. they are in a quiescent state (Schultz et al., 1978). Activated satellite cells exhibit an altered morphology. The cytosolic fraction of the cells will have a larger volume and cell extensions will start to form to facilitate movement, there will be an increase in the number of organelles and a reduction in heterochromatin level (Hawke and Garry, 2001, Schultz, 1976, Schultz and McCormick, 1994); (Figure 1.3). Based on their morphology and position several lines
of evidence have confirmed the presence of skeletal muscle satellite cells in a number of vertebrate species including humans (Illa et al., 1992, Ishikawa, 1966, Muir et al., 1965).

**Figure 1.3.** Satellite cell location in an adult muscle fibre.

In uninjured muscle fibres, the satellite cell is quiescent and rests in an indentation in the adult muscle fibre. The satellite cells can be distinguished from the myonuclei by a surrounding basal lamina and more abundant heterochromatin. When the fibre becomes injured, the satellite cells become activated and increase their cytoplasmic content. The cytoplasmic processes allows for chemotaxis of the satellite cell along the myofibre. Taken from (Schultz and McCormick, 1994).
1.4.2 Molecular markers of satellite cells

Satellite cells can be identified using immunohistochemistry and immunocytochemistry, where antibodies bind to a specific antigens, and are viewed under a microscope. Marker specific to each phase of satellite cell activation exist (figure 1.4). Quiescent satellite cells are most commonly identified by their characteristic expression of the paired box transcription factor 7 gene (Pax7) mRNA and protein (Day et al., 2007, Zammit et al., 2006b). Myoblasts can be obtained from muscle tissue and cultured in vitro where they also express muscle specific markers such as Pax7, Myf5 and MyoD. When induced to differentiate, they stop proliferating, begin expressing myogenin and fuse together forming multinucleated cells called myotubes. Myotubes are post-mitotic and expresses terminal markers of muscle differentiation such as sarcomeric myosin heavy chain (MHC) (Zammit et al., 2006a). Although a comprehensive list of satellite cell markers are presented in Table 1.1, only satellite cell markers used in the experimental sections of this study will be discussed in detail below.

Several antibodies have been reported to recognise satellite cells in adult skeletal muscle during various stages of myogenesis. The first antibodies used to distinguish satellite cells from other cells were Leu19/CD56 which recognises the neural cell adhesion molecule (NCAM) (Illa et al., 1992, Schubert et al., 1989). NCAM is expressed during early myogenesis and in adult satellite cells. A range of researchers have reported that NCAM is a useful marker for satellite cells in biopsy samples of human muscle. This is a consequence of, the fact that it binds to quiescent as well as activated satellite cells without binding to myonuclei (Mackey et al., 2010, Mackey et al., 2009, Malm et al., 2000).
Figure 1.4. Schematic representation of satellite cell activation and progression through the myogenic program. Quiescent satellite cells, underneath the basal lamina of muscle fibres, express Pax7 and Myf5. Upon activation, they up-regulate MyoD and divide to produce a pool of muscle precursor cells (mpc). Satellite cell progeny then follow one of two fates. They may down-regulate MyoD and self-renew to give rise to a Pax7 expressing satellite cell. Alternatively, they may differentiate, down-regulating Pax7, Myf5, and MyoD and expressing MRF4 and myogenin, eventually fusing either to form new or to repair damaged myofibres. Taken from (Boldrin et al., 2010).
Table 1.1. Markers of satellite cells
Modified and adapted from (Boldrin et al., 2010).

<table>
<thead>
<tr>
<th>Satellite cells marker</th>
<th>Stage of expression</th>
<th>Species</th>
<th>References</th>
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<tr>
<td>Pax7</td>
<td>Quiescent and activated satellite cells</td>
<td>Mouse and human</td>
<td>(Dellavalle et al., 2007, Pawlikowski et al., 2009, Seale et al., 2000)</td>
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<tr>
<td>Pax3</td>
<td>Quiescent satellite cells</td>
<td>Mouse</td>
<td>(Relaix et al., 2006)</td>
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<td>Myf5</td>
<td>Quiescent and activated satellite cells</td>
<td>Mouse and human</td>
<td>(Beauchamp et al., 2000, Dellavalle et al., 2007, Sabourin et al., 1999, Yablonka-Reuveni et al., 1999)</td>
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<tr>
<td>MyoD</td>
<td>Activated and differentiated satellite cells</td>
<td>Mouse and human</td>
<td>(Dellavalle et al., 2007, Pawlikowski et al., 2009, Sabourin et al., 1999, Yablonka-Reuveni et al., 1999)</td>
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<td>MNF</td>
<td>Quiescent and activated satellite cells</td>
<td>Mouse</td>
<td>(Garry et al., 1997)</td>
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<td>C-met</td>
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<td>Mouse and human</td>
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<td>(Tanaka et al., 2009)</td>
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<td>Mouse</td>
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<td>(Pallafacchchina et al., 2010)</td>
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<td>(Fukada et al., 2004, Mitchell et al., 2005)</td>
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Desmin is an intermediate filament protein found in high abundance in the cytoskeleton of muscle cells (Capetanaki et al., 2007, Lazarides and Hubbard, 1976). Desmin is the most widely used marker for human myoblasts in culture as there is a reliable, commercially available monoclonal antibody. Desmin is present in human and animals proliferating muscle precursor cells in culture, in regenerating muscle fibres at 24 hours or more after injury and following the fusion of myoblasts (Allen et al., 1991, Bockhold et al., 1998, Brocks et al., 1991, Jacquemin et al., 2004, Schafer et al., 2006).

MyoD belongs to a family of proteins known as the myogenic regulatory factors (MRFs) and is required for determination of myoblasts into the myogenic lineage during embryonic development (Cao et al., 2006, Megeney et al., 1996). MyoD is not normally expressed in adult myofibres or quiescent satellite cells, but is expressed upon satellite cell activation. In cultured human myoblasts, it continues to be expressed during differentiation and after fusion into myotubes (Dellavalle et al., 2007). Both in vivo and in vitro studies have demonstrated that MyoD-deficient myoblasts have a reduced differentiation potential presented as an impaired ability to fuse, resulting in a reduction in the number of myotubes that can be generated (Yablonka-Reuveni et al., 1999, Megeney et al., 1996, Montarras et al., 2000, Sabourin et al., 1999). These results suggest that MyoD not only is an important marker for satellite cells, but is also responsible for inducing myogenic differentiation. Myogenin, also a member of the myogenic regulatory factor family, is expressed during the early phases of differentiation (Dellavalle et al., 2007). Myogenin is implicated in cell cycle arrest and regulates phenotypic differentiation and subsequent cell fusion (Andres and Walsh, 1996). Myosin heavy chain (MHC) is a muscle specific protein which forms the thick myofilaments in skeletal muscle fibres, it is composed of two heavy chains and four
light chains (Cripps et al., 1999). MHC is expressed during early and late stages of myogenic differentiation (Soulez et al., 1996).

Finally, Ki67 is a protein used as a marker of proliferation (Gerdes et al., 1984, Silvestrini et al., 1988). The Ki67 antigen is present in cells, including satellite cells, during all phases of the cell cycle, and is often used to define the growth fraction of satellite cells population (Gerdes et al., 1984, Mackey et al., 2009, Ono et al., 2010). The identity of the Ki67 antigen has not been reported.

### 1.4.3 The role of satellite cells in adult muscle

The role of satellite cells in muscle regeneration and repair was explained in section 1.3.6.

Despite the strong relationship between muscle regenerative ability and the presence of satellite cells, the relevance of satellite cell contribution to hypertrophy of human skeletal muscle remains controversial. It has been shown in rodents that when satellite cell proliferation is inhibited by gamma irradiation, there is a reduced hypertrophic response to overload (Phelan and Gonyea, 1997, Rosenblatt and Parry, 1992). In contrast, recent studies using genetic ablation of the satellite cell pool in adult mice have provided evidence that satellite cells are not always essential for hypertrophy (McCarthy et al., 2011). Studies in human muscle that examine the relationship between fibre cross sectional area, myonuclear number and satellite cell activation; suggest that existing myonuclei are transcriptionally able to sustain an area of muscle fibre equivalent to 2000µm², known as the myonuclear domain (Cheek, 1985, Petrella et al., 2006). At the onset of hypertrophy, individual myonuclei increase their transcriptional
activity, and consequently their myonuclear domain will enlarge to support moderate enhancement of cytoplasmic volume, this allows the fibre to increase by up to 26% or until a certain limit of the myonuclear domain has been reached (Kadi et al., 2004b, Petrella et al., 2006). Any subsequent hypertrophy beyond this limit in theory requires the addition of new myonuclei and satellite cell fusion.

1.4.4 The origin of satellite cells

The origin of satellite cells has been a topic of long-lived debate. It is widely accepted that satellite cells are originally derived from the somites, as evidence by several study. Early experiments using a quail-chick chimera model point out that satellite cells derive developmentally from the somites (Armand et al., 1983). More recently, this concept has also been confirmed using green fluorescent protein lineage tracing and the quail–chick grafting techniques demonstrated that satellite cell originate from somites and in particular from a Pax3/7 positive population of cells located in the central region of the dermomyotome of the somite (Gros et al., 2005). However, different origins have been proposed for satellite cells such as the dorsal aorta, other blood vessels or the bone marrow (De Angelis et al., 1999, Ferrari et al., 1998, Gussoni et al., 1999, Seale et al., 2001). Thus, these results suggested that satellite cells are not homogeneous cells and may actually derive from sources other than the somite during embryonic development. As discussed previously, it has been demonstrated that satellite cells are essential for muscle regeneration. It is thus unlikely that non muscle stem cells make any significant contribution to adult muscle regeneration although they may occasionally be identified in regenerating muscle following major insult.
1.4.5 Effects of ageing on satellite cells

During early postnatal growth, satellite cells account for 30-35% of sublaminar muscle nuclei in mice, declining to approximately 5-7% in adults (Allbrook et al., 1971, Cardasis and Cooper, 1975, Schultz, 1976). Some controversy surrounds the loss of satellite cells with age, as some studies document relatively equal numbers in young and old individuals (Dreyer et al., 2006, Roth et al., 2000). Others report a decline in satellite cells content with increasing age (Kadi et al., 2004a, Renault et al., 2002, Verdijk et al., 2007). Verdijk et al. (2007) reported that satellite cell loss was restricted to the type II muscle fibres and one possible explanation is that satellite cell loss is fibre type specific. The fibre type composition of the specific muscle being examined or that is found in the biopsy material under examination may determine whether an age associated decrease in satellite cell numbers is seen.

There is no agreement as to whether defective regeneration of aged muscles is determined by changes in systemic factors or by impairment of the satellite cells themselves. The first experiments to suggest a “systemic” impairment to muscle repair and regeneration were performed by Carlson and Faulkner (1989) who showed that cross age transplantation experiments of the right EDL muscle between young (2-3 months) and old (24 months) inbred Wistar rats resulted in two fold increases in muscle mass and strength when elderly muscle was grafted onto a younger animal as compared to autografted to elderly muscle. This study suggested that the poor regeneration of muscles in old animals is a function of the environment for regeneration provided by the old host and not a function of the aged muscles themselves. Long-term reinervation deficiencies in the elderly hosts were subsequently implicated in this phenomenon (Carlson et al., 2001).
Further support for the findings of Faulkner and Carlson (1989), were reported in a parabiotic study where pairs of mice were connected by the circulatory system, so that the regenerating tissues of one animal were exposed to the circulating factors of another (Conboy et al., 2005). This was performed pairs of animals of the same age (isochronic parabioses) or different ages (heterochronic parabiosies). In this study, parabiotic pairings of young and old mice were followed by exposure of the muscle to dry ice (cryogenic) injury which this resulted in satellite cell activation. The young isochronic parabionts regenerated well whereas the old isochronic parabionts regenerated poorly as shown by failure of myotube formation, and prominent fibrosis at the site of injury. There was a significant improvement in the regeneration of muscles in elderly mice which were paired with younger mice compared to aged isochronic pairings. The appearance of newly formed fibres (identified by the expression of neonatal myosin heavy chain) in the elderly animals attached to a young partner was similar to that seen in young mice.

The molecular mechanism implicated in the above response and shown to be directly linked with impaired muscle regeneration is the Notch signalling pathway (Conboy et al., 2003). In young isochronic and heterochronic parabionts there was a marked up-regulation of cell surface protein Delta-1 (an activator of the Notch signalling pathway) in satellite cells, whereas Delta-1 induction was lacking in the old, isochronic parabionts (Conboy et al., 2005). Thus, heterochronic parabiosis not only improves the proliferative response of satellite cells, it also restores the key molecular signalling in these cells that is essential for muscle regeneration. A subsequent study suggested that the cause of impaired satellite cell proliferation is an imbalance between Notch and
TGF-β/pSmad signalling pathway which are suggested to inhibit satellite cell regenerative potential (Carlson et al., 2008, Carlson et al., 2009b).

Strong evidence implying involvement of a circulating factor in satellite cell determination and proliferation comes from in vitro experiments in which a mouse muscle was extracted from young C57-BL/6 male mice, and cultured in serum obtained from elderly (22-24 months) or young (2-3) mouse (Carlson and Conboy, 2007). Two fold increases in desmin, BrdU and Myf5 expression were found in cells cultures in young serum as opposed to elderly serum, indicating that greater muscle proliferation in a more youthful circulating environment. More recently, in vitro experiments in which human myoblasts from young and old subjects were cultured in the presence of young or aged blood sera (isochronic and heterochronic) (Carlson et al., 2009b), showed similar results to that obtained in mice. Cells exposed to young human serum had increased expression of the proliferating muscle markers desmin and Brdu compared to cells cultured in elderly human serum. In contrast, However, Bayer et al. (2012) found that no difference in tendon cell proliferation and collagen expression when cells were exposed to human serum from young and elderly subjects.

The results of the experiments described above thus suggest that there is an age associated decline in the properties of the circulatory environment which impairs the regeneration potential of aged satellite cells. Ageing has been shown to result in increased Wnt signalling in serum, which is associated with increased fibrosis (Brack et al., 2007), increased serum levels of TGF-β, which inhibits satellite cell behaviour (Carlson et al., 2009a), reduced serum levels of IGF-1 which act as a potent mitogen of satellite cells in culture (Allen and Boxhorn, 1989, Goldspink and Harridge, 2004).
Numerous other causes have been suggested to affect the efficiency of skeletal muscle regeneration within aged environment. These include reduced capillarization (Ryan et al., 2006), thickening of the basal lamina (Snow, 1977a), and increase in collagen content within the muscle (Alnaqeeb et al., 1984).

In contrast to the in vivo and in vitro observations discussed above, it has been shown that human myoblasts from old donors can contribute myonuclei very efficiently to regenerating mouse muscle in vivo (Schafer et al., 2006). Single fibre grafting experiments in mice have shown that satellite cells from old animals can contain equivalent in vivo myogenic and self-renewal potential to that of young animals (Collins et al., 2007), and studies involving transplantation of whole muscles have not reported differences in myotube formation between elderly and young muscles grafted into elderly or young animals (Smythe et al., 2008). This suggests no differences in the regeneration of muscle and formation of myotubes with age. There was, however, a delay in myotube formation observed in the older animals. A more recent study by Shavlakadze et al. (2010) similarly showed a delay in the onset of regeneration after autografting muscle into a geriatric mouse host, though again the long term formation of new muscle was unaffected and there was no effect of the geriatric host on regenerating muscle. Other animal studies which have used a variety of different models including denervation (Dedkov et al., 2003), cross transplantation in mdx mice (Boldrin et al., 2009) as well as chronic low-frequency electrical stimulation (Putman et al., 2001) have similarly reported no age-related impairment to muscle repair.
Several studies have found age associated differences when compared in vitro differentiation of primary human cells from donors of different ages. Fulle et al. (2005) compared human muscle cells obtained from a newborn (5 day old), young (29 years old) and old (71 years old) subjects with 85, 71 and 47% of desmin cells respectively. They found that the percentage of MHC positive cells (Fusion index) had decreased in the elderly subject after 5 days in culture when compared to newborn and young subjects. This status was suggested to be due to a decrease in antioxidant capacity of Catalase and Gluthatione transferase in satellite cells derived from the elderly compared to that in cells isolated from young individuals. Subsequent experiments by this group have shown a 20% decrease of fusion index (as measured after 7 days of in vitro differentiation and expressed as the percentage of nuclei in myosin heavy chain positive cells containing at least two nuclei) in elderly subjects (83 ± 6 years old, n=3) when compared to young subjects (30 ± 2 years old, n=3); (Beccafico et al., 2007). They also have shown that myotubes derived from elderly subjects are thinner and contain few myonuclei compared to the ones derived from the young individual.

Beccafica et al. (2010) isolated myoblasts from the vastus lateralis muscle of 10 young (29 ± 6 years old) and 11 old (77 ± 6 years old) and cultured them in either growth media or differentiation media. These authors showed that myoblasts from older subjects displayed lower levels of Brdu incorporation when compared to younger subjects thus suggesting impaired proliferation in the elderly cells. Western blotting analysis revealed no expression of the early myogenic differentiation marker; myogenin; or the terminal differentiation marker, MHC in myoblasts derived from older subjects compared to younger subjects. In addition, when compared to those of younger subjects, the older myoblasts displayed significantly higher levels of S100B, an
intracellular protein known to inhibit myoblast differentiation, and lower levels of membrane-bound RAGE, a receptor promoting myoblast differentiation.

Corbu et al. (2010) isolated myoblasts from the vastus lateralis muscle of 3 young (from 4 months to 16 years old) and 6 old (74-76 years old) subjects. These authors showed that the myogenic potential seemed to be compromised in the aged of human muscle cells. However, it may be difficult to draw parallels between the old subjects (74-76 years old) and the young controls aged from 4 months to 16 years old used in this study; the hormonal profile during childhood and puberty is higher in growth factors than in adulthood, which might explain the marked increase in myogenicity seen in the younger cells.

In another human study, Lorenzon et al. (2004) compared human muscle cells obtained from a 2 year old, 21 year old, 48 year old and 76 year old. They found fusion index and number of nuclei per myotube were decreased between the 21 and 76 year old subjects after 4, 6 and 9 days in culture. Furthermore, myotubes formed from satellite cells extracted from aged donors show a delay in the maturation of the excitation-contraction coupling mechanism when compared to young donor cells. Jacquemin et al. (2004) compared cells from biopsy tissue extracted from a 5 day old, 17 year old and 65 year old human. They found fusion index and the mean number of nuclei per myotube were decreased between the 17 and 65 year old subjects after 3 days in culture. Whilst a further in vitro study showed that myoblasts extracted from muscles of elderly people do not fuse as efficiently as those from younger subjects (Pietrangelo et al., 2009). This was suggested to be due to accumulation of oxidative damage, altered turnover of cytoskeleton and extracellular matrix degradation, and activation of atrophic pathways.
through the FOXO-dependent program following analysis of mRNA expression profiles in these cells from microarray analysis.

Critically, several of the studies described above draw conclusions from comparison of very small numbers of subjects, often only one from each age group (Fulle et al., 2005, Jacquemin et al., 2004, Lorenzon et al., 2004) or from comparing populations that do not have equivalent proportions of cells expressing desmin (Beccafico et al., 2007, Pietrangelo et al., 2009) or have not been characterised in terms of desmin content (Beccafico et al., 2010). Desmin content reflects the proportion of myogenic cells in the culture and therefore determines differentiation potential of a population independently of age. Studies comparing only one subject from each age group do not provide strong enough evidence on their own of age related effects, since in the human population there are very large variations in factors other than age (i.e. genetic and lifestyle factors) that could provide an explanation for the effects observed.

1.5 In vitro ageing

1.5.1 Replicative senescence

Serial passage of normal somatic cells ultimately results in a state of permanent growth arrest called cellular senescence. Such a phenomenon also known as replicative senescence was first described by Leonard Hayflick and Paul Moorhead who observed that normal human fibroblasts had a limited life span after being serially passaged in culture (Hayflick and Moorhead, 1961). They described three phases in the growth history of human diploid cell strains (Figure 1.5). Phase I lasts 1 to 3 weeks and is described as the primary culture phase when the cells have been freshly freed from
intact tissue and cover the surface of the culture flask. Phase II lasts 2 to 10 months and represents the period when cells divide in culture. Phase III occurs when cell replication progressively slows until the cells ultimately stop dividing and enters replicative senescence. Even though initial experiments were with fibroblasts, replicative senescence has been observed in other cell types including satellite cells, keratinocytes, endothelial cells and lymphocytes (Decary et al., 1997, Kang et al., 1998, Spaulding et al., 1999, Wagner et al., 2001).

Figure 1.5. History of cell strains and the phenomenon of cell alteration during proliferative senescence. Taken from (Hayflick and Moorhead, 1961).
Senescent muscle cells differ in a number of ways from early passage cells. Morphologically, senescent cells display a larger and more flattened morphology with an increased in microfilament content (Renault et al., 2000). Senescent cells can be identified in vitro by a specific marker of senescence; senescence associated β-galactosidase (Dimri et al., 1995, Gruber et al., 2010) and these cells display dramatic changes in chromatin structure, with formation of senescence associated heterochromatin foci (SAHF) (Narita et al., 2006). Furthermore, senescent cells are characterised by an irreversible G1 growth arrest with up-regulation of the CDK inhibitors p16 and p21 (Stein et al., 1999). Although unable to replicate, senescent cells can remain metabolically active for a long periods of time and are resistant to apoptosis (Gruber et al., 2010, Hampel et al., 2005).

One mechanism that is suggested to lead to replicative senescence is telomere shortening. Telomeres are TTAGGG repeats located at the end of linear chromosomes that protect the DNA ends from degradation and recombination. It has commonly been thought that telomere loss acts as a mitotic clock that signals senescence when one or more telomeres reach a critical length (Chan et al., 2001, de Lange, 2005, Mouly et al., 2005). In eukaryotes, telomere shortening occurs with every round of cell division due to the inability of DNA polymerase to copy the ends of linear molecules (Blasco, 2005, Bodnar et al., 1998). Eventually telomeres become critically short, which in turn induces a DNA damage response characterized by the formation of double strand DNA breaks containing the phosphorylated histone H2AX, that activate the p53 tumor suppressor protein resulting in up-regulation of cell cycle inhibitors and telomere-initiated senescence (Celeste et al., 2002, de Lange, 2005, von Zglinicki et al., 2005).
1.5.2 Replicative potential and senescence in human myoblasts

Myoblasts obtained from healthy people appear to show different proliferative potential depending on the age of the individual at the time of the isolation. Myoblasts extracted at birth can undergo 60-70 population doublings (PD) in culture before entering replicative senescence. Myoblasts obtained from a five month old individual they underwent 43 PD whereas and after 20 years the number of PD was 15-25 before cells entered replicative senescence (Decary et al., 1997). Interestingly, the dramatic decrease observed in the in vitro replicative potential of myoblasts occurs from birth to 20 years of age followed by a period of relative stability thereafter (Decary et al., 1997). This initial decrease in replicative potential is thought to be related to the rapid growth of skeletal muscle during childhood and adolescence. Stabilization of the in vitro replicative potential of adult myoblasts is thought to coincide with decreased muscle growth.

Senescent myoblasts have been used as a model to study muscle ageing. Senescent myoblasts exhibit a lower fusion index and fewer nuclei per myotube than proliferating (early passage) or “young” cells (Bigot et al., 2008). The expression of two fibroblastic markers, CTGF (connective tissue growth factor) and collagen IV also increase in senescent myoblasts. Machida et al. (2004) have shown decreased proliferation and differentiation potential in muscle-derived stem cells after passaging procedure (Machida et al., 2004). They showed that the percentage of cells that were positive for the myogenic markers desmin, MyoD and Pax7 was reduced from 90% to 55%, and the percentage of nuclei in myotubes decreased from 46.7% to 12.5% after the third passage. More recently, C2C12 mouse skeletal myoblasts were used to investigate the impact of serial passaging on the behaviour of myoblasts (Sharples et al., 2011).
Following multiple divisions, these cells displayed a reduced levels of muscle creatine kinase (marker of differentiation) compared to control cells. Furthermore, these cells had significantly reductions of IGF-1, MyoD and myogenin together with elevated IGFBP5. Taken altogether, these studies suggest that serial passaging procedure has a detrimental effect on myoblast performance, but it remains to be determined if this reflects the ageing process in vivo.

1.5.3 Senescence-Associated Secretory phenotype (SASP)

The conditioned medium of senescent cells in vitro is enriched with secreted proteins (Krtolica and Campisi, 2002, Shelton et al., 1999). The SASP concept was first described by the Campiai group, when they realized that secreted factors from senescent fibroblasts promote the development of age-related epithelial cancer. A series of recent papers have identified a range of cytokines involved in SASP (Coppe et al., 2010, Coppe et al., 2008, Rodier et al., 2009). Coppe et al. (2008) compared proteins secreted by pre-senescent human fibroblast with senescent human fibroblast cells by incubating each culture in serum free-medium. Using antibody arrays, the study highlighted the increased secretion of IL-6, IL-7, IL-8, IGFBPs and TNF-receptors in senescent conditioned medium when compared to pre-senescent conditioned medium. Furthermore, SASP induction was observed in a number of different cell types, including human epithelial cells (Coppe et al., 2008), and human oral keratinocytes (Kim et al., 2011b). Current interest in the senescent secretome has revealed several molecules that are important players in extracellular matrix (ECM) remodelling. A group of factors that have been identified as potential regulators of the ECM and
secreted by senescent cells which include IGF1, Wnt, Plasmin and TGF-β (Kuilman and Peeper, 2009).

Among the factors identified in the SASP, three are of particular relevance to the present work as they have been shown to affect myoblast behaviour. These are TGF-β, TNF-α, and IL-6.

### 1.5.3.1 Transforming Growth Factor Beta (TGF-β)

TGF-β belongs to a family of proteins that includes nodals, activins, bone morphogenic proteins (BMP), anti-Mullerian hormone, myostatin and other related factors found in vertebrates, insects, and nematodes (Massague, 1990, Massague, 1998). Five unique isoforms are found in different species: TGF-β 1, 2 and 3 are mammalian, TGF-β4 are avian, and TGF-β5 are amphibians isoforms (Roberts and Sporn, 1992). TGF-β was first described as sarcoma growth factor, secreted by transformed fibroblasts (de Larco and Todaro, 1978). TGF-β1 is also produced by several cell types including: platelets, lymphocytes, macrophages, neutrophils, injured skeletal muscle, and C2C12 mouse skeletal myoblasts (Assoian et al., 1987, Assoian et al., 1983, Grotendorst et al., 1989, Haugk et al., 1995, Henningsen et al., 2010, Kehrl et al., 1986, Peters et al., 2005). TGF-β1 is a pleiotropic cytokine which appears to play an important role in cell proliferation, differentiation, migration, angiogenesis, apoptosis, inflammation and scarring in various tissues (Heldin et al., 2009, Lawrence, 1996, Massague, 1998, Massague and Chen, 2000).
TGF-βs are synthesised as homodimeric pro-proteins (proTGF-β) that are proteolytically processed in the Golgi apparatus by furin-type enzymes. Furin cleaves the dimeric TGF-β-propeptides (the latency-associated protein “LAP”) from the mature TGF-β homodimer which remains non-covalently bound to the growth factors. This complex is known as the small latent complex (SLC). Once the SLC has been targeted to the extracellular matrix (ECM), LAP covalently binds to latent TGF-β binding protein (LTBP) to form the large latent complex (LLC) via disulfide bonds (Figure 1.6). LTBP may function to facilitate secretion of the TGF-β-LAP complex and to promote targeting of TGF-β to the ECM, where activation takes place. The TGF-βs are primarily stored in this latent form (L-TGF-β) in the ECM and require activation before being able to bind to their cell surface receptors and exert an effect on their target (Annes et al., 2003, Massague and Chen, 2000, Rifkin, 2005).

TGF-βs act through binding to TGF-β type I and TGF-β type II receptors that form a heteromeric complex with the ligand (Derynck and Feng, 1997). These receptors are serine/threonine kinase receptors. In the complex, the binding of TGF-β to the type II receptor, activates and recruits the type I receptor to the complex to initiate a TGF-β signalling response to the nucleus (Verrecchia and Mauviel, 2002). Subsequently, type I receptor phosphorylates transcriptional factors of the Smad family which are translocated to the nucleus to mediate the effects of TGF-β at the cellular level (Figure 1.7) (Chaudhury and Howe, 2009). Furthermore, TGF-βs also activate different non-Smad pathways, including PI3K, Ras, Par6, Jnk/p38/MAPK pathways, some of which regulate important cellular processes (Chaudhury and Howe, 2009).
Figure 1.6. The TGFβ large latent complex (LLC). The LLC comprises TGFβ (black), LAP (red) and LTBP. TGFβ and LAP are proteolytically separated at the site indicated by the arrowhead. After processing, TGFβ remains noncovalently associated with LAP. LAP and LTBP are joined by disulfide bonds (light blue lines). The LLC is covalently linked to the extracellular matrix (ECM) through an isopeptide bond (green) between the N-terminus of LTBP (somewhere between EGF-like domain and the hinge domain, arrow) and a currently unidentified matrix protein. The hinge domain (arrow) of LTBP is a protease-sensitive region that allows LLC to be proteolytically released from the ECM. Taken from (Annes et al., 2003).
**Figure 1.7.** Schematic Representation of Canonical and Non-canonical TGFβ Signaling Pathway. Taken from (Chaudhury and Howe, 2009).
The ability of TGF-β1 to inhibit myoblast differentiation and to depress proliferation has been documented (Allen and Boxhorn, 1987, Greene and Allen, 1991, Massague et al., 1986). There is some evidence to suggest that TGF-β1 levels become elevated with age and this has been suggested to be the mechanism by which satellite cell activation is impaired in older muscle (Carlson et al., 2009a, Carlson et al., 2009b). Culturing C2C12 myoblasts in the presence of 5 ng/ml of human recombinant TGF-β1 has been shown to increase proliferation and inhibit differentiation (Schabort et al., 2009). Molecular analysis has indicated that this effect of TGF-β1 results from the increased nuclear localisation of proliferating cell nuclear antigen (PCNA), MyoD degradation, and decreased p21, myogenin and MHC expression. In this experiment the inhibition of differentiation was reversible once TGF-β1 was eliminated. Subsequent studies have shown that TGF-β1 decreases fusion of C2C12 myoblasts in an isoform-independent manner (Schabort et al., 2011).

More recently, human myoblasts were cultured in the presence of 25 ng/ml human recombinant TGF-β1 vs. control (Carlson et al., 2009b). It was found that myoblast proliferation and differentiation was dramatically reduced by TGF-β1. It has been postulated that TGF-β1 is capable of maintaining satellite cell in quiescence following isolation for cell culture, a process which normally involves their activation, in a dose dependent manner (3, 10, and 30 ng/ml) (Rathbone et al., 2011). Beggs et al. (2004) demonstrated that during proliferation and differentiation, treatment with TGF-β1 (1 ng/ml) increased the phosphorylation of Smad2/3 and decreased MyoD and myogenin expression. This suggest that Smad acts as downstream of TGF-β1 to inhibit the function of myoblasts.
1.5.3.2 Tumour Necrosis Factor Alpha (TNF-α)

TNF-α, an endogenous cytokine, is secreted by a number of cells including: fibroblast, muscle, macrophages, neutrophils, adipose tissue, vascular smooth muscle and vascular endothelium (De Rossi et al., 2000, Hotamisligil et al., 1995, Liu et al., 2000, Zoico and Roubenoff, 2002). TNF-α is released after proteolytic cleavage by the TNF converting enzyme (TACE, a member of the a-disintegrin-and-metalloproteinase (ADAM) family), and mediated its effects via two distinct receptors: type 1 (TNFR1) and a type 2 (TNFR2) (Sethi and Hotamisligil, 1999).

TNF-α is a pleiotropic pro-inflammatory cytokine, which has been considered a pathological factor in different condition such as muscle wasting (Bhatnagar et al., 2010). The muscle wasting occurs via the activity of several important pathways (Figure 1.8). TNF-α activates caspase-8 (apoptosis pathway), the ubiquitin proteasome pathway (protein breakdown and muscle atrophy pathway), MAPK family pathway, and reduces the rate of protein synthesis which ultimately compromised skeletal muscle regeneration (Gomes et al., 2001, Li et al., 1999, Llovera et al., 1997, Mercier et al., 2002, Moresi et al., 2008, Phillips and Leeuwenburgh, 2005, Reid and Li, 2001).

Langen et al. (2004) demonstrated that in C2C12 myoblasts, 10 ng/ml TNF-α initially activated proliferation by increasing cell cycle progression and subsequently reduced differentiation. This effect was associated with activation of the NF-κB pathway, which has a role in destabilization of MyoD (Guttridge et al., 2000). This effect was reversible once TNF-α was removed (Langen et al., 2001). It has also been shown that creatine kinase, MyoD and myogenin expression are reduced in the presence of 20 ng/ml of TNF-α when compared with control cells. Similar results were reported by Foulstone et
al. (2004), using primary culture human myoblasts (20 ng/ml), and Saini et al. (2008), using C_{2}C_{12} myoblasts (1.25 ng/ml). However, this effect is concentration dependent, as 0.05 ng/ml of TNF-α has been shown to stimulate myoblast differentiation, while 0.5-5 ng/ml are inhibitory (Chen et al., 2007).

Figure 1.8. TNF-α signalling pathway. Taken from (Mocellin et al., 2005).
1.5.3.3 Interleukin-6 (IL-6)

IL-6, a pleiotropic cytokine, was first discovered by Weissenbach and others in human fibroblasts (Weissenbach et al., 1980). IL-6 is produced by a number of cells including: skeletal muscle cells under various conditions, adipocytes, macrophages, neutrophils, endothelial cells and osteoblasts (Akira et al., 1993, Corbel and Melchers, 1984, De Rossi et al., 2000, Heinrich et al., 1990, Nagaraju et al., 1998, Pedersen et al., 2001). Exercise rapidly increases the nuclear transcriptional rate of IL-6 within the contracting muscles, with a 10- to 20-fold increase in mRNA content observed following 30 minutes of “non-damaging” exercise and a possible peak (up to 100-fold) immediately at the end of the exercise bout (Keller et al., 2001). Furthermore, The cytokine IL-6 is produced and release by muscle fibres into the circulation during exercise (Penkowa et al., 2003). At the membrane of target cells, IL-6 exerts its effect by binding to the membrane bound receptor α subunit (IL-6Rα) and association of the receptor with gp130 to trigger signalling transduction (Taga et al., 1989). Cellular effects are induced through the JAK/STAT pathway (Figure 1.9) (Heinrich et al., 1998, Ni et al., 2004). In response to IL-6 binding, JAK becomes activated which then activates and phosphorylates STAT3 at a Tyrosine residue at position 705. Two activated STAT3 molecules will homodimerise and translocate to the nucleus to activate transcription.

IL-6 is a pro-inflammatory cytokine and IL-6 infusion or over-expression in mice increases muscle atrophy (Bonetto et al., 2011, Strassmann et al., 1992, Tsujinaka et al., 1995). Following 14 days of intramuscular infusion of IL-6, loss myofibrillar content and decreased total protein content have been observed in tibialis anterior in rats (Haddad et al., 2005). Furthermore, it has been observed that chronic IL-6 exposure on young animals can also slow the rate of muscle growth (Bodell et al., 2009).
In contrast to the previous finding, IL-6 knockout mice demonstrated a blunted hypertrophic muscle growth compared with wild-type mice as a result of reduced myonuclear number as well as satellite cells proliferation (Serrano et al., 2008). The ablation of IL-6 decreases the expression of myogenin in C2C12 myogenic cells, whereas the administration of IL-6 (2 ng/ml) markedly increases the expression of this protein (Baeza-Raja and Munoz-Canoves, 2004). A recent study reported that recombinant IL-6 (10 ng/ml) promoted proliferation of human myoblasts (Wang et al., 2008).

The contrasting effects of IL-6 on muscle remain to be explained, but one possibility is that endogenous levels produced during non-inflammatory situations are beneficial whereas when IL-6 is overproduced in scenarios where inflammation or other pathological process is occurring is detrimental.

Figure 1.9. IL-6 signalling pathway. Taken from (Ni et al., 2004).
1.7 Summary

Sarcopenia is the age-related loss of muscle mass. Sarcopenia is a prevalent condition which can contribute to a reduced quality of life and increased mortality risk in the elderly. Satellite cells are the muscle stems which are used in muscle maintenance, repair and hypertrophy. There is growing interest in the behaviour of these cells in regard to muscle ageing. Once removed from their anatomical location satellite cells (muscle precursor cells or myoblasts) can be studied in culture and repetitively passaged until they reach proliferative senescence (a state where they are no longer able to divide). This approach has been used as a model of muscle ageing. However, there is uncertainty as to how in vitro ageing of cells reflects of the behaviour of these cells aged in vivo, i.e. when obtained from elderly people.

Two models have been proposed to explain defective regeneration of aged muscles: 1) systemic factors impair satellite cell responses or 2) satellite cells themselves exhibit a decline in function with age. The overall aim of this thesis was to study the behaviour of human myoblasts in regard to their ability to proliferate and differentiate in vitro by obtaining cells from muscle biopsy samples from subjects of different ages and to examine the effects of senescence and age-associated inflammatory factors on these cells.
1.8 Hypotheses

The work in this thesis therefore aims to test three main hypotheses:

1- Myoblasts aged in vivo (obtained from old subjects) display a similar cellular phenotype to senescent myoblasts (aged in vitro) and these are distinct from that of myoblasts obtained from young subjects. (Chapter 3 and 4)

2- Inflammatory cytokines inhibit proliferation and differentiation of human myoblasts in a dose dependent manner. (Chapter 5)

3- Culture of human myoblasts in elderly human serum will inhibit proliferation of human myoblasts compared to culture in young human serum. (Chapter 6)

To address my hypotheses the specific aims were of the thesis are listed below:

1- To investigate differences between young and old myoblasts in term of their optimal proliferation and differentiation in vitro.

2- To compare the differentiation potential of cells obtained from young and old people with those of cells that reached proliferative senescence (i.e. were “aged” in culture).

3- To analyse the cytokine profile (secretome) of cells from young, old and senescent myoblasts during differentiation.

4- To elucidate the role of aged-associated inflammatory cytokines in the behaviour of human myoblasts.

5- To investigate the effects of young or old human serum to sustain or inhibit proliferation of human myoblasts.
Chapter 2: Methods
This chapter describes the general techniques used, further specific details can be found within the relevant chapters.

2.1 Human subjects and ethical approval

All subjects gave written informed consent to take part in the studies reported in this thesis. Before inclusion, all experimental protocol was described to potential subjects in detail and time was allocated to allow for the individual to ask any questions that they may have had regarding any aspect of the study. Following this meeting, subjects were asked to complete a healthy questionnaire as defined by (Greig et al., 1994) and only healthy individuals were included in the study. Ethical approval was given by the London Research Ethics Committee and all experimental procedures were performed in a Human Tissue Authority licensed laboratory in accordance with the Human tissue Act (2004) and the Declaration of Helsinki.

2.2 Muscle biopsy procedure

Muscle samples were obtained from the vastus lateralis muscle by using the needle muscle biopsy technique (Bergstrom, 1962). Briefly, subjects rested in a supine position. The skin overlaying the area to be sampled was shaved, if required, and thoroughly cleaned with chlorhexadine solution and then anaesthetised (2% lignocaine). After allowing a short time for the anaesthetic to take effect, a small scalpel blade was used to make a 0.5 cm incision through the skin and muscle fascia. A biopsy needle was then inserted through the incision into the muscle belly, and suction applied via a 50 ml syringe. Each biopsy provided 100-300 mg of muscle tissue and was immediately
placed in ice cold Solution A (7.2mg/ml HEPES, 7.6mg/ml NaCl, 224μg/ml KCl, 1.98mg/ml D-Glucose, 1.134μg/ml Phenol red, ddH2O, pH 7.6; Sigma Aldrich).

2.3 Blood sampling and serum extraction

Following the biopsy procedure, blood (A maximum of 50ml) was drawn from an antecubital vein into a 10-ml serum vacutainer tube (BD vacutainer ref. 367958). After coagulating for 30 min at room temperature, the blood was centrifuged at 3000g for ten minutes. Serum was immediately pipetted off and divided into aliquots of one millilitre and stored at -70ºC. A fresh aliquot was subsequently thawed for each experiment.

2.4 Cell culture

All cell manipulations were performed in a positive pressure tissue culture hood. Prior and subsequent to each manipulation, the tissue culture hood surfaces were cleaned with 1% Virkon solution followed by 70% ethanol.

2.4.1 Satellite cell extraction

The muscle biopsy material was moved into the sterile tissue culture hood and washed with Solution A to remove any surface blood. It was then stripped of visible connective and fat tissue using sterile scalpels and the remaining tissue was cut into pieces less than one millimetre in diameter on a petri dish containing 5ml ATE solution (solution A + 5mg/ml Trypsin EDTA, Invitrogen). A wide bore pipette (25 ml) was then used to transfer the enzyme solution containing minced muscle, to a sterile 35 ml Wheaton flask
containing a magnetic stirrer. The dish was washed twice with 5ml ATE solution to collect any muscle fragments which were not aspirated on the first attempt, and placed into the flask making the total volume up to 15 ml. The muscle fragment suspension was heated in a stirring water bath at 37°C for 15 min with gentle agitation to dissociate muscle cells. The supernatant containing suspended cells was aspirated and transferred to a sterile 50 ml falcon tube (BD Bioscience, Belgium) containing 15 ml DMEM (Invitrogen), 10% foetal calf serum (FCS) Gold (PAA) and 1% PSG (100 units/ml Penicillin, 100 μg/ml Streptomycin and 292 μg/ml L-Glutamine, Gibco). Fresh ATE solution (15ml) was added to the remaining muscle and the entire procedure repeated twice. The three supernatants were centrifuged for six minutes at 650g. Following centrifugation, the supernatant containing enzymes was discarded and the cell pellet was re-suspended in skeletal muscle growth medium (Promocell, Germany; containing 50 μg/ml Foetal Calf Serum (FCS), 50 μg/ml Fetuin (bovine), 10 ng/ml Epidermal Growth factor, 1 ng/ml Basic fibroblast Growth Factor, 10 μg/ml insulin and 0.4 μg/ml Dexamethasone) supplemented with 10% FCS and PSG (100 units/ml Penicillin, 100 μg/ml Streptomycin and 292 μg/ml L-Glutamine) or basal medium (Promocell, Germany). At this point in the procedure the cells are referred to as freshly isolated cells.

2.4.2 Estimation of cell viability

A Neubauer haemocytometer was used in order to make an estimate of viable cell number, 10 μl of well mixed cell suspension, 4 μl of 0.5% trypan blue and 6 μl of skeletal muscle growth medium were mixed in a sterile 1.5ml microcentrifuge tube. The solution was placed in the cell incubator (37°C, 5% CO₂) for 5 min. 10 μl of this
solution were loaded into the haemocytometer. Cells in the four corner grids were counted under a Nikon model inverted microscope at x 100 magnification. Viable cells identified as iridescent, with rounded morphology. Non-viable cells lost their membrane integrity and hence were trypan blue positive. The resultant mean of 4 grids was calculated, which represented average cell numbers occupying 0.1 mm³. The percentage of viable cells was calculated as:

\[
\% \text{ viability} = \frac{\text{number of viable cells counted}}{\text{total number of cells counted}} \times 100
\]

The total number of cells was calculated as:

\[
\text{Cells.mL}^{-1} = \text{Average cells in 4 grids} \times \text{dilution factor (2)} \times 10^4 \text{.mL}^{-1}
\]

2.4.3 Expansion of freshly isolated cells

Freshly isolated cells resuspended in GM were transferred to sterile T75 flask. Cells were cultured in a humidified incubator at 37°C and 5% CO₂. The medium was refreshed on the third day after the extraction procedure and subsequently every 48 hours. Cells were passaged every 48-96 hours depending on when they reached 50% confluence as described in Section 2.4.4. The cells were expanded for two weeks and frozen in liquid N₂. Cells that were cultured for two weeks, stored in liquid nitrogen and thawed are referred to in this thesis as committed myoblasts.

2.4.4 Passaging procedure

Cells were passaged every 48-92 hrs or at 50% confluence. Culture medium was removed and placed in a 50 ml Falcon tube. The flask was then gently washed twice with warmed (37°C) Phosphate buffer saline (PBS, Invitrogen) to remove any serum,
and this solution also added to the 50 ml tube. To enzymatically detach cells from their culture flask, a pre-warmed (37°C) solution of 5mg/ml trypsin EDTA (Sigma) in PBS was added and the flask was transferred to the incubator for 2-5 mins. Dissociation of the cells from the base of the flask was checked under the microscope, if necessary flask was gently tapped to facilitate cell release. The cells were then transferred to the 50 ml tube containing used media and PBS. The cell suspension was centrifuged (6 mins at 650g) and the cell pellet was resuspended in a volume of GM or basal medium. A small volume (10µl) was removed to check the total cell number and viability (Section 2.4.2).

2.4.5 Thawing procedure

Growth medium was placed in vented T75 flasks. Flasks were incubated in the cell culture incubator for 30 minutes. Following removal from liquid nitrogen tanks, cell vials were immediately placed in a water bath at 37°C and allowed to thaw as quickly as possible. Vials were cleaned with 70% ethanol and transferred to the culture hood. A small amount of pre-warmed growth medium was transferred from the flask to the cryovial containing the cells and the liquid was gently aspirated. All medium from the cryovial was added dropwise into the tissue culture flask and the cell suspension mixed gently. The flask was placed in the incubator and monitored for cell adherence over the next 24 hours.
2.5 experiment with freshly isolated cells

2.5.1 Culture of cells in human serum

Freshly isolated cells were resuspended in basal medium. Cells were plated in duplicate wells of a 24 well plate, coated with ECM matrix (8 mg/ml, Sigma), at a density of 100,000 cells per well. To each well was added an equal volume of 2X GM containing all supplements at twice the working concentration described in section 4.2.1 or of basal medium supplemented with 30% human serum and 2% PSG. This resulted in final serum and antibiotic concentrations were halved and were at the appropriate working concentrations. Media were changed 72 hours after initial isolation and every 48 hours thereafter. Before discarding, old media was transferred to a sterile 1.5ml microcentrifuge tube and centrifuged at 650g for six minutes to harvest any floating myoblasts. The supernatant was discarded and the cell pellet was re-suspended in fresh medium and added to the appropriate culture wells. Cells were fixed at 3, 5 and 7 days after isolation.

2.5.2 Culture with human recombinant cytokines

Freshly isolated cells were resuspended in basal medium. Cells were plated in duplicate wells of a 24 well plate, coated with ECM matrix (8 mg/ml, Sigma), at a density of 100,000 cells per well. Equal volumes of 2X GM (control) or 2X GM supplemented with twice the desired final cytokine concentration and 2% PSG were then added to each well such that final cytokine, serum and antibiotic concentrations were halved. For differentiation assays on freshly isolated cells, three days after plating in GM “at this time cells appear attached to the plates” as described above the medium was aspirated and replaced with differentiation medium (DM), the skeletal muscle cell basal medium
supplemented with 0.5% BSA and 1% PS (100 units/ml Penicillin, 100 μg/ml Streptomycin) and no cytokines (control) or with 1 ng.ml⁻¹ TNF-α, TGF-β or IL-1β. Media were changed every 48 hrs. Before discarding, old media was transferred to a sterile 1.5ml eppendorf tube and centrifuged at 650g for six minutes to harvest any floating myoblasts. The supernatant was discarded and the cell pellet was re-suspended in fresh medium and added to the appropriate culture wells. Cells were fixed after 7 days in differentiation medium.

2.6 Committed human myoblast culture

After thawing (Section 2.4.5), cells in this thesis are referred to as committed myoblasts. The cells were cultured in GM and the medium was changed every 48 hrs. Once the cells were 50% confluent after a total of 48-92 hrs, they were counted passaged once (Section 2.4.4) and divided for experimental procedures as outlined below.

2.6.1 Population Doubling Time

The cells were cultured in growth medium as described above. Media was replaced with fresh GM every 2 days. Cells were passaged as described in section 2.4.4 at 50% confluence. Cell pellet was resuspended in GM and counted as described in section 2.4.2. For population doubling experiments cells were plated at a density of 2.5 X 10³ cells/cm² in 35mm culture dishes in triplicate. The number of population doubling at every passage was calculated as PD = log (N₁/N₀)/log2 where N₁ is the final cell number and N₀ is the initial number of cells seeded. Proliferative senescence was defined as
failure of cells to divide for three consecutive weeks (Bigot et al., 2008, Di Donna et al., 2003).

### 2.6.2 Proliferation and differentiation assays

Cells were passaged once (Section 2.4.4) and cultured in GM to 50% confluence. At this point, cells were plated in 96 well dishes at a density of 1000 cells/well. Cells were fixed after 24 hr and different antigens were examined by immunocytochemistry (Section 2.8). For differentiation assays, cells were plated in duplicate in 96 well dishes at a density of 7000 cells/well. Media was replaced after 24 hr with differentiation medium (DM), containing basal medium supplemented with 10 µg/ml insulin (Sigma), 10 µg/ml transferin (Sigma) and 1% PS (100 units/ml Penicillin, 100 µg/ml Streptomycin). Cells were cultured for a further 7 days in differentiation medium, with aliquots of cells being fixed every 24 hr.

### 2.6.3 Collection of conditioned media

At early passage (young and old cells) and late passage (senescent cells), aliquots of 7000 cells were plated into a 96 well dishes in growth medium. After 24 hr, differentiation was induced by simply replacing growth medium with differentiation medium. Conditioned media (CM) were collected after two 48 hour incubation periods after placing the cells in DM: The period between day 0- and day 2 of differentiation (i.e. the first 48 hours in DM) and between days three and five of differentiation (i.e. 72-120 hours). CM was immediately stored at -70ºC.
2.7 Reconstitution of cytokines

10 µg recombinant human TNF-α (Calbiochem, Nottingham, UK) was purchased and initially prepared as x 1000 stock solution at 100 ng µl⁻¹ TNF-α containing sterile distilled water. It was stored at -20ºC until required.

Recombinant human TGF-β was purchased from R&D systems, Inc. The 2 µg pellet was reconstituted in 20 µl of sterile HCL making the stock solution concentration of 0.10 µg µl⁻¹. It was stored at -20ºC until required.

Recombinant human IL-1β, IL-6 and IL-8 were purchased from R&D systems, INC. The 5 µg pellet was reconstituted in 50 µl of sterile PBS making the stock solution of 0.10 mg/ml. It was stored at -20ºC until required.

2.8 Immunocytochemistry

2.8.1 Fixation procedure

At the end of all experiments, cells were fixed in their wells by the addition of an equal amount of 2X PFA medium (7.4% paraformaldehyde +/- 0.4% Triton) to the experimental culture medium for 10 mins. The fixative and medium were then aspirated off and the wells were washed 2X for 5 mins with PBS/0.01%NaN₃ to remove any excess paraformaldehyde and triton and stored at 4ºC for up to one week.
2.8.2 Immunocytochemistry procedure

After fixation cells were incubated in blocking solution, 1% bovine serum albumin fraction V ≥ 96% (BSA; sigma) in PBS, 0.01% NaN₃ for one hour at room temperature. The primary antibodies used and their working dilutions are described in detail in table 2.1. Primary antibodies were diluted in 1% BSA and incubated overnight at room temperature. Wells were washed 2X for 5min in PBS/NaN₃, and then incubated for 1hr at room temperature in appropriate secondary antibody (diluted 1:1000 in 1% BSA). Wells were washed 2X for 5mins in PBS, 0.01% NaN₃. Nuclei were stained by incubating for 10 mins in 1μg/ml Hoechst 33258 solution (Sigma).

Secondary antibodies were Alexa fluor 594 goat anti-rabbit, Alexa flour 594 goat anti-mouse and Alexa fluor 488 goat anti-mouse (Invitrogen).

2.8.3 Analysis

All samples were blinded and coded to the investigator. Images of at least five randomly selected fields of view were selected and photographed using a digital camera mounted to a Zeiss Axiovert 135 inverted fluorescence microscope at 10 or 32X objectives. A grid system was used to avoid re-sampling of a counted area. Two wells were studied per condition and the mean value used for analysis. At least 250 nuclei within each field of view were counted and analysed.
Table 2.1. Primary antibodies used

<table>
<thead>
<tr>
<th>Clone name</th>
<th>Supplier</th>
<th>Dilution</th>
<th>Antigen Recognise</th>
</tr>
</thead>
<tbody>
<tr>
<td>D33</td>
<td>DakoCytomation, Denmark</td>
<td>1:250</td>
<td>Desmin</td>
</tr>
<tr>
<td>F5D</td>
<td>Developmental Studies Hybridoma Bank</td>
<td>1:100</td>
<td>Myogenin</td>
</tr>
<tr>
<td>MF20</td>
<td>Developmental Studies Hybridoma Bank</td>
<td>1:100</td>
<td>Sarcomeric MHC</td>
</tr>
<tr>
<td>NCL-MyoD1</td>
<td>Novocastra Laboratories (UK) LTD</td>
<td>1:50</td>
<td>MyoD1</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Biocare Medical</td>
<td>1:250</td>
<td>Ki-67</td>
</tr>
<tr>
<td>CD56</td>
<td>Becton Dickinson</td>
<td>1:100</td>
<td>NCAM</td>
</tr>
<tr>
<td>TE-7</td>
<td>Millipore, UK</td>
<td>1:200</td>
<td>Fibroblast</td>
</tr>
<tr>
<td>γ-H2AX</td>
<td>Abcam</td>
<td>1:500</td>
<td>DNA Double-strand breaks</td>
</tr>
</tbody>
</table>
2.9 Luminex based assays

The Luminex assay was employed to investigate concentration of cytokines, chemokines and growth factors in serum and in conditioned media. This multiplex immunoassay is based on xMAP® detection technology, combining a flow cytometer, a fluorescent-dyed beads, lasers and digital signal processing. The assay was prepared and used according to the manufacturer’s protocol using High Sensitivity Human Cytokine Magnetic Bead (cat. # HSCYTMAG-60SK, Millipore) and TGF-β1 Single Plex (cat. # TGF-β-64K-01, Millipore).

The principle of this technology is the ability to measure several proteins simultaneously in a single sample (Figure 2.1). Microspheres are identified by loading them with different concentrations of two fluorescent dyes. Different antibodies are linked to the surface of microspheres with a particular identity. The microspheres are first incubated with the sample, then biotin conjugated secondary antibodies and finally streptavidin-conjugated phycoerythrin (Figure 2.1). The instrument (FLEXMAP3D) works much like a flow cytometer and focuses the beads into a stream where they pass two lasers in single file. One excites the dyes with which the microsphere were loaded identifying the bead and thus the antibody bound to it, and the second excites the phycoerythrin and allows quantification of the bound antigen. Colour signal is captured, translated digitally into real time and the data is then quantified. The technology has the ability to detect at least 0.13 pg/ml of analytes in a volume as small as 25 μl per well.
1. Bead with capture antibody

2. Capture antibody binds analyte

3. Fluorescence labelled reporter antibody binds to capture analyte

4. Bead ID and reporter quantity determined by laser detector

**Figure 2.1.** Basic Principle of Luminex Assay.

Adapted from www1.ic.ac.uk/resources.
2.9.1 Procedure

1. An aliquot of culture supernatant or serum was thawed prior to the experiments.
2. Standards were prepared by serial dilution according to the manufacturer’s protocol.
3. The filter was pre-wet by pipetting 200 µl of 1X Wash Buffer into each well of either Microtiter Filter Plate or Magnetic plate.
4. The plate was sealed and mixed on a plate shaker for 10 minutes at room temperature.
5. Washing buffer was removed by vacuum (filter plate) or aspiration with a multichannel pipette (Magplates).
6. The bead bottle was sonicated for 30 seconds and vortexed for 1 minute.
7. 25 µl of the mixed beads were added to each well.
8. 25-50 µl of assay buffer were added to the background and the sample wells depending on the kit.
9. Equal volumes (25-50 µl depending on the kit) of each sample, standard and control were added to appropriate wells.
10. Where serum was being measured, 25-50 µl of matrix solution were added to the background, standards, and control wells.
11. The plate was covered with aluminium foil and incubated with agitation on a plate shaker overnight at 4ºC.
12. The fluid was removed by vacuum aspiration aspiration with a multichannel pipette (Magplates).
13. The plate was washed with 200 µl washing buffer two times.
14. 25-50 µl of detection antibody was added to each well.
15. The plate was sealed with aluminium foil, and incubated on a plate shaker for one hour at room temperature.
16. 25-50 µl Streptavidin-Phycoerythrin was added to each well.
17. The plate was covered with aluminium foil, and incubated with agitation on a plate shaker for 30 minutes at room temperature.

18. The content was removed by vacuum aspiration with a multichannel pipette (Magplates).

19. The plate was washed with 200 µl washing buffer two times.

20. 100-150 µl of sheath fluid was added to each well. The plate was covered with aluminium foil and resuspended the beads on a plate shaker for five minutes.

20. The plate was read.

2.10 Measurement of myotube area

Myotubes were measured using Adobe Photoshop Cs version 8 as described by Agley et al. (2012). It must be noted that the image analysis of myotube area was a surrogate based on MHC expression. In brief, digital images of MHC stained cells were taken from five non-overlapping fields per well using Zeiss Axiovert 135 inverted fluorescence microscope at 10 X objective. Scale bars were inserted into every image and the files were saved in Tiff format. Without re-sizing the image, the measuring tool was used to measure the number of pixels spanning the scale bar. By dividing the known linear distance of the scale bar (100 µm) by the pixel value, individual pixel width in µm was obtained. As pixels are perfect squares, multiplying the width of a single by itself (pixel height) will give its area in µm². This calibration factor was entered into an excel spreadsheet. The myotube area was selected based on MHC staining, using the colour range select panel. The number of pixels corresponding to this area was then quantified with histogram function. This pixel value was entered into the excel spreadsheet and multiplied by the calibration factor to yield a close approximation of total myotube area in µm².
2.11 Evaluation methods used

Several different methods of myoblasts extraction have been used for human muscle. Generally satellite cells are extracted by the explants or digestion methods. The digestion method protocol used in this thesis involves the incubation of muscle fragments with the digestive enzymes trypsin (Morgan; eurobiobank 2004). This help to dissociate the mononucleated satellite cells from their associated myofibres. Two pilot experiments were performed in order to see which medium and coating conditions were suitable for the extraction of muscle cells from biopsy tissue.

1- Cell expansion was compared using Ham F-10 (Invitrogen) or DMEM (Invitrogen) or Promocell to see which medium suitable for muscle cells, cells were assessed for their time to 50% confluence and total cell yield (Table 2.2). Promocell medium produced the highest yield of cells and were the first to reach 50% confluency.

2- ECM matrix coating condition was compared with plastic (uncoated) plates in order to see whether ECM matrix suitable for the attachment of muscle cells extracted from muscle biopsy (Table 2.3). This experiment indicates that ECM matrix coated dishes were adequate in promoting the attachment of muscle stem cells compared to uncoated dishes.

Both Promocell medium and ECM coated dishes will be used in future experiment.

β-galactosidase assay (Millipore) was used as described in Dimri et al. 1995 to define senescence culture and the method gave no result.
### Table 2.2. Comparison of medium condition

<table>
<thead>
<tr>
<th>Medium</th>
<th>Time to 50% confluence</th>
<th>Total cell yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ham F-10</td>
<td>No cells present after one week</td>
<td>None after one week</td>
</tr>
<tr>
<td>DMEM</td>
<td>After one week cells were at approximately 10% confluency</td>
<td>None after two weeks</td>
</tr>
<tr>
<td>Promocell</td>
<td>10 days</td>
<td>3.5 million after 10 days</td>
</tr>
</tbody>
</table>

### Table 2.3. Comparison of coated condition

<table>
<thead>
<tr>
<th>Attachment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastic (uncoated)</td>
<td>10-20 cells attached in total at 7 days</td>
</tr>
<tr>
<td>ECM matrix</td>
<td>Cells start to attach at day 3 and continue up to day 7</td>
</tr>
</tbody>
</table>
Chapter 3: Senescence, but not *in vivo* ageing, is associated with delayed differentiation and increased DNA damage in human primary myoblasts.
3.1 Introduction

The serial passaging of normal somatic cells ultimately results in a state of permanent growth arrest called replicative senescence. This was initially shown for fibroblasts by Hayflick and Moorhead (1961) but also more recently, for human satellite cells (Decary et al., 1997). The senescing myoblasts have been used as a model to study muscle ageing. For example, senescent myoblasts obtained from an infant donor showed a delayed differentiation response compared to proliferating myoblasts from the same donor (Bigot et al., 2008). Serially passage C2C12 cells in vitro exhibited a reduced in MyoD and myogenin expression which are associated with decreased IGF expression (Sharples et al., 2011). However, there is uncertainty as to how in vitro ageing of cells reflects the physiological process of ageing of similar cells in vivo, e.g. when proliferating cells obtained from young and elderly subjects are compared.

Satellite cell activation and proliferation have been shown to be impaired in older animals in response to injury and represents one of the key age-specific defects in muscle repair (Conboy et al., 2003, Scime et al., 2009). There is also some evidence that myoblasts obtained from muscles of elderly people do not differentiate as efficiently in vitro when compared with those from younger subjects (Beccafico et al., 2007, Beccafico et al., 2010, Jacquemin et al., 2004, Lorenzon et al., 2004, Pietrangelo et al., 2009). Critically, these conclusions are drawn from comparison of very limited numbers of subjects, often only one from each age group (Jacquemin et al., 2004, Lorenzon et al., 2004), and from populations that often have a low proportion of myoblasts (Pietrangelo et al., 2009) or have not been characterised in terms of desmin content (Beccafico et al., 2010).
3.2 Aim

There were two aims to the study presented in this chapter. The first was to examine differences between myoblasts obtained from young and old people in terms of their proliferation and differentiation *in vitro*. The second was to compare the behaviour of myoblasts obtained from young and old people with those “aged” (senesced) in culture during differentiation.

3.3 Methods

The methods for myoblast extraction are detailed in the general methods chapter (Section 2.4.1), as are methods for expanding, storing, thawing, culturing, calculating population doubling time and passaging (Section 2.4 and 2.6). Measurement of myotube area was carried out as described in Section 2.10.

3.3.1 Subjects

Prior to acceptance in the study, participants completed a health questionnaire and were only included if they fulfilled the “healthy” or “medically stable” criteria (Greig et al., 1994). The total muscle biopsies were collected from 5 young (aged 23-25 years) and 4 elderly (aged 67–82 years) subjects and used as specified in the figure legends.
3.3.2 Culturing human myoblasts

Following thawing, cells were cultured in a skeletal muscle cell growth medium (GM) until they reached senescence (defined as failure to divide for three consecutive weeks). The proportion of desmin (myoblasts) and Ki67 (proliferating) positive cells over time in culture was monitored. At several time points during the culture period (4, 16, 28 days and when the cells reached senescence) cells were monitored for expression of MyoD (myogenic regulatory factor involved in the specification and activation of satellite cells) and differentiation potential. For differentiation experiments, cells were plated in 96 well dishes at a density of 7000 cells/well. GM was replaced after 24 hours with a differentiation medium (DM) and cultured for a further 7 days in DM, with aliquots of cells being fixed every 24 hours in 4% paraformaldehyde + 0.2% triton. Cells expressing markers of differentiation (myogenin and myosin heavy chain, MHC) were identified using immunocytochemistry as described in chapter 2 (section 2.8). Fusion index was measured after 7 days and expressed as the percentage of nuclei in myosin heavy chain positive cells containing at least two nuclei.

3.3.3 DNA damage staining

Aliquots of cells (1000 cells) from non-senescent and senescent populations were plated into 96 well dishes in growth medium. The cells were fixed after 24 hrs with 4% paraformaldehyde + 0.2% triton and stained for γ-H2AX (DNA damage marker).
3.4 Statistics

Statistical comparisons were made using IBM SPSS 19 software, and P values \( \leq 0.05 \) were considered significant. Statistical significance for interactions between groups (Young, Elderly, 16 days, 28 days and Senescent) and time was determined using mixed two-way repeated measure analysis of variance (ANOVA) with post-hoc Bonferroni. One-way ANOVA with post-hoc Tukey comparisons was conducted to confirm statistical significance between variable of interest e.g. between groups. Results for repeated measure and one-way ANOVA are expressed using the F-Ratio. Data are representative of three experiments performed in duplicate. All values are shown as individual data points or as mean values ± SE. T-tests were used when comparing young and elderly cells with a single condition. ANOVA was used when performing multiple comparisons of elderly, young and senescence cells across more than one time condition.

3.5 Results

3.5.1 Desmin content of young and elderly subjects

The percentage of desmin positive cells was evaluated in myoblast populations obtained from frozen stocks of early passage primary cultures obtained from biopsies of five young and four elderly subjects. Representative images of desmin and Ki67 are shown in Figure 3.1. The cell preparations used here were similar in terms of the desmin expression (Table 3.1). The data showed no aged related differences in cultures obtained from young and elderly subjects (83 ± 8%, young vs. 81.5 ± 11%, elderly).
3.5.2 Replicative potential and desmin content of human myoblasts over time in culture.

To compare the proliferative potentials and desmin content of myoblasts isolated from young and old muscle biopsies. Cells were continually passaged in growth medium until they reached replicative senescence. This was defined as failure to divide for three consecutive weeks. Upon thawing and expansion, the samples behaved in a similar manner irrespective of the age of the donor: Several populations of cells (two from young subjects and three from elderly subjects) maintained their initial desmin content (50-94%) over time in culture (Figure 3.2a) and underwent 5-12 mean population doublings (MPDs; C, E, F, G and H, Figure 3.3). Others populations, however, lost their desmin content over time (50-95% starting, 0% at senescence; Figure 3.4a) and underwent 15-20 MPDs (A, B, D and I, Figure 3.1). Cells that did not maintain desmin content were discarded from analysis of γ-H2AX, MyoD and differentiation (Section 3.5.4-3.5.7).

Table 3.1. Percentage of desmin positive cells in young and old subjects.

<table>
<thead>
<tr>
<th>Age</th>
<th>% of Desmin&lt;sup&gt;++&lt;/sup&gt; cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>51</td>
</tr>
<tr>
<td>25</td>
<td>86</td>
</tr>
<tr>
<td>23</td>
<td>94</td>
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<td>80</td>
<td>93</td>
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<td>67</td>
<td>50</td>
</tr>
<tr>
<td>77</td>
<td>89</td>
</tr>
</tbody>
</table>
**Figure 3.1.** Images taken from (a) young (from a male subject aged 20), (b) elderly (from a male subject aged 77) and (c) senescent myoblasts stained for desmin (green), Ki67 (red) and Hoechst 33342 (blue) after 24 hours in growth medium. Scale bar = 20 µm. Young and elderly cells were similar in term of desmin and Ki67 expression. Senescent cells display a larger and more flattened morphology with an increased in microfilament content.
**Figure 3.2.** Time course of (a) Desmin, (b) Ki67 and (C) Desmin+Ki67 expression for cells that maintained desmin, in human myoblasts obtained from young (n=2) and elderly (n=3) subjects over time in culture.
**Figure 3.3.** *In vitro* expansion of human satellite cells isolated from subjects of different ages. The number of population doubling at every passage was calculated as $PD = \log \left( \frac{N_1}{N_0} \right) / \log 2$ where $N_1$ is the final cell number and $N_0$ is the initial number of cells seeded.
Figure 3.4. Time course of (a) Desmin, (b) Ki67 and (C) Desmin+Ki67 expression for populations that lost desmin. Cells were obtained from three young subjects and one elderly subject over time in culture.
3.5.3 Myoblast proliferation from young and elderly subjects.

The relative proportion of Ki67$^{+}$ cells measured 24 hours after plating was similar between cells obtained from young (55.8 ± 2.9%) and elderly (54.8 ± 3.3%) individuals (Figure 3.5a). The percentage of proliferating muscle cells (Ki67 and desmin positive) was also similar between cells obtained from young (47.8 ± 5%) and elderly (54.8 ± 3.3%) subjects after 24 hours in growth medium (Figure 3.5b). Upon serial passaging of cells that maintained desmin over time, the proportion of proliferating muscle cells decreased gradually until the cells finally stopped proliferating after 28 to 35 days. Although there was some heterogeneity between populations (Figure 3.2c), this was the case in both young and elderly individuals and no age related differences are apparent. The time course of Ki67 expression in cells that lost desmin expression is shown in Figure 3.4 b and c. Again there was some heterogeneity between populations and no apparent age effect, although there was only one cell population from an elderly subject in this group. It can be seen by comparing Figure 3.3 that populations that lost desmin had longer proliferative potentials and this was reflected in their expression of Ki67 (Figure 3.2b and 3.4b).

3.5.4 Expression of MyoD in growth medium from young and elderly myoblasts compared to in vitro ageing.

After 24 hr culture in GM, MyoD expression was found to be similar between cells obtained from young and elderly donors (8.6 ± 2.1%, young vs. 7.8 ± 1.31%, old; Figure 3.6). After this initial analyses the two young and three old cell populations that maintained desmin expression were analysed as a group of n=5 populations. There was a gradual decline in MyoD expression in all populations as cells approached senescence.
(i.e. after 16 and 28 days in culture) but the difference from young and old cells only reached significance ($F_{(4, 19)} = 4, P = 0.02$) when cells reached senescence (Figure 3.6).

**Figure 3.5.** Similar proliferation in young and old human myoblasts.
Myoblasts from young (n=5) and elderly (n=4) subjects were cultured in growth medium for 24 hr and stained for desmin and Ki67. No significant differences were observed between young and elderly myoblasts ($P \leq 0.05$). Data are mean value ± SE.
Figure 3.6. Changes in MyoD expression with time in culture, in human myoblasts obtained from young (n=5) and elderly (n=4) subjects analysed 24 hours after plating. Thereafter, young and old cells were analysed as a group (n=5) after 16 and 28 days and when they reached replicative senescence. Only when the cells reached senescence was there a significant reduction in the expression of MyoD compared to young and elderly cells. *, P = 0.02 statistically significant difference from young and elderly subjects. Data are mean value ± SE.
3.5.5 Myoblast differentiation from young and elderly subjects compared with in vitro ageing.

Figure 3.7 shows representative images from young and elderly biopsies of myogenin+ki67 expression. Typical staining patterns of myogenin and MHC+MyoD are shown in Figure 3.8 & 3.9.

Figure 3.10a shows quantitation of expression of myogenin and Ki67 in the populations that maintained desmin over time in culture. A significant proportion of myogenin+ve cells were also Ki67+ve after 24 (day 1) and 48 hours (day 2) in differentiation medium (DM). This was the case in both the young and elderly myoblasts, indicating that myoblasts are capable of expressing myogenin before complete cell cycle arrest.

Myoblasts derived from young and elderly subjects expressed MyoD in proliferation medium (Figure 3.6 and Figure 3.11b). Myogenin expression was apparent within 24 hours of change to differentiation medium (Figure 3.10b) and preceded MHC expression which was only detected after 48 hours of differentiation (Figure 3.11a). All MHC +ve cells were also MyoD +ve. Interestingly, at the onset of fusion (day 3 of differentiation), all MyoD +ve nuclei were found within MHC +ve myotubes (Figure 3.11c). There was a delay in the onset of myogenin and MHC expression (Figure 3.10b & 3.11) when cells reached senescence.

The proportion of cells expressing MyoD, myogenin, MHC and co-staining for both MHC and MyoD significantly increased during differentiation, F \(_{(1, 19)}\) = 843, P < 0.0005 for myogenin, F \(_{(1, 19)}\) = 1396, P < 0.0005 for MHC, F \(_{(1, 19)}\) = 638, P < 0.0005 for MyoD, F \(_{(1, 19)}\) = 1396, P < 0.0005 for MHC and MyoD co-staining.
No significant differences were observed in the percentage of MyoD+ve, myogenin+ve, Ki67+ve/myogenin+ve, MyoD+ve/MHC+ve and MHC+ve cells between young and elderly myoblast at any point during differentiation.

Cells cultured for several passages and examined 16 and 28 days after thawing showed a significant and progressive decrease in the percentage of MyoD+ve (F(4, 19) = 133, P < 0.0005), myogenin+ve (F(4, 19) = 73, P < 0.0005), MyoD+ve/MHC+ve (F(4, 19) = 112, P < 0.0005), and MHC+ve (F(4, 19) = 112, P < 0.0005), cells at each time point during differentiation (Figure 3.10b & 3.11) and shown there was a significant interaction between groups and time.

3.5.6 Fusion index and myotubes size of young and old cells compared with senescent cells.

Seven days after differentiation was induced in the cells, fusion index (FI) and size of myotubes were determined. Myotube size was measured by quantitating the area in each field of view positive for MHC.

The data presented in Figure 3.12a show that there was no difference in the FI between cells from the young and elderly subjects (88.8 ± 2.1%, young vs. 84 ± 1.9%, old). However, in the senescent cells the FI was significantly lower compared to cells from both young and elderly people at the earliest passage, F(1, 11) = 1244, P < 0.0005.
The data presented in Figure 3.12b shows that there was also no difference in myotube area between young and elderly cells at the earliest passage, but myotube area was significantly decreased in the senescent cells, $F_{(1, 11)} = 9.11, P = 0.005$. Representative images of MHC staining after seven days of differentiation are shown in Figure 3.13.

**Figure 3.7.** Images taken from of (a) young (from a male subject aged 23) and (b) elderly (from a male subject aged 80) stained for myogenin (yellow arrow), Ki67+Myogenin (green arrow) and Hoechst 33342 (white arrow) at 2 days in differentiation medium. Scale bar = 20 µm. A significant proportion of myogenin+ve cells were also Ki67+ve after 24 (day 1) and 48 hours (day 2) in differentiation medium (DM). This was the case in both the young and elderly myoblasts.
Figure 3.8. Images taken from of (a) young (from a male subject aged 23), (b) elderly (from a male subject aged 80) and (c) senescent myoblasts stained for myogenin (yellow arrow) and Hoechst 33342 (white arrow) at 7 days in differentiation medium. Scale bar = 20 µm. Young and elderly cells were similar in term of myogenin expression. There was a decrease in the onset of myogenin expression when cells reached senescence.
Figure 3.9. Images taken from of (a) young (from a male subject aged 25), (b) elderly (from a male subject aged 80) and (c) senescent myoblasts stained for MyoD+MHC (yellow arrow) and Hoechst 33342 (white arrow) at 7 days in differentiation medium. Scale bar = 20 µm. Young and elderly cells were similar in term MHC and MyoD expression. There was a decrease in the onset of MHC and MyoD expression when cells reached senescence.
Figure 3.10. Time course of (a) ki67+myogenin and (b) myogenin expression, in human myoblasts obtained from young (n=5) and elderly (n=4) subjects. Cells were either differentiated at early passage (in vivo ageing model) or at several time points during the culture period (16 and 18 days, n=5) until they reached replicative senescence (n=5). Data are mean value ± SE. *, P < 0.0005 statistically significant difference from young and elderly subjects.
Figure 3.11. Time course of (a) MHC, (b) MyoD and (c) MyoD+MHC expression, in human myoblasts obtained from young (n=5) and elderly (n=4) subjects. Cells were either differentiated at early passage (in vivo ageing model) or at several time points during the culture period (16 and 18 days, n=5) until they reached replicative senescence (n=5). Data are mean value ± SE. *, P < 0.0005 statistically significant difference from young and elderly subjects.
**Figure 3.12.** Fusion index (a) and measurement of myotube total area (b), in human myoblasts obtained from young (n=5) and elderly (n=4) subjects. Cells were either differentiated at early passage (*in vivo* ageing model) or when they reached replicative senescence (n=5). Data are mean value ± SE. *, P < 0.0005 & **, P = 0.005 statistically significant difference from young and elderly subjects.
Figure 3.13. Images taken from of (a) young (from a male subject aged 24), (b) elderly (from a male subject aged 82) and (c) senescent myoblasts stained for MHC (yellow arrow) and Hoechst 33342 (white arrow) at 7 days in differentiation medium. Senescent myoblasts were thinner and contain few myonuclei compared to the ones derived from the young and elderly people.
3.5.7 DNA damage.

Analysis of γ-H2AX (DNA damage Marker) expression in young and elderly myoblasts revealed very few positive cells in either the young or elderly myoblasts analysed at the earliest point in the culture (<10%, Figure 3.14, 3.15). In senescent cultures, 90% of myoblasts stained positive for γ-H2AX (Figure 3.15). These differences were significant, $F_{(1, 11)} = 2510$, $P < 0.0005$.

Figure 3.14. Images taken from of (a) early passage (white arrow) and (c) senescent myoblasts (yellow arrow) stained for γ-H2AX (red) and Hoechst 33342 (blue) after 24 hours in growth medium. Scale bar = 20 μm. A markedly higher number of senescent cells were positive for γ-H2AX (a marker of DNA double strand breaks) compared to early passage cells.
Figure 3.15. A DNA damage in senescent cells vs. early passage cells, in human myoblasts obtained from young (n=5) and elderly (n=4) subjects. Cells were either stained with γH2AX at early passage or when they reached replicative senescence (n=5). Data are mean value ± SE. *, P < 0.0005 statistically significant difference from young and elderly subjects.
3.6 Discussion

Skeletal muscle regeneration and repair during life depends on the proliferation and differentiation of satellite cells. Any age-related changes in regenerative capacity intrinsic to the satellite cells themselves could thus be due to impaired proliferation or differentiation. This study revealed two main findings. Firstly, proliferative potential, differentiation potential and differentiation time course were similar between cells from the young and elderly subjects at the earliest passage analysed. The second major finding was that there was a progressive decrease in the ability of cells to express markers of differentiation with increasing time in culture. When the cells reached senescence, expression of myogenin and MHC was delayed by two-four days following transfer to differentiation medium when compared to early passage cells. In addition, a markedly higher number of senescent cells were positive for γ-H2AX (a marker of DNA double strand breaks) compared to early passage cells.

3.6.1 Young vs. old myoblasts

Marked heterogeneity in proliferation potential and desmin content was found in myoblasts obtained from different subjects, even when these are of the same age group. There does not appear to be a relationship between myogenic purity (proportion of desmin positive cells) of a population and the age of the donor which is in agreement with findings of others (Beccafico et al., 2007, Pietrangelo et al., 2009, Schafer et al., 2006). Furthermore, there was no clear association between MPD and donor age. A replicative lifespan of 5-20 MPD observed for young and elderly myoblasts in this study is consistent with previous estimates of the replicative capacity for human myoblasts. Decary et al. (1997) observed replicative capacity of 6-19 population doublings for
young and elderly human myoblasts in culture, and Pietrangelo et al. (2009) reported that young and elderly human myoblasts reach senescence after 15-20 population doublings. Expression of markers of proliferation decreased gradually until the cells finally stopped proliferating. Other investigators who have found a progressive declined in the number of proliferating cells with time in culture (Renault et al., 2000).

Several populations of cells maintained their initial desmin content whereas others lost their desmin content over time in culture. The results are in agreement with an earlier observation in human myoblasts showing that the number of population doubling and desmin expression varies independently of age (Schafer et al., 2006). Recent research has demonstrated that primary muscle precursor cells can be stratified into distinct populations based on their proliferative characteristics (Rossi et al., 2010, Rouger et al., 2004). This finding is relevant to the study of human primary muscle cultures where it is possible that the balance of fast proliferating to slow proliferating muscle precursor cells may ultimately determine culture purity in the presence of contaminating fibroblasts. Fast proliferating cells may outstrip the growth of fibroblasts, or whereas slow proliferating may be outgrown by them.

Many experiments support the finding that proliferation and differentiation of human muscle precursor cells are not affected by the age of the donor. Recent in vitro experiments show that muscle precursor cells obtained from elderly animals can proliferate and differentiate similarly to that of young animals (Dumke and Lees, 2011). Furthermore, it has been shown that human myoblasts from an elderly donor can contribute myonuclei very efficiently to regenerating mouse muscle in vivo (Schafer et al., 2006). Single fibre grafting experiment have shown that satellite cells from old
animal can possess an equivalent \textit{in vivo} myogenic and self-renewal potential to that of young animals (Collins et al., 2007). Whole muscle grafting experiments have shown that although there is a slight delay in myotube formation, no difference is observed between elderly and young muscle grafted into elderly or young animals (Smythe et al., 2008).

In contrast to the present results and to the observations discussed above, other studies have found age associated difference when compare differentiation of primary human cells from donors of different ages. Lorenzon et al. (2004) compared human muscle cells obtained from a 2 year old, 21 year old, 48 year old and 76 year old. They found fusion index and number of nuclei per myotube were decreased between the 21 and 76 year old subjects after 4, 6 and 9 days in culture (Lorenzon et al., 2004). There was also a delay in the maturation of the excitation-contraction coupling mechanism when myoblasts from the elderly donor were compared to those from the young donors (Lorenzon et al., 2004). Fulle et al. (2005) compared human muscle cells obtained from a newborn (5 day old), young (29 years old) and old (71 years old) subjects with 85, 71 and 47\% of desmin cells respectively. They found that the percentage of MHC positive cells (Fusion index) had decreased in the elderly subject after 5 days in culture when compared to newborn and young subjects. Subsequent experiments by this group have shown a 20\% decrease of fusion index (as measured after 7 days of \textit{in vitro} differentiation and expressed as the percentage of nuclei in myosin heavy chain positive cells containing at least two nuclei) in elderly subjects (83 ± 6 years old, n=3) when compared to young subjects (30 ± 2 years old, n=3); (Beccafico et al., 2007). They also have shown that myotubes derived from elderly subjects are thinner and contain few myonuclei compared to the ones derived from the young individual.
Beccafica et al. (2010) isolated myoblasts from the vastus lateralis muscle of 10 young (29 ± 6 years old) and 11 old (77 ± 6 years old) and cultured them in either growth media or differentiation media. These authors showed that myoblasts from older subjects displayed lower levels of Brdu incorporation when compared to younger subjects thus suggesting impaired proliferation in the elderly cells. Western blotting analysis revealed no expression of the early myogenic differentiation marker; myogenin; or the terminal differentiation marker, MHC in myoblasts derived from older subjects compared to younger subjects. In addition, when compared to those of younger subjects, the older myoblasts displayed significantly higher levels of S100B, an intracellular protein known to inhibit myoblast differentiation, and lower levels of membrane-bound RAGE, a receptor promoting myoblast differentiation. Several other studies showed that human myoblasts extracted from muscles of elderly subjects do not fuse as efficiently in vitro as those from younger subjects (Jacquemin et al., 2004, Pietrangelo et al., 2009). There are a number of concerns which need to be borne in mind when drawing conclusions from some of these studies. For examples, some studies which have either identified age related impairments in human myoblast differentiation have compared only one individual from each age group (Jacquemin et al., 2004, Lorenzon et al., 2004). When populations from several young and several elderly donors have been compared this relationship is less clear and cells from several elderly subjects do show robust differentiation (Pietrangelo et al., 2009). Therefore any previous differences reported could be due to individual variability in factors (genetic or lifestyle factors) other than age. Further concerns centre on comparing populations that do not have equivalent proportions of cells expressing desmin (Beccafico et al., 2007, Pietrangelo et al., 2009), which have compared cells which have not been characterised in terms of desmin content (Beccafico et al., 2010).
In differentiation experiments it is particularly important to monitor the desmin content of the population carefully because this can vary over time in culture as demonstrated by data in Figures 3.2 and 3.4 and by Schafer et al. (2006). It is possible to increase the myogenic purity of a population by magnetic cell sorting with the cell surface marker NCAM (Pietrangelo et al., 2009, Schafer et al., 2006). Such purification result in an increase in fusion index for cells from a young subject but not from an elderly subject (Pietrangelo et al., 2009) and this was taken as demonstration of age related impairment. Morphological differences were also reported for myotubes formed from old and young myoblasts with the former being smaller and thinner (Pietrangelo et al., 2009). In the present study, a quantitative approach was undertaken to compare myotubes from young and elderly subjects. The result shows that there is no significant difference in myotube area between young and elderly myoblast.

3.6.2 Non-senescent vs. senescent myoblasts

Replicative senescence is characterised by an irreversible growth arrest with associated morphological and physiological alterations (Campisi and d'Adda di Fagagna, 2007). This phenomenon has been used as a model to study ageing in fibroblast and has been proposed as a model of ageing in myoblasts since myoblasts from an infant donor aged in vitro showed a delayed differentiation response, a lower fusion index and fewer nuclei per myotube compared to proliferating myoblasts from the same donor (Bigot et al., 2008). However, there is uncertainty as to how in vitro ageing of cells reflects the physiological ageing of similar cells in vivo. In the present experiment it was not possible to separate cells obtained from young and elderly people. In agreement with Bigot and co-workers (2008) senescent myoblasts exhibited a delay in myogenic
differentiation, a lower fusion index and a reduced in myotube area compared to early passage cells from both young and elderly donors. The results in the present study have not been able to show a difference in the senescent profile of young (20-23 years old) compared to elderly (67-82 years old) subjects. Undoubtedly in vivo ageing is associated with a reduction in various physiological function of muscle. It is unsure how the senescent in vitro relates to the findings found in vivo. It could be that if the cells from very old subjects were studied e.g. approaching 100 years old, then the characteristics of these cells pre in vitro senescence would more akin to those in the present study observed following multiple passaging to senescence in vitro may have not been observed. More experiments need to be done in order to definitively define the relationship between in vitro senescence and ageing in vivo.

The observed reduced expression of MyoD in senescent myoblasts could be directly responsible for reduction differentiation potential as reflected by a decreased and a delay in myogenin and MHC expression. MyoD is a myogenic regulatory factor that required for myogenic commitment and differentiation (Berkes and Tapscott, 2005, Buckingham et al., 2003, Cooper et al., 1999). Furthermore, it has been shown that MyoD plays an integral role in regulating myogenin transcription and that myogenin subsequently cooperates with MyoD to activate expression of another set of genes (Cao et al., 2006). Both in vivo and in vitro studies have demonstrated that MyoD-deficient myoblasts show a reduced differentiation potential as determined by decreased ability to fuse and a reduction in the number of generated myotubes (Yablonka-Reuveni et al., 1999, Megeney et al., 1996, Montarras et al., 2000, Sabourin et al., 1999).
Ageing and cellular senescence are characterised by increased DNA damage (Sedelnikova et al., 2004, Rube et al., 2011). The precise mechanism that may contribute to the cellular senescence has not been defined, the shortening of telomeres is thought to act as a mitotic clock that signals senescence when one or more telomeres reach a critical length (Mouly et al., 2005). In eukaryotes, telomere shortening occurs during ageing due to the inability of DNA polymerase to copy the ends of chromosomes with fidelity (Bodnar et al., 1998). The dysfunction of telomeres results in a DNA damage response characterized by the formation of double strand DNA breaks containing the phosphorylated histone H2AX (Celeste et al., 2002). To investigate whether DNA damage accumulates with both in vivo ageing and in vitro senescence in human myoblasts, γ-H2AX antibody staining was used to characterise DNA double strand breaks in the myoblasts from the donors of different ages, as well as in senescent myoblasts. Young and elderly myoblasts exhibited a similar expression of γ-H2AX, while senescent myoblasts displayed a much higher DNA damage. Senescence of human myoblasts accompanied by increased generation of reactive oxygen species, reduced mitochondrial mass and a decreased whole cell ATP level all of which can lead to increased damage of DNA (Minet and Gaster, 2012).

Many studies on human fibroblasts have demonstrated an inverse relationship between donor age and replicative lifespan (Macieira-Coelho and Ponten, 1969, Schneider and Mitsui, 1976). This effect is inconsistent with a high number of healthy donor samples (Cristofalo et al., 1998). In human myoblasts, however, there appears to be no correlation between the age of the donor and the number of mean population doublings achieved before senescence (data presented Figure 3.3); (Decary et al., 1997, Mouly et al., 2005, Pietrangelo et al., 2009, Renault et al., 2000). Furthermore, it has also been
shown that myoblasts obtained from very elderly subjects (>80 years of age) behaved similarly to cell populations obtained from very young subjects (2-5 years of age) in terms of their mean population doublings (Schafer et al., 2006). One explanation that has been proposed is that satellite cells in healthy muscle would not normally undergo a significant number of divisions in vivo once growth has ceased and therefore would maintain their proliferative potential throughout the lifespan of the individual (Decary et al., 1997). A similar maintenance of differentiation capacity would also be coherent with this explanation.

In conclusion, the data presented herein and in other studies in animals and humans suggest that skeletal muscle derived myoblasts obtained from old donors have both a robust proliferative and differentiation potential in vitro. It is possible that genetic and lifestyle heterogeneity between individuals is a larger contributor than age to observed variations of human myoblast behaviour in vitro and in vivo. The data suggest that serial passage and replicative senescence, but not in vivo ageing, result in a delay in myoblast differentiation and increase DNA damage. This questions the use of senescence in culture as a model of in vivo ageing. This study also demonstrates that careful monitoring of passage number and desmin content is critical for the comparison of myogenicity between different myoblast populations. The data would also indirectly support a role for environmental rather than cell intrinsic factors in impaired muscle regeneration in older animals.
Chapter 4: Secretory profile of human myoblast during differentiation
In the previous chapter, a series of experiments were designed to characterise the intrinsic behaviour of cells from young, old and senescent myoblasts by using a cell culture approach and immunocytochemistry. The work described in this chapter examines the release of cytokine by human myotubes.

4.1 Introduction

Skeletal muscle is an endocrine active organ producing a variety of cytokines, the so-called myokines, which are involved in regulation of muscle homeostasis (Pedersen and Febbraio, 2008). Skeletal muscle has recently been shown to be a major source of IL-6 especially during exercise, where it plays a role in glucose and lipid metabolism in muscle (Ostrowski et al., 1998, Carey et al., 2006). In addition to IL-6, other potential myokines expressed and secreted by contracting skeletal muscle have been identified, including IL-8 and IL-15 (Chan et al., 2004, Nielsen et al., 2007). Recent studies have demonstrated that IL-4, IL-6, IL-7, IL-8, IL-13, TNF-α and TGF-β1 can be detected in medium conditioned by human myotubes differentiated from satellite cells (Haugen et al., 2010, Jacquemin et al., 2007, Lafreniere et al., 2006, Peterson and Pizza, 2009, Tsivitse et al., 2005). Furthermore, several factors have been detected in medium conditioned during differentiation of murine C2C12 skeletal muscle cells (Henningsen et al., 2010). A quantitative proteomics platform was used in the study to identify and quantitatively analyse 635 secreted proteins including 35 growth factors, 40 cytokines and 36 metalloproteidases. These results suggest that myokines could potentially be involved in local autocrine/paracrine interactions within muscle tissue.
Ageing has been associated with systemic low-grade inflammation, the so-called inflammaeing. Inflammaeing is defined, in part, by increased serum and plasma levels of a variety of pro-inflammatory cytokines and growth factors, including IL-6, TNF-α, and TGF-β1 (Carlson et al., 2009a, Dobbs et al., 1999, Njemini et al., 2011, Paolisso et al., 1998, Wei et al., 1992). The impact of ageing on the cytokine secretion from human skeletal muscle cells remains uncharacterised.

In the previous chapter data was shown which suggested that proliferative senescence is not a representative model for ageing of myoblasts as myoblasts from old people were not different from those of young subjects. However, there is evidence that senescent cells accumulate in vivo with increasing age and are found at sites of age related pathology (Dimri et al., 1995, Paradis et al., 2001, Vasile et al., 2001). Senescent cells secrete several cytokines which leads to altered cellular function and may also have an impact on overall tissue function. Senescent cells secrete high levels of IL-6, IL-8, IL-1β and TGF-β all of which could be involved in the proliferative arrest and disrupted differentiation (chapter 3); (Coppe et al., 2010, Orjalo et al., 2009, Parrinello et al., 2005, Tremain et al., 2000).

Based on the data obtained in Chapter 3, it is predicted that no differences will be observed in the secretory profiles of young and old myoblasts at early passages, but that differences will be observed between early passage and senescent myoblasts. The cytokines chosen for examination include IL-4, IL-6, IL-7, IL-8, IL-13, TNF-α, and TGF-β1 which have been observed in differentiating myotubes and IL-6, IL-8, IL-1β and TGF-β which have been observed in the senescent secretome as mentioned above.
Two time periods were chosen for analysis. The first 48 hours after placing cells in differentiation medium and the period between 72-120 hours. As shown in Chapter 3, Ki67 is still detectable in the first 24 hours, the same time that myogenin is first detected but no myotubes are observed. At day three, MHC expression starts. MHC expression coincides with myoblast fusion and myotube formation. Therefore the phenotype of cells changes dramatically between the two periods chosen for examination. An advantage of measuring cytokine production during differentiation is that the medium contains no serum.

4.2 Aim

There are two major aims of this study

1- To define the secretory profile of cells from young and old myoblasts during differentiation.

2- To compare the secretory profile of non-senescent and senescent myoblasts during differentiation.

4.3 Methods

The methods for myoblast extraction are detailed in the general methods chapter (Section 2.4.1), as are methods for expanding, storing, thawing and culturing (Section 2.4). The methods for differentiation of young, old and senescent cells are detailed in Section 2.6.2.
4.3.1 Collecting condition medium

At early passage (young and old cells) and late passage (senescent cells), aliquots of 7000 cells were plated into a 96 well dishes in growth medium. After 24 hr, differentiation was induced by simply replacing growth medium with differentiation medium. Conditioned media (CM) were collected after two 48 hour incubation periods after placing the cells in DM: The period between day 0- and day 2 of differentiation (i.e. the first 48 hours in DM) and between days three and five of differentiation (i.e. 72-120 hours). CM was immediately stored at -70°C.

4.3.2 Luminex assay

The Luminex assay was carried out as described in general methods chapter (section 2.9). A panel of nine cytokines was investigated at different time points. In brief, an aliquot of culture supernatant was thawed prior to the experiments. 25-50 μl of each sample, the manufacturer provided positive control, negative control as well as standard curve samples were added to wells of the plate containing 25-50 μl of assay buffer and 25-50 μl of diluted beads (1:2.5). The plate was sealed, covered with aluminium foil to protect it from light and incubated with agitation on a plate shaker overnight at 4°C. The sample was then removed and washed 2X with washing buffer provided in the kit. 25-50 μl of detection antibodies was added to each well and incubated for one hour at room temperature to bind the bead-cytokine complexes. Then Streptavidin-Phycoerythrin was added and incubated for thirty minutes at room temperature. The sample was then removed and washed 2X with washing buffer. One hundred microlitres of sheath fluid was then added to each well and the plate was read in a Luminex plate reader, where a red laser excited the fluorochromes in the beads and, the identity of the bead and
corresponding antigen were determined. At the same time a green laser excited the phycoerythrin coupled antibodies to determine the amount of cytokine associated with the specific bead. Samples were tested in duplicate and the average concentration for each sample was taken.

4.4 Statistics

Statistical comparisons were made using IBM SPSS 19 software, and P values ≤ 0.05 were considered significant. Statistical significance for interactions between group (Young, Elderly and Senescent) and time was determined using mixed two-way repeated measure analysis of variance (ANOVA) with post-hoc Bonferroni. If there were significant interactions present, one way-ANOVA tests were conducted to confirm statistical significance between variable of interest, e.g. between groups, and paired-sample t tests undertaken for variables of interest within group and time. Results for repeated measure and one-way ANOVA are expressed using the F-Ratio, whereas results foe t-tests are expressed as t-statistics. All values are shown as mean ± SE.
4.4 Results

An example of Luminex cytokine standard curve is presented in Figure 4.1. The data was plotted as Median Fluorescence Intensity (MFIs) vs. Calculated cytokine concentration (pg/ml) and R^2 = 0.99.

IL-6
Logistic5p: y = a+(b-a)/(1+((x/c)^d))^f

Figure 4.1. A representative standard curve created for IL-6.
A standard curve was created from the standards included, using 5PL-(parametric logistic) regression fit using the manufacturer's software.
4.4.1 Cytokine secretion of differentiated human myotube

The levels of TNF-α, IL-1β, IL-4, IL-7, IL-10 and IL13 were below the detection limits of the assay (0.13 pg/ml) in all young, elderly and senescent myoblasts.

4.4.1.1 TGF-β1

TGF-β1 was detected during the first 48 hours of incubation in DM for all groups observed. The concentration of TGF-β1 in the CM significantly declined in the second 48 hour incubation period measured during differentiation, $F_{(1, 11)} = 141, P < 0.0005$, (confirmed by a Paired t test, $t_{13} = 6.3, P < 0.0005$). There was also a significant interaction between groups and time, $F_{(2, 11)} = 19.3, P < 0.0005$. There were no significant differences between young and elderly myoblasts in the level of expression of TGF-β1 at any of the time points examined (Figure 4.2a). Significant differences were found when senescent myoblasts were compared to young and elderly myoblasts at 0-48 hours, $F_{(2, 11)} = 43, P < 0.0005$, and at 72-120 hours, $F_{(2, 11)} = 7.6, P = 0.008$, (Figure 4.2a). Figure 4.2b shows the individual values for TGF-β1 expression for each time point.
4.4.1.2 IL-6

IL-6 was detected in conditioned medium from some cells at the time intervals examined (Figure 4.3b). There was no time effect and no significant interaction between groups and time for IL-6 expression. There were no statistically significant differences in the amount of IL-6 detected among senescent, young and elderly myoblasts at any time periods examined (Figure 4.3a). Figure 4.3b shows the individual values for IL-6 expression for each time point.

4.4.1.3 IL-8

The mean concentration of IL-8 significantly declined with time for all groups during myoblasts differentiation, \( F_{(1, 11)} = 5.1, P = 0.045 \), (confirmed by a Paired t test, \( t_{13} = 2.2, P = 0.043 \)). There were no significant differences between young and elderly cells in the amount of IL-8 at any of the time periods examined (Figure 4.4a). The level of IL-8 at 0-48 hours had a trend to be higher in senescent myoblasts compared to young and elderly myoblasts, \( F_{(2, 11)} = 4, P = 0.05 \). The difference between senescent and early passage myoblasts was however statistically significant at 72-120 hours, \( F_{(2, 11)} = 5.5, P = 0.022 \), (Figure 4.4a). Figure 4.4b shows the individual values for IL-8 expression for each time point.
Figure 4.2. (a) TGF-β1 levels for young, old and senescent myoblasts during differentiation. Medium was conditioned for 48 hours for two different periods of differentiation. The concentration was measured in media from muscle cells obtained from young (n=5) and elderly (n=4) subjects. Cells were either differentiated at early passage (in vivo ageing model) or when they reached replicative senescence (n=5). Data are mean value ± SE. *, P < 0.0005 & **, P = 0.008 statistically significant difference from young and elderly subjects. (b) Individual data points are shown for TGF-beta expression during myoblast differentiation. Individual points are represented by the following symbols: ■23 yr, ◆25 yr, ▲23 yr, ●24 yr, X20 yr, ○82 yr, □80 yr, ◻67 yr and △77 yr.
Figure 4.3. (a) IL-6 levels for young, old and senescent myoblasts during differentiation. Medium was conditioned for 48 hours for two different periods of differentiation. The concentration was measured in media from muscle cells obtained from young (n=5) and elderly (n=4) subjects. Cells were either differentiated at early passage (*in vivo* ageing model) or when they reached replicative senescence (n=5). Data are mean value ± SE. (b) Individual data points for are shown for IL-6 expression during myoblast differentiation. Individual points are represented by the following symbols: ■ 23 yr, ♦ 25 yr, ▲ 23 yr, ● 24 yr, X 20 yr, ○ 82 yr, □ 80 yr, ♣ 67 yr and △ 77 yr.
Figure 4.4. (a) IL-8 levels for young, old and senescent myoblasts during differentiation. Medium was conditioned for 48 hours for two different periods of differentiation. The concentration was measured in media from muscle cells obtained from young (n=5) and elderly (n=4) subjects. Cells were either differentiated at early passage (in vivo ageing model) or when they reached replicative senescence (n=5). Data are mean value ± SE. *, p = 0.022 statistically significant difference from young and elderly subjects. (b) Individual data points are shown for IL-6 expression during myoblast differentiation. Individual points are represented by the following symbols: ■23 yr, ◆25 yr, ▲23 yr, ●24 yr, X20 yr, ○82 yr, □80 yr, →67 yr and △77 yr.
4.5 Discussion

This is the first study on the effect of ageing and senescence on the cytokine secretion during human myoblast differentiation. This study revealed two main findings. Firstly, there was no significant difference between young and old myoblasts in the level of expression of detectable cytokines at any of the time periods examined. Secondly, when the cells reached senescence, secretion of TGF-β1 and IL-8 was increased compared to early passage myoblasts.

It is now widely accepted that skeletal muscle cells cultures express several cytokines which are involved in maintaining a balance between growth and differentiation of satellite cells (Charge and Rudnicki, 2004). Detection of IL-4, IL-6, IL-7, IL-8, IL-13, TNF-α and TGF-β1 has been reported in condition medium during differentiation of human skeletal muscle primary cultures (Haugen et al., 2010, Jacquemin et al., 2007, Lafreniere et al., 2006, Nagaraju et al., 1998, Peterson and Pizza, 2009, Tsivitse et al., 2005), but none of these studies used subjects older than 35 years of age. Nine different cytokines were tested in the media from young, old and senescent myotubes using a Luminex based assay. IL-6, IL-8 and TGF-β1 were detectable above baseline. In the present study, TGF-β1, IL-6 and IL-8 are predominantly secreted during the first 48 hours in differentiation medium as the concentration of TGF-β1 and IL-8 decreased dramatically with time. This suggests that myoblasts and not myotubes produce the most TGF-β1 and IL-8, and may indicate that production of these molecules is highest in cells in GM rather than DM. The level of cytokines is GM was not measured. Additionally, there was no age related difference in TGF-β1, IL-6 and IL-8 expression in myoblast differentiation. The data reported herein are in agreement with earlier observations showing that no age related difference in TNF-α, IL-6, IL-1β and TGF-β
mRNA expression in human skeletal muscle (Hamada et al., 2005, Pedersen et al., 2004). Taken together the data indicate that these TGF-β1, IL-6 and IL-8 should be classified as a myokines and may exert their effect in an autocrine or paracrine fashion.

On the other hand, some of the cytokines previously thought to be skeletal muscle derived (IL-4, IL-7, IL-13 and TNF-α) and produced during differentiation were below the detection range in our culture. This could be explained in several ways. First, methods for cell extraction and digestion and, assays for detection of cytokines and, in the composition of differentiation medium differed between the present and previous studies. Secondly, some studies (Jacquemin et al., 2007, Lafreniere et al., 2006, Peterson and Pizza, 2009, Tsivitse et al., 2005) examined myoblasts derived from only one donor individual. Although not examined in this or any of the studies cited herein, it is possible that production of the complete cytokine profile detected in muscle in vivo depends on interactions with certain cell types such as fibroblasts and neurones. Finally, skeletal muscle cells might not produce these mediators during differentiation in vitro cell cultures.

Interestingly senescent cells showed differences in TGF-β and IL8, but not IL6, when compared to early passage cells. It has been shown that persistence of DNA damage induces senescent growth arrest and, IL-6 and IL-8 secretion (Rodier et al., 2009, Rodier et al., 2011). Experiments in the previous chapter showed that senescent myoblast populations displays a markedly high proportion of DNA damaged compared to early passage myoblasts, so it could be suggested that senescence-associated inflammatory cytokine secretion is a consequence of DNA damage occurring during in vitro passage and not in vivo ageing.
TGF-β is an important cytokine that is involved in regulating skeletal muscle myogenesis (Zentella and Massague, 1992, Carlson et al., 2009a). In tissue culture experiments, it has been shown that TGF-β1 is secreted during myoblast proliferation and differentiation (Henningsen et al., 2010, Lafyatis et al., 1991, Nagaraju et al., 1998). Other studies have also demonstrated that fusing satellite cells and newly formed myotubes contain strong TGF-β immunoreactivity during skeletal muscle regeneration, providing evidence for TGF-β role in myoblast fusion (McLennan and Koishi, 1997). In contrast to these findings, TGF-β1 is also capable of inhibiting satellite cell proliferation and differentiation (Allen and Boxhorn, 1987, Carlson et al., 2009b). The present study shows that senescent cells secrete high concentrations of TGF-β1 (250-300 pg/ml) in the first 48 hours of differentiation. Senescent cells neither express myogenin, MHC nor fuse. Thus it could be suggested that TGF-β1 production and inability of senescent cells to differentiate are related and this examined in Chapter 5.

IL-8 belongs to a large family of chemokines that attract primarily neutrophils and act as angiogenic factors (Pedersen and Febbraio, 2008). The role of IL-8 within the muscle is still not fully characterised. IL-8 mRNA has been shown to be enhanced during exercise and IL-8 protein expression within skeletal muscle fibres in the recovery from exercise (Akerstrom et al., 2005, Chan et al., 2004). Recent evidence demonstrated that IL-8 protein is also detected in media during differentiation of human skeletal muscle derived myoblasts (Peterson and Pizza, 2009, Tsivitse et al., 2005), but no direct effect on muscle cells has been reported. The effect of IL-8 on proliferation and differentiation of human myoblasts will be examined in the next chapter.
IL-6 is a pleiotropic cytokine that is secreted and produced by skeletal muscle cells under various conditions where it plays an important role in insulin sensitivity and lipolysis (Carey et al., 2006, Weigert et al., 2005, Wolsk et al., 2010). Exercise rapidly increases the nuclear transcriptional rate of IL-6 within the contracting muscles, with a 10- to 20-fold increase in mRNA content observed following 30 minutes of “non-damaging” exercise and a possible peak (up to 100-fold) immediately at the end of the exercise bout (Keller et al., 2001). Furthermore, the cytokine IL-6 is produced and released by muscle fibres into the circulation during exercise (Penkowa et al., 2003). The muscle-derived IL-6 therefore appears to act in an endocrine manner and may help to explain some of the beneficial effects associated with exercise.

There is evidence that, in addition to its endocrine effects, IL-6 may be involved in regulating processes occurring locally within the muscle producing it. Exogenous IL-6 has been shown to promote myoblast proliferation and differentiation (Baeza-Raja and Munoz-Canoves, 2004, Okazaki et al., 1996, Wang et al., 2008). IL-6 expression is induced during differentiation of C2C12 myoblasts and this is controlled by the p38/NF-κB pathway (Baeza-Raja and Munoz-Canoves, 2004). Moreover, the ablation of IL-6 in myoblasts decreases the expression of differentiation-associated genes such as myogenin. Conversely, overexpression of IL-6 increases the extent of myogenic differentiation (Baeza-Raja and Munoz-Canoves, 2004). IL-6 is also necessary for myofibre growth under certain conditions. In young mice, growing myofibres as well as associated satellite cells actively produce IL-6 (Serrano et al., 2008). When IL-6 expression is eliminated genetically in these mice, hypertrophy is adversely affected in vivo. Taken together, these results suggest that this cytokine may be involved in both satellite cell proliferation and fusion, as well as playing a role in muscle hypertrophy. It
might thus have been expected that IL-6 production would decrease in senescent cells but this was not observed. This shows that maintenance of IL-6 expression is not sufficient to sustain differentiation in senescent cells. Alternatively the myogenic inhibitory effects of TGF-β1, discussed above, could be dominant as the concentration of this cytokine was significantly higher at time periods measured.

To conclude, myotubes have been used as an in vitro model of skeletal muscle for many years, and are therefore well suited for finding, identifying and studying new muscle derived cytokines (myokines) in vitro. Investigation of the secretory profile of human myoblast during differentiation provides clear evidence that there are several muscle-derived myokines that have direct effects on muscle cells. This strongly suggests that these molecules can act locally and may exert their effect in an autocrine or paracrine manner in vivo. No significant difference was observed between young and elderly myoblasts in the level of expression of TGF-β1, IL-6 and IL-8 at any of the time intervals measured. This study revealed that only senescent myoblasts display a markedly higher TGF-β1 and slightly higher IL-8 secretion compared to early passage myoblasts. Further studies are required to explore the role of cytokines on the behaviour of human myoblasts (Chapter 5).
Chapter 5: Effects of inflammatory cytokines on the behaviour of human myoblasts
Several cytokines have been proposed to affect the behaviour of satellite cells within the aged environment. These include TGF-β1, TNF-α, IL-1β and IL-6. In addition, senescent myoblasts secreted high levels of TGF-β1 and IL-8 compared to early passage myoblasts from both young and elderly people (chapter 4). The work described in this chapter examines the effects of these cytokines on the behaviour of human myoblasts.

5.1 Introduction

In normal adult muscle tissue satellite cells exist in a quiescent state, but upon muscle injury these cells becomes activated and proliferate to give rise to a population of myogenic precursors which are capable of repairing or replacing the damaged muscle (Bischoff, 1975, Konigsberg et al., 1975, Morgan and Partridge, 2003, Moss and Leblond, 1971, Snow, 1977b). Soluble endocrine, paracrine and autocrine factors have been identified which play a crucial role in muscle regeneration by either stimulating or inhibiting proliferation and differentiation of myoblasts (Table 5.1). These include TGF-β, TNF-α, IL-1β and IL-6. It has been shown that plasma/serum (circulating) and local tissue cytokine levels become elevated with age (Table 5.2) and this has been suggested as a mechanism by which satellite cell activation is impaired in older muscle.

Previous studies performed concerning TGF-β, TNF-α, IL-1β and IL-6 and their effect on satellite cell behaviour are contradictory (Table 5.1). The majority of studies only focus on one specific concentration of these cytokines which is higher than the physiological range. Therefore, the major aim of this study was to provide a more comprehensive assessment of the effects of these physiologically relevant doses of these cytokines on the behaviour of freshly isolate or committed human myoblasts.
5.2 Aim

The effects of the cytokines were examined at different time points in the culture procedure: 1) immediately following enzymatic digestion and isolation when the majority of cells have not started to express desmin and are termed freshly isolated cells; 2) after expansion for one to two weeks of culture when the cells are expressing desmin and termed committed. All of the committed myoblasts used here are from same populations used in Chapters 3 and 4.

Table 5.1. Studies on the effect of cytokines on the proliferation and differentiation of myoblasts.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Model</th>
<th>Dosage</th>
<th>Finding</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>Human</td>
<td>25 ng/ml</td>
<td>Inhibit proliferation and differentiation</td>
<td>(Carlson et al., 2009b)</td>
</tr>
<tr>
<td></td>
<td>C_{2C12}</td>
<td>5 ng/ml</td>
<td>Promote proliferation and inhibit differentiation</td>
<td>(Schabort et al., 2009)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>C_{2C12}</td>
<td>0.1-10 ng/ml</td>
<td>Inhibit differentiation</td>
<td>(Langen et al., 2001)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Human</td>
<td>10 ng/ml</td>
<td>Promote proliferation and no effects on differentiation</td>
<td>(Wang et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>C_{2C12}</td>
<td>2 ng/ml</td>
<td>Induce differentiation</td>
<td>(Baeza-Raja and Munoz-Canoves, 2004)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>C_{2C12}</td>
<td>10 ng/ml</td>
<td>Promote proliferation and inhibit differentiation</td>
<td>(Langen et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>20 ng/ml</td>
<td>Stimulate proliferation and inhibit differentiation</td>
<td>(Foulstone et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>C_{2C12}</td>
<td>0.05 ng/ml</td>
<td>Stimulate differentiation</td>
<td>(Chen et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5-5 ng/ml</td>
<td>Inhibit differentiation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C_{2C12}</td>
<td>0.001-10 nM</td>
<td>Inhibit proliferation and differentiation</td>
<td>(Layne and Farmer, 1999)</td>
</tr>
</tbody>
</table>
Table 5.2. Reference values for serum or plasma concentration of young and elderly people

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Young</th>
<th>Elderly</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>0-1500 pg/ml</td>
<td>500-3000 pg/ml</td>
<td>(Carlson et al., 2009a)</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.89 pg/ml</td>
<td>2.09 pg/ml</td>
<td>(Ferrucci et al., 2005, Njemini et al., 2011)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0-10 pg/ml</td>
<td>10-120 pg/ml</td>
<td>(Paolisso et al., 1998)</td>
</tr>
</tbody>
</table>

5.3 Methods

5.3.1 Subjects

Prior to acceptance in the study, participants completed a health questionnaire and were only included if they fulfilled the “healthy” or “medically stable” criteria (Greig et al., 1994). Muscle biopsies were collected from 8 young (aged 23-27 years) individuals.

5.3.2 Treatment of myoblasts with cytokines

5.3.2.1 Treatment of freshly isolated cells with cytokines

The methods for myoblast extraction are detailed in the general methods chapter (Section 2.4.1), as are methods for expanding, storing, thawing and culture after thawing in growth medium (Section 2.4 and 2.6). The methods for treatment of freshly isolated myoblasts with cytokines are detailed in Section 2.5.2 and described briefly below. Cell pellets obtained after enzymatic digestion were resuspended in serum free medium, counted and plated in 24 well plates containing culture medium with double
concentrations of FCS, PSG and cytokines relative to the desired final concentration. For differentiation experiments, cells were initially plated in growth medium for three days and then transferred to differentiation medium with 0.5% BSA with or without (control) cytokines as specified in the figures. DM was changed every 48 hours and the cells fixed for immunocytochemical analysis on the seventh day of differentiation.

5.3.2.2 Treatment of committed myoblasts with cytokines

Cells were thawed, passaged once (Chapter 2, Section 2.4), and cultured in skeletal muscle cell growth medium to 50% confluence. At this point, cells were plated in 96 well dishes at a density of 1000 cells/well in the presence or absence of human recombinant TGF-β1, TNF-α, IL-1β, IL-6 or IL-8 at concentrations ranging between 1 pg/ml and 10 ng/ml. Cells were fixed after 3 days and different antigens were examined by immunocytochemistry (Chapter 2, Section 2.8). For differentiation assays, cells were cultured in a skeletal muscle cell growth medium to 50% confluence and plated in 96 well dishes at a density of 7000 cells/well. Media were replaced after 24 hr with differentiation medium containing 0.5% BSA and 1% PS (100 units/ml Penicillin, 100 μg/ml Streptomycin) in the presence or absence of human recombinant TGF-β1, TNF-α, IL-1β, IL-6 or IL-8 at concentrations ranging from 1 pg/ml to 1 ng/ml as specified in the text and figures. Cells were fixed after 3 days and myogenin expression was examined by immunocytochemistry (Chapter 2, Section 2.8).
5.4 Statistics

Statistical comparisons were made using IBM SPSS 19 software, and P values ≤ 0.05 were considered significant. One-way analysis of variance (ANOVA) with post-hoc Dunnett comparisons to control was conducted to confirm statistical significance between variables of interest, e.g. between treated and non-treated cells. Statistical significance for interactions between groups (Control, 100 pg/ml TGF-β1 and 300 pg/ml TGF-β1) and time was determined using mixed two-way repeated measure ANOVA with post-hoc Bonferroni. Results for repeated measure and one-way ANOVA are expressed using the F-Ratio. All values are expressed as mean ± SE.

5.5 Results

5.5.1 Effects of cytokines on proliferation and myogenic commitment of freshly isolated myoblasts

5.5.1.1 TGF-β1 and TNF-α

Typical staining patterns of desmin and Ki67 are shown in Figure 5.1. Freshly isolated human myoblasts were cultured in the presence or absence of human recombinant TGF-β1 (1 pg/ml, 100 pg/ml, 1 ng/ml, 2 ng/ml and 25 ng/ml) or human recombinant TNF-α (1 pg/ml, 100 pg/ml, 1 ng/ml, 10 ng/ml and 50 ng/ml) and monitored for three days (Figure 5.2a-b). The number of cells expressing desmin at this time was significantly lower in the treated cultures and this appeared to be a dose dependent response to both TGF-β1, F (5, 24) = 24, P < 0.0005, and TNF-α, F (5, 18) = 30, P < 0.0005. Only concentrations above 1ng/ml were effective as no statistically significant differences
were observed in the percentage of desmin expressing cells between control and treated cells at concentrations of 1-100 pg/ml. No significant differences were observed in the percentage of Ki67 expressing cells between treated and untreated myoblasts.

### 5.5.1.2 IL-1β, IL-6 and IL-8

There were not sufficient values to carry out statistical analysis. Data are presented as mean percentage of the two experiments for IL-1β and as percentage for one experiment for IL-6 and IL-8 (mean of two wells).

Freshly isolated human myoblasts were cultured in the presence or absence of human recombinant IL-1β, IL-6 or IL-8 (1 pg/ml, 100 pg/ml, 1 ng/ml and 10 ng/ml) and monitored for three days (Figure 5.3a-c). It was observed that the number of desmin expressing cells was lower for IL-1β treated cells than control and the response appeared to be dose-dependent with effects only observed at concentrations above 1ng/ml. The percentage of Ki67 expressing cells was similar between control and IL-1β treated cultures. Neither IL-6 nor IL-8 had any effect on desmin or Ki67 expression.
Figure 5.1. Images taken from of freshly isolated myoblasts in the absence (a) or (b) presence of TGF-β1, (c) TNF-α, (d) IL-1β, (e) IL-6, or (f) IL-8 at 1 ng/ml stained for desmin (green), Ki67 (red) and Hoechst 33342 (blue). Scale bar = 20 µm. The number of desmin expressing cells was lower for TGF-β1, TNF-α and IL-1β treated cells than control.
Figure 5.2. Effects of TGF-β1 and TNF-α on the proliferation and commitment of freshly isolated myoblasts. Freshly isolated human myoblasts were cultured in the presence or absence (a) of human recombinant TGF-β1 (n=5) (1 pg/ml, 100 pg/ml, 1 ng/ml, 2 ng/ml and 25 ng/ml) or (b) human recombinant TNF-α (n=4) (1 pg/ml, 100 pg/ml, 1 ng/ml, 10 ng/ml and 50 ng/ml) and monitored for three days. Data are mean value ± SE. * P < 0.0005 statistically significant difference from control.
Figure 5.3. Effects of IL-1β, IL-6 and IL-8 on the proliferation and commitment of freshly isolated myoblasts. Freshly isolated human myoblasts were cultured in the presence or absence (a) of IL-1β (n=2), (b) IL-6 (n=1) or (c) IL-8 (n=1) (1 pg/ml, 100 pg/ml, 1 ng/ml and 10 ng/ml) and monitored for three days.
5.5.1.3 Reversibility of the effects of treatment with inflammatory cytokines

To investigate whether the effect of TGF-β1, TNF-α or IL-1β on desmin expression was reversible, freshly isolated human myoblasts were cultured in the presence or absence of the cytokines at 1ng/ml. After three days, the medium was removed and the cells were culture for an additional two days in growth medium. The data presented in Figure 5.4 shows that desmin expression was completely restored 2 days after removal of TGF-β1, TNF-α or IL-1β.

5.5.2 Effects of cytokines on NCAM expression in freshly isolated myoblasts

Representative images of NCAM are shown in Figure 5.5. Freshly isolated human myoblasts were cultured in the presence or absence of 1ng/ml human recombinant TGF-β1, human recombinant TNF-α, or human recombinant IL-1β and examined after three days (Figure 5.6). The number of cells expressing NCAM was similar under all conditions tested.
Figure 5.4. Reversibility effects on desmin expression after removal of inflammatory cytokines. Freshly isolated human myoblasts were cultured in the presence or absence (a) of human recombinant TGF-β1 (n=3), human recombinant TNF-α (n=3) or (b) human recombinant IL-1β (n=2). After three days, TGF-β1, TNF-α or IL-1β was removed and the cells were culture for an additional 2 days in growth medium. Data are mean value ± SE. * P < 0.0005 statistically significant difference from control and after removal of cytokine.
Figure 5.5. Images taken from of freshly isolated myoblasts in the absence (a) or (b) presence of TGF-β1, or (c) TNF-α at 1 ng/ml stained for NCAM (green) and Hoechst 33342 (blue). Scale bar = 20 µm. The number of cells expressing NCAM was similar under all conditions tested.
Figure 5.6. Inflammatory cytokines did not affect NCAM expression.

Freshly isolated human myoblasts were cultured in the presence or absence of human recombinant TGF-β1 (n=3), or human recombinant TNF-α (n=3), or human recombinant IL-1β (n=3) (1 ng/ml) and examined after three days. No significant differences were observed in the presence and absence of the cytokines. Data are mean value ± SE.
5.5.3 Differentiation of freshly isolated myoblasts

Representative images of myogenin are shown in Figure 5.7. The number of cells expressing myogenin was significantly lower following treatment with both TGF-β1 and TNF-α, F (2, 6) = 63, P < 0.0005, (Figure 5.8). The same inhibition of myogenin expression was observed following treatment with IL-1β (figure 5.8) but there were not sufficient values to carry out statistical analysis. Data are mean percentage of two experiments.

Figure 5.7. Images taken from of freshly isolated myoblasts in the absence (a) or (b) presence of TGF-β1 at 1 ng/ml stained for myogenin (yellow arrow) and Hoechst 33342 (white arrow). Scale bar = 20 µm. The number of cells expressing myogenin was significantly lower following treatment with TGF-β1.
Figure 5.8. % of cells labelled for myogenin following treatment with TGF-β1, TNF-α or IL-1β. Freshly isolated human myoblasts were first cultured in growth medium for three days and subsequently transferred to differentiation medium in the presence or absence of 1 ng/ml human recombinant TGF-β1 (n=3), human recombinant TNF-α (n=3) or human recombinant IL-1β (n=2) and monitored for seven days. Data are mean value ± SE. * P < 0.0005 statistically significant difference from control.
5.5.4 Proliferation and commitment of committed myoblasts

In contrast to freshly isolated myoblasts, the committed myoblasts showed no difference in desmin expression following three days of treatment with TGF-β1, TNF-α, or IL-1β at concentration of 1 pg/ml and 10 ng/ml (Figure 5.10a-b). IL-6 and IL-8 also had no effect on desmin expression as was observed with the freshly isolated myoblasts. The expression of Ki67 was similar between committed myoblasts in the presence or absence of all the cytokines tested (Figure 5.10a-b). Typical staining patterns of desmin and Ki67 are shown in Figure 5.9.

Figure 5.9. Images taken from of established myoblasts in the absence (a) or (b) presence of TGF-β1 at 10 ng/ml stained for desmin (green), Ki67 (red) and Hoechst 33342 (blue). Scale bar = 20 µm. The number of desmin and Ki67 expressing cells was similar between cells treated with TGF-β1 and control.
Figure 5.10. Profile of desmin and Ki67 expression in committed myoblasts cultured in the presence or absence of inflammatory cytokines. Myoblasts from young (n=3) subjects were cultured in growth medium for 3 days in the presence or absence (a) of TGF-β1, TNF-α, IL-1β or (b) IL-6 or IL-8 and stained for desmin and Ki67. No significant differences were observed between treated and untreated myoblasts (P ≤ 0.05). Data are mean value ± SE.
5.5.5 Differentiation of committed myoblasts

Representative images of myogenin are shown in Figure 5.11. Committed human myoblasts were first cultured in growth medium for 24 hours and subsequently transferred to differentiation medium in the presence or absence of human recombinant TGF-β1, TNF-α or IL-1β at 1 pg/ml, 100 pg/ml and 1 ng/ml and monitored for three days (Figure 5.12a-c). The number of cells expressing myogenin was significantly lowered when cells were treated with 1 ng/ml of TGF-β1, F (3, 8) = 113, P < 0.0005, TNF-α, F (3, 8) = 27, P < 0.0005, or IL-1β, F (3, 8) = 49, P < 0.0005, when compared to control cells. TNF-a and IL-1β were also effective at inhibiting myogenin expression at concentrations of 100pg/ml but not at 1 pg/ml. IL-6 and IL-8 had no effect on myogenin expression at concentrations up to 1ng/ml (Figure 5.13).
Figure 5.11. Images taken from of established myoblasts in the absence (a) or (b) presence of TGF-β1, (c) TNF-α, (d) IL-1β, (e) IL-6, or (f) IL-8 at 1 ng/ml stained for myogenin (yellow arrow) and Hoechst 33342 (white arrow). Scale bar = 20 μm. The number of myogenin expressing cells was lower for TGF-β1, TNF-α and IL-1β treated cells than control.
Figure 5.12. % of cells labelled for myogenin following treatment with (a) TGF-β1, (b) TNF-α, (c) IL-1β. Committed human myoblasts (n=3) were first cultured in growth medium for 24 hours and subsequently transferred to differentiation medium in the presence or absence of human recombinant TGF-β1 or TNF-α or IL-1β at 1 pg/ml, 100 pg/ml and 1 ng/ml and monitored for three days. Data are mean value ± SE. *, P < 0.0005, **, P = 0.003 & #, P = 0.002 statistically significant difference from control.
Figure 5.13. % of cells labelled for myogenin in the presence or absence (a) IL-6 (b) IL-8. Committed human myoblasts (n=3) were first cultured in growth medium for 24 hours and subsequently transferred to differentiation medium in the presence or absence of human recombinant IL-6 or IL-8 at 1 pg/ml, 100 pg/ml and 1 ng/ml and monitored for three days. No significant differences were observed between treated and non-treated myoblasts (P ≤ 0.05). Data are mean value ± SE.
5.5.6 Time course inhibition of myogenin in the presence of 300 pg/ml

**TGF-β1**

Experiments in the previous chapter showed that senescent myoblasts secreted high concentrations of TGF-β1 (250-300 pg/ml) compared to both cells from young and elderly subjects (100-150 pg/ml). In order to examined whether these concentrations are inhibitory, committed human myoblasts were cultured in growth medium for 24 hours and subsequently transferred to differentiation medium in the presence or absence of human recombinant TGF-β1 at 100 pg/ml or 300 pg/ml. Cells were cultured for a further 7 days in differentiation medium, with aliquots of cells being fixed every 24 hr. In parallel, after 3 days, 300 pg/ml TGF-β1 was removed and the cells were culture for an additional 4 days in the presence of TGF-β1 at 100 pg/ml (Figure 5.15). There was a significant interaction between groups and time, $F_{(2, 6)} = 208$, $P < 0.0005$, during myoblasts differentiation. The number of cells expressing myogenin was decreased in the presence of TGF-β1 at 300 pg/ml when compared to cells treated with 100 pg/ml and control cells. The data presented in Figure 5.15 shows that myogenin expression was completely restored 24 hours after reduction of TGF-β1 from 300 pg/ml to 100 pg/ml. Representative images of myogenin are shown in Figure 5.14.
Figure 5.14. Images taken from of established myoblasts in the absence (a) or (b) presence of TGF-β1 at 300 pg/ml stained for myogenin (green) and Hoechst 33342 (blue) after 7 days in differentiation medium. Scale bar = 20 µm. The number of cells expressing myogenin was significantly lower following treatment with TGF-β1.
Figure 5.15. Time course of myogenin expression in the presence or absence of TGF-β1. Committed human myoblasts (n=3) were first cultured in growth medium for 24 hours and subsequently transferred to differentiation medium in the presence or absence of human recombinant TGF-β1 at 100 pg/ml or 300 pg/ml. Cells were cultured for a further 7 days in differentiation medium, with aliquots of cells being fixed every 24 hr. In some wells 300 pg/ml TGF-β1 was substituted with 100 pg/ml after three days and the cells were cultured for an additional 4 days in under this condition. Data are mean value ± SE. *, P < 0.0005 statistically significant difference from control.
5.5 Discussion

This is the first study on the effect of inflammatory cytokines on the behaviour of freshly isolated myoblasts at very early stages following enzymatic dissociation of muscle from biopsy tissue. It is also the first to test the effect of physiologically relevant doses of TGF-β1, TNF-α, IL-1β, IL-6 and IL-8 on committed myoblasts. This study revealed several findings. Firstly, none of the cytokines tested had any effect on the expression of the proliferation marker Ki67 on any of the cells tested. Secondly, desmin and myogenin expression were significantly inhibited in freshly isolated myoblasts exposed to TGF-β1, TNF-α, or IL-1β, and this effect was reversible. Thirdly, myogenin but not desmin expression was significantly inhibited in committed myoblasts in the presence of TGF-β1, TNF-α, or IL-1β. Taken together the data suggest the cytokines have no effect on proliferation of myoblasts but do inhibit progression of both myogenic commitment and differentiation in these cells. Furthermore, the cytokines inhibit myogenic progression but are unable to reverse it. The latter point is supported by two observations: 1) the cytokines do not inhibit NCAM expression in freshly isolated cells since satellite cells express this marker in the quiescent state in vivo; 2) the cytokines do not inhibit desmin expression in committed myoblasts, at least in populations that show stable expression of this protein.

The reported findings regarding the effects of TGF-β1 and TNF-α on myoblast proliferation are contradictory. Some studies suggesting these cytokines promote (Langen et al., 2001, Langen et al., 2004, Schabort et al., 2009) and others that they inhibit (Carlson et al., 2009b, Layne and Farmer, 1999) proliferation of myoblasts. The discrepancies may be attributed to differences in the doses used. In the present study, it
was shown that TGF-β1 or TNF-α do not affect myoblast proliferation in a dose dependent manner in both freshly isolated and committed myoblasts.

The available data does show that differentiation of human myoblasts in culture is negatively affected by TGF-β1 and TNF-α in a dose dependent manner. It has been shown that either TGF-β1 or TNF-α inhibit the transcriptional activity of MyoD and myogenin (Langen et al., 2001, Liu et al., 2001, Martin et al., 1992, Rathbone et al., 2011, Sharples et al., 2010), suggesting a mechanism by which TGF-β1 or TNF-α could regulate differentiation of myoblasts. MyoD is a myogenic regulatory factor required for myogenic commitment and differentiation (Berkes and Tapscott, 2005, Buckingham et al., 2003, Cooper et al., 1999). Furthermore, it has been shown that MyoD plays an integral role in myogenin activation and myogenin subsequently cooperates with MyoD to activate expression of other sets of genes (Cao et al., 2006). Both in vivo and in vitro studies have demonstrated that MyoD-deficient myoblasts show a reduced ability to fuse, a reduction in the number of generated myotubes, and consequently a reduced differentiation potential (Yablonka-Reuveni et al., 1999, Megeney et al., 1996, Montarras et al., 2000, Sabourin et al., 1999). Although MyoD expression was not analysed in the experiments presented herein, the above provides an explanation for the inhibition of myogenin expression during differentiation of both freshly isolated and committed myoblasts treated with TGF-β1 and TNF-α.
TGF-β1 and TNF-α have also been shown to induce expression of cyclin D1, which is involved in progression from the G1 to S phases of the cell cycle and to negatively affect cell cycle exit thereby leading to inhibition of myoblast differentiation (Langen et al., 2004, Rao and Kohtz, 1995). Both the absence of an effect of TGF-β1 and TNF-α on Ki67 expression as well as their inhibitory effects on myogenin expression could potentially be explained by this mechanism. Continued Ki67 expression in serum free DM in the presence of the cytokines was not investigated, however, and it is thus not possible to assert conclusively whether inhibition of cell cycle arrest was a contributory factor to the lack of myogenic progression observed.

IL-1β is a pleiotropic pro-inflammatory cytokine, which has been considered a pathological factor in different conditions such as inflammatory myopathies and diabetes (Authier et al., 1997, Lundberg et al., 2000, Maedler et al., 2002). The physiological concentration of IL-1β in serum at rest has been reported to be 2.04 ± 4.93 pg/ml in young and 2.52 ± 7.41 in elderly subjects (Kim et al., 2011a). IL-1β is produced by skeletal muscle where it plays an important role in muscle protein breakdown by activating p38 MAPK and NF-κB, and the muscle specific ubiquitin ligases atrogin-1/MAFbx and MuRF-1 (Authier et al., 1997, Jozsi et al., 2000, Li et al., 2009). To the author knowledge only one group has investigated the effect of IL-1β on the differentiation of myoblasts into myotubes (Langen et al., 2001). Culturing C2C12 myoblasts in the presence of 0.1-10 ng/ml IL-1β has been shown to inhibit differentiation. In agreement with this observation, the present study shows that IL-1β inhibits desmin expression, sustains proliferation and inhibits differentiation of freshly isolated human myoblasts at doses of 100 pg/ml but not at 1pg/ml, closer to the reported serum concentrations. The inhibition of desmin expression in freshly isolated myoblasts
was reversible after removal of IL-1β from the medium. It is possible that IL-1β acts through similar mechanisms to TGF-β1 and TNF-α by either inhibiting MyoD expression or inducing cyclin expression and action, but this possibility has not been tested.

It has been proposed that TGF-β1 induces trans-differentiation of myoblasts into fibroblasts (Cencetti et al., 2010). In order to examine whether TGF-β1, TNF-α, or IL-1β affected the phenotype of myoblasts, freshly isolated human cells were cultured in the presence or absence of the cytokines and stained for NCAM after three days of culture. NCAM is a marker of satellite cells used for their identification in histological sections of resting muscle (Mackey et al., 2010, Mackey et al., 2009) as well as for magnetic sorting of myoblasts cultures (Pietrangelo et al., 2009). The results showing that NCAM expression was similar between treated and untreated myoblasts, indicates that none of the cytokines tested induced trans-differentiation myoblasts into a different cell type. Rather, it appears that by reversibly inhibiting expression of the muscle regulatory factors without affecting proliferation, these cytokines can maintain the cells in an immature state. The length of time over which treatment with these cytokines can maintain this immature state and still be fully reversible, without affecting the proliferation or differentiation potential, of the myoblasts remains to be determined.

The three cytokines may act through similar signalling mechanisms to exert their effect. E-box and MEF2 binding sites are indispensable for desmin and myogenin expression (Du et al., 2003, Li and Capetanaki, 1994). It has been shown that TGF-β through Smad3 inhibits E-box and MEF2 dependent transcription which subsequently lead to inhibition of myoblasts differentiation (Liu et al., 2001, Liu et al., 2004). More recently,
TNF-α and IL-1α have been shown to induce secretion of Activin A which subsequently inhibits human myoblast differentiation via ALK/SMAD2/3 signalling (Trendelenburg et al., 2012).

Conflicting literature exists on the effect of IL-6 on skeletal muscle. IL-6 is a pleiotropic cytokine that is secreted and produced by skeletal muscle cells under various conditions where it plays an important role in insulin sensitivity and lipolysis (Carey et al., 2006, Weigert et al., 2005, Wolsk et al., 2010). It has been shown that IL-6 levels become elevated with age and this has been associated with the loss of muscle mass and strength (Njemini et al., 2011, Pedersen et al., 2003, Visser et al., 2002). There is evidence to suggest that it induces hypertrophy (Serrano et al., 2008) as well as atrophy (Haddad et al., 2005). Exogenous IL-6 has been shown to promote myoblast proliferation and differentiation (Baeza-Raja and Munoz-Canoves, 2004, Wang et al., 2008). The data show that IL-6 has no effect on proliferation and differentiation of either freshly isolated or committed human myoblasts. The discrepancies can be attributed wholly to differences in doses used and different methodological techniques. The results suggest that IL-6 does not affect muscle proliferation or differentiation either at physiological (1pg/ml) or supra-physiological (0.1-10ng/ml) doses. Differences in cell extraction and digestion, different differentiation medium and different muscle type (vastus lateralis muscle vs. temporal muscle) between this study and others (Baeza-Raja and Munoz-Canoves, 2004, Wang et al., 2008) could potentially explain the discrepancy in findings.
IL-8 belongs to a large family of chemokines that attract primarily neutrophils and act as angiogenic factors (Pedersen and Febbraio, 2008). IL-8 mRNA has been shown to be enhanced during exercise and IL-8 protein expression within skeletal muscle fibres in the recovery from exercise (Akerstrom et al., 2005, Chan et al., 2004). The physiological concentration of IL-1β in serum at rest has been found be 23.9 ± 29.7 pg/ml in young and 27.6 ± 43.9 in elderly subjects (Kim et al., 2011a). The direct effect of IL-8 on muscle cells has not been reported. The present study shows that IL-8 has no effect on proliferation and differentiation of human satellite cells, suggesting that IL-8 has no role to play in muscle cell behaviour.

It must also be stated that the definition of physiological doses for all the cytokines studied in the present chapter is based on the concentrations detected in serum. The effective concentrations of cytokines to which satellite cells are exposed in skeletal muscle tissue have not been determined and may be higher than serum concentrations by such mechanisms such as binding to extracellular matrix proteins or localized secretion by the myofibres. Other possibilities are that the in vivo effects the cytokines examined are modulated by the presence of other molecules that could decrease the sensitivity threshold of the cells compared to that observed in vitro, or that the cytokines would act cooperatively to exert their effects. Even taking these arguments into consideration it is difficult to justify the total absence of any effect over the 1000-fold concentration range of IL-6 and IL-8 examined here on the proliferation and differentiation responses of human myoblasts shown here.
In conclusion, the data presented herein show that TGF-β1, TNF-α, and IL-1β interfere with myogenic progression in a dose dependent manner and suggest that TGF-β1, TNF-α, and IL-1β might act *in vivo* to sustain proliferation. Possible mechanisms of action include increased cyclin expression with subsequent reductions in cell cycle exit or effects on transcription of muscle specific genes. The sustained elevation of these cytokines *in vivo* would result of an inability of satellite cells to regenerate skeletal muscle after damage.
Chapter 6: Effects of human serum on the behaviour of human myoblast
6.1 Introduction

Several studies have undertaken which suggest that muscle extrinsic factors arising from the systemic environment may affect muscle repair (Carlson and Faulkner, 1989, Carlson and Conboy, 2007, Carlson et al., 2009b). The first experiments to suggest a “systemic” impairment to muscle repair and regeneration were performed by Carlson and Faulkner (1989) who showed that the mass and function of long term (60 days) transplanted rat muscles were impaired by the age of the host, not by the age of the transplanted muscle. A parabiotic study has also reported that the systemic environment is critical to the success of regeneration by aged satellite cells and suggested that inhibitory factors might be present in serum (Conboy et al., 2005). More recently, in vitro experiments have demonstrated that sera from elderly rodents or humans inhibit myoblast activation and subsequently proliferation and fusion (Brack et al., 2007, Carlson and Conboy, 2007, Carlson et al., 2009b). Findings such as these have been attributed to reduced serum levels of IGF-1, an imbalance between Notch and TGF-β/pSmad pathway signalling, and increased levels of circulating TGF-β1 (Carlson et al., 2009a, Carlson et al., 2008, Reeves et al., 2000). Therefore it seems to be that a loss of regenerative potential in the satellite cells depends on the factors present or absent in the extra-cellular environment.

Ageing has been associated with increased serum concentrations of inflammatory cytokines (Carlson et al., 2009a, Dobbs et al., 1999, Paolisso et al., 1998, Schmitt et al., 2005, Wei et al., 1992), specifically TGF-β1, TNF-α and IL-6. Experiments in the previous chapter showed that TGF-β, TNF-α, and IL-1β robustly inhibit desmin expression at early time points in freshly isolated cells. The study presented in this
chapter was contributed data to a paper which was published in the Journal of Gerontology.

6.2 Aim

There were two aims to the study. The first aim was to assess the ability of freshly isolated myoblasts extracted from human muscle biopsies to proliferate in culture when exposed to serum from either young or elderly subjects. Proliferating muscle cells were identified by their expression of the antigens for Ki67 and desmin. The second aim was to measure the concentration of inflammatory cytokines in serum that was obtained from young and elderly subjects by using Luminex based assays.

6.3 Methods

The methods for myoblast extraction are detailed in the general methods chapter (Section 2.4.1). Methods for human myoblast isolation in human sera are outlined below (Section 2.5.1). The protocol for human serum extraction is outlined in the general methods chapter (Section 2.3). The Luminex assay was carried out as described in general methods chapter (Section 2.9).

6.3.1 Subjects

Prior to acceptance in the study, participants completed a health questionnaire and were only included if they fulfilled the “healthy” or “medically stable” criteria (Greig et al., 1994). The total sera were collected from 6 young (aged 23-29 years) and 6 elderly (aged 69–82 years) subjects and used as specified in the figure legends.
6.3.2 Human myoblast cultured in human sera

A muscle biopsy was obtained from a young male (aged 27 years). Cells were isolated and were immediately resuspended in basal medium. Cells were plated in duplicate wells of a 24 well plate, coated with ECM matrix (8 mg/ml, Sigma), at a density of 100,000 cells per well. Growth medium (2X GM, control) or 2X growth medium supplemented with 30% human serum and 2% PSG was then added to each well such that final serum and antibiotic concentrations were halved. Media were changed 72 hours after initial isolation and every 48 hours thereafter. Before discarding, old media was transferred to a sterile 1.5ml microcentrifuge tube and centrifuged at 650g for six minutes to harvest any floating myoblasts. The supernatant was discarded and the cell pellet was re-suspended in fresh medium and added to the appropriate culture wells. Cells were fixed at 3, 5 and 7 days after isolation and analysed for desmin, Ki67 and both desmin+Ki67.

6.4 Statistics

Statistical comparisons were made using IBM SPSS 19 software, and P values ≤ 0.05 were considered significant. Statistical significance for interactions between group (Young and Elderly) and time was determined using mixed two-way repeated measure ANOVA with post-hoc Bonferroni. If there were significant interactions present, independent t tests were conducted to confirm statistical significance between variable of interest e.g. between groups, and paired-sample t tests undertaken for variable of interest within group and time. Results for repeated measure are expressed using the F-Ratio. All values are expressed as mean ± SE.
6.5 Results

6.5.1 Characterisation of human myoblasts in growth medium

Typical staining patterns of desmin and Ki67 are shown in Figure 6.1. To determine the time course of desmin and Ki67 expression immediately after isolation from muscle biopsies, cells were cultured in GM and monitored for three, five and seven days (Figure 6.2). Desmin expression was observed in 50% of cells after three days and 85% after seven days (Figure 6.2a). Ki67 expression was observed in 65% at day three and 90% at day 7 (Figure 6.2b). All desmin positive cells also expressed Ki67 at each time point (Figure 6.2c). At day 3 the wells contained an average of 1000 cells indicating that 1% of plated cells (100000 plated per well) had adhered. At day 5 the wells contained an average of 3000 cells and by day 7 the total number was >3000 cells, indicating that the number of adherent cells increased with time in culture.

6.5.2 Effects of human serum on freshly isolated cells

Human myoblasts were cultured in human serum (6 young vs. 6 elderly individuals) and monitored for three, five and seven days (Figure 6.3a-c). The number of cells expressing desmin, Ki67 and co-staining for both desmin and Ki67 was significantly increased with time in culture for all groups, F (2, 9) = 11.122, P = 0.004 for desmin, F (2, 9) = 39.34, P < 0.0005 for Ki67, F (2, 9) = 21, P < 0.0005 for both desmin and Ki67. No significant differences were observed in the percentage of desmin+, Ki67+ and Ki67+desmin+ cells at any of the time point examined between young and elderly serum. Figure 6.4a-c shows the individual values for desmin and Ki67 co-expression for each serum or for GM. High proportions of cells expressing desmin also express Ki67 irrespective of whether they are cultured in human serum or GM.
Figure 6.1. Images taken from of human myoblasts. Cells from young subject (27 years) were cultured in the human serum obtained from young (from a male subject aged 24 years) (a) and elderly (from a male subject aged 82) (b) subjects. Cells were fixed at days 7 and stained for desmin (green), Ki67 (red) and Hoechst 33342 (blue). Scale bar = 20 μm. The number of desmin and Ki67 expressing cells was similar between young and elderly serum.
Figure 6.2. Time course of (a) desmin (b) Ki67 and (c) desmin and Ki67 expression in cells freshly isolated in growth medium.
Figure 6.3. Time course of (a) desmin (b) Ki67 and (c) desmin and Ki67 expression in cells freshly isolated in human serum. Cells were obtained from 27 year old and cultured in media containing serum obtained from young (closed symbols, solid lines, n=6, aged 23–29 years) or elderly (open symbols, dashed lines, n=6, aged 69–82 years) subjects. Values are mean ± SE. No difference was observed between young and old sera conditions. Day 3 is significantly different from days 5 and 7 (* P < 0.0005) for desmin and Ki67 expression and significantly different from day 7 only for both desmin and Ki67 expression (° P < 0.0005) for both serum conditions. Day 5 is significantly different from day 7 for desmin and, both desmin and Ki67 expression (** P = 0.01) for both serum conditions.
Figure 6.4. Individual data points for culture in young serum (YS) or old serum (OS) are shown for desmin and Ki67 positive cells at days 3 (1), 5 (b) and 7 (c), blue squares indicate responses in GM. Individual serum and gender ages are denoted by the following symbols: elderly: ♦ 82 yr, □ 69 yr, ▲ 80 yr, △ 77 yr, × 74 yr, ● 82 yr, young: ▲ 27 yr, × 24 yr, ○ 23 yr, ♠ 24 yr, ● 25 yr, △ 25 yr.
6.5.3 Cytokine levels in serum

There were no statistically significant differences in the serum level of TGF-β1, TNF-α, IL-6, IL-8, IL-7, IL-13 and IL-10 between young (n=6) and elderly (n=6) subjects (Table 6.1). The level of IL-4 and IL-1β were below the detection limits of the assay (0.13 pg/ml) in all young and elderly subjects. Figure 6.5a-g shows the individual values for cytokine levels for each serum.

Table 6.1. Serum level of cytokines in young and old subjects. Values are given as mean ± SE.

<table>
<thead>
<tr>
<th>Cytokine name</th>
<th>Serum concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>1297.0 ± 72.0</td>
</tr>
<tr>
<td>TNF-α</td>
<td>7.0 ± 3.0</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.3 ± 0.8</td>
</tr>
<tr>
<td>IL-8</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>IL-7</td>
<td>17.0 ± 3.0</td>
</tr>
<tr>
<td>IL-13</td>
<td>4.0 ± 2.0</td>
</tr>
<tr>
<td>IL-10</td>
<td>7.0 ± 3.0</td>
</tr>
</tbody>
</table>
Figure 6.5. Individual data points for (a) TGF-β1, (b) TNF-α, (c) IL-6 and (d) IL-8 level in human serum obtained from young (n=6, aged 23–29 years) or elderly (n=6, aged 69–82 years) subjects.
Figure 6.6. Individual data points for (a) IL-7, (b) IL-13 and (c) IL-10 level in human serum obtained from young (n=6, aged 23–29 years) or elderly (n=6, aged 69–82 years) subjects.
6.5 Discussion

The present study revealed two main findings. Firstly, the proliferation of myoblasts was not affected by the age of the circulatory milieu. Secondly, the serum levels of TGF-β1, TNF-α, IL-6, IL-8, IL-7, IL-13 and IL-10 showed no significant differences between young and elderly individuals.

The system of freshly isolated cells examines the early events of myoblast culture, including cell cycle entry. The time course of this response was studied in one cell population and observed that sera from both young and elderly subjects was equally effective in promoting Ki67 and desmin expression when measured at Days 3, 5 and 7 (Figure 6.3) of proliferation. In parallel, the time course of Ki67 and desmin expression was studied in the absence of human sera (i.e. only in GM, Figure 6.2). Expression of both Ki67 and desmin increased with time in culture and virtually all desmin positive cells eventually expressed Ki67 showing that myogenic commitment and proliferation are closely related at this early stage. It must be noted that this assay cannot determine whether the increase in proliferating muscle cells between day 3 and day 5 is due to increased desmin expression in proliferating cells, increased proliferation in desmin positive cells or more robust proliferation of the cells which were double labelled at day 3.

In this study serum concentration from young and elderly subjects was measured by a Luminex based assay. No differences were seen in the levels of TGF-β1, TNF-α, IL-6, IL-8, IL-7, IL-13 and IL-10 between the two groups. This could then explain why the cells behave the same in young and elderly serum. Some controversy surrounds the cytokine levels with age, as some studies demonstrated no age related difference (Bayer
et al., 2012, Kim et al., 2011a, Pedersen et al., 2004), while others found an increased in elderly subjects when compared to young subjects (Carlson et al., 2009a, Ferrucci et al., 2005, Zhu et al., 2009). A likely reason for such a discrepancy is that in the latter studies the participants were not pre-screened for confounding medical conditions, which could increase inflammation. In contrast, the subjects used in the present study and those by (Kim et al., 2011a, Pedersen et al., 2004) were generally healthy. Although this study is based on more subjects than any previous studies, the variation between individuals potentially impact on outcomes. That said, the fact that there were no differences despite there being heterogeneity between individuals, suggests that if there were any age related differences, a large number of cell populations would need to have been studied to demonstrate this statistically. Whilst this possibility cannot be excluded the data presented here suggest that that there are no age related differences.

It was shown that TGF-β1 inhibits the proliferation and differentiation of skeletal muscle cells in vitro (Figure 5.2 & 5.8 in Chapter 5). Recent reports demonstrated that skeletal muscle satellite cells cultured in young and elderly serum are highly sensitive to TGF-β1 (Carlson et al., 2009a, Carlson et al., 2009b). The present thesis shows that there is no significant difference in TGF-β1 levels between the sera of young and old subjects (1297 ± 72 pg/ml vs. 1062 ± 218 pg/ml) and these concentrations should be inhibitory (Figure 5.2 & 5.8 in Chapter 5). TGF-β1 is secreted in latent forms in a large latent complex which is attached to the extracellular matrix and release of TGF-β from this complex is required to allow receptor binding and initiation of the desired signalling pathway and subsequent response (O'Kane and Ferguson, 1997, Munger et al., 1997, Gleizes et al., 1997). There is no conclusive evidence to demonstrate whether TGF-β1 is active or not active in human serum. Taken together these results suggest that serum
TGF-β1 levels are not active. Specifically, “if all endogenous young and old sera TGF-β1 were active, it would readily suppress satellite cell proliferation in vitro” (Carlson et al., 2009a).

There are various differences in the findings between this study and other recent studies. The findings in this study showed that muscle proliferation and differentiation is not impaired with age. In contrast to the data herein, it has previously been demonstrated from studies in mice and humans that serum from aged individuals decreases the ability of myoblasts to proliferate, or express the muscle markers, Pax7, Myf5, MyoD, and desmin (Brack et al., 2007, Carlson and Conboy, 2007, Carlson et al., 2009b). Whilst the difference between this study and those using a mouse cell /serum model (Brack et al., 2007, Carlson and Conboy, 2007) would not be the first instance of a species difference, there are some methodological differences between the protocol used in this study and that used by Carlson and Conboy (2007) and Carlson et al. (2009b). Most significantly, the cells used for most mouse cultures were associated with living myofibres when isolated from tissue. The mouse muscle was also severely injured two days prior to myofibre extraction (Carlson and Conboy, 2007), thus the satellite cells were already activated in vivo. The biopsy in this study was obtained from resting muscles and the cells are only activated during the biopsy and cell preparation procedures. The human myoblast isolation procedures outlined in this study are comparable with those of many laboratories (Dellavalle et al., 2007, Schafer et al., 2006). Other methodological differences between this study and other experiments (Brack et al., 2007, Carlson and Conboy, 2007, Carlson et al., 2009b) include the use of different enzymes for tissue digestion (trypsin as opposed to collagenase), the use of different markers to measure cell proliferation (Ki67 as opposed to BrdU), different
time points for initial measurements (72 versus 48 h), different serum concentrations (15% versus 10%) and the fact that the sera were not pooled by age group in any of the present assays. Pooling of serum may represent the major source of difference in observations presented here and in (Carlson et al., 2009b), which eliminating the possibility for comparison between individual serum donors. It must be noted that individual serum samples were used in this study. Further work is needed to clarify what effects these methodological differences may have. However, the results would suggest that the source of any age related inhibition of satellite cell response is not serum borne.

Whilst there are data showing impaired satellite cell behaviour (Brack et al., 2007, Carlson and Conboy, 2007, Conboy et al., 2005), it is noteworthy that there are a number of other studies which suggest that satellite cell proliferation and myogensis occur as effectively in older as in young animals. For example, recent work examining whole muscle grafts transplanted between young (8 weeks) and old (13-21 months) mice suggest that there are minor age-associated delays in inflammation and neovascularisation in response to injured muscle with no detrimental effect on muscle regeneration (Smythe et al., 2008). Other animal studies which have used a variety of different models including chronic low-frequency electrical stimulation (Putman et al., 2001), denervation (Dedkov et al., 2003) as well as cross transplantation in mdx mice (Boldrin et al., 2009) have similarly observed no age related impairment to muscle repair. Taking the above observations into account, it is also possible to speculate that there is a slower activation of young cells cultured in old serum that might be detectable before day three of culture, but the data presented show that even if this were the case, these initial differences have no effect on later myogenic responses.
In human exercise studies there is also some debate as to whether there is an age related impairment to the function of satellite cells. There is some evidence that in response to an acute bout of exercise, the increase in satellite cell number may be blunted in older male individuals (Dreyer et al., 2006). Furthermore, a short period (4 weeks) of strength training immediately following a two weeks period of cast immobilization, resulted in a blunted satellite cell response in the older muscles (Carlson et al., 2009b). These short term responses seem in contrast to the results of a number of longer (12-16 weeks) strength training studies, which suggest that satellite cell number can be successfully increased in older individuals (Mackey et al., 2007, Roth et al., 2001, Verdijk et al., 2009, Verney et al., 2008) with no age related impairments (Roth et al., 2001). These data coupled with the demonstrated effectiveness of strength training regimens to increase muscle mass and improve function, even in very elderly people (Fiatarone et al., 1990, Harridge et al., 1999).

Taken altogether the literature has a body of work which suggests that age-related impairments in satellite cell mediated repair exist in animal muscles following severe damage. There are no equivalent human models for such extreme damage. There is also emerging evidence in animals suggesting that there is no age-related impairment but suggesting that there may be delayed response. The results of this study suggest that there is no effect of the aged circulatory milieu on the behaviour of freshly isolated human myoblasts in culture.
Chapter 7: General Discussion and Conclusions
7.1 Main findings

The data presented in this thesis demonstrated no difference in the behaviour of cells obtained from both either young or elderly people at least in terms of the parameters measured which related to proliferation and differentiation. However, dramatic differences were observed in cells that had undergone replicative senescence. A marked reduction in differentiation potential was observed in the senescent cells, as evidenced by a decrease in the expression levels of myogenin after three days of differentiation and MHC after five days of differentiation. In addition, senescent cells exhibited higher expression of the DNA damage marker γ-H2AX and increased TGF-β secretion (Chapter 3 and 4). The work presented in this thesis also indicated that TGF-β1, TNF-α, and IL-1β interfere with myogenic behaviour in a dose dependent manner (Chapter 5), but that young and healthy elderly sera contain similar amounts of these cytokines and thus seemed equally effective in promoting proliferation and desmin expression in human myoblasts independent of the age of the subject (Chapter 6).

7.2 Intrinsic behaviour of human myoblasts

Replicative senescence of cells in culture has previously been used as a model to study the ageing process in human muscle (Bigot et al., 2008). However, the relevance of this approach to in vivo ageing remains unclear. Chapter 3 and 4 are the first study to investigate whether in vivo muscle ageing differs from in vitro muscle ageing by studying primary human muscle cells obtained from both young and elderly people, before and after they reached replicative senescence. Several parameters associated with ageing process were compared: ability to undergo differentiation; presence of DNA damage; and production of cytokines. The progression of differentiation of human
primary myoblasts was shown to be decreased and delayed by two-four days in senescent compared to early passage cells. However, the differentiation rates of cells from older subjects were similar to those of cells from young subjects at early passages and did not exhibit the characteristics of the senescent cell populations. Only the senescent cells demonstrated altered cell morphology, exhibited significant DNA damage and high levels of TGF-β1 and IL-8 secretion. These data suggest that passage number is a critical factor to control in such experiments as reductions in differentiation potential is significantly affected after sixteen days of “in vitro ageing”. This important as a number of human myoblast studies involve a cell expansion period of indeterminate duration (Beccafico et al., 2007, Beccafico et al., 2010, Corbu et al., 2010, Fulle et al., 2005, Lorenzon et al., 2004, Pietrangelo et al., 2009).

The data reported in Chapter 4 show that senescent cells secrete high concentrations of TGF-β1 (250-300 pg/ml) and this concentration inhibits myoblast fusion. It could thus be suggested that delay and decrease differentiation in senescent myoblasts is determined by TGF-β1 production since by day 3-5 myogenin and MHC expression is such that it corresponds with a reduction in TGF-β1 secretion to 100pg/ml, a non inhibitory concentration.

The major question is whether the changes observed in replicative senescence reflect in vivo ageing. Many studies on human fibroblasts have demonstrated an inverse relationship between donor age and replicative lifespan (Macieira-Coelho and Ponten, 1969, Schneider and Mitsui, 1976). This effect is inconsistent with a high number of health donor samples (Cristofalo et al., 1998). The work in this thesis and that of others did not discern any correlation between the age of the donor and the number of mean
population doublings before myoblasts senesced (Decary et al., 1997, Mouly et al., 2005, Pietrangelo et al., 2009, Renault et al., 2000). It has also been shown that myoblasts extracted from elderly biopsies (>80 years) behave similarly to those obtained from very young cell populations (2-5 years) in terms of their mean population doublings (Schafer et al., 2006). One explanation that has been proposed for the contrast in behaviour between myoblasts and skin fibroblasts is that satellite cells in healthy muscle would not normally undergo a significant number of divisions in vivo once growth has ceased and therefore would maintain their proliferative potential throughout the lifespan of the individual (Decary et al., 1997). A similar maintenance of differentiation capacity would also be coherent with this explanation.

The data reported in Chapter 3 show a large heterogeneity in proliferation capacity and desmin content of myoblasts obtained from different subjects, even when these are of the same age group. In this regard Schafer et al. (2006) found that the number of population doublings and proportion of desmin expression in human myoblasts varies independently of age. There also does not appear to be a relationship between myogenic purity (proportion of desmin positive cells) of a population and the age of the donor (Beccafico et al., 2007, Pietrangelo et al., 2009, Schafer et al., 2006).

Two key differences were noted between the work in this thesis and those who identified age related impairment of human myoblast differentiation:

i. Some studies draw conclusions from comparisons of very small numbers of subjects, often only one from each age group (Fulle et al., 2005, Jacquemin et al., 2004, Lorenzon et al., 2004).
ii. Other studies draw conclusions from comparing populations that do not have equivalent proportions of cells expressing desmin (Beccafico et al., 2007, Pietrangelo et al., 2009) or have not been characterised in terms of desmin content (Beccafico et al., 2010).

Desmin content reflects the proportion of myogenic cells in the culture and therefore determines differentiation potential of a population independently of age. Studies comparing only one subject from each age group do not provide strong enough evidence on their own of age related effects, since in the human population there are very large variations in factors other than age (i.e. genetic and lifestyle factors) that could provide an explanation for the effects observed.

As mentioned above, the data show that proliferation and differentiation of human myoblasts are not affected by the age of the donor. Supporting evidence for the above include recent in vitro experiments with myoblasts from elderly animals showing that these can proliferate and differentiate similar to that of young animals (Dumke and Lees, 2011). Furthermore, it has been shown that human myoblasts from an elderly donor can contribute myonuclei very efficiently to regenerating mouse muscle in vivo (Schafer et al., 2006). Collins et al. (2007) found that elderly mouse satellite cells are also not impaired in their ability to regenerating muscle in vivo.
7.3 Behaviour of human myoblasts in the presence of cytokines

Circulating levels of IL-6, TNF-α and TGF-β seem to increase with advancing age (Carlson et al., 2009a, Dobbs et al., 1999, Njemini et al., 2011, Paolisso et al., 1998, Wei et al., 1992). Local production of TGF-β, TNF-α and IL-1β also increase in aged skeletal muscle (Leger et al., 2008, Przybyla et al., 2006, Carlson et al., 2009b). The exact effect of these cytokines on skeletal muscle satellite cells is not clear and the results from different studies are contradictory (Table 5.1). However, the concentrations of cytokines administered differ between studies with the majority only assessing the effect of one concentration of cytokine on myoblasts. In Chapter 5, a comprehensive characterization of the dose-dependent effects of IL-6, IL-8, IL-1β, TNF-α, and TGF-β was tested on human myoblasts proliferation and differentiation. The data show that TGF-β1, TNF-α, and IL-1β are potent inhibitors of desmin and myogenin expression in freshly isolated primary muscle cells during the early stage of activation. Cell proliferation was not impaired as evidenced by the expression of Ki67, but commitment to the myogenic lineage was suspended (as evidenced by impaired desmin expression) even though the cells were expressing the satellite cell marker NCAM. Importantly, inhibition of desmin expression in freshly isolated myoblasts was reversible after removal of TGF-β1, TNF-α, and IL-1β from the medium. In committed myoblasts these cytokines do not inhibit desmin but do inhibit differentiation as indicated by decreased myogenin expression. Taken together the data suggest the cytokines have no effect on proliferation of myoblasts, but do inhibit progression of both myogenic commitment and differentiation. The three cytokines may act through similar mechanisms to exert their effect. Although not tested in the present thesis this may indicate that the cytokines could inhibit cell cycle withdrawal and therefore reduce differentiation. Thus sustained elevation of these cytokines would seem to result of in an inability of satellite cells to
regenerate skeletal muscle after damage. Interestingly, the cytokines IL-6 and IL-8 have no effect on proliferation and differentiation of human satellite cells, suggesting that IL-8 and IL-6 may have no role to play in muscle repair.

7.4 Behaviour of human myoblasts in the presence of human serum

Freshly isolated cells cultured in media containing human sera to examine the onset and progression of Ki67 and desmin expression showed no effect of the age of the serum measured at 3, 5 and 7 days of proliferation. The results suggest that human myoblast behaviour is not negatively affected by culture in an “elderly environment”. This might be explained by the fact that serum levels of TGF-β, TNF-α, IL-6, IL-8, IL-7, IL-13 and IL-10 were found to be similar between young and healthy elderly subjects.

This thesis shows that TGF-β1 inhibits the proliferation and differentiation of skeletal muscle cells in vitro even at low concentrations (300 pg/ml; Figure 5.2, 5.8, 5.12 & 5.15 in Chapter 5) and as mentioned above there is no significant difference in TGF-β1 levels between the sera of young and old subjects. However, these concentrations (1297 ± 72 pg/ml vs. 1062 ± 218 pg/ml) should be inhibitory (Figure 5.2 & 5.8 in Chapter 5). Importantly, there is no conclusive evidence to demonstrate whether TGF-β1 is active or not active in human serum because TGF-β1 is secreted in latent forms in a large latent complex which is attached to the extracellular matrix and release of TGF-β from this complex is required to allow receptor binding and initiation of the desired signalling pathway and subsequent response (O’Kane and Ferguson, 1997, Munger et al., 1997, Gleizes et al., 1997). Taken together these results might suggest that serum TGF-β1 levels are not active. Specifically, “if all endogenous young and old sera TGF-β1 were
active, it would readily suppress satellite cell proliferation *in vitro*” (Carlson et al., 2009a).

The data in this thesis on freshly isolated cell cultures are in contrast with results from studies in mice (Brack et al., 2007, Carlson and Conboy, 2007) and humans (Carlson et al., 2009b) where serum from elderly donors decreased the ability of myoblasts to incorporate BrdU and express the muscle markers, Pax7, Myf5, MyoD, and desmin.

Three key differences were noted between the *in vitro* myoblasts experiments performed by Brack et al. (2007), Carlson and Conboy (2007) and Carlson et al. (2009) and the human protocol used in this thesis:

i. The mouse muscle tissue was severely damaged two days prior to satellite cell extraction, thus the satellite cells were already activated *in vivo* prior to their culture *in vitro*. There are no equivalent human models for such extreme damage. Biopsies were obtained from resting muscles and satellite cells were only activated during the biopsy and cell preparation procedures.

ii. Satellite cells in the mouse experiments were cultured with their associated myofibres intact. It was not possible to include whole human fibres in the established culture protocol.

iii. Pooling of serum may represent the major source of difference in observations presented here and compared with Carlson et al. (2009) since one or two badly performing sera would “contaminate” the whole batch suggesting that there is a difference between young and elderly subjects when in fact only one or two outliers may show a diminished response.
Satellite cells behaviour *in vivo* is moderated by its myofibre and basal lamina as well as neuronal factors, cell-cell interaction with fibroblasts and adipocytes and other factor within the “niche” (Gopinath and Rando, 2008). Whilst studying the behaviour of cells in culture has a number of experimental advantages, the extent to which their behaviour in culture can be extrapolated to behaviour *in vivo* remains unclear. The precise composition of the satellite cell niche *in vivo* is likely to be only estimated crudely by culture conditions. It is possible that satellite cell niche may not have been accurately recreated in this study which could be a contributing factor to the lack of response noted.

Satellite cells can be identified in cryosections of human muscle biopsy samples and a number of studies have demonstrated that satellite cell number can be increased in elderly people following a period of strength training (Mackey et al., 2007, Roth et al., 2001, Verdijk et al., 2009, Verney et al., 2008). In those studies that had a comparable group of young subjects, no age related differences in satellite cell number have been reported (Roth et al., 2001). These data coupled with the demonstrated effectiveness of strength training regimens to increase muscle mass and improve function, even in very elderly people (Fiatarone et al., 1990, Harridge et al., 1999), suggest that satellite cell behaviour, in the longer term is not limiting muscle adaptation to normal resistance exercise training in later life.

Finally, it must be noted that following enzymatic digestion and isolation of satellite cells, it is possible that only “good” cells were isolated from the elderly biopsies and that *in vivo* “bad” cells might be poorly functioning. However, given the similarity in yield between young and old biopsies this seems unlikely.
In conclusion, the data in this thesis show that whilst a senescent phenotype can be readily obtained in vitro, the behaviour of early passage cells in regard to the characteristics relevant for muscle regeneration (proliferation and differentiation) are similar between cells obtained from young and elderly people. This coupled with other evidence, suggests that the satellite cells in vivo are functional in later life and may not be critically involved in the mechanism responsible for sarcopenia. However, their sensitivity to inflammatory cytokines suggests that if these were present in vivo from other tissues (myofibres, connective tissue, nervous tissue) they might affect the myogenic behaviour of the cells.

7.5 Future direction

The results of the work presented in this thesis raise a number of possibilities for future research. These include:

I. Marked heterogeneity in desmin content was found in myoblasts obtained from different subjects, several populations of cells maintained their initial desmin content whereas others lost their desmin content over time in culture. It is possible that the balance of fast proliferating to slow proliferating muscle precursor cells may ultimately determine culture purity in the presence of contaminating fibroblasts. Fast proliferating cells may outstrip the growth of fibroblasts, or whereas slow proliferating may be outgrown by them. Nevertheless, it will be important for future work to enrich cultures with myogenic cells and eliminate fibroblasts and other non-myogenic cells using a separation technique (Magnetic sorting).

II. This thesis showed that proliferative potential, differentiation potential and differentiation time course were similar between cells from the young and
elderly subjects at the earliest passage analysed. When the cells reached senescence, expression of myogenin and MHC was delayed by two-four days following transfer to differentiation medium when compared to early passage cells. To confirm no ageing effect gene expression profiles of human myoblasts and telomere length should be analysed to determine if these differ when comparing ageing in vivo to replicative senescence of human myoblasts in vitro.

III. The work presented in this thesis indicated that TGF-β1, TNF-α, and IL-1β interfere with myogenic behaviour in a dose dependent manner and this effect was reversible. The next step would be to study the signalling pathways involved to determine the interaction between TGF-β1, TNF-α, and IL-1β which affect skeletal muscle. The length of time over which treatment with these cytokines can maintain this immature state and still be fully reversible, without affecting the proliferation or differentiation potential, of the myoblasts also remains to be determined. Furthermore, it might be important for future work to inhibit TGF-β1, TNF-α, and IL-1β to determine the effect on human myoblasts using blocking agents and to increase the myogenic efficiency of muscle cells.
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