The effects of testosterone and ageing on skeletal muscle growth and androgen receptor expression

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Abstract

Aging is associated with a significant reduction in skeletal muscle mass, termed sarcopenia. As life expectancies and elderly populations continue to increase, so does the prevalence of sarcopenia. Sarcopenia has been associated with depression, frailty, and severe reductions in quality of life in older adults, as well as placing significant strain on healthcare systems. Therefore, there is a clear requirement for effective, preventative treatments, which can easily be self-administered by older adults. Prior studies have suggested that reductions in androgen receptor content occur with aging, and that this may be integral to an age-associated loss of skeletal muscle mass. Testosterone, an androgenic steroid hormone instrumental in the development and maintenance of skeletal muscle mass also decreases with age. Testosterone may potentially increase androgen receptor content, thereby increasing skeletal muscle growth, and responsiveness to other treatments for sarcopenia such as exercise programmes. This thesis aimed to investigate the effects of various testosterone administration protocols on skeletal muscle growth and androgen receptor expression *in vitro* and determine how these effects differ in aged skeletal muscle tissue. The main findings from this work suggest that 50 nM and 100 nM testosterone accelerates differentiation, inhibits atrophy, and increases androgen receptor mRNA expression *in vitro*, with greater effects occurring following repeated doses. Conversely, these effects were not present in skeletal muscle cells which had undergone replicative ageing via population doubling, suggesting that there is a limit to the number of doublings cells can undergo before becoming immune to the effects of testosterone administration. Preliminary findings also demonstrated a testosterone induced increase in cellular metabolic activity, which may indicate an increase in cellular viability. In conclusion, this thesis provides evidence for a testosterone induced increase in androgen receptor expression, whilst also demonstrating an ageing induced resistance to the effects of testosterone administration in skeletal muscle.

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Chapter 1

General Introduction

1.1 General Introduction

Skeletal muscle is a contractile organ directly and indirectly attached to bone and a key component of the human body, enabling locomotion and structural support (Chal and Pourquie, 2017). Skeletal muscle comprises 40% of total body weight in humans, contains 50-75% of all body proteins and accounts for 30-50% of whole-body protein turnover (Frontera and Ochala, 2015). Skeletal muscle contributes to a number of bodily functions, including the conversion of chemical energy into mechanical energy to produce muscular contraction, characterized by muscle shortening. The shortening of muscle produces force resolved at the tendon, pulling the skeleton and resulting in body movement and the maintenance of posture and position (Mayeuf-Louchart and Duez, 2015). Small constant adjustments in skeletal muscle are required to maintain the body upright. Skeletal muscle also contributes significantly to energy metabolism by storing amino acids, carbohydrates and other substrates, as well as protecting the body, acting as a barrier to external traumas and supporting the weight of the body's organs (Hargreaves and Spriet. 2020; Westerblad, Bruton and Katz, 2010). Furthermore, skeletal muscle is a major regulator of systemic glucose homeostasis, by regulating insulin-stimulated glucose uptake and glucose disposal, preventing the onset of insulin resistance, obesity and type II diabetes (Buczkowska and Dworzecki, 2003; Yang, 2014). Aging is associated with a reduction in skeletal muscle fibre size and number, motor units and muscular strength. Between the ages of 24 and 80, approximately 40% of skeletal muscle mass is lost, resulting in a condition called ‘sarcopenia’ (Morley et al., 2001). However, the rate of muscular atrophy increases significantly at 50 years of age, and further accelerates at 65 resulting in the majority of muscular atrophy occurring between 50 and 80, leading to 50% of people aged 80 and over suffering from sarcopenia (Baumgartner et al., 1999; Morley, 2012). A strong association between muscle mass and mortality exists in older adults, with adults with a low skeletal muscle mass index (SMMI < 10.75kg/m² in males and < 6.75kg/m² in females) having twice the risk of all-cause mortality as those

with a normal or high SMMI (Chuang et al., 2014; Metter et al., 2002). Sarcopenia has been associated with an increased risk of disability and frailty and decreased mobility, leading to significant reductions in quality of living (Landi et al., 2012). Sarcopenia significantly increases fall risk in older adults, as well as increasing the severity of consequences of falls (Landi et al., 2012; Pijnappels et al., 2008). Whilst the consequences of sarcopenia are well researched, significant work is still required in determining the specific causes of sarcopenia and how to best treat them in order to improve the quality of life and avoid further complications in older adults.

1.2 Skeletal Muscle Structure, Function and Regeneration

Skeletal muscle structures are surrounded by a connective tissue called the epimysium, which houses bundles of skeletal muscle fibres. These bundles of fibres consist of myofibers, which are surrounded by the connective tissue endomysium and encased in perimysium (Frontera and Ochala, 2015). Myofibers are responsible for the generation of force within skeletal muscle and can vary in cross-sectional area, length and fibre type (Type I, type IIa and type IIx) (Scott et al., 2001; Zierath and Hawley, 2004). Each myofibre contains an arrangement of the protein's actin and myosin, forming myofibrils (**Fig 1.1**). Within a myofibril exists an array of sarcomeres in series and parallel, which enable whole skeletal muscle contraction (Sanger et al., 2005).

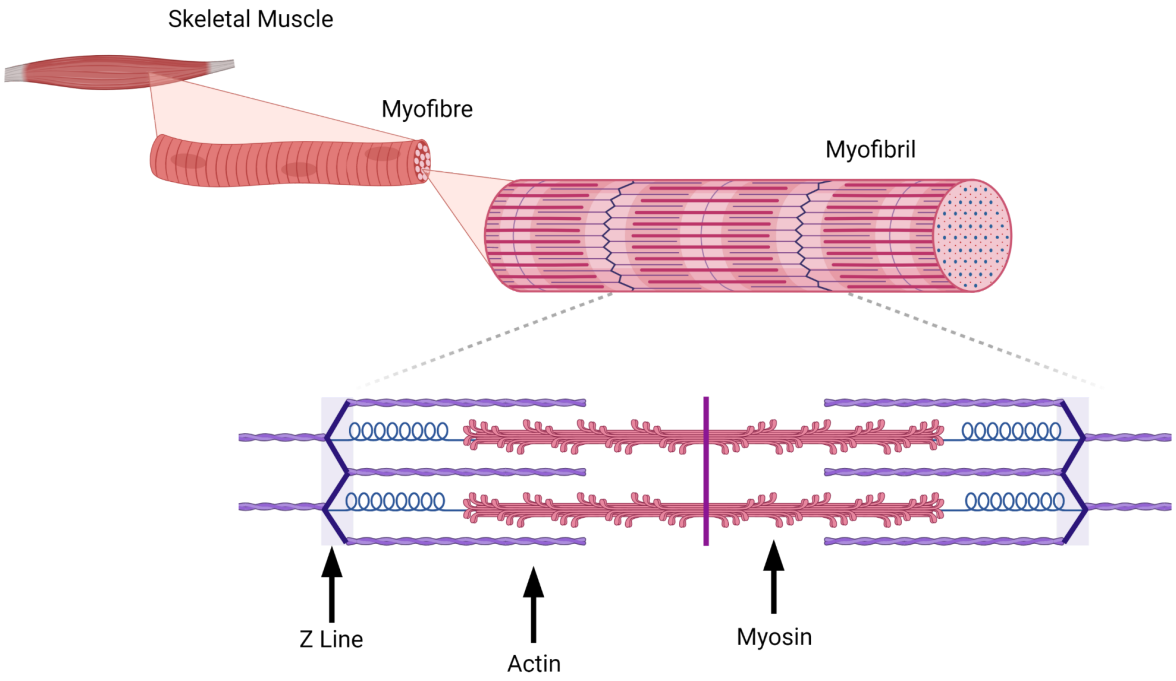

Fig 1.1. Skeletal muscle structure from whole muscle to ultrastructure. Created with BioRender.com

Skeletal muscle contraction originates with a nerve impulse in the motor cortex of the brain, this impulse is transmitted to the neuromuscular junction (NMJ), causing the release of acetylcholine (ACh) from the intracellular vesicles and into the synaptic junction. ACh binds to its receptor and produces a miniature membrane potential (MEPP), when enough MEPP's are generated and depolarize the sarcolemma an action potential is produced (AP). The AP travels along the sarcolemma and into the T-tubules, depolarising them and allowing for the electrical signal generated at the sarcolemma to be transmitted to within the muscle fibre (Jayasinghe and Launikonis, 2013). This results in the release of calcium from the sarcoplasmic reticulum (SR) into the sarcoplasm of the muscle fibre. Due to this modulation of calcium, actin-myosin cross-bridging occurs, where calcium ions bind to the troponin isoform C, conformationally changing the tropomyosin that surrounds actin and enabling myosin to interact with it (Zot and Potter, 1987). Myosin heads bind to the actin monomers and rotate, stretching the myosin

filament and exerting force on the actin, resulting in the movement of actin between myosin filaments (Lan and Sun, 2005; Piazzesi et al., 2002). The force produced via actin-myosin cross-bridging is transferred throughout the length of the muscle as each sarcomere is linked in series at the Z line of actin filaments, resulting in a whole muscle contraction.

Skeletal muscle is capable of self-repairing severe muscle damage caused by repeated skeletal muscle contractions as described above. Minor damage caused by daily activity is repaired by the recruitment of intracellular vesicles, however more extensive muscle damage requires the migration and fusion of satellite cells (Grounds, 1991). The repair of mature myofibres is reliant on the activation, proliferation and differentiation of satellite cells. To repair myofibres, satellite cells must first commit to becoming myoblasts, this can occur via a variety of signaling pathways including Wnt signaling, notch signaling and sphingolipid signaling (Abreu, 2018; Girardi and Grand, 2018; Otto et al., 2008; Saba and Garza-Rodea, 2013; Wen et al., 2012). Upon their activation, satellite cells leave quiescence and enter the cell cycle, where cells undergo stochastic and asymmetric division to replenish the pool of satellite cells and prepare satellite cells to commit to becoming myoblasts (Fu, Wang and Hu, 2015). Myogenic regulatory factors (MRFs), a group of transcription factors responsible for targeting myogenic loci, then commit satellite cells to a myogenic fate (Hernandez-Hernandez et al., 2017). Through various autoregulatory and cross-regulatory mechanisms, MRFs are capable of regulating their levels of expression to orchestrate myogenesis (Asfour, Allouh and Said, 2018). An early increase in myogenic factor 5 (Myf5) and myoblast determination protein 1 (MyoD) expression are followed by increases in Myogenin and myogenic factor 6 (MRF4) expression and a decrease in Myf5 and MyoD to commit satellite cells to muscle repair (Marsh et al., 1997). The activated myoblasts then migrate to the damaged muscle tissue and fuse with damaged myofibres, contributing their myonuclei to the existing fibres.

The maintenance and growth of skeletal muscle is reliant on a state of positive net protein turnover, where protein synthesis outweighs protein breakdown (Millward et al., 1975). A number of factors influence the development of skeletal muscle mass and strength including physical activity, diet, age and hormones (Baumgartner et al., 1999). Physical activity, and more specifically resistance training, is one of the most commonly mentioned determinants of skeletal muscle mass, as countless studies have demonstrated resistance training induced increases in muscle protein synthesis and consequently skeletal muscle mass (Bandy et al., 1990; Damas et al., 2018). Whilst people typically think of progressive overload and weight training when considering physical activity aimed at developing muscle, in older adults simple daily physical activity such as walking or participating in leisure sports can be sufficient to induce increases in muscle protein synthesis and aid the maintenance of skeletal muscle mass, especially in those suffering from sarcopenia or frailty who are unable to participate in resistance training (Steffl et al., 2017).

Often associated with resistance training, dietary protein intake contributes significantly to skeletal muscle mass by ensuring the supply of essential amino acids and stimulating protein synthesis (Landi et al., 2016). Inadequate protein intake results in increased protein degradation and a consequent increase in muscle breakdown (Garlick, et al., 1991). Alongside protein intake, an adequate caloric intake is vital for the efficient maintenance and growth of skeletal muscle, particularly in individuals participating in resistance training (Aragon and Schoenfeld, 2020; Slater et al., 2019).

Intramuscular sex steroid concentrations, primarily testosterone in males and estrogen in females, have been repeatedly associated with skeletal muscle mass, with prior studies showing estrogen, testosterone and dihydrotestosterone to be predictors of muscle strength, power, and size (Pollanen et al., 2015). Furthermore, exercise has been shown to induce upregulation of serum sex steroid hormone levels,

resulting in increased protein synthesis and a consequent increase in muscle mass (Sato and Iemitsu, 2014). Similarly, insulin-like growth-factor I (IGF-I) is strongly associated with skeletal muscle mass, due to enhancing satellite cell proliferation and differentiation (Clemmons, 2009).

Aging significantly affects skeletal muscle, with 40% of skeletal muscle mass being lost between the ages of 24-80. This is in part a result of lifestyle changes associated with aging, especially in older adults, with the onset of retirement often incurring reductions in caloric intake, dietary protein intake and total physical activity (Berger et al., 2005; Stephens and Toohey, 2018). Furthermore, a number of hormonal changes occur with aging (Hermann and Berger, 2001). Whilst factors such as diet or physical activity can be altered through lifestyle changes, hormone levels are significantly harder to modify, presenting a significant challenge in the case of aging and sarcopenia. IGF-I has been shown to decrease with age, similarly, aging induced skeletal muscle atrophy is often associated with reductions in free testosterone, with prior studies demonstrating the effectiveness of free testosterone as a predictor of sarcopenia in older males (Milewicz et al., 2013; Vanbillemont et al., 2009; Yuki et al., 2013).

1.3 Testosterone

Testosterone (17β-Hydroxyandrost-4-en-3-one: $C_{19}H_{28}0_2$) is a naturally occurring androgenic steroid hormone responsible for the regulation of many physiological processes within the body. Testosterone is the primary male sex hormone and is instrumental in the development of male reproductive tissues, muscle mass and bone metabolism (Mooradian, Morley and Korenman, 1987). Testosterone is an androstanoid composed of 17beta-hydroxy and 3-oxo groups (**Fig 1.2**). Physiological serum testosterone levels for healthy, young men range from 400-1000 ng/dL. Testosterone can exist in a ‘free’ or ‘bound’ state. Approximately 98% of the body’s testosterone is bound to either sex hormone binding globulin (SHBG) or albumin, the remaining 2% of testosterone is not attached to any proteins and is referred to as

'free' (Shea, Wong and Chen, 2014). It should be noted that both free testosterone and serum albumin bound testosterone are regarded as bioavailable forms of testosterone.

With aging and a consequent increase in SHBG, bioavailable testosterone reduces at a rate of 2-3% per year in adult males, with bioavailable testosterone declining from 83-257 ngl/dl in 20-year-old males to 40-168 ng/dl in 60-year-old males (Stanworth and Jones, 2008). Total testosterone levels decline at an average rate of 1% a year in adult males, with total testosterone peaking in 18-year-olds ranging from 300-1200 ng/dl (Salameh et al., 2010). Individuals displaying total testosterone levels below 300 ngl/dL are diagnosed with hypogonadism, an inability to maintain sufficient levels of testosterone typically either as a result of a problem with the testes or pituitary gland signaling (Dillon et al., 2010; Fabbri et al., 2016).

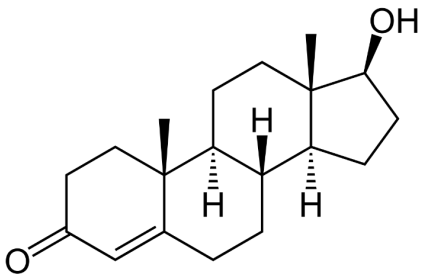

Fig 1.2 – The chemical structure of testosterone. Testosterone is composed of 17beta-hydroxy and 3-oxo groups.

1.3.1 Testosterone production

Testosterone production occurs primarily in the gonads, in Leydig cells in the interstitial space of the testes in males and in the theca cells in the ovaries in females (Swerdloff, Wang and Bhasin, 1992; Walker, 2011). Small amounts of testosterone are also secreted from the adrenal cortex, with a further

30-50% of androgen production occurring in peripheral tissues, including muscle, bone and fat (Luu-The and Labrie, 2010). Gonadal testosterone production is controlled via the hypothalamic-pituitary-gonadal-axis (HPGA), which is responsible for controlling reproduction and aging. The HPGA signals the central nervous system to innervate the secretion of gonadotropin-releasing-hormone (GnRH) which stimulates the production and release of luteinizing hormone (LH) from the pituitary gland (Bruni et al., 1977). LH triggers the biosynthesis of testosterone from cholesterol or acetate within the male Leydig cells by binding to G-protein coupled membrane receptors (Franchimont, 1983). The binding of LH activates adenylate cyclase, causing an increase in cAMP, leading to an increase in synthesis of steroidogenic enzymes (Payne and Hales, 2004). Increased levels of testosterone cause signaling to the hypothalamus to suppress GnRH secretion and reduce anterior pituitary GnRH responsiveness, thereby reducing testosterone biosynthesis (Sheckter, Matsumoto and Bremner, 1989) (**Fig 1.3**). This feedback loop maintains consistent levels of testosterone. Testosterone within peripheral tissue is synthesized locally from dehydroepiandrosterone (DHEA) and DHEA sulphate using various steroidogenic enzymes.

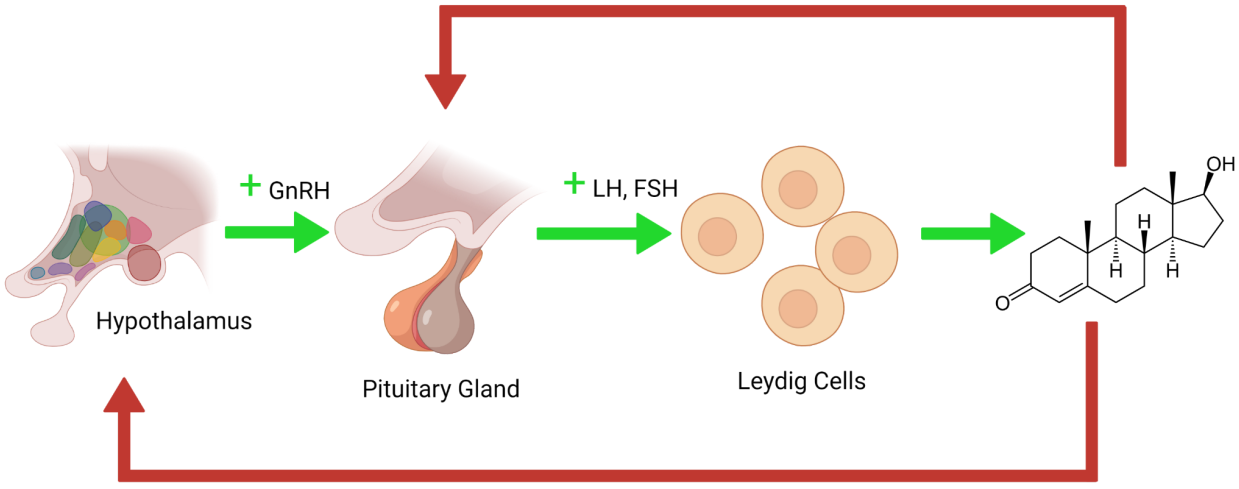

Fig 1.3 – Testosterone production and feedback loop. GnRH, gonadotropin-releasing-hormone; LJ, luteinizing hormone; FSH, follicle stimulating hormone. HPGA signaling causes the release of GnRH, stimulating the release of LH and FSH from the pituitary gland. LH triggers biosynthesis of testosterone within leydig cells. Increases in testosterone levels signal to the hypothalamus to reduce GnRH secretion and to the pituitary gland to reduce GnRH responsiveness, leading to a reduction in testosterone biosynthesis. Created with BioRender.com

Testosterone has been shown to elicit a hypertrophic response in skeletal muscle by upregulating protein synthesis and downregulating protein degradation via transcriptional changes (Urban et al., 1995; Wolfe et al., 2000). This change in protein turnover underpins increases in skeletal muscle cross sectional area (CSA) with testosterone administration (Sinha-Hikim et al., 2006).

1.3.2 Testosterone Signaling Pathways

The primary mechanism by which testosterone is thought to elicit its effects in muscle is via interaction with the androgen receptor (AR), which resides in an un-ligand bound form within the cytoplasm of target cells as a multi complex protein (Grino, Griffin and Wilson, 1990; Loy, Sim and Long, 2003; Spiering et al., 2009). 5-a reductase enzymes first aromatize testosterone to form dihydrotestosterone (DHT), which binds to un-ligand-bound ARs within the cytoplasm, causing several conformational changes in the AR (Liao et al., 2002). These ARs then dissociate from their associated multi-protein complexes, allowing them to recruit co-activators. The ligand bound ARs then translocate to the nucleus and interact with androgen response elements (AREs) within the genome as a transcription factor (**Fig 1.4**) (Bennet et al., 2010). These AREs include IGF-I, myoblast determination protein 1 (MyoD) and myosin heavy chains and all perform specific functions relating to cell proliferation, differentiation, and maturation, which are fundamental for muscle growth (Shaffer et al., 2004). Whilst testosterone typically requires conversion to

DHT to have a significant effect on AR signaling, high concentration doses of testosterone are capable of having similar effects on the AR to DHT (Grino, Griffin and Wilson, 1990). Testosterone is likely able to overcome its weaker androgenic potency, compared to DHT, by enacting mass action on the AR, although it should be noted that the cell culture in the study by Grino, Griffin and Wilson used fibroblast monolayers, not myoblasts.

Testosterone also mediates satellite cell activation via the androgen receptor, as well as indirectly via increases in IGF-I, with prior studies demonstrating increases in satellite cell activation following testosterone administration and decreases in activation following castration in numerous species and muscle tissues (Chen, Zajac and MacLean, 2005; Nnodim, 2001). Activated satellite cells are referred to as myoblasts, which are capable of fusing into existing muscle fibres to increase their size or fusing together to form new muscle fibres (Pallafacchina, Blaauw and Schiaffino, 2012). Satellite cells also comprise the predominant location for androgen receptor expression, therefore an increase in satellite cells within the muscle further increases the availability of androgen receptors for testosterone to interact with (Kadi et al., 1999; Sinha-Hikim et al., 2004).

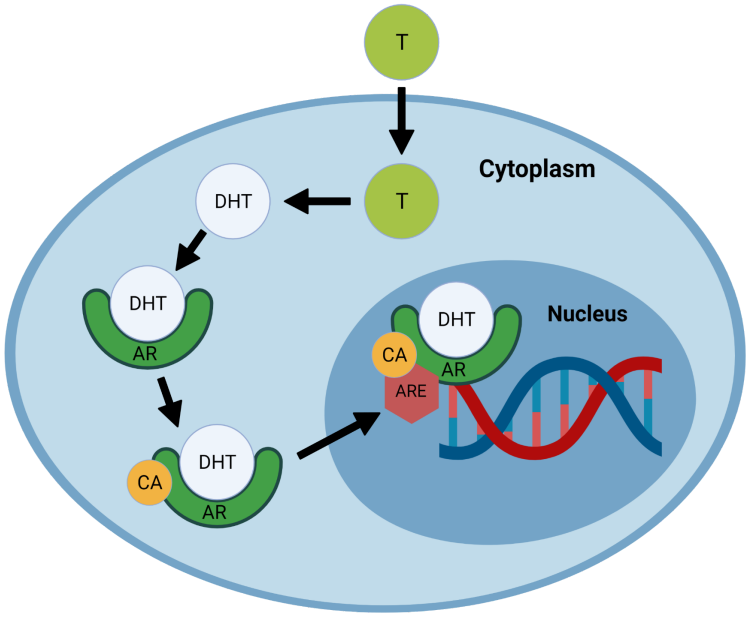

Fig 1.4 – Testosterone androgen receptor interaction. T, Testosterone; DHT, Dihydrotestosterone; AR, Androgen Receptor; CA, Co-activators; ARE, Androgen Response Elements. T enters the cytoplasm and is converted to DHT by 5-a reductase enzymes, DHT then binds to the AR and recruits co-activators. AR bound DHT translocates to the nucleus and interacts with AREs as transcription factors. Created with BioRender.com

IGF-I Downstream Pathways

IGF-I signaling forms the basis for a number of signaling pathways regulating skeletal muscle growth, primarily the PI3K/Akt/mTOR pathway. Testosterone induced increases in IGF-I concentrations increase IGF-I binding to its receptor, causing phosphoinositide 3-kinase (PI3K) activation and releasing a free phosphate group (Ashton et al., 1995; Egerman and Glass, 2014; Hobbs et al., 1993). This phosphate group phosphorylates protein kinase B (Akt), which in turn activates the mechanistic target of rapamycin (mTOR) (Basualto-Alarcon et al., 2013) (**Fig 1.5**). Akt also leads to the inhibition of the ubiquitin-proteasome and autophagy-lysosome systems which are responsible for protein degradation,

as well as increasing notch activation, resulting in increased satellite cell activation. Increases in mTOR phosphorylation activate the enzyme P70S6K, which activates the ribosomal protein S6K, consequently increasing ribosomal biogenesis and protein synthesis. mTOR also releases the inhibitory effect of 4E-BP1 on the translation initiator eIF-4E, thereby increasing translation and protein synthesis (Gautsch et al., 1998).

Ubiquitin ligase-proteasome and Autophagy-lysosome Systems

Whilst the majority of pathways influenced by testosterone increase skeletal muscle hypertrophy by increasing protein synthesis, some inhibit protein degradation to alter protein turnover. Both the ubiquitin ligase-proteasome and autophagy-lysosome pathways are responsible for targeting proteins for degradation. Testosterone's activation of Akt inhibits Forkhead box transcription factors (FoxOs), preventing FoxOs activation of both the ubiquitin ligase-proteasome and autophagy-lysosome pathways and decreasing protein degradation (**Fig 1.5**). Research by Pires-Oliveira et al. (2009) found that testosterone deprivation in mice resulted in a 31-fold and 18-fold increase in mRNA expression of the ubiquitin ligases atrogin-1 and Murf-1, acute testosterone administration then repressed mRNA expression to control levels in the previously testosterone deprived mice. Similar research by Zhao et al. (2008) also found that testosterone administration blocked dexamethasone induced increases in the ubiquitin ligase MAFbx mRNA expression, reducing muscular atrophy. Research by Serra et al. (2013) has shown similar effects of testosterone deprivation on autophagy in mice, with castrated mice showing increasing levels of autophagy markers and lysosome enzymatic activity compared to controls. The same study also demonstrated that testosterone administration in mice inhibited the lysosome and proteasome pathways via the IGF-I pathway. Both the ubiquitin ligase-proteasome and autophagy-lysosome pathways are also inhibited as a result of testosterone inhibiting adenosine

monophosphate-activated protein kinase-a (AMPKa) activation, which would normally increase autophagy and ubiquitin-ligase activity (Rooy et al., 2016).

P38MAPK/JNK

Testosterone induced increases in IGF-I have been shown to modulate phosphorylation of 3 specific mitogen activated protein kinase (MAPK) subfamily proteins (JNK, P38MAPK and ERK) (Park et al., 2004; Pronsato and Milanesi, 2016; Wu et al., 2010). C-Jun N-terminal kinase (JNK) is primarily involved in inhibition of cell growth and promotes cellular apoptosis. Increases in IGF-I have been shown to decrease JNK phosphorylation, thereby reducing its activation and preventing cell apoptosis and inhibition of growth. Extracellular signal regulated kinase (ERK) increases promotion of cell growth. P38 MAPK has a number of roles in cell growth, dependent on the isoforms being phosphorylated; increases in P38-α activation promote differentiation and inhibit proliferation, whereas increases in P38-β activation have the opposite effect on satellite cells. Research by Brown et al. (2009) found that testosterone administration increased P38MAPK activation and inhibited JNK activation in mouse skeletal muscle within 2 weeks of starting treatment, suggesting that testosterone administration may increase skeletal muscle growth by inhibiting cell apoptosis and promoting cell growth and differentiation. This is further supported with research by Kovacheva et al. (2010) who demonstrated that age associated increases in JNK activation resulted in significant decreases in gastrocnemius muscle weight in mice. Testosterone administration in the aged mice reduced JNK activation and increased gastrocnemius muscle weight, along with increasing satellite cell differentiation and proliferation.

Notch Signaling

The notch signaling pathway is responsible for cell-cell communication, neuronal function, and myogenic progression. Testosterone induced Increases in notch signaling activation have been shown to increase

satellite cell proliferation and activation, with research in mice suggesting that testosterone administration may increase notch signaling both directly and indirectly via the akt pathway (Kovacheva et al., 2010). MRF activation has also been shown to mediate notch signaling, with increases in P38MAPK and the inhibition of JNK leading to increased notch activation (Dubois et al., 2012; Roy et al., 2016) (**Fig 1.5**). Therefore, a testosterone induced modulation of MRF activity is likely to lead to an increase in notch signaling. Testosterone administration has also been shown to increase expression of Delta 1, the ligand which binds to activate notch receptors, increasing the potential for notch activation (Brown et al., 2009).

Myostatin Repression

Myostatin is a myokine that acts on muscle cells' autocrine function to inhibit myogenesis, preventing cell growth and differentiation. Myostatin promotes the formation and activation of the protein complexes SMAD 2,3 and 4 which inhibit Akt activation, preventing downstream mTOR activation (Rodriguez et al., 2014) (**Fig 1.5**). Myostatins inhibition of Akt also increases FoxO activation, thereby increasing protein degradation via the ubiquitin ligase-proteasome and autophagy-lysosome systems (Elkina et al., 2011). Myostatin also downregulates paired-box-protein 7 (Pax7), leading to reduced satellite cell activation (McFarlane et al., 2008). Testosterone prevents myostatins inhibition of muscle growth by repressing myostatin directly and indirectly via an increase in the autocrine glycoprotein follistatin (Braga et al., 2012; Kovacheva et al., 2010; Singh et al., 2009). Interestingly, research by Dubois et al. (2014) demonstrated an increase in myostatin via AR binding in response to testosterone administration in mice levator ani muscle. Dubois proposed this was a self-regulating mechanism to prevent significant growth in response to large increases in testosterone, which is supported by prior research by Lakshman et al. (2009) demonstrating increases in serum myostatin expression following testosterone administration, which reduced over the course of the treatments. Lakshman's research also

found that despite increasing myostatin, testosterone administration still significantly increased skeletal muscle mass. Prior research by Kawada, Okuno and Ishii (2006) demonstrated a decrease in myostatin content within mice soleus, gastrocnemius and plantaris muscle following testosterone administration, therefore it may be that whilst testosterone administration increases serum myostatin as a regulatory measure, it decreases myostatin content within the muscle. It should also be noted that all three studies were carried out in different muscles, with Dubois' research using the pelvic muscle, and as previously demonstrated, different muscle types respond differently to testosterone administration, particularly regarding AR binding.

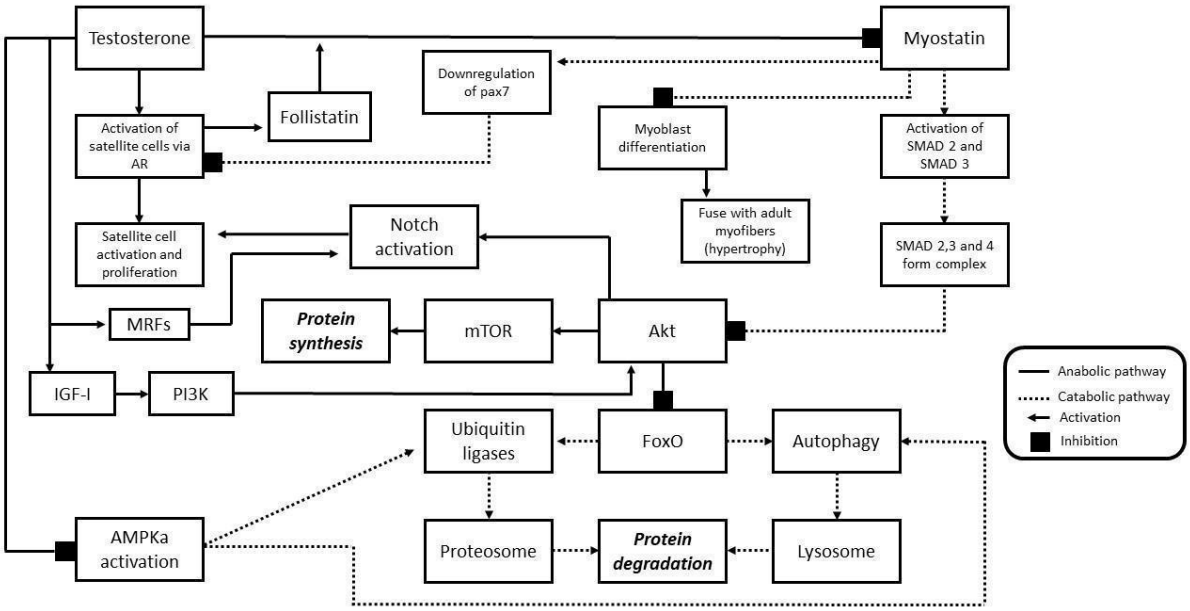

Fig 1.5 – Interaction of testosterone with skeletal muscle signaling pathways. AR, Androgen receptor; IGF-I, Insulin-like growth factor 1; MRF, Myogenic regulatory factors; PI3K, Phosphoinositide 3-kinase; Akt, Protein kinase B; mTOR, Mammalian target of rapamycin; AMPKa, 5' adenosine monophosphate-activated protein kinase a; FoxO, Forkhead box O proteins; Pax7, Paired box protein 7.

1.3.3 Exogenous testosterone supplementation

Pharmaceutical derivatives of testosterone have been developed in order to treat older adults with conditions such as hypogonadism. However, whilst extensive research has been invested in physiological testosterones effect on skeletal muscle, little attention has been paid to the effects of exogenous testosterone administration until recently. This may be due to testosterone administration being commonly associated with illicit use as a performance enhancing drug, often with adverse health implications (Westerman et al., 2016; Wood and Stanton, 2012). There are a number of different testosterone derivatives, typically referred to as esters, the two most commonly prescribed, and utilised in research being testosterone enanthate and testosterone cypionate (Whitsel et al., 2001). The main difference between esters is the number of carbon and hydrogen atoms, with different esters possessing different half-lives. Esterification improves the solubility of testosterone in oil, allowing for a slower release of testosterone from the injection site (Mackey, Conway and Handelsman, 1995). The more carbon atoms present in an ester, the more soluble in oil it is. Testosterone cypionate possesses 8 carbon atoms in its ester group, making it one of the most soluble esters currently developed (Gooren and Behre, 2008). Testosterone can be delivered via an injectable, transdermally as a patch or cream or sublingually by dissolving a tablet under the tongue (Borst et al., 2014). Testosterone is typically injected to avoid liver toxicity complications and to enable greater control over dosage. Transdermal delivery is required daily in small doses via a patch or cream to maintain a steady level of testosterone at all times, this avoids the peaks and drops in testosterone sometimes experienced using injectable testosterone. Transdermal patches however are significantly more expensive than injectables and often cause skin irritation. Sublingual administration under the tongue avoids the liver toxicity problems commonly associated with oral administration of testosterone, however, requires administration every 12 hours.

Research has also led to the development of subcutaneous testosterone pellets, small pellets implanted under the skin designed to slowly release 1-3mg of testosterone a day (McCullough et al., 2012). Typically pellets only need to be replaced every 3-4 months and multiple pellets can be implanted in order to increase dosages. However, trials have reported problems with dosing, with many patients requiring upwards of 10 pellets implanted to achieve a reasonable dose (McCullough, 2014). Whilst the most effective method of administering testosterone is clearly via an injectable, many elderly patients are apprehensive or unable to regularly self-administer injectables, therefore the further development of pellets, sublingual droplets and transdermal creams and patches may be crucial in improving testosterone administration and the uptake of testosterone therapy in older adults suffering from sarcopenia.

1.4 Androgen Receptors

The androgen receptor consists of three main domains: the N terminal domain (NTD), the DNA binding domain (DBD) and the ligand binding domain (LBD) (**Fig 1.6**). The DBD consists of two zinc fingers that recognise specific DNA sequences and allow direct binding of the AR to promoter and enhancer regions of AR-regulated genes, allowing the NTD and LDB to stimulate or repress transcription of these genes. The LDB mediates interactions between the AR and chaperone proteins, whilst also interacting with the N-terminus of the AR to stabilise bound androgens. The AR also contains several signal sequences, including two transcriptional activation functions, AF-1 and AF-2. AF-1 resides in the NTD whilst AF-2 resides in the LBD. AF-1 contains two transcription activation units, tau-1 and tau-5, which are required for full AR activity. AF-2 consists primarily of the H12 helix, which acts as a lid following agonist binding, closing the ligand binding pocket (LBP).

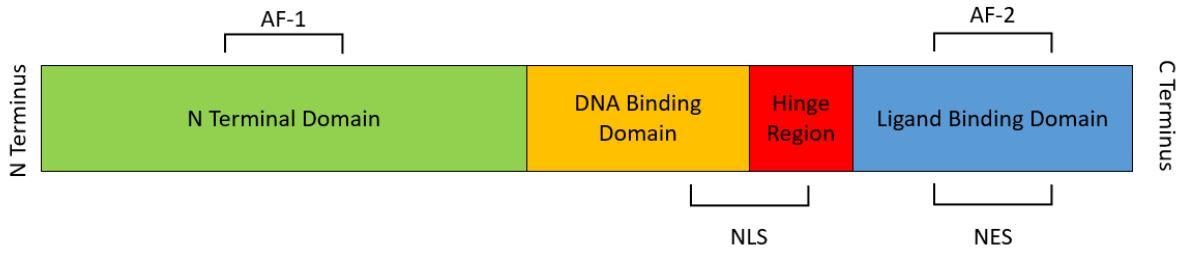

Fig 1.6 – Androgen receptor structure and domains. AF-1, activation function 1; NLS, nuclear localisation signal; AF-2, activation function 2; NES, nuclear export signal.

Whilst increases in testosterone via supplementation have typically been associated with increased skeletal muscle mass, recent research suggests that greater physiological levels of testosterone are not associated with increased muscle hypertrophy (Herbst & Bhasin, 2004; Morton et al., 2018). Morton et al. (2018) suggest that increased skeletal muscle hypertrophy instead correlates with levels of intramuscular AR content, with AR content being the limiting factor in skeletal muscle growth. Testosterone binds to the AR to signal skeletal muscle hypertrophy, with ARs becoming saturated and unable to further bind once they have reached their limit. Increased levels of physiological testosterone may have a potential to increase skeletal muscle hypertrophy, however if ARs become fully saturated at lower levels of testosterone then increases in testosterone will have a reduced effect on hypertrophy as there will be no available ARs for testosterone to bind to. Prior research investigating the effect of serum testosterone on prostate cancer has suggested a similar saturation model, showing that below the normal physiological levels of testosterone there is a saturation point, after which any increases in serum testosterone have little effect on benign prostate growth (Morgentaler and Traish, 2009). Previous research further supports this theory, showing that ARs in most tissues are saturated at lower levels of testosterone, independent of physiological testosterone concentrations (Dahlberg, Snochowski and Gustafsson, 1981). Despite this, studies have shown that administration of exogenous testosterone

increases skeletal muscle hypertrophy regardless of physiological testosterone levels. Therefore, it may be that exogenous testosterone has a different mechanism of action on skeletal muscle to physiological testosterone, however prior research suggests that this is not the case.

Bhasin et al. (2001) demonstrated that physiological testosterone and supraphysiological testosterone conform to the same single-log linear dose response curve in terms of their effect on fat free mass and muscle mass in older adult males. Alternatively, it may be possible that large doses of exogenous testosterone increase AR content by inducing greater expression of the AR gene, via an overload effect, in addition to potentially binding to ARs to induce hypertrophy. Prior studies suggest that testosterone may influence AR content, as when rats were castrated they experienced a significant decrease in AR content (Dahlberg, Snochowski and Gustafsson, 1981; Michel and Baulieu, 1980). However, a number of similar studies show contrasting results, demonstrating a significant increase in maximum AR binding post gonadectomy, although this may be due to different tissue and muscle phenotype ARs responding differently to modulations in sex steroid hormones (Krieg, 1976; Rance and Max, 1984). As the Rance study only investigated the tibialis anterior, a primarily fast twitch muscle, whilst the Dahlberg and Michel studies used primarily slow twitch muscles, it appears that the effect of castration, and potentially aging, on skeletal muscle AR content may depend on muscle phenotype. Regardless of changes in AR content induced by gonadectomy, post castration treatment with testosterone pellets has been shown to significantly increase AR content in striated rat muscle (Michel and Baulieu, 1980). As the majority of literature utilising testosterone treatments to investigate their effect on AR content used recently castrated rats, it may be beneficial to examine the effect of testosterone treatments in non-castrated rats, to establish if exogenous testosterone actually increases AR content or if it just rescues levels of AR content to a baseline, pre-castration level in rats. It may be that significant

reductions in free testosterone could reduce AR content, therefore age associated increases in SHBG may reduce AR content via decreasing free testosterone levels.

Table 1.1 – Summary of prior studies demonstrating the effects of androgenic steroid treatment and castration on androgen receptor content

Author	Year	Tissue	Treatment	Effect on Androgen Receptor
Antonio et al.	1999	Rat levator ani, bulbocavernosus & plantaris	Castration followed by 50mg/kg body weight DHT/day for 7 days	Castration decreased levator ani and bulbocavernosus AR protein content significantly and non-significantly in plantaris, DHT treatment increased AR protein content significantly in levator ani and bulbocavernosus and non-significantly in plantaris
Boesel et al.	1980	Rat prostate	2.5mg testosterone propionate	Testosterone increased ventral prostate nuclear AR receptor content without changing cytoplasmic AR content
Carson et al.	2002	Rat soleus & plantaris	6mg/kg body weight nandrolone decanoate/week for 4 weeks	Nandrolone decanoate increased soleus AR protein content by 250% and 940% in 5-mo-old and 25-mo-old rats but had no significant effect on plantaris AR protein content
Dahlberg, Snochowski & Gustafsson	1981	Rat and mouse skeletal muscle homogenate	Neonatal castration	Castration decreased skeletal muscle AR concentrations to that of female rats
Doumit et al.	1996	Pig skeletal muscle myoblasts	10^{-7} M testosterone	Testosterone increased immunoreactive AR incrementally after 6, 12 and 24 hours and reduced differentiation
Kumar & Thakur	2004	Mouse brain	Gonadectomy followed by 2mg testosterone propionate	No effect of gonadectomy, testosterone decreased AR mRNA levels
Lee et al.	2003	Rat soleus	6mg/kg body weight nandrolone decanoate/week for 4 weeks	ND had no effect on adult Soleus AR protein concentration but increased adult plantaris AR protein concentration
Menard & Harlan	1993	Rat brain	Castration and 2mg/kg ND/day for 14 days	ND increased brain AR immunoreactivity, castration decreased brain AR immunoreactivity
Michel & Baulieu	1980	Rat quadriceps femoris	20mg testosterone pellet implanted for 15 days	Testosterone pellets significantly increased AR concentration

Sinha-Hikim et al.	2004	Human skeletal muscle myoblasts	100nM testosterone OR 30nM DHT	Testosterone and DHT modestly increased AR protein content and increased AR mRNA by 70%

1.4.1 Tissue Specificity in Nuclear Receptors

Whilst there is limited data on the tissue specific effects of hormone administration on androgen receptor expression and content, prior research on receptors from the same nuclear superfamily may support the theory of tissue-specificity in ARs. Studies have demonstrated tissue specific effects in vitamin D receptors, where treatment with vitamin D increased vitamin D receptor (VDR) mRNA expression in the epidermis but not in the kidneys (Zineb et al., 1998). This is similar to the suggestion that testosterone can increase androgen receptor expression in specific tissues but not others. Studies have also shown that vitamin D administration exerts tissue and cell specific effects in nuclear VDRs, suggesting that the effects of vitamin D administration are mediated at a tissue and cell specific level (Campbell et al., 2010). Progesterone receptors have also shown tissue specific effects in response to progesterone administration (Batra and Losif, 1989). Cytosolic progesterone receptors in the uterus displayed an increase in content 24 hours post progesterone administration, whilst receptors in the vagina and urethra showed no change. Progesterone administration also increased the apparent dissociation constant in nuclear progesterone receptors in the vagina and urethra, whilst having no effect on nuclear receptors in the uterus after 24 hours. The same study also found that progesterone decreased cytosolic estrogen receptor concentration in the uterus but not in the vagina or urethra, providing strong evidence for tissue-specific effects of progesterone administration on receptor content. Thyroid hormone receptors have displayed similar behaviour to VDRs, with thyroid hormone administration inhibiting 11-beta-Hydroxysteroid dehydrogenase (11 beta-HSB) gene expression in the liver and pituitary at a pre-translational level but having no effect in the kidney and distal colon (Whorwood, Sheppard and Stewart, 1993). Previous research has also found thyroid hormone to have tissue specific effects in the brain, small intestine and bone, which are suggested to be modulated by tissue specific co-factors (Brent, 2000; Forrest et al., 1996). Therefore, the effects of testosterone on AR

content may be highly tissue specific, as prior studies have demonstrated similar tissue specificity in receptors belonging to the same nuclear superfamily. If the response of AR content is tissue specific, then further studies are required to determine the level of specificity in skeletal muscle, as the response of AR content to testosterone may vary as far as the fibre type level.

1.5 Sarcopenia

The term sarcopenia was first used in 1988, to describe an age associated loss of skeletal muscle mass (Rosenberg, 1997). In 2018, the European Working Group on Sarcopenia in Older People (EWGSOP) revised Rosenberg's definition of sarcopenia, changing it to include either reduced levels of muscular strength or reduced physical function (Cruz-Jentoft et al., 2019). This new definition encompassed research from a number of studies demonstrating that a significant loss of muscle mass was not always prevalent in cases of sarcopenia, whilst reductions in leg extension power and handgrip strength were, suggesting that aging has a greater impact on muscular strength than muscle mass (Baumgartner et al., 1999). However, it should be noted that the Baumgartner study excluded participants deemed unhealthy, thereby potentially excluding participants with severe cases of sarcopenia, as sarcopenia has been shown to lead to frailty, depression, disability and a number of other conditions (Landi et al., 2012). Changes in muscular strength in older adults have also been shown to precede and be greater than changes in muscle mass, therefore it may be possible that Baumgartner only assessed participants with early signs of sarcopenia, especially as only one third of the study population was aged 80 or older (Doherty, 2001; Goodpaster et al., 2006). Sarcopenia has been associated with significant decrements in quality of life, with decreases in skeletal muscle strength in older adults being associated with increased fracture risk, decreased cognitive function and reductions in stair-climb ability and walking speed (Rizzoli et al., 2013). Decrements in skeletal muscle function impose restrictions on everyday activities, with many suffering from sarcopenia being unable to participate in physical activity and other forms of social

engagement. It is important to reduce the effects of muscle wasting in older adults, to enable them to live a more fulfilling life and prevent the occurrence of depression, cognitive decline and other serious mental conditions often found in retirees as a result of social isolation.

Beyond the implications for quality of life, sarcopenia imposes a significant financial burden on healthcare systems. Recent estimates suggest that sarcopenia costs the National Health Service (NHS) an annual excess of £2.5 billion per year (Pinedo-Villanueva et al., 2019). Furthermore, research conducted in Portuguese hospitals found that sarcopenia increased hospitalisation costs by 58.5% for patients aged under 65 and 34% for patients aged 65 and over (Sousa et al., 2016). Sarcopenic patients being treated for unrelated conditions also experienced longer stays in hospital compared to non-sarcopenic patients being treated for the same conditions. The results from Sousa's study are notable as they are the first to utilise the updated EWGSOP definition of sarcopenia encompassing reductions in muscle strength and physical function rather than just reductions in muscle mass. Previous research accounting only for reductions in muscle mass estimated the direct healthcare costs of sarcopenia in the United States to be $18.5 billion per year, this estimate is likely to be significantly higher if adjusted to use the EWGSOP definition (Janssen et al., 2004). As life expectancy continues to increase, both the risk of sarcopenia and the severity of sarcopenia in older adults increases due to continuing decrements in muscle mass (Mathers et al., 2015). This will undoubtedly result in an increased burden on healthcare systems; therefore it is crucial to develop treatments to prevent and manage sarcopenia in older adults.

1.5.1 Causes of Sarcopenia

Sarcopenia occurs as a reduction in protein turnover, caused by a slower rate of protein synthesis or increased rates of protein degradation. A number of factors commonly affect protein turnover in the elderly, including nutrition, lifestyle and hormonal changes.

Caloric Intake

Aging has been associated with a decrease in total caloric intake, resulting in significant weight loss (Morley, 2007). An adequate caloric intake is required to maintain skeletal muscle mass, and with a reported 25.4% of older persons aged 65 and over and 49.5% of older persons aged 85 and over consuming inadequate calories, the risk of malnourishment and undernutrition is significantly increased in older persons (Cuervo et al., 2008; Gaskill et al., 2008). Inadequate caloric intake causes vital organs to become dependent on muscle to provide calories for survival via protein catabolism, resulting in significant decrements in muscle mass (Agarwalla et al., 2015; Rigaud et al., 2000). Research has also shown that whilst hospitalized, older persons are not provided with adequate calories for survival, resulting in an increased risk of developing sarcopenia, potentially creating a negative cycle for older persons routinely hospitalized as a result of injuries sustained due to sarcopenia (Sullivan et al., 1999). Research also suggests that older persons who previously practiced intentional calorie restriction and dieting are more likely to develop anorexia following retirement, leading to significant reductions in calorie intake (Morley et al., 1989; Morley, 2001).

Protein Intake

Dietary protein is crucial in maintaining healthy levels of skeletal muscle mass, as it ensures the supply of essential amino acids and stimulates protein synthesis (Landi et al., 2016). Sufficient protein ingestion also reduces the risk of preoperative complications and increases bone mineral density. A reduction in dietary protein leads to increased protein degradation, consequently reducing net protein turnover and increasing muscle breakdown (Garlick et al., 1991). Recent research has demonstrated an association between reduced protein intake and sarcopenia in populations aged 60 and greater (Chen et al., 2016). Prior studies have associated a decrease in protein intake with an increased risk of sarcopenia, suggesting that older persons consuming less than 1.2g/kg/day of protein are at a significantly greater

risk of developing sarcopenia, this is likely to account for a significant number of cases of sarcopenia, as studies have demonstrated that 22-38% of men aged 50 and over fail to consume the recommended 0.8g/kg/day of protein (Kerstetter et al., 2003; Morley, 2012). Many older adults fail to achieve the recommended protein intake due to dietary changes that come with retirement, as many retirees forgo a healthy diet and become increasingly sedentary. Furthermore, age associated reductions in physical function reduce many older adults' ability to shop for, and prepare, foods that meet macronutrient targets. Aging and sedentary behaviour in older adults have also been shown to induce an “anabolic resistance”, where the muscle protein synthesis response to protein intake and physical activity is blunted and muscular atrophy occurs (Breen et al., 2011). This increases the need for a schedule of regular protein intake throughout the day in order to maintain muscle protein synthesis, which many older adults are unaware of or do not adhere to. Whilst some older adults may consume the recommended daily protein intake, it is unlikely that they are distributing protein consumption throughout the day in order to maximise muscle protein synthesis.

Micronutrient Intake

Research has shown micronutrient intake to play an important role in the prevention and management of sarcopenia in elderly populations, although not as significant as caloric and protein intake, with sarcopenic subjects exhibiting significantly lower micronutrient intakes compared to non-sarcopenic controls, particularly in vitamin D and vitamin B12 (Verlaan et al., 2017). Interestingly, despite consuming significantly less micronutrients, the sarcopenic subjects appeared to have adequate intakes of all micronutrients apart from vitamin D; suggesting either vitamin D is the determining micronutrient in the management of sarcopenia, or micronutrient intake required to manage sarcopenia is significantly greater than that which is currently considered ‘adequate’ in older persons.

Aging has been associated with a reduction in serum 25-hydroxyvitamin D (25OHD) levels, with participants experiencing a mean decrease of nearly 8% over 7 years, despite increases in vitamin D supplementation (Mirfahkraee et al., 2017). Prior longitudinal research further supports this, demonstrating an 18% decrease in 25OHD levels over 12 years in elderly participants with a mean starting age of 70 (Perry et al., 1999). 25(OH)D is the hydroxylated form of vitamin D, therefore reductions in serum 25(OH)D are a suitable indicator of reduced vitamin D (Vieth, 1999). This significant decrease in 25(OH)D is likely a result of age associated reductions in the skin's ability to photosynthesize vitamin D, as well as a reduction in dietary vitamin D intake and exposure to sunlight commonly associated with retirement in the elderly (Holick 1987; Parfitt et al., 1982; Slingerland et al., 2007). Studies have shown increased levels of 25(OH)D in older persons are associated with increased leg strength, improved body composition and reduced risk of sarcopenia, however this may not be solely due to increased 25(OH)D and instead be a result of increased physical activity, as participants engaging in regular physical activity are likely to have greater 25(OH)D levels as a result of increased exposure to sunlight that would not be experienced by sedentary participants. (Marantes et al., 2011; Visser, Deeg and Lips, 2003). Neither the Marantes nor the Vissers study recorded participants' levels of physical activity, therefore it is not possible to determine which of these conclusions is accurate based on these studies alone. However, more recent studies assessing the effectiveness of vitamin D supplementation have shown significant increases in muscle function and body composition in participants treated with vitamin D, suggesting vitamin D does play a role in improving body composition, but has no significant effect on muscular strength in normal populations (Abiri et al., 2016). Although, some studies do suggest that vitamin D can increase muscle strength specifically in vitamin D insufficient elderly patients, with baseline 25(OH)D levels below 25nmol/L, suggesting that vitamin D may play a role in managing and preventing sarcopenia (Stockton et al., 2011).

Vitamin B12 deficiency often occurs in older persons as a result of poor nutrition or following a vegan or vegetarian diet, with over 20% of older adults exhibiting insufficient levels of Vitamin B12 (Andres et al., 2004). Vitamin B12 deficiency has been associated with reduced lean body mass and skeletal muscle mass, suggesting Vitamin B12 deficiency may be a factor responsible for the development of sarcopenia (Ates Bulut et al., 2017). Sarcopenic older adults have also been shown to consume 22% less Vitamin B12 and possess Vitamin B12 serum concentrations 15% lower than non-sarcopenic older adults (Verlaan. et al., 2017). Vitamin B12 deficiency has also been associated with an increased risk of depression, particularly in older adults, with Vitamin B12 deficient older adults becoming twice as likely to be severely depressed as non-deficient subjects (Penninx et al., 2000; Sanchez-Villegas et al., 2009; Tiemeier et al., 2002). Depression can often lead to social isolation and reduced physical activity Vitamin D exposure, thereby reducing muscle mass and strength and further increasing the risk of sarcopenia (Roshanaei-Moghaddam et al., 2009).

Physical Activity/Sedentary Behaviour

Aging is associated with a reduction in physical activity (PA) participation, with studies showing a linear decline in activity levels with age (Berger et al., 2005; Kallinen and Markku, 1995). As older adults enter retirement, they become more sedentary due to no longer having to commute to work or engage in PA at work (Phillips et al., 2001). Phillips also found that adults with manual jobs were more likely to achieve PA recommendations than sedentary workers during middle age, however upon retirement they were less likely to achieve these recommendations. Some studies have attributed age-related declines in PA to a dislike of the activities deemed appropriate to older age, as activities often prescribed to older adults tend to be much less physically demanding (Berger et al., 2005). Conversely, the same study also found that older adults avoided PA due to reductions in physical abilities and a fear of injury, therefore it may be important to change the perception of PA for older adults in order to increase engagement. Other

studies have shown that leisure time PA increases slightly after the transition to retirement, although it is uncommon for this increase in leisure time PA to make up for the loss of work-related PA associated with retirement (Berger et al., 2005; Evenson et al., 2002). Leisure-time PA is also dependent on a multitude of other factors, including socio-economic status, access to facilities and prior experience of PA pre-retirement (Shankar, McMunn and Steptoe, 2010).

Recent research has shown that physical activity, specifically in older adults, has a dose response relationship with lean mass, lower limb strength and grip strength (Foong et al., 2015; Mijnarends et al., 2016). However, research has shown that despite engaging in regular PA, declines in muscle mass still occur in active older adults, therefore, whilst increases in PA can delay and manage the effects of sarcopenia, it is unable to prevent it (Marcell, 2003; Mijnarends et al., 2016). Studies have also shown that decreases in PA are not directly associated with strength changes in older adults, therefore PA may not be a contributing factor to sarcopenia, although it may be a possible treatment (Hughes et al., 2001). However, levels of PA in the Hughes study were self-reported, therefore not as accurate or reliable as methods of measuring PA used in similar studies. As studies have shown that physical activity increases androgen receptor mRNA expression, potentially increasing skeletal muscle androgen receptor content, it may be possible that retirement induced decreases in PA also contribute to age associated reductions in skeletal muscle AR content and consequently lead to sarcopenia (Ahtiainen et al., 2011; Bamman et al., 2001).

Hormonal Changes

Studies have shown reduced levels of testosterone and increased concentrations of SHBG, a regulator of free testosterone, in elderly males compared to young and middle-aged males (Campbell et al., 2006; Vanbillemont et al., 2009). Research determining reference values for sex hormones in the elderly

demonstrated a continuous increase in SHBG and decreases in testosterone between the ages of 60 and 90 in elderly males (Milewicz et al., 2013). Reduced free testosterone in older adults is associated with reduced skeletal muscle mass and strength, suggesting that age associated reductions in testosterone are a contributing factor in the development of sarcopenia (Baumgartner et al., 1999; Iannuzzi-Sucich et al., 2002; Vermeulen, Goemaere & Kaufman, 1999). Increased levels of physiological testosterone are also associated with reduced fall risk in older males, likely due to increased muscle strength reducing sway and increasing the ability to recover from gait perturbations (Orwoll et al., 2006; Pijnappels et al., 2008).

Prior research suggests that increases in SHBG lead to reductions in free testosterone and reduces cellular uptake of testosterone (Hakkinen and Parkarinen, 1993; Kaiser et al., 1995; Li et al., 2016). Studies have found that aging increases the proportion of SHBG-bound testosterone, which is not available for intracellular transport, thereby reducing free testosterone levels and inhibiting muscle growth (Korenman et al., 1990). However, research in 400 healthy males, aged 40-80 years, found that higher levels of SHBG were associated with higher total testosterone, suggesting that age related increases in SHBG are not responsible for the age-related decline in testosterone seen in elderly males (Ronde et al., 2005). Although, it should be noted that all participants in the Ronde study were living independently and healthy, therefore this may have led to a health selection bias, especially in the higher age groups. Interestingly, recent in vivo studies in male mice have found that despite SHBG increasing total concentrations of testosterone, increased SHBG resulted in the development of hypogonadism, leading to reduced free testosterone levels (Laurent et al., 2016). Research in obese males presented similar findings, showing a positive correlation between SHBG and total testosterone (Osuna et al., 2006). Therefore, it appears that age-related increases in SHBG may not decrease total testosterone levels, but increase binding of testosterone to SHBG, resulting in a decrease in free testosterone and

consequent reduction in muscle growth. As research has found SHBG to exponentially increase with age, free testosterone should also be expected to exponentially decrease with age, greatly increasing the risk of sarcopenia with age (Liu et al., 2007).

Growth hormone (GH) secretion declines with age, with significant decrements occurring from 50 years onwards (Bartke et al., 2003; Thompson, 2007). GH is secreted from the pituitary gland in a pulsating pattern, regulated by hypothalamic hormones. Research has shown that age, diet and exercise all affect the magnitude and frequency of these pulses (Velloso, 2008). Aging, specifically the reaching of retirement age, is associated with decreases in quality of diet and physical activity, suggesting that older persons possess significantly lower levels of GH partly due to lifestyle changes (Berger et al., 2005). GH stimulates the synthesis of IGF-I, which stimulates protein synthesis in skeletal muscle, therefore reductions in GH secretion are likely to lead to decreases in muscle mass and consequently increase the risk of sarcopenia. Research in adults with GH deficiency syndrome has shown that physiological levels of GH are associated with levels of lean body mass and skeletal muscle mass, suggesting that individuals with higher natural levels of GH have increased levels of muscle mass (Beshyah et al., 1995; Cuneo et al., 1992; Simpson et al., 2002). GH supplementation has been shown to prevent catabolism in malnourished older persons, as well as increasing protein synthesis by 55% in aged rats (Kaiser, Silver and Morely, 1991; Sonntag, Hylka and Meites, 1985). However, GH use in ill, malnourished elderly patients has been found to increase mortality, potentially due to a modulation of immune function (Takala and Zandstra, 1999). GH supplementation also increased the length of hospital and time spent in intensive care in surviving patients in the Takala study, suggesting that whilst it may aid to increase muscle mass, the side effects of GH make its use in sarcopenia patients unproductive.

IGF-I has been shown to stimulate myoblast proliferation and induce myogenic differentiation, resulting in the formation of myotubes, the basis for skeletal muscle (Duan, Ren and Gao, 2010). IGF-I also contributes to muscle growth by suppressing the expression of atrophy related ubiquitin ligases, specifically atrogin-1 and muscle-ring-finger-1, thereby preventing muscular atrophy (Sacheck et al., 2004). Increases in IGF-I have been associated with increased muscle protein and RNA content in vitro, and increased strength and muscle mass in vivo (Duan, Ren and Gao, 2010; Sacheck et al., 2004). Both aging and reduced caloric intake results in reduced IGF-I levels (Sonntag, Lenham and Ingram, 1992). Similar studies show supporting results, with aged mice with lower levels of IGF-I demonstrating a reduction in protein synthesis compared to aged mice with greater levels of IGF-I and young mice (Willis and Parkhouse, 1994). Prior research suggests that age-related decrements in IGF-I are a result of a decrease in the number of IGF-I receptors causing the development of an IGF-I resistance, leading to a reduced rate of IGF-I induced protein synthesis in aged rats compared to young rats. (Dardevet et al., 1994; Willis et al., 1997). Conversely, limited research suggests that the existence of an IGF-I receptor gene polymorphism resulting in decreased IGF-I receptors may prolong human longevity, though it is yet to be determined if there is a direct correlation between aging and IGF-I levels in humans (Bonafe et al., 2003; Giacomoni and Rein, 2004).

1.5.2 Revising the Definition for Sarcopenia

Based on the findings of this literature review, it may be beneficial to further revise the definition for sarcopenia. The current EWGSOP definition allows for diet and PA to result in the diagnosis of sarcopenia, however reductions in muscle mass and strength can occur at any age as a result of these factors, whilst sarcopenia is an age-related condition. Therefore, sarcopenia should be diagnosed on the basis of age-related hormonal changes, rather than lifestyle changes, resulting in a revised definition:

‘A reduced level of muscle mass AND strength or physical function as a result of age associated hormonal changes.

By separating lifestyle and hormonal changes, treating muscular atrophy in older adults can be divided into two distinct pathways: those requiring hormonal or other drug-based treatments and those requiring lifestyle interventions. This is likely to result in a significant reduction in the burden placed on healthcare systems by sarcopenia and also prioritises prevention over treatment in the case of non-hormonal muscular atrophy in older adults. Muscular atrophy as a result of lifestyle changes can be prevented via proper exercise and nutrition, however hormonal changes require expensive treatments, therefore there should be a clear distinction between the two forms of muscle loss in older adults.

1.5.3 Current treatments for sarcopenia

Sarcopenia treatment is currently limited, and sarcopenia is widely accepted as a normal part of aging. Most treatments rely on resistance training programmes in order to increase skeletal muscle mass and strength, however these programmes are often difficult for elderly populations to complete, especially those exhibiting severe cases of sarcopenia or other disabling conditions (Roth, Ferrel and Hurley, 2000). Little is known about how long the effects of resistance exercise on sarcopenia last following the cessation of a training programme (Malafarina et al., 2012). In some cases, testosterone replacement therapy has been prescribed to increase muscle size and strength, however numerous complications currently make its use contradictory (Malafarina et al., 2012). Currently, the most common form of TRT is injectable testosterone enanthate or cypionate, due to their slower release into the body requiring less frequent administrations (Whitsel et al., 2001). Testosterone enanthate and cypionate are typically injected once every 1-3 weeks depending on dose and the individual. Growth hormone has also been utilised to reduce the effects of sarcopenia; however, it appears to have little effect on physical function

in elderly populations, merely increasing muscle size (Zachwieja and Yarasheski, 1999). Growth hormone has also been associated with numerous detrimental side effects, including edema, carpal tunnel syndrome, gynecomastia and intracranial hypertension (Reed, Merriam and Kargi, 2013).

Currently the most effective treatment for sarcopenia appears to be maintaining a high protein diet and resistance training regime throughout old age, before sarcopenia symptoms become severe enough to inhibit physical function (Bamman et al., 1998; Paddon Jones et al., 2008; Paddon Jones and Rasmussen, 2009). Recent research suggests that CRISPR/cas9 myostatin disruption and stem cell therapy may provide potential long-term treatments for sarcopenia, however significantly more research is required before either of these methods can be applied, along with the navigation of substantial ethical roadblocks (Tompkins et al., 2017; Wei et al., 2016).

1.5.4 Aging and Androgen Receptor Content

Existing research suggests that aging causes a decrease in AR protein content and gene expression (Gonzalez-Cadavid et al., 1976; Poole et al., 2011; Tohgi et al., 1995). However, the effect of aging on the AR is both species and tissue specific, with the majority of the available literature examining rat prostate, liver and penile tissue, all of which demonstrate a significant decrease in AR mRNA expression and content.

Extensive research has shown a significant decrease in AR mRNA and protein content in rat prostates, with studies consistently showing decreases in protein content of approximately 50% between young and old rats (Boesel et al., 1980; Shain et al., 1983). Further research has also shown that reductions in rat prostate AR content are specific to species of rats, suggesting that whilst some species may experience significant reductions in AR content and expression with age, others may not (Shain & Boesel,

1977). Similar research found a 30% decrease in prostate AR mRNA in rats aged 24 months compared to 3 months, but no difference in prostate AR mRNA in dogs aged 10 years compared to 1 year (Prins et al., 1996). Prins et al. suggest that this difference between species is due to increased stromal cell AR expression in canines overriding typical age associated regulation of prostate growth, leading to prostatic hyperplasia. However, this explanation does not account for why different species of rats experience different reductions in AR content. Literature investigating the effects of aging on prostate AR content may be of significant use to this thesis, as prior research has suggested that the physico-chemical characteristics of rat prostate ARs are similar to rat skeletal muscle ARs (Krieg, 1976).

Studies have shown a decrease in AR content and mRNA post-puberty in rat penile tissue, this decrease continues with age throughout the rat's lifespan, suggesting that it is not merely an acute post-puberty decrease (Gonzalez-Cadavid et al., 1991; Rajfer et al., 1980). Similar research in rat livers found that AR mRNA is almost undetectable in pre-pubertal and senescent male rats, with middle aged rats exhibiting levels of AR mRNA approximately 100-fold the other age groups (Song et al., 1991; Supakar et al., 1993). Liver AR activity has been shown to correlate with urinary output of alpha-2u-globulin (A2U) (Roy, 1974). Production of A2U ceases in immature and senile rats, and therefore may be the cause of the significantly greater decrease in AR content seen in the liver compared to any other tissue (Roy, Milin and McMinn, 1974).

There are a lack of studies investigating the effects of age on skeletal muscle AR protein content. However, limited studies into rat muscle suggest that changes in AR content are muscle specific; research using rat soleus and plantaris muscle found a 65% reduction in soleus muscle AR content in 25-month-old rats compared to 5-month-old rats, although, they found no change in plantaris AR content between the two groups (Carson et al., 2002). Carson suggests that this may be due to aging

affecting fast and slow twitch muscles differently, although provides no explanation for why the fast twitch muscle was not affected by aging. Prior studies have shown that plantaris AR protein content is not influenced by circulating testosterone levels, therefore it may be that age associated reductions in free testosterone caused a decrease in the 25-month-old rats soleus AR content but not plantaris (Antonio, Wilson & George, 1999).

The limited research examining human skeletal muscle AR content is divided, with Poole et al. showing aging causes a significant decrease in skeletal muscle AR mRNA, with 20-year-old males exhibiting a lower body muscle AR mRNA expression approximately 3x greater than that found in 60-year-old males (Poole et al., 2011). However, research conducted by Ahtiainen et al. (2011) found no difference in vastus lateralis AR expression between similar age groups. The study fails to mention the specific lower body muscle that biopsies were taken from, therefore it may be that this difference in results is due to samples being obtained from different muscle phenotypes, similar to Carson et al. 's findings in rat muscle. Prior research supports Ahtiainen et al. findings, demonstrating no significant relationship between age and human vastus lateralis AR mRNA (Marcell et al., 2001). However, Marcell et al.'s research only examined elderly subjects with hormone levels below the average, as the subjects selected for the study were intended to participate in further research investigating the effects of exogenous testosterone administration, thereby limiting the variability of the group. Aside from the above two studies, little research exists investigating the effect of age on human skeletal muscle AR content or mRNA expression. Some papers have shown that aging reduces AR content and mRNA expression in the hippocampal subregions of the brain and in foreskin in humans, however, based on the previously mentioned findings in rats, it is unlikely that human skeletal muscle AR will react in exactly the same way to aging as other tissue (Roehrborn et al., 1987; Tohgi et al., 1995).

Aging clearly reduces AR content and mRNA expression in rats, however the degree to which this decrease occurs appears to be tissue specific, with the greatest declines occurring in the liver whilst the plantaris muscle appears to experience no decline in AR content at all. There are a number of clear gaps in the current literature surrounding the effects of aging on androgen receptor content, predominantly whether aging reduces AR mRNA expression and protein content in vitro in skeletal muscle cells and whether this reduction is applicable to human models or if it is specific to mice and rats. It may also be beneficial to categorise different tissues based on aging's effect on their AR content and expression, similarly to how tissue can be categorised based on androgen responsiveness, however as available data is so limited for most tissues this is currently not possible.

1.5.5 Myoatlas Analysis of Androgen Receptor Expression

To further investigate the effect aging has on tissue specific androgen receptor expression, the Myoatlas tool was used to compare AR gene expression in mouse soleus and tibialis anterior muscle. Myoatlas is a tool developed by the Millay Lab at the Cincinnati Children's Hospital, available at https://research.cchmc.org/myoatlas. The tool consists of a database of over 45,000 nuclei from wild-type mouse Tibialis Anterior muscle, which have been profiled across their entire lifespan using single-muscle RNA-sequencing. The nuclei have been filtered and grouped based on cell identity and gene expression profile, enabling the investigation of expression of genes of interest across all populations of cells and myonuclei. Using the Myoatlas tool, AR gene expression was investigated in soleus and tibialis anterior muscle in rats aged 5-30 months, to determine if AR expression was age and fiber type specific. Myoatlas data demonstrated that AR gene expression is greater in 5-month-old rat TA in satellite cells compared to 30-month-old rat TA and 5-month-old rat soleus (**Fig 1.7A**). Ar gene expression was also greater in 5-month-old TA in type IIb myonuclei compared to 30-month-old TA and 5-month-old soleus (**Fig 1.7B**). Conversely, AR gene expression was greater in 5-month-old soleus and

30-month-old TA in type IIx myonuclei compared to 5-month-old TA (**Fig 1.7C**). These data further support the suggestion that the effects of age on androgen receptor gene expression are both muscle and cell type specific, and demonstrate that androgen receptor expression reduces with age, potentially reducing androgen receptor content.

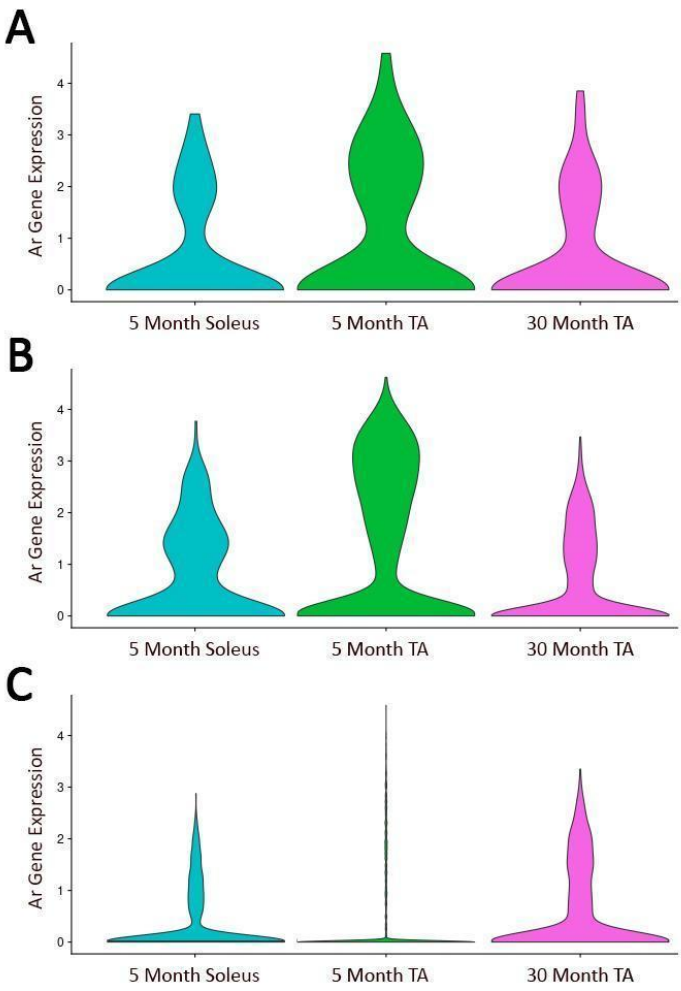

Fig 1.7. **A**) AR gene expression level in 5 and 30-month-old mouse soleus and tibialis anterior satellite cells**. B**) AR gene expression level in 5 and 30-month-old mouse soleus and tibialis anterior type IIb myonuclei. **C)** AR gene expression level in 5 and 30-month-old mouse soleus and tibialis anterior type IIx

myonuclei. AR gene expression was visualised using the MyoAtlas webtool, available at https://research.cchmc.org/myoatlas/.

1.6 Summary

This literature review has described evidence that maintaining adequate skeletal muscle mass is key for living an independent, healthy lifestyle into older age. Age associated muscle wasting imposes significant decrements in the quality of life in an expanding elderly population. Sarcopenia also places a serious financial and personnel burden on healthcare systems, with sarcopenic patients requiring longer and more expensive treatment than non-sarcopenic patients being treated for the same condition. A number of factors have been shown to influence sarcopenia, including dietary, physical activity and hormonal changes. Lifestyle changes such as diets and physical activity levels can be resolved via exercise and nutrition programmes, however hormonal changes can be much harder to address. With aging comes a significant reduction in free testosterone levels, likely induced by increases in SHBG, which has been associated with skeletal muscle wasting. Research has demonstrated the effectiveness of testosterone administration in restoring skeletal muscle size, strength and function in older adults, resulting in the use of testosterone replacement therapy in patients suffering from hypogonadism and severe sarcopenia. Prior studies have demonstrated an aging associated reduction in androgen receptor content, although this effect appears to be both species and tissue specific. Recent research has also demonstrated a tissue specific effect of testosterone to increase or restore androgen receptor content following castration. Therefore, testosterone may be capable of increasing skeletal muscle androgen receptor content in older adults, increase skeletal muscle hypertrophy and reduce the effects of sarcopenia.

1.7 Aims and Objectives

1.7.1 Aims

1. To determine the effect of testosterone administration on skeletal muscle androgen receptor expression
2. To determine the morphological and transcriptional effects of testosterone administration at different timepoints throughout differentiation
3. To determine whether aging affects skeletal muscle androgen receptor content and the anabolic effect of testosterone

1.7.2 Objectives

To achieve aim 1) single and repeated doses of testosterone will be administered to C2C12 skeletal muscle myoblasts to determine the effects of testosterone treatment on skeletal muscle androgen receptor expression, gene expression and differentiation.

To achieve aim 2) testosterone will be administered to C2C12 myoblasts at various points throughout differentiation, to examine the morphological and transcriptional effects of treatment timing.

To achieve aim 3) an *in vitro* model of aged skeletal muscle will be used to determine the differing effects of testosterone administration in young and aged muscle. This model will be used in conjunction with methods optimised in study 1.

1.7.3 Hypotheses

It was initially hypothesised that testosterone would have differing effects dependent upon the stage of differentiation that testosterone was administered in. Furthermore, it was hypothesised that testosterone would incur increases in cellular metabolic activity, potentially indicative of increases in

cellular viability. Repeated doses of testosterone were hypothesised to incur greater effects compared to single doses and testosterone was also hypothesised to rescue the effects of aging on myotube size and myonuclear accretion. Based on recent *in vitro* research, testosterone was initially hypothesised to have no effect on AR expression

Chapter 2

General Methods

2.1 Microscopy and image processing

Microscope images were captured using a Leica DM116000B microscope (Leica Biosystems, Wetzlar, Germany). All image processing was completed using the Leica Application Suite for Windows (Leica Biosystems, Wetzlar, Germany). Morphological image analysis was completed using Image J version 1.53e (Wayne Rasband and contributors, National Institutes of Health, USA).

2.2 Statistics and figures

Data collation and organisation was performed using Microsoft Excel 2016 version 2107 (Microsoft Corporation, Redmonds, Washington, USA). All statistical analysis was performed using IBM SPSS Statistics version 27 (IBM, Armonk, NY, USA). All figures were created using Graphpad Prism version 9 (Graphpad Software, Inc., San Diego, USA).

2.2 Plasticware

Cell populations were cultured on T75 cm^2 culture flasks (Corning, NY, USA) and experiments performed on 95mm^2 6-well plates (Corning, NY, USA). Eppendorf tubes for sample collection and storage were purchased from Eppendorf (Eppendorf, Hamburg, Germany). Cryogenic vials were purchased from Simport Scientific (Simport Scientific, Saint-Mathieu-de-Beloeil, Canada)

2.3 Chemicals and reagents

0.2% Gelatin was prepared by mixing 1g porcine gelatine powder (Sigma-Aldrich Company Ltd, Dorset, UK) in 500ml distilled H_2O. Gelatin was labelled with the date of preparation, autoclaved and stored at room temperature.

1X Phosphate buffered saline (PBS) was prepared by dissolving 5 PBS tablets (Sigma-Aldrich Company Ltd, Dorset, UK) in 500ml distilled H_2O. PBS was labelled with the date of preparation, autoclaved and stored at room temperature.

Growth media (GM) was prepared by mixing 395ml Dulbecco's modified Eagle's medium (DMEM) (Lonza, Verviers, Belgium) with 100ml fetal bovine serum gold (FBS) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 5ml penicillin-streptomycin solution (penstrep) (Lonza, Verviers, Belgium) in a 500ml DMEM bottle. Growth media was labelled with the date of preparation and stored at 4°C. Differentiation media (DM) was prepared by mixing 485ml DMEM with 10ml horse serum (HS) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 5ml penstrep in a 500ml DMEM bottle. Differentiation media was labelled with the date of preparation and stored at 4°C.

Testosterone (T1500-1G) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, UK). TRI Reagent solution was purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Chloroform, isopropanol and ethanol were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, UK). Trypan blue was purchased from Hyclone Laboratories (Hyclone Laboratories Inc, Logan, Utah, USA). Tetrazolium dye used for MTT assays was purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, UK). Trypsin was purchased from Lonza (Lonza, Verviers, Belgium) Methanol and triton were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, UK).

Antibodies used for immunocytochemistry and primers used for PCR were purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Specific primers are detailed in section 2.8.5.

Testosterone was prepared by adding 7.2mg testosterone to 5ml of DMSO to create a 5 mM stock solution. The stock solution was then allotted into 50µl Eppendorf and stored at -20°C. Working stock was kept for a month before being replaced by a fresh stock to maintain potency.

2.4 Cell Culture

All cell culture experiments were performed under a Kojair Biowizard Silverline class II hood (Kojair Tech Oy, Vippula, Finland). Cells were incubated at 37°C and 5% CO_2 in a HERAcell 150i CO_2 incubator (Thermo Scientific Inc. Cheshire, UK). All liquids, waste media and supernatant were discarded via extraction pump (Charles Austen Pumps Ltd. Surrey, UK). Solutions were prepared using dH_2O from a MilliQ water purification system (Merck KGaA. Darmstadt, Germany).

T75 flasks were gelatinised with 5ml of 0.2% gelatin, left at room temperature for 10 minutes and then incubated at 37°C for 10 minutes before excess gelation was aspirated. C2C12 cells were brought up in 1ml cryovials at 1 x 10^6 cells from liquid nitrogen and thawed. 1ml cell suspension and 14ml GM was added to the gelatinised T75 flasks. T75 flasks were labelled with experiment code, cell type, date and passage number then incubated at 37°C in a humidified 5% CO_2 atmosphere.

Once cells were approximately 80% confluent, they were washed twice with 5ml PBS per T75 to remove any serum. 1ml trypsin was added to each T75 and incubated at 37°C for 5 minutes to detach cells from the gelatin. Cell detachment was confirmed under the microscope. 4ml GM was added per T75 flask to neutralise trypsin, then homogenised and collected into a 50ml falcon tube. Cell suspension was prepared in a 1:1 dilution with trypan blue stain (totaling 40µl suspension) and dispersed into a haemocytometer for cell counting as detailed in section 2.4.1. For experimental procedures, cells were

seeded at 50,000 cells/cm^2 in 2ml GM per well onto pre-gelatinised six well plates and incubated at 37°C for 24 hours to attach.

Seeding density was calculated using the following equation:

Required ml trypsinized cells = Required seed (no. cells/ml) / cell count (no. cells/ml) x Required ml of media

Once cells had proliferated to approximately 80% confluency they were washed with 1ml PBS per well and treated with 2ml DM per well. DM was topped up every 48 hours with 200µl DM per well. Cells were induced to differentiate for a 10 day time course for each experiment (**Fig 2.1**).

2.4.1 Cell Counting by Trypan Blue Exclusion

Cells were counted using a haemocytometer. Cells were prepared as a 1:1 suspension in 0.4% Trypan blue stain (totaling 40µl suspension; 20µl cell suspension and 20µl Trypan blue) and loaded into the haemocytometer. Cells were counted in all eight corner grids under a microscope at x10 objective. Viable cells were classified as round and clearly visible, whilst non-viable cells were larger and trypan blue positive due to a loss of membrane integrity. The mean of all 8 grids was calculated to determine average cell number per 0.1 mm^3, this value was then multiplied by 2 to account for the dilution factor of 1:1 and multiplied by 10^4 to calculate the number of cells in 1ml of cell suspension.

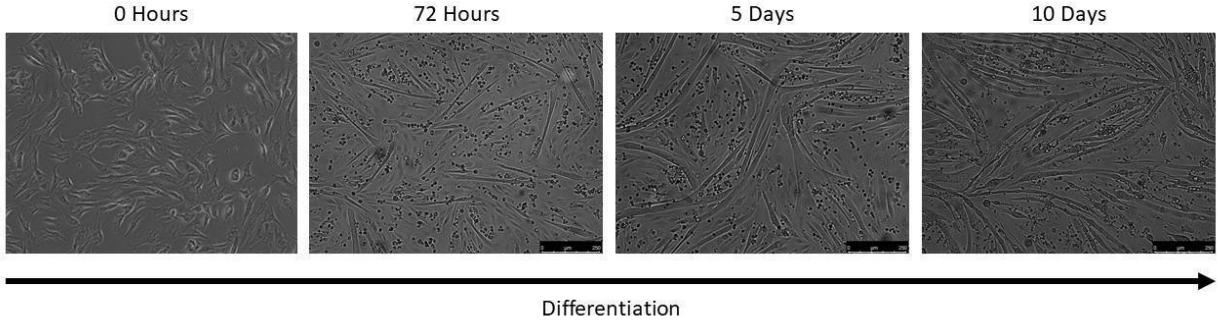

Fig 2.1 Brightfield images (10x objective) of cultured C2C12 skeletal muscle myoblasts over the course of 10 days differentiation

2.4.2 Multiple Population Doublings

Young C2C12 myoblasts (as low a passage number as possible to ensure accurate estimation of population doubling numbers) were seeded at $1x10^6$ cells in T75 flasks in GM and incubated until approximately 80% confluent. Cells were then trypsinized and seeded onto new T75s at $1x10^6$ for 48 hours, with doubling time and cell numbers being recorded throughout. This cycle was repeated until the desired number of population doublings was reached.

2.5 MTT (diphenyl tetrazolium bromide) Assay

2.5.1 General Principle

The MTT assay is a colorimetric assay used to measure cellular metabolic activity. NAD(P)H- dependent oxidoreductase enzymes in viable cells reduce tetrazolium dye in the MTT solution into insoluble formazan crystals, identifiable by their purple colour. Absorbance of the formazan solution can be quantified by measuring at a wavelength of 500-600nm using a spectrophotometer. The assay measures cellular activity as the enzymatic conversion of the MTT solution to formazan crystals occurring in the mitochondria.

2.5.2 MTT Assay

Tetrazolium dye was added to PBS at a concentration of $5mg.ml^{-1}$ to create an MTT solution. At relevant timepoints, media was aspirated, cells were washed twice with 2ml PBS/well and 2ml fresh DM/well was added. 200µl MTT solution/well was added (10% of total media per well) and cells were incubated for 3

hours until visible purple formazan crystals formed at the base of each well. DM and MTT solution was then aspirated and six wells were incubated at 37°C until dry. 1ml DMSO/well was added to six wells to produce a vibrant purple solution which is visible at a wavelength of 570 nm. Six well plates were then placed into a Spark multimode microplate reader (Tecan Trading AG, Männedorf, Switzerland) measured at a wavelength of 570 nm and the results outputted to Microsoft Excel.

2.6 Imaging and Image Analysis

2.6.1 Coordinate based Imaging

At the first relevant time point for each study, cells were imaged under a with a 10x objective, the coordinates for each image were recorded and at all subsequent timepoints the same cells were reimaged at the same coordinates. This allowed for a more accurate measure of the effects of testosterone and age on myotube number and diameter, by analysing the percentage increase in diameter and number in each condition following treatment, rather than comparing raw numbers between conditions. Ten images were taken per condition, per time point with each image being taken from a different well on a six well plate.

2.6.2 Measuring Myotube Number

Myotube number was measured in ImageJ using the multi-point tool which allows for the selection and counting of multiple regions of interest (**Fig 2.2**). Myotubes were classified as multi nucleic structures

with a visible outer membrane. Myotubes that were not entirely visible within the image frame were still classified as myotubes and included in the count.

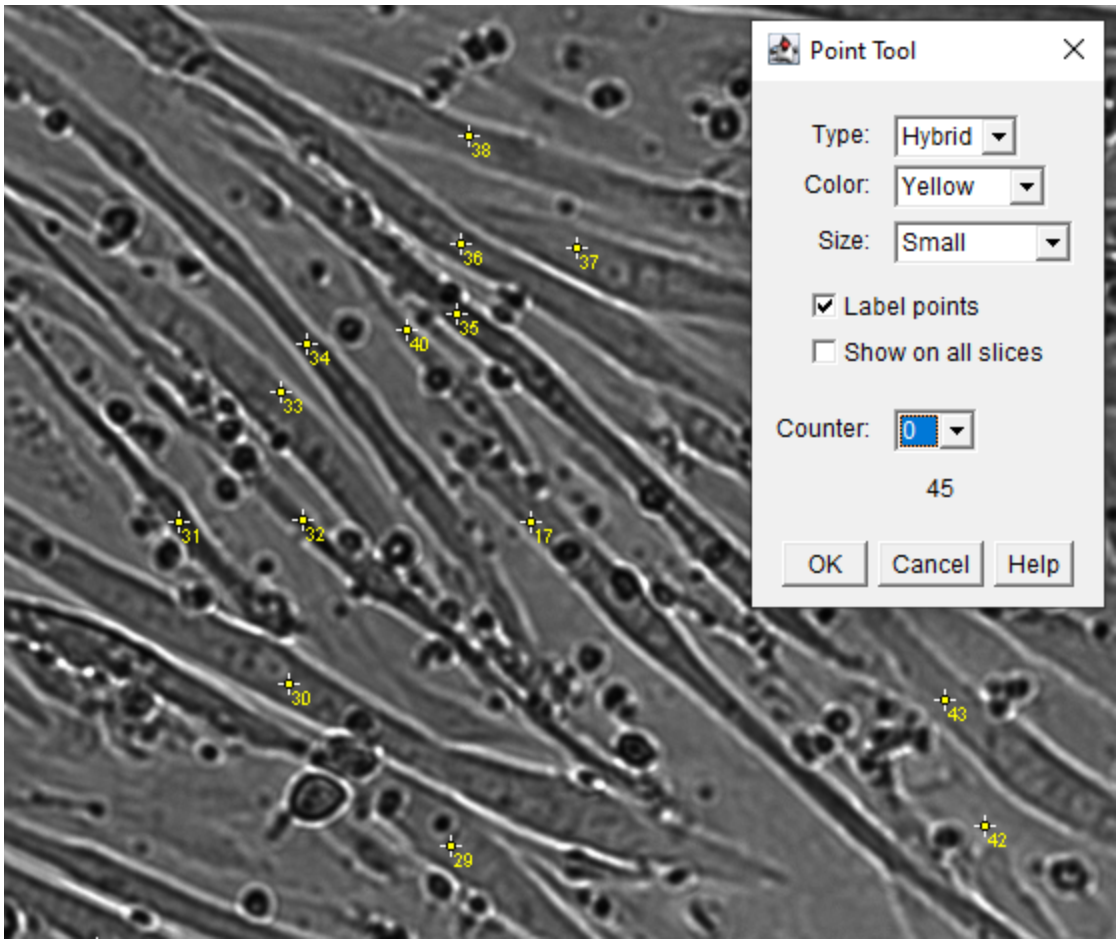

Fig 2.2 Example segment of 10x objective live image with myotubes selected as regions of interest using the multi-point tool.

2.6.3 Measuring Myotube Diameter

Myotube diameter was measured in ImageJ. Myotubes were measured at 3 equidistant points along their length using the straight-line tool and then an average diameter was calculated for each myotube (**Fig 2.3**). Diameter was only measured in myotubes that were completely visible within the image frame.

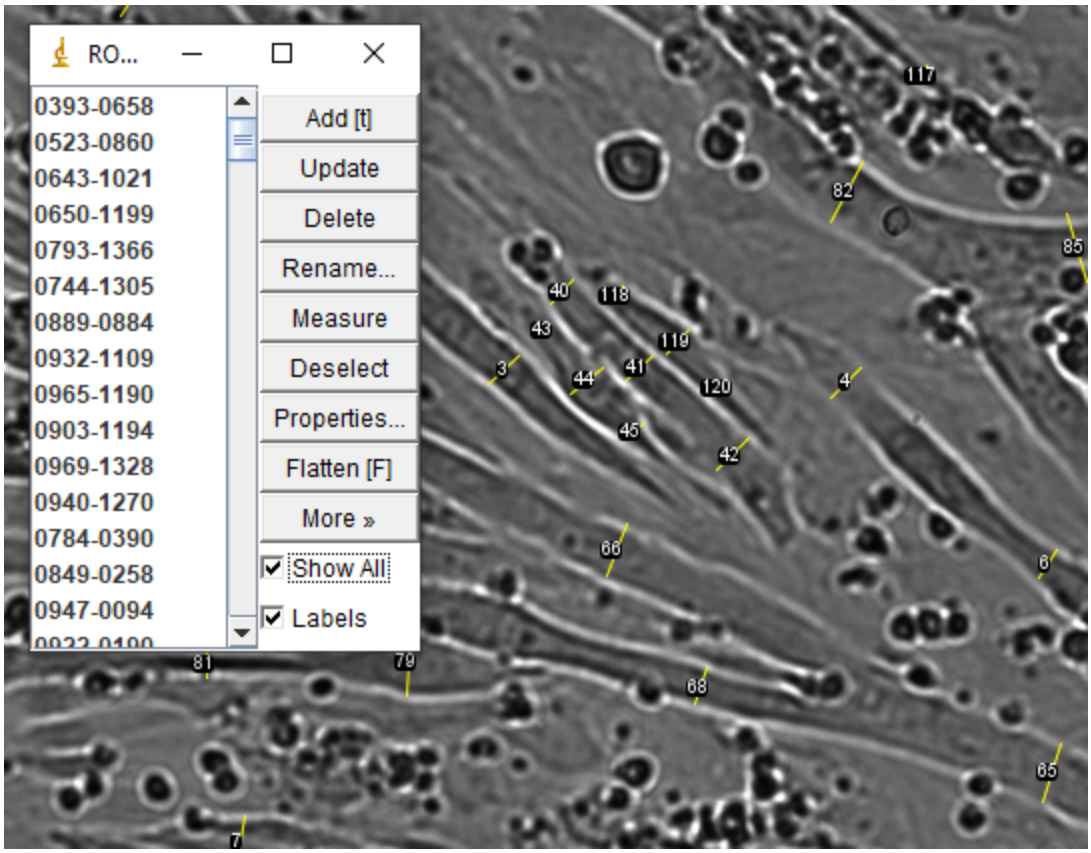

Fig 2.3 Example segment of 10x objective live image with myotube diameter measurements completed using the straight-line measurement tool and region of interest manager

2.7 Immunocytochemistry

2.7.1 General Principle

Immunocytochemistry allows for the detection of specific antigens via the use of an antibody. Antibodies consist of two functional domains, resembling the letter Y. The antigen binding fragment domain (Fab) forms the arms of the Y, with the end of each arm forming a region responsible for antigen binding, called the antigen binding site. The bottom of the Y contains the Fc domain, which other antibodies recognise. This Y shaped structure allows for the direct detection of antigens using a single fluorophore-labelled antibody, or indirect detection via the binding of a fluorophore labelled secondary antibody raised against the Fc domain of an unlabeled primary antibody. When exposed to specific light wavelengths the secondary antibody containing a fluorochrome emits fluorescence (**Fig 2.4**). In fluorescence microscopy, a wavelength of light is focused on the antibody labelled specimen to excite the electrons of the fluorochrome molecules to a higher energy state (known as the stokes shift), the relaxation following this excitation results in the emission of light in the form of a lower-energy photon in the visible light region. This fluorescent light can then be filtered through a dichroic mirror and visualised using a microscope. Images captured during this emission of fluorescence can then be analysed and quantified to compare levels of specific antigens.

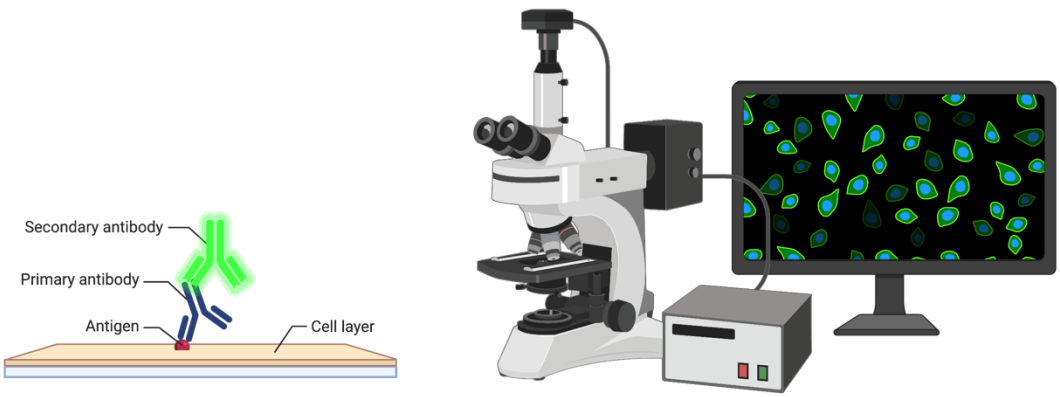

Fig 2.4 Schematic representation of antigen immunolabeling and fluorescent microscopy, showing the binding of a primary and secondary antibody to an antigen and the visualisation of fluorescence emitting cells via a fluorescent microscope.

2.7.2 Specimen Fixation

At relevant time points, cells were washed 2x with 1ml cold TBS per well. Methanol was diluted with TBS at 3 concentrations (25%, 50%, 100% methanol). Following washing, 500µl 25% methanol per well was added to the six well plates and left at room temperature for 5 minutes. The methanol was aspirated and replaced with 50% methanol for a further 5 minutes before being replaced with 100% methanol for a further 5 minutes. Cells were washed briefly with 1ml TBS per well following methanol treatments and 2ml TBS/well was added to the six well plates. Six well plates were then wrapped in parafilm and labelled with the experiment code, condition, timepoint and date. Samples were stored at 4°C until analysis.

2.7.3 Immunofluorescence

Cells were incubated in six wells in 500µl 0.1% Triton X-100 PBS for 10 minutes then washed in PBS 3 times for 5 minutes. Cells were incubated in 500µl 10% serum from species the secondary antibody was raised in, in PBST (PBS + 0.1% Tween 20) for 30 minutes. Cells were incubated overnight at 4°C. Solution was decanted and cells were washed in PBS 3 times for 5 minutes. Cells were incubated in 500µl secondary antibodies in 1% BSA for 1 hour in the dark. Solution was decanted and cells were washed 3 times with PBS for 5 minutes in the dark. Cells were then incubated in nuclear stain if applicable for 10 minutes before media was aspirated and cells were washed with twice PBS and stored at 4°C.

Immunostained cells were imaged under a Leica DM116000B microscope (Leica Biosystems, Wetzlar, Germany) at 10x objective using blue colour channels for DAPI and green for MyoHC. Six images were taken per condition per time point.

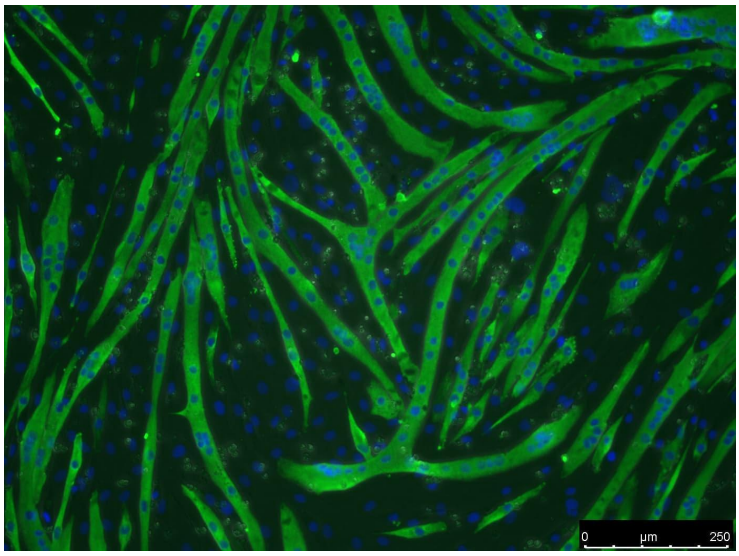

Fig 2.5 Myotubes stained for DAPI and MyoHC and imaged at 10x objective under a microscope using blue colour channels for DAPI and green for MyoHC.

2.7.5 Measuring Number of Nuclei Per Myotube

In Immunostained images, myotubes were classified as multi nucleic structures with a visible outer membrane and expressing MyoHC. To calculate the average number of nuclei per myotube, the number of myotubes expressing MyoHC was analysed using the multi-point tool in ImageJ. A blue DAPI stain was then overlain over the image and the number of nuclei within each myotube were counted. The number of nuclei incorporated into myotubes was then divided by the number of myotubes to calculate the average number of nuclei per myotube.

2.7.6 Measuring Fusion Index

To calculate the fusion index for each stained image, the total number of nuclei in each DAPI overlay was counted. The number of nuclei incorporated into myotubes was then divided by the total number of nuclei to calculate the fusion index. The fusion index was multiplied by 100 to be presented as a percentage.

2.8 Polymerase Chain Reaction (PCR)

2.8.1 General Principle

PCR relies on the amplification of mRNA isolated from samples. Amplification of target genes is achieved using the following steps:

1. Isolated mRNA is synthesized into complementary DNA (cDNA) via reverse transcription using reverse transcriptase and nucleoside triphosphates (dNTPs) (**Fig 2.6Ai**).
2. cDNA is heated, causing it to denature into two single stranded DNA molecules (ssDNA)
3. Sequence specific primers (SSPs) anneal at the 3’ end of both ssDNA strands (**Fig 2.6Aii**).
4. RNA polymerase transcribes the target gene (**Fig 2.6Aiii**).

In order to detect the amplified product, cDNA is labelled with a fluorescence emitting molecule during each cycle (**Fig 2.6B**). Fluorescence is then proportionally increased with the amplification of cDNA molecules and detected in real time (**Fig 2.6C**).

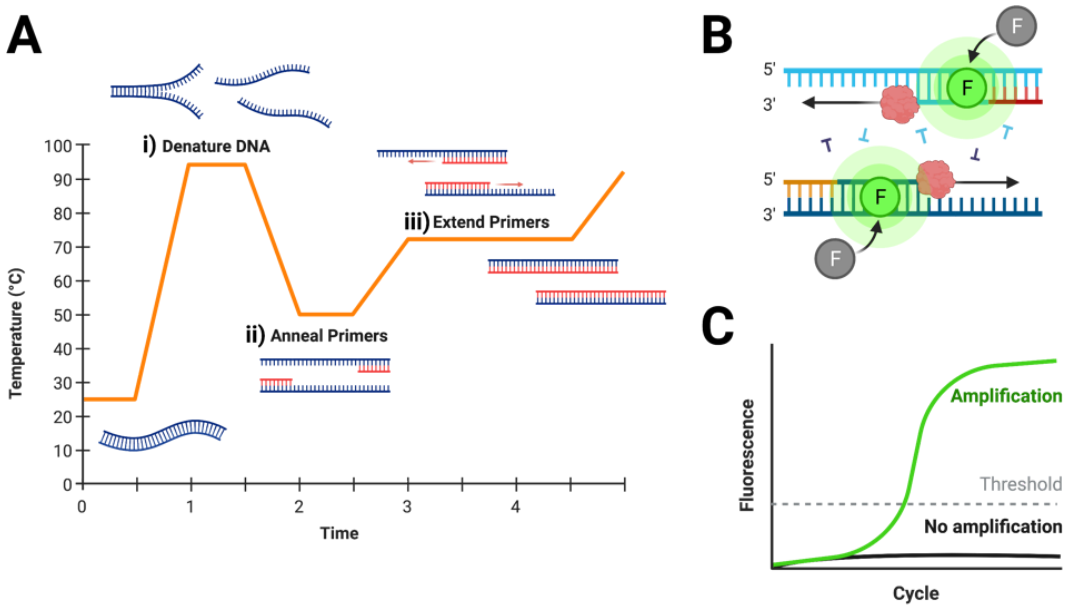

Fig 2.6 Schematic representation of the principle steps of real time quantitative polymerase chain reaction.

2.8.2 RNA Isolation and Quantification

At relevant time points, cells were washed 3x with 1ml PBS per well then lysed with 200µl Trizol per well for 1 minute at room temperature before being scraped and collected in 1.5ml Eppendorf tubes. Eppendorf tubes were labelled with the experiment code, condition, time point and date and stored at -20°C.

All of the following procedures were carried out using RNA free pipette tips, eppendorf tubes and pipettes designed specifically for RNA use. For phase separation, 200µl chloroform for every 1ml Trizol was added to lysates in 1.5ml eppendorf tubes. Eppendorf tubes were then vortex mixed for 15 seconds and left at room temperature until the lysate separated into 3 distinct phases: the aqueous phase containing RNA, interphase containing DNA and the bottom organic phase containing proteins and lipids.

Samples were centrifuged at 12,000 rcf for 15 minutes at 2-8°C. The aqueous phase was then pipetted from samples and 500µl isopropanol per 1ml Trizol was added to samples and inversion mixed. Samples were incubated at room temperature for 10 minutes then centrifuged at 12,000 rcf for 15 minutes at 2-8°C. Supernatant was pipetted from samples leaving just a pellet. Pellets were washed with 1ml 75% ethanol per 1ml Trizol and centrifuged at 7,500 rcf for 5 minutes at 2-8°C twice. Supernatant was pipetted from samples and pellets were air dried for 15 minutes at room temperature. Pellets were resuspended in 20µl DEPC H_2O, centrifuged on a bench top centrifuge and incubated at 55-60°C on a heating block until the pellet was completely dissolved.

RNA concentration and purity were assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at wavelengths of 260 and 280nm. The spectrophotometer was wiped clean using dH_2O prior to use and between samples. 1µl of RNA isolate was pipetted onto the spectrophotometer. RNA quality (ng/µl nucleic acid concentration), 260:280 and 260:230 ratios, and A260 and A280 measurements were all recorded from the spectrophotometry. Only samples with a 260:280 ratio between 1.9 and 2.15 were used for PCR amplification.

2.8.3 Polymerase Chain Reaction (PCR)

RNA was prepared in PCR specific RNA free reaction tubes. 3µl RNA sample was mixed with 5µl Quantifast SYBR Green (Qiagen, Hilden, Germany), 0.5µl of specific forward and reverse primers, 0.1µl Fast RT mix (Qiagen, Hilden, Germany) and 0.9µl water. mRNA was amplified on a rotor-gene Q PCR machine (QIAGEN inc, Hilden, Germany). Double stranded cDNA was synthesized at 50°C for 10 minutes. Complementary DNA was then denatured at 95°C for 10 seconds into ssDNA, before primer annealing and extension occurred at 60°C for 30 seconds. This cycle of denaturing, annealing and extension was

repeated for 40 cycles. The SYBR green within the RNA mix emits a fluorescent dye upon binding DNA molecules, the rotor gene Q software detects this emission of fluorescence and generates an amplification curve throughout the course of the cycles of denaturing, annealing and extension.

2.8.4 PCR Quantification

Changes in mRNA expression were assessed using a relative quantification method. This method determines changes in steady state mRNA levels of a target gene and expresses these changes relative to the levels in a reference gene. To calculate levels of expression the ΔΔCt method was used. This method measures the differences between threshold cycles using a calibrator sample, usually a sample from an untreated control or zero-hour time point (Livak & Schmittgen, 2001).

The following equation was used to calculate ΔΔCt:

ΔCt = Ct (gene of interest) - Ct (housekeeping gene)

ΔΔCt = ΔCt (treated sample) - ΔCt (calibrator gene)

2.8.5 Primer Sequences for Genes of Interest

Table 2.1 Primer sequences for *Mus musculus* for genes of interest used in RT-qPCR in chapters 3 and 4.

Gene	Primer Sequence		Amplicon Length (bp)	Accession Number
Ar	AAAGAGCCGCTGAAGGGAAA	GGAGACGACAAGATGGGCAA	99	NM_013476.4
Myostatin	TACTCCGAATAGAAGCCATAA	GTAGCGTGATAACGTCATC	194	NM_010834.3
MyoG	GTGCCCAGTGAATGCAACTC	CGAGCAAATGATCTCCTGGGT	94	NM_031189.2
Myod1	CATTTCAACCCAGAGAAC	GGCGATAGAAGCTCCATA	125	NM_010866.2
RP2β	GGTCAGAAGGGAACTTGTGGTAT	GCATCATTAAATGGAGTAGCGTC	197	NM_153798.2

Chapter 3

The morphological and transcriptional effects of testosterone administration at different stages of differentiation in C2C12 skeletal muscle cells

3.1 Abstract

Aim: This study aimed to determine the morphological and transcriptional effects of testosterone administration in the early and late stages of differentiation in skeletal muscle myoblasts.

Method: C2C12 myoblasts were induced to differentiate into myotubes with 100 nM testosterone administration occurring at 0 hours (when cells were undifferentiated myoblasts) or 7 days differentiation (when cells were differentiated myotubes). Control cells were treated with an equivalent dose of DMSO at 0 hours differentiation. After 72 hours and 10 days differentiation samples were fixed for morphological measures (myotube number, diameter and branched myotube number) and lysed for mRNA (Ar and myostatin gene expression). Cellular activity was measured at 10 days differentiation using an MTT assay.

Results: Testosterone treatment in undifferentiated myoblasts significantly increased myotube number ($P < 0.001$) and branched myotube number ($P < 0.001$), however had no significant effect on myotube diameter. Treatment also significantly increased Ar expression after 72 hours ($P = 0.032$), but then reduced Ar expression after 10 days ($P = 0.046$), as well as significantly reducing myostatin expression after 10 days ($P = 0.042$). Testosterone treatment in differentiating myotubes significantly increased branched myotube number ($P < 0.001$) and myotube diameter ($P < 0.001$), however had no significant effect on myotube number, Ar expression or myostatin expression. Testosterone administration at both time points significantly increased metabolic activity ($P = 0.039$ for cells treated as undifferentiated myoblasts, $P = 0.014$ for cells treated as differentiating myotubes).

Conclusions: These findings suggest that whilst testosterone primarily accelerates myoblast fusion and increases commitment to terminal differentiation when administered to undifferentiated myoblasts, testosterone administration in differentiated myotubes works to inhibit atrophy. This study provides data

demonstrating the effects of testosterone administration in differentiated myotubes, which when combined with prior research forms a more complete picture of the effects of testosterone treatment throughout skeletal muscle cells lives. This study also provides pilot data suggesting that testosterone treatment may increase cellular viability, although this needs to be confirmed through more specific cell viability assays.

3.2 Introduction

Testosterone has been shown to elicit a hypertrophic response in skeletal muscle by upregulating the rate of protein synthesis and downregulating the rate of protein degradation (Urban et al., 1995; Wolfe et al., 2000). This change in protein turnover has been shown to increase skeletal muscle cross sectional area (CSA), strength and function in older adult males (Sinha-Hikim et al., 2006). *In vitro* testosterone administration also elicits a number of morphological effects in C2C12 skeletal muscle myoblasts and myotubes with prior studies demonstrating increases in myotube diameter, number and myonuclear accretion following a single 100 nM dose (Deane et al., 2013; Hughes et al., 2016). Furthermore, testosterone has demonstrated protective effects on C2C12 cells against dexamethasone induced atrophy, as well as increasing skeletal muscle regeneration post cardiotoxin induced injury (Serra et al., 2013; Zhao et al., 2008). The effects of testosterone may be mediated by transcriptional changes, as Hughes et al. (2016) and Deane et al. (2013) both show increases in MyoG expression 72 hours post testosterone treatment. This reported increase in MyoG expression is likely to account for the increase in myotube number, due to increasing myoblast fusion and committing myoblasts to differentiation (Lee, 2002).

Despite multiple papers demonstrating the effects of testosterone administration in undifferentiated C2C12 myoblasts, little research exists showing the effects of testosterone administration in differentiated myotubes. Prior work by Hughes (2014) has demonstrated that testosterone administration in both undifferentiated myoblasts and differentiating myotubes can induce morphological and transcriptional changes. Hughes (2014) reported that 50 nM testosterone treatment increased myotube number and size following administration in undifferentiated myoblasts, however when administered to differentiating myotubes only myotube size increased, until a significantly larger

500 nM dose of testosterone was administered. These findings suggest that testosterone administration may accelerate myoblast commitment to differentiation and increase myotube size in the early stages of myotube differentiation. However, there is currently little research investigating the effects of testosterone administration in the later stages of myotube differentiation. Whilst Hughes (2014) demonstrated increases in myoblast activation, these effects may not be present in terminally differentiated myoblasts where many of the myoblasts have already formed myotubes which are unable to undergo further cell division. Therefore, there is a gap in our understanding of how testosterone administration affects skeletal muscle cells at different administration points throughout differentiation of muscle cells.

This study aimed to compare the effects of testosterone administration in 1) undifferentiated myoblasts and 2) myotubes nearing peak differentiation in a 2D cell culture model. Morphological (myotube number and diameter) and transcriptional (Ar and myostatin mRNA expression) data were collected along with preliminary cellular metabolic activity data in the form of an MTT (3-(4,5-Dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide) assay.

Based on previous research by Deane et al. (2013) and Hughes (2014), it was hypothesised that testosterone administration in undifferentiated myoblasts would increase myotube number and inhibit myostatin expression. Whilst in differentiating myotubes it was hypothesised that testosterone administration would only increase myotube diameter. Neither treatment was hypothesised to increase Ar expression. Testosterone treatment was also hypothesised to increase cellular metabolic activity, as prior work has suggested testosterone treatment may improve mitochondrial function (Pitteloud et al. 2005; Pongkan et al. 2015).

3.3 Methodology

Experimental Design

C2C12 skeletal muscle cells were cultured and induced to differentiate in the presence of either 100 nM DMSO (referred to as CON cells) or 100 nM testosterone at 0 hours differentiation (referred to as early differentiation treatment, or EDT, cells) or 100 nM testosterone after 7 days differentiation (referred to as late differentiation treatment, or LDT, cells). Morphological measures (myotube number, diameter and branched myotube number) were taken after 72 hours and 10 days differentiation. Cells were isolated for RNA after 72 hours and 10 days differentiation to measure androgen receptor and myostatin gene expression. Cells were also subjected to an MTT assay after 10 days differentiation to measure cellular metabolic activity.

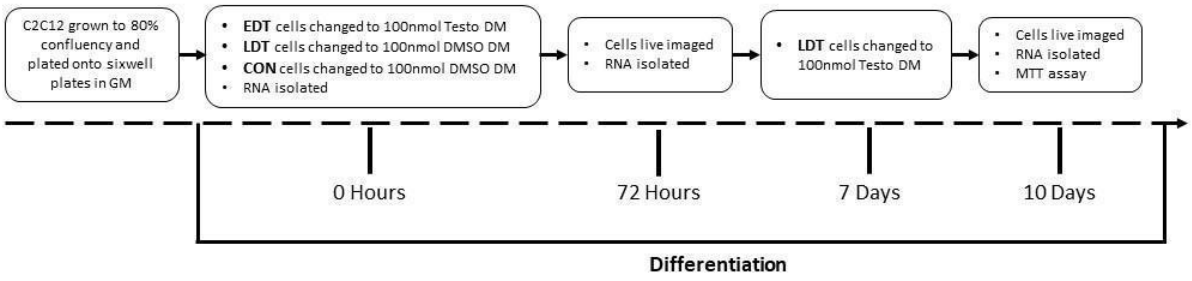

Fig 3.1. Schematic representation of the experimental design used to examine the effects of testosterone administration during early and late differentiation of muscle cells.

Cell Culture

C2C12 skeletal muscle myoblasts were resurrected, cultured and seeded onto 6-well plates as described in section 2.4.

Cell Treatment

All cell treatments were prepared as outlined in section 2.5. At 0 hours differentiation EDT cells were changed from growth media to differentiation media treated with 100 nM testosterone and CON and LDT cells were changed to differentiation media treated with 100 nM DMSO. After 7 days differentiation LDT cells were changed to differentiation media treated with 100 nM testosterone.

Live Imaging

After 72 hours and 10 days differentiation, myotubes were live imaged under a microscope at 10x objective as described in section 2.7.1. Myotube number, diameter and branched myotube number were measured as outlined in sections 2.7.2-3

Polymerase Chain Reaction (PCR)

After 72 hours and 10 days differentiation, myotubes were lysed, isolated for RNA and diluted as described in sections 2.9.2-5. PCR was then performed as detailed in section 2.9.6. The relative mRNA expression of Ar and myostatin was quantified using the $\Delta\Delta$Ct method as described in section 2.9.7 against the reference gene RP2B and a calibrator sample of control treatment after 0 hours differentiation. Primer sequences for genes of interest are detailed in section 2.9.8.

MTT Assay

After 10 days differentiation, differentiation media was aspirated and replaced with a tetrazolium dye solution as described in section as described in sections 2.6.2-3. Cellular metabolic activity was then measured using a plate reader and MTT assay as described in section 2.6.3.

Statistical Analysis

All data are expressed as means ± SD. Statistical significance was set at the 0.05 level of confidence. Treatment dependent effects were investigated using a one-way between subjects ANOVA, with Bonferroni *post hoc* analysis used to assess significant effects.

3.4 Results

Cellular Metabolic Activity

There was a significant effect of testosterone treatment on cellular metabolic activity after 10 days differentiation, where testosterone treatment in undifferentiated myoblasts (EDT) and differentiated myotubes (LDT) increased cellular activity compared to control myotubes treated with DMSO (EDT 120.4 vs CON 100%; *P* = 0.039) (LDT 125.8 vs CON 100%; *P* = 0.014) (**Fig 3.2**).

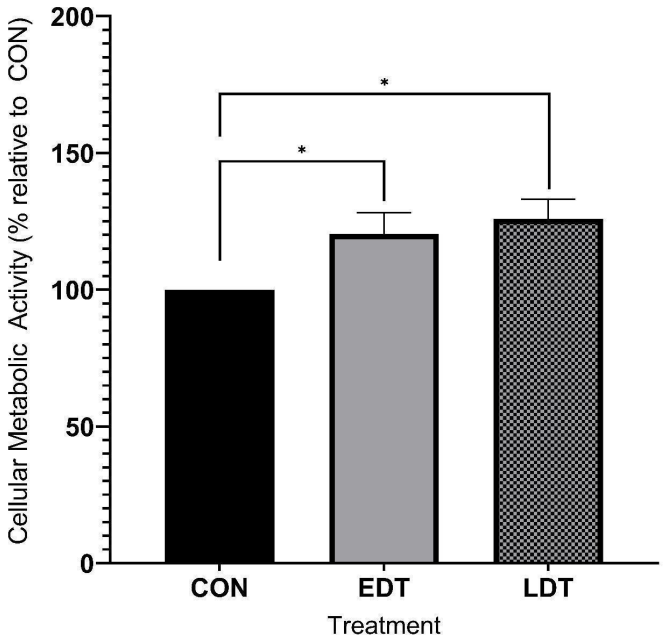

Fig 3.2. The effects of 100nM testosterone treatment in undifferentiated myoblasts (EDT) and differentiating myotubes (LDT) on cellular activity after 10 days differentiation versus control. * $P \leq 0.05$. n = 3

Myotube Morphology

Testosterone treatment in undifferentiated myoblasts significantly increased myotube number per field after 10 days differentiation, however testosterone treatment in differentiated cultures had no significant effect on myotube number (CON 52 ± 6.9 myotubes vs EDT 81 ± 7.2 myotubes vs LDT 52 ±4.5 myotubes; $P < 0.001$ for CON vs EDT and EDT vs LDT) (**Fig 3.3A, Fig 3.4**).

Testosterone treatment in undifferentiated myoblasts and differentiating myotubes significantly increased branched myotube number after 10 days differentiation (CON 1.0 ± 1.3 branched myotubes vs EDT 3 ± 1.7 branched myotubes vs LDT 3 ± 1.4 branched myotubes; $P = 0.011$ for EDT vs CON, $P = 0.001$ for LDT vs CON) (**Fig 3.3C, Fig 3.4**).

Testosterone treatment in differentiating myotubes significantly increased myotube diameter after 10 days differentiation compared to control myotubes and myotubes treated with testosterone at the onset of differentiation (CON 3.7 ± 1µm vs EDT 3.6 ± 0.9µm vs LDT 4.2 ± 1.2µm vs; $P < 0.001$ for LDT vs CON and LDT vs EDT) (**Fig 3.3B, Fig 3.4**).

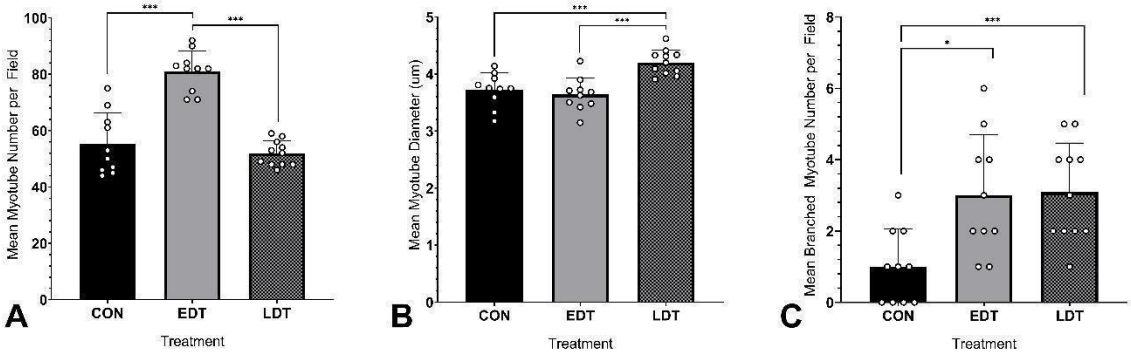

Fig 3.3. The effects of 100nM testosterone treatment on myotube morphology after 10 days differentiation **A)** The effects of testosterone treatment in undifferentiated myoblasts (EDT) and differentiating myotubes (LDT) on myotube number per field **B)** The effects of testosterone treatment in undifferentiated myoblasts (EDT) and differentiating myotubes (LDT) on myotube diameter **C)** The effects of testosterone treatment in undifferentiated myoblasts (EDT) and differentiating myotubes (LDT) on branched myotube number per field. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. n = 3.

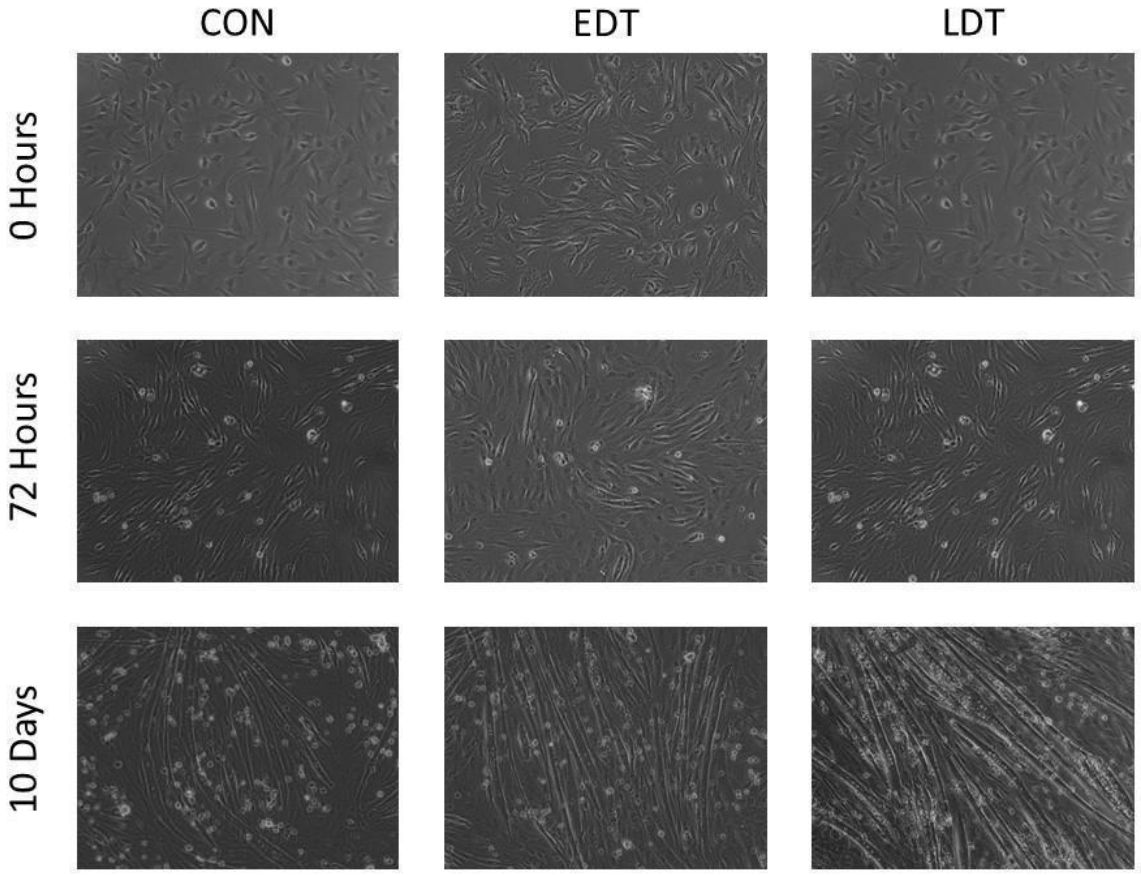

Fig 3.4. 10x objective brightfield images at 0 hours, 72 hours and 10 days differentiation of C2C12 skeletal muscle myoblasts treated with 100nM DMSO (**CON**), 100nM testosterone at 0 hours differentiation (**EDT**) and 100nM testosterone at 7 days differentiation (**LDT**).

mRNA Expression

There was no significant effect of testosterone treatment on Ar mRNA expression in differentiating myoblasts (**Fig 3.5A**). Testosterone treatment in undifferentiated myoblasts significantly increased Ar mRNA expression after 72 hours differentiation (EDT 2.36 ± 0.16 vs CON 1.92 ± 0.10; P = 0.032).

However, after 10 days of differentiation Ar expression had significantly decreased below control values (EDT 0.88 ± 0.15 vs CON 1.32 ± 0.23; *P* = 0.046) (**Fig 3.5B**).

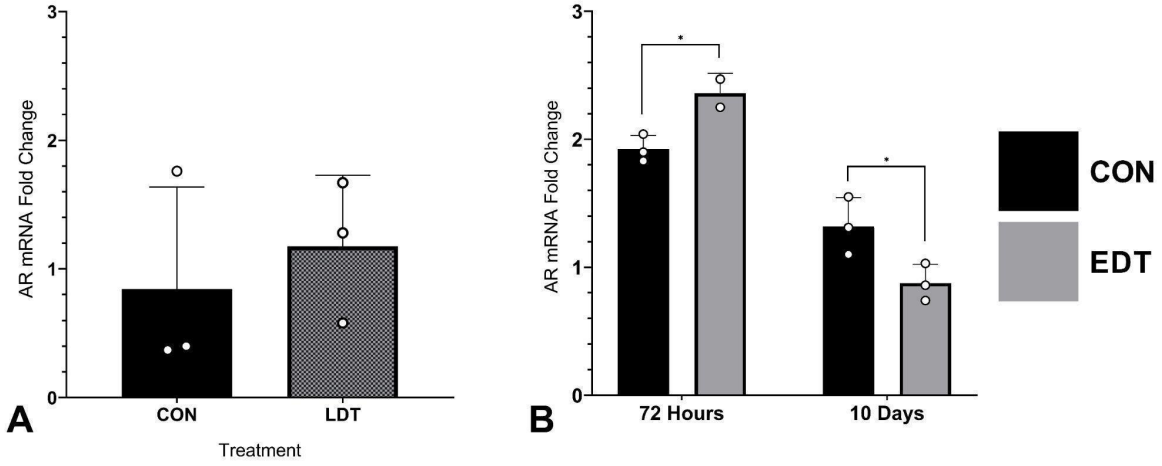

Fig 3.5. The effects of 100nM testosterone treatment on mRNA expression **A)** The effects of testosterone treatment in differentiating myotubes (LDT) on Ar expression after 72 hours 10 days differentiation. **B)** The effects of testosterone treatment in undifferentiated myoblasts (EDT) on Ar gene expression after 10 days differentiation. * $P \leq 0.05$. n = 3

There was no significant effect of testosterone administration at 7 days differentiation in already differentiated myotubes (**Fig 3.6A**). Testosterone treatment in undifferentiated myoblasts significantly decreased myostatin expression after 10 days differentiation (CON 5.8 ± 1.6 vs EDT 2.2 ± 1.5; *P* = 0.042) (**Fig 3.6B**).

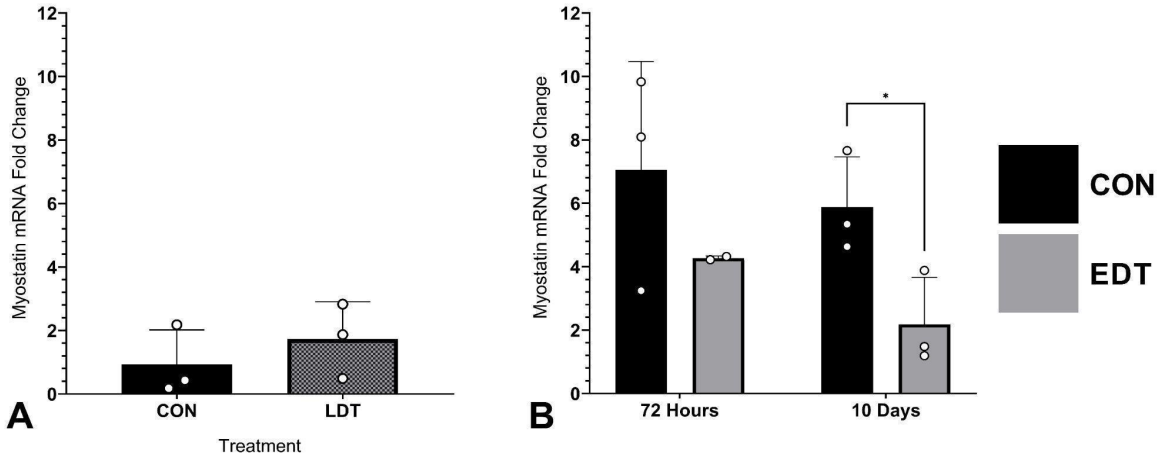

Fig 3.6. The effects of 100nM testosterone treatment on myostatin mRNA expression **A)** The effects of testosterone treatment in differentiating myotubes (LDT) on myostatin expression after 72 hours and 10 days differentiation. **B)** The effects of testosterone treatment in undifferentiated myoblasts (EDT) on myostatin gene expression after 10 days differentiation. * $P \leq 0.05$. n = 3

3.5 Discussion

Testosterone treatment in undifferentiated myoblasts increases myotube number but not diameter

Treatment with 100 nM testosterone at 0 hours differentiation significantly increased the total number of myotubes per field after 10 days differentiation compared to control treatment. This supports prior research by Hughes et al. (2016) and Deane et al. (2013) who reported significant increases in myotube number after 7 days differentiation following 100 nM testosterone administration. However, in contrast to the previously mentioned studies, testosterone treatment failed to have any effect on myotube diameter, whilst Deane et al. (2013) and Hughes et al. (2016) both observed significant increases in diameter post testosterone administration. Previous research by Basualto-Alarcon et al. (2013) has also demonstrated increases in myotube size after 5-7 days differentiation following 100 nM testosterone

administration at 0 hours differentiation. As the myotubes in this study had a further 3 days of differentiation compared to those measured in similar studies, they may have reached peak size before 10 days and started to atrophy as numerous studies have previously suggested that myotube growth peaks at 5-7 days differentiation (Gwag et al., 2013; Hsieh et al., 2020; Veliça and Bunce, 2010). Therefore, it seems likely that the myotubes measured in this study had atrophied significantly and may not be representative of testosterone's effects on myotube size.

Further work in chapter 4 will take images throughout the time course of differentiation, to determine exactly when myotubes reach peak differentiation and begin to atrophy in a 2D cell culture model. Furthermore, chapter 4 will discuss the use of alternative cell culture models, such as 3D bioengineered muscle, to prevent myotubes from atrophying after 5-7 days differentiation.

Testosterone treatment in differentiating myotubes increases myotube diameter but not number

Late testosterone treatment increased myotube diameter but not myotube number, the inverse of the effects seen following early testosterone treatment. Whilst there is a significant lack of research in treating fully differentiated myotubes with testosterone, these findings agree with prior work from Hughes (2014) demonstrating an increase in myotube diameter but not number following treatment in the later stages of differentiation with 50 nM testosterone. Testosterone may enact different effects on myotube development dependent upon the stage of differentiation that treatment is rendered. Based on these preliminary findings, it appears that testosterone treatment at the onset of differentiation causes an increase in myoblast fusion whilst testosterone treatment in already differentiated myotubes acts to further increase existing myotube size rather than encourage the formation of new myotubes. This supports the current consensus that skeletal muscle increases in size via an increase in the CSA of existing fibres rather than an increase in fibre number. If myotubes reach peak size after 7 days as

previously suggested, then it appears that testosterone treatment in differentiating myotubes works to sustain myotube growth, or at least inhibit atrophy as suggested in prior studies, resulting in significantly greater myotube diameter in testosterone treated cells (Roy et al., 2016; Serra et al., 2013; Zhao et al., 2008). However, further research would be required in order to determine the relative increase in myotube diameter between 7- and 10-days differentiation in control and testosterone treated cells.

Hughes (2014) reported that testosterone treatment maintained IGF-I expression for a further 24 hours post-treatment whilst IGF-I expression in control myotubes reduced. Prior research from Jacquemin et al. (2004) demonstrated that the addition of IGF-I induced a 20% increase in human myotube diameter when applied 72 hours after the onset of differentiation, therefore it seems likely that the testosterone induced increase in IGF-I plays a key role in increasing myotube diameter. Increases in IGF-I cause downstream signaling effects leading to an inhibition of the ligase-proteasome and autophagy-lysosome systems, which are responsible for protein degradation, as well as an increase in protein synthesis via mTOR. IGF-I induced increases in protein synthesis and inhibition of protein degradation are therefore likely to play a key role in the reported increase in myotube diameter following testosterone treatment. Hughes (2014) also reported an increase in myogenin 24 hours after testosterone treatment, which could further contribute to the increased myotube diameter observed in this study as increases in myogenin have been shown to induce hypertrophy in mature myotubes (Cretoiu et al., 2018).

Following these findings, further work should measure the percentage change in myotube diameter between 7- and 10-days differentiation, to determine if testosterone is prolonging myotube growth, or inhibiting atrophy as previous studies suggest. It is also necessary to determine at which time point myotubes reach peak size, in order to examine the effects of testosterone on myotube formation and growth before they begin to atrophy. Furthermore, whilst Hughes (2014) demonstrated that 50 nM

testosterone had no effect on myotube number in differentiating myotubes, they also reported that a larger 500 nM dose did incur an increase in myotube number. Therefore, further work should determine if this effect of larger doses persists into the later stages of differentiation examined in this study. It may be that the larger dose of testosterone administered by Hughes (2014) was successful in increasing myotube numbers due to there being a large number of myoblasts not yet committed to differentiation, which would not be present in the later stages of differentiation.

Testosterone treatment in undifferentiated myoblasts and differentiating myotubes increases myotube branching

To the authors knowledge, this study is the first to measure the effects of testosterone treatment on the branching of differentiated myotubes *in vitro*. Testosterone treatment in both undifferentiated myoblasts and differentiating myotubes increased the number of branched myotubes, although whether this branching represents an increase in hypertrophy is yet to be determined. Prior research has proposed that *in vitro* myotube branching occurs when the end of one myotube contacts the lateral surface of another (Fear, 1977). Therefore, the observed increase in branched myotubes may simply be a result of the increased myotube number and diameter following testosterone treatment increasing the probability of myotubes making contact and fusing with each other. Previous research has demonstrated splitting in pathological human muscle and muscle fibers in response to exercise, injury, and other stimuli (Bray & Banker, 1970; Schmalbruch, 1976). However, whether the branching myotubes observed in this study split or not cannot be determined from these results alone, although splitting seems unlikely without the application of an additional stimulus such as exercise.

Testosterones effect on myotube branching will be further explored using coordinates-based imaging to monitor the activity of branched myotubes over the entire period of differentiation in chapter 4. Future

work should focus on determining the cause of myotube branching *in vitro* and if it actually occurs *in vivo,* as if branching does not occur *in vivo* then there is little use in further investigating the effects on branching in 2D cell culture models, especially when structured 2D and 3D bioengineered models, the use of which is becoming more common in cell culture research, do not cause myotube branching.

Testosterone treatment in undifferentiated myoblasts causes initial peaks in Ar mRNA expression but then reduces Ar mRNA expression below control levels following differentiation

After 10 days of differentiation, Ar expression was significantly reduced in the cells treated with testosterone as undifferentiated myoblasts compared to control cells. This is contrary to prior research by Hughes (2014) and Hughes et al. (2016) demonstrating no effect of testosterone treatment on Ar expression after 7 days differentiation. As previously mentioned, the myotubes in this study appear to have reached peak size and begun to atrophy by 10 days which may explain some variance between the two studies. This will be investigated further in chapter 4 by collecting samples at regular time points throughout differentiation. Testosterone administration may also downregulate Ar expression in the later stages of differentiation to prevent excessive inappropriate hypertrophy. Alternatively, as testosterone treatment increased Ar expression initially at the 72-hour time point, it may be that the effects of the single dose of testosterone had worn out by 10 days. This may have caused Ar expression to decrease due to a negative feedback loop, as there would no longer be a need for an increased Ar content to facilitate the binding of the increased testosterone levels. This will also be further explored in chapter 4 using a repeat dose protocol.

The majority of research shows Ar associated increases in hypertrophy following testosterone administration; therefore, it is unlikely a permanent reduction in AR expression is occurring as a result of testosterone treatment (Basualto-Alarcon et al., 2013; Wu and Gore, 2010; Yin et al., 2020). Recent work

by Morton et al. (2018) demonstrated a linear relationship between Ar content and increases in lean body mass following resistance training, whilst levels of intramuscular and circulating testosterone had no effect on changes in lean body mass. This suggests that androgen receptor saturation may be the limiting factor in skeletal muscle hypertrophy, rather than testosterone abundance, therefore increases in AR content induced by testosterone administration may significantly increase the potential for skeletal muscle hypertrophy. Whilst prior studies have demonstrated an increase in skeletal muscle AR expression following testosterone treatment, the majority of these studies were conducted in hypogonadal males or in castrated animals (Ghanim et al., 2018; Michel and Baulieu, 1980). Hughes (2016) reported that testosterone treatment increased AR expression back to normal levels in population doubled cells, therefore it may be that testosterone is capable of rescuing reduced Ar expression caused by age or castration associated reductions in serum testosterone, however it is unable to permanently increase Ar content beyond normal physiological levels. This is further supported by Ferrando et al. (2002) who reported that testosterone treatment increased Ar expression in elderly patients with low levels of serum testosterone within the first month of treatment, however after six months of treatment AR expression had returned to normal levels.

Further studies will expand on the effects of testosterone treatment in aged muscle, as chapter 4 will explore the effects of testosterone administration on replicatively aged skeletal muscle cells. Future work should also investigate the long-term effects of testosterone treatment on AR content *in vivo*, as if testosterone is capable of permanently increasing AR content then this may constitute a further mechanism by which testosterone increases skeletal muscle hypertrophy. Whilst prior studies suggest that testosterone treatment can only increase AR content back to normal levels in hypogonadal patients, it may be that larger doses such as those utilised by bodybuilders are required to increase AR content beyond normal physiological levels. Therefore, further studies could also determine if AR content

increases in a dose dependent manner following testosterone treatment in hypogonadal and healthy subjects. Furthermore, if AR content is being increased to meet the demands of testosterone binding as previously suggested, then potentially greater doses would need to be administered with time in order to continually increase the demand for AR content.

Testosterone treatment reduces myostatin expression in undifferentiated myoblasts but not in differentiating myotubes

Testosterone treatment in undifferentiated myoblasts caused a non-significant reduction in myostatin mRNA expression after 72 hours differentiation, which may have played a role in the reported increase in myotube number, as myostatin has been shown to inhibit myoblast activation, proliferation and differentiation via inhibiting activation of the Akt/mammalian target of rapamycin (mTOR)/p70S6 protein synthesis pathway (Joulia, 2003; McFarlane et al., 2008; Morissette et al., 2009; Rodriguez et al., 2014). Testosterone treatment also significantly reduced myostatin mRNA expression after 10 days, therefore it is surprising that there was no corresponding increase in myotube diameter as prior studies have demonstrated an increase in myotube size following myostatin inhibition, as a result of preventing myostatins blockade of genes induced during differentiation (Trendelenburg et al., 2009). Testosterone administration may induce a decrease in AR expression in the later stages of differentiation to prevent too great of a hypertrophic effect, whilst simultaneously reducing myostatin expression to prevent muscle loss. Similar research by Hughes (2014) found that 50 nM testosterone had no significant effect on myostatin expression after 72 hours differentiation, but significantly reduced expression after 7 days differentiation. Conversely, Deane et al. (2013) reported that testosterone treatment had no effect on myostatin expression after 72 hours or 7 days differentiation, suggesting that testosterone induced hypertrophy is independent of myostatin. Alternatively, if testosterone administration accelerated early

myoblast fusion then reductions in myostatin expression may have occurred within the first 48 hours of differentiation, with myostatin expression returning to control levels by 72 hours differentiation to prevent excessive hypertrophy, as previously suggested by Dubois et al. (2014) and Lakshman et al. (2009). Surprisingly, whilst Hughes (2014) used a smaller dose of 50 nM testosterone, Deane used the same 100 nM dose of testosterone as this study, therefore dosage is unlikely to be responsible for the differing results. Later research by Hughes et al. (2016) reported that 100 nM testosterone administration had no significant effect on myostatin expression after 72 hours or 7 days, therefore further research is clearly necessary to determine the effects of testosterone administration on myostatin expression *in vitro*.

Unlike in undifferentiated myoblasts, treatment in differentiating myotubes had no effect on myostatin mRNA expression, this is in agreement with limited similar research, as Hughes (2014) reported no significant effect of 50 nM or 500 nM testosterone treatment on myostatin expression in differentiated myotubes. Langley et al. (2002) demonstrated that myostatin inhibits myoblast differentiation via a downregulation of MyoD expression, causing myoblasts to fail to differentiate into myotubes, therefore, testosterone treatment may only significantly affect myostatin expression in undifferentiated myoblasts. This is further supported by the reported decreases in myostatin expression in undifferentiated myoblasts but not in differentiating myotubes. Whilst this study provides preliminary data for the effects of testosterone administration timing on gene expression, it is important to acknowledge that due to the small sample size used and the high degree of variability within the datasets, the reliability of these findings is questionable and requires further research.

Future research should first prioritise determining how the effects of testosterone on myostatin expression differ at different administration timepoints throughout differentiation, as the current

literature appears undecided. Further work could then also determine how these changes in myostatin expression affect myotube number and size, and the mechanisms by which this occurs, as whilst some studies report that an inhibition of myostatin increases myotube diameter, results from this study suggest that this did not occur.

Testosterone treatment in undifferentiated myotubes and differentiating myoblasts increases cellular metabolic activity

After 10 days of differentiation, testosterone treatment in the undifferentiated myoblasts and differentiating myotubes had significantly increased cellular metabolic activity, suggesting a potential increase in cellular viability. Recent research has demonstrated the potential of testosterone to influence mitochondrial health, specifically in skeletal muscle tissue, which is likely to be reflected in cellular viability. Gorgey et al. (2020) reported that testosterone treatment improved mitochondrial health in skeletal muscle post spinal cord injury, in the form of an increase in mitochondrial citrate synthase, a marker of mitochondrial density. Pronsato et al. (2020) demonstrated similar findings, with physiological doses of testosterone protecting C2C12 cells from H_2O_2 induced apoptosis. These effects were attenuated following treatment with the AR inhibitor flutamide, suggesting that testosterone acts via the androgen receptor to up-regulate mitochondrial gene expression. Prior studies have also demonstrated negative effects of testosterone deprivation on mitochondrial health and shown similar effects of testosterone therapy in improving mitochondrial function, albeit not in skeletal muscle tissue (Pitteloud et al. 2005; Pongkan et al. 2015). For example, hypogonadal participants show impaired mitochondrial function compared to those with normal testosterone levels (Pitteloud et al. 2005) and orchidectomized rats develop cardiac mitochondrial dysfunction, which could be attenuated via testosterone treatment (Pongkan et al. 2015).

Conversely, prior research has reported testosterone induced apoptosis in some tissues, although not currently in healthy skeletal muscle. Lopes et al. (2014) demonstrated that 1000 nM testosterone treatment in rat vascular smooth muscle cells reduced mitochondrial respiration and induced apoptosis. This echoes previous research by Kanda et al. (1996) who found that cell viability in monocytes was reduced following treatment with 1000 nM testosterone. However, Kanda also demonstrated that smaller doses of testosterone, such as the 100 nM dose used in this study, had no significant effect on cell viability. Similarly, Sinha-Hikim et al. (2007) reported that testosterone therapy had no effect on muscle cell death in cardiotoxin injured mice. Other research showing testosterone induced apoptosis were conducted in neuronal (Estrada et al., 2006), gastric (Amani et al., 2019) and granulosa cells (Zhao, 2013). Therefore, it appears that the effects of testosterone on mitochondrial function may be tissue as in some tissue testosterone reportedly induces apoptosis, whereas in skeletal muscle tissue it appears to perform the opposite effect and protect and upregulate mitochondrial gene expression. These effects are also likely dose dependent, and further work is clearly necessary to determine the specific doses in different tissues that testosterone administration becomes damaging.

As far as the author is aware, this study is the first to directly compare the effects of testosterone treatment on cellular metabolic activity in myoblasts vs differentiated myotubes. The MTT assay results suggest that there is no difference in effect on cell activity between treating myotubes at 0 hours and 7 days differentiation. Prior research using different drug treatments has demonstrated dose-timing dependent effects on myotubes, due to changes in the associated receptors localisation as myoblasts differentiate into myotubes (Han et al., 2007; Montenegro et al., 2019). The lack of difference in cell viability between testosterone treatments could therefore be unexpected as studies have demonstrated differences in AR localisation between myoblasts and myotubes (Wannenes et al., 2008). Pronsato et al., (2012; 2020) suggest an alternative explanation, as prior research by their team demonstrated the

potential existence of non-classically localised ARs within the mitochondria in myoblasts. Therefore, testosterone may exert non-genomic actions via these ARs to influence mitochondrial dynamics in myoblasts and myotubes. However, research has also provided evidence that the effects of testosterone on proteins associated with mitochondrial dynamics may act in an AR-independent manner. Wang et al., (2015) treated C2C12s with testosterone in the presence of flutamide, an antiandrogen, and discovered that testosterone still exerted the same effects on mitochondrial dynamics, suggesting that testosterone acted via an AR-independent mechanism.

Whilst these results suggest that testosterone administration likely increases skeletal muscle cell viability, an MTT assay alone is inadequate to confirm these findings, as the assay only measures cellular metabolic activity, which has been shown to differ depending upon the stage of differentiation that cells are in, and not the number of viable cells. Furthermore, whilst the MTT assay is designed to measure cellular activity as the conversion of MTT to formazan within the mitochondria, prior work has suggested that this reaction may also occur outside of the mitochondria, reducing the strength of this assay in determining mitochondrial activity (Bernas and Dobrucki, 2002; Berridge and Tan, 1993). Future work should utilise a more robust array of cellular viability assays, performed at different points throughout the time course of differentiation. Foremost, the use of a propidium iodide (PI) assay would be highly beneficial, as the PI assay would allow for the quantification of dead or damaged cells within the samples. As prior studies have demonstrated a dose response effect of testosterone inducing apoptosis in different tissues, further work should also determine the effects of differing doses in skeletal muscle tissue (Kanda et al., 1996).

Chapter Summary

This chapter has demonstrated morphological and transcriptional differences in how C2C12 skeletal muscle cells respond to testosterone treatment, dependent on the stage of differentiation that testosterone is administered. These findings extend prior work from Hughes et al. (2016) by demonstrating the effects of testosterone administration in fully differentiated myotubes. Combined with Hughes et al.'s (2016) previous research documenting the effects of testosterone administration in undifferentiated myoblasts and myotubes in the early stages of differentiation, this work provides a more complete picture of the morphological effects of testosterone administration at each stage of skeletal muscle cell differentiation. As previously reported by Hughes et al. (2016) and supported by this chapter, testosterone administration at the onset of differentiation appears to increase myoblast activation and fusion, resulting in an increased number of myotubes, likely as a result of inhibiting myostatin expression. This chapter furthers Hughes et al.'s (2016) work by demonstrating that testosterone administration near the peak of differentiation appears to either delay myotube atrophy or induce further increases in myotube hypertrophy, whilst having little to no effect on androgen receptor or myostatin expression. This chapter also provides preliminary evidence for testosterone treatment increasing cellular metabolic activity as well as demonstrating the effectiveness of the testosterone treatments planned to be used in further experiments.

Chapter 4

The effects of repeated testosterone administration in young and aged C2C12 skeletal muscle cells

4.1 Abstract

Aim: This study aimed to determine the effects of repeated testosterone administration in young skeletal muscle myoblasts and myoblasts having undergone multiple population doublings to replicate the effects of aging in satellite cells.

Method: C2C12 myoblasts, passage 15 and 30, were induced to differentiate in the presence of a single 100 nM dose of testosterone, or repeated doses of 100 nM testosterone every 48 hours. At each timepoint (72 hours, 5 days, 7 days and 10 days differentiation) cells were live imaged for morphological measures (myotube number, diameter and branched myotube number) and mRNA was collected for gene expression analysis by PCR (AR, MyoG and MyoD mRNA expression). Myonuclear accretion (nuclear fusion index and the average number of nuclei per myotube) was assessed after 7 days of differentiation using immunocytochemistry. Cellular metabolic activity was measured after 10 days of differentiation using an MTT assay.

Results: Repeated testosterone treatment significantly increased cellular metabolic activity in young cells ($P = 0.014$). Similarly, testosterone administration significantly increased myotube number ($P < 0.001$) and branched myotube number ($P = 0.023$) in young cells. Repeated testosterone treatment appeared to accelerate differentiation in young cells, by inducing greater increases in myotube diameter between 72 hours and 5 days ($P = 0.001$), but then atrophying at a greater rate between 7- and 10-days differentiation. Testosterone administration had no effect on any morphological measures in population doubled cells. There was no significant effect of testosterone treatment on mRNA expression in the young or population doubled cells.

Conclusions: These findings suggest that testosterone treatment is capable of increasing cellular metabolic activity, and potentially cellular viability, as well as accelerating differentiation, with repeated doses of testosterone incurring greater effects. This study also builds on prior research demonstrating that whilst testosterone may be able to rescue the effects of replicative aging in skeletal muscle cells there is a limit to the number of population doublings myoblasts can undergo before becoming irreparable and immune to the effects of testosterone administration.

4.2 Introduction

Ageing is associated with a systemic reduction in muscular strength and mass termed sarcopenia. Sarcopenia affects a significant proportion of the elderly population, with 14% of older adults aged between 65-70 years and 53% of adults aged 80 and above currently suffering from it (Baumgartner et al., 1999; Morley, 2012). Sarcopenia severely affects quality of life, with reductions in muscle function preventing many from completing simple daily tasks leading to a reduction in overall physical activity and significant reductions in mental health (Landi et al., 2012). Sarcopenia is caused by a reduction in protein turnover, induced by a multitude of lifestyle mediated-factors such as changes in dietary protein and calorie intake and reduced physical activity, alongside hormonal changes associated with aging (Berger et al., 2005; Chen et al., 2016; Sullivan et al., 1999). This progressive reduction in physical activity and nutrient intake often leads to a further decrease in muscular function, preventing individuals from undertaking physical activity and dietary interventions which would ultimately improve their condition. Beyond the implications for quality of life, sarcopenia imposes a significant financial burden on healthcare systems. Recent estimates suggest that sarcopenia costs the NHS an annual excess of £2.5 billion per year (Pinedo-Villanueva et al., 2019). Furthermore, research conducted in Portuguese hospitals found that sarcopenia increased hospitalisation costs by 58.5% for patients aged under 65 and 34% for patients aged 65 and over (Sousa et al., 2016). Sarcopenia also increased hospital stay times in patients treated for unrelated conditions when compared to their non-sarcopenic counterparts.

One of the most significant hormonal changes associated with aging is a reduction in testosterone levels. Prior longitudinal research has demonstrated that serum testosterone levels decrease at a rate of approximately 1% per year after the age of 30 (Wang et al., 2008). As a result, patients suffering from sarcopenia have previously been treated with regular testosterone administration, due to testosterones

anabolic effect in skeletal muscle tissue increasing muscular size and strength (Malafarina et al., 2012). Numerous studies have demonstrated increases in skeletal muscle size and function in hypogonadal elderly patients treated with testosterone protocols (Sih et al., 1997; Katznelson et al., 1996). Research conducted by Sih et al. (1997) administered testosterone cypionate biweekly for 12 months, resulting in an increase in bilateral grip strength in hypogonadal, elderly men. More recently, Shankar et al. (2010) demonstrated that transdermal gel, delivering 50mg per day of testosterone, increased lower body skeletal muscle strength and lean body mass over the course of a six-month period. Previous research has also demonstrated observed increases in fat free mass and stair-climb power following a testosterone treatment protocol (Kenny et al., 2010; Storer et al., 2017; Travison et al., 2011). Therefore, there is a clear application for testosterone therapy in the treatment of sarcopenia in an aging population.

Appropriate pre-clinical models are required to define mechanistic insights of testosterone therapy in aged muscle. Elderly skeletal muscle satellite cells possess a reduced differentiation capacity, with decreased Mrf5, MyoD and myogenin expression following multiple proliferative divisions (Bigot et al., 2008). Previous work by Sharples et al. (2011) demonstrated an impairment in skeletal muscle cell differentiation following multiple population doublings of skeletal muscle myoblasts *in vitro*. Using this 'aged' cell model, research by Deane et al. (2013) and Hughes et al. (2016) reported that testosterone was able to rescue some of the effects of aging in population doubled C2C12 myoblasts, with testosterone significantly increasing myotube diameter and myonuclear accretion, as well as increasing MyoG mRNA expression. Whilst prior studies have investigated the effects of single dose testosterone treatments in young and 'aged' C2C12 myoblasts, to the authors knowledge no studies have investigated

the effects of repeated doses of testosterone in young and population doubled cells as would typically be observed following testosterone therapy *in vivo* (Deane et al., 2014; Hughes et al., 2016).

This study aimed to compare the effects of testosterone administration in young and population doubled myoblasts throughout the time course of C2C12 differentiation and build on prior research by Deane et al. (2013) and Hughes et al. (2016) by using repeated exposures to testosterone and analysing effects at regular intervals throughout the course of differentiation. Morphological (myotube number, diameter and myonuclear accretion) and transcriptional (AR, MyoG and MyoD mRNA expression) data were collected along with preliminary cellular metabolic activity data in the form of an MTT assay. Based on the findings from chapter 3 and prior work by Hughes (2014), it was hypothesised that repeated doses of testosterone would incur greater increases in myotube number, diameter and cellular metabolic activity compared to a single dose, as well as greater transcriptional changes in the form of increased AR and MyoG expression in the young cells. Testosterone treatment was also hypothesised to rescue the effects of population doubling on myotube size and myonuclear accretion.

4.3 Methodology

Experimental Design

Young cells (approximately passage 15) and replicatively aged cells (approximately passage 30) were cultured and induced to differentiate in the presence of a single 100 nM dose of DMSO (referred to as CON cells), a single 100 nM dose of testosterone (referred to as TCON cells) or repeated doses of testosterone (referred to as TEXP cells). Morphological measures (myotube number, diameter and branched myotube number) and mRNA expression (AR, MyoG and MyoD expression) were analysed after 72 hours, 5 days, 7 days and 10 days differentiation. Measures of myonuclear accretion (the nucleic

fusion index and average number of nuclei per myotube) were taken after 7 days of differentiation. Cells were also subjected to an MTT assay after 10 days differentiation to measure cellular metabolic activity.

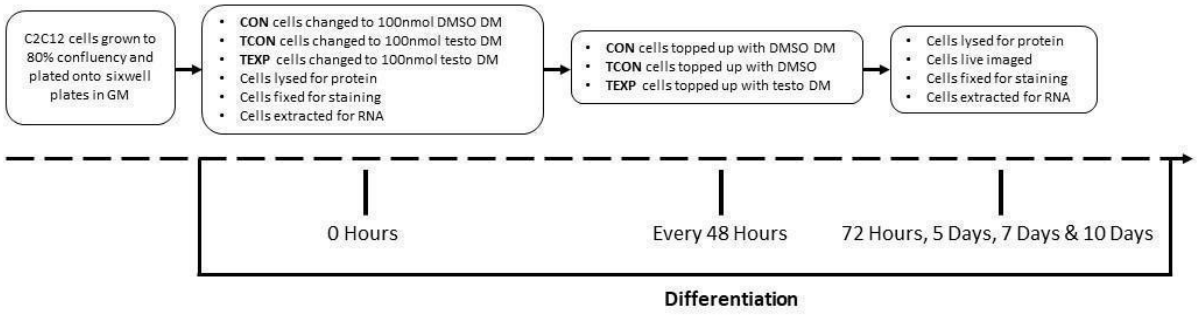

Fig 4.1. Schematic representation of the experimental design used to examine the effects of repeated testosterone administration in young and replicatively aged skeletal muscle myoblasts.

Cell Culture

C2C12 skeletal muscle myoblasts were seeded onto 6-well plates as described in section 2.4. Two different 'ages' of cells were used for this study. Young cells were approximately passage 15 and replicatively aged cells were approximately passage 30. Replicatively aged cells were acquired from a previous study, therefore the exact number of population doublings the cells had undergone was unknown. The methodology for the population doubling of cells is described in section 2.4.2.

Cell Treatment

All cell treatments were prepared as outlined in section 2.5. At 0 hours differentiation CON cells were changed from growth media to differentiation media treated with DMSO, and TCON and TEXP cells were changed to differentiation media treated with 100nM testosterone. Every 48 hours CON and TCON cells were topped up with regular differentiation media, TEXP cells were topped up with testosterone treated differentiation media.

Live Imaging

At each timepoint (72 hours, 5 days, 7 days, 10 days), myotubes were live imaged under a microscope at 10x objective as described in section 2.7.1. Myotube number, diameter and branched myotube number were measured as outlined in sections 2.7.2-3.

Immunocytochemistry

After 7 days of differentiation, cells were fixed and immunostained for MF20, a marker of myosin heavy chain only expressed in differentiating myoblasts and myotubes. Nuclei were counterstained with DAPI as described in sections 2.8.2-3. Immunostained samples were then imaged and the nucleic fusion index and average number of nuclei per myotube were measured as described in sections 2.8.4-6.

Polymerase Chain Reaction (PCR)

After 72 hours, 5 days, 7 days and 10 days differentiation, myotubes were lysed, isolated for RNA and diluted as described in sections 2.9.2-5. PCR was then performed as detailed in section 2.9.6. The relative mRNA expression of AR and myostatin was quantified using the $\Delta\Delta$Ct method as described in section 2.9.7 against the reference gene POL2R and a calibrator sample of control treatment after 0 hours differentiation. Primer sequences for genes of interest are shown in section 2.9.8.

MTT Assay

After 10 days differentiation, differentiation media was aspirated and replaced with a tetrazolium dye solution as described in section as described in sections 2.6.2-3. Cellular metabolic activity was then measured using a plate reader and MTT assay as described in section 2.6.3.

Statistical Analysis

All data are expressed as means ± SD. Statistical significance was set at the 0.05 level of confidence. For cellular metabolic activity and myonuclear accretion, treatment dependent effects were determined using a one-way between subjects ANOVA. Significant main effects were assessed using Bonferroni adjustment *post hoc* analysis. For morphological measures and mRNA expression, time and treatment dependent effects were determined using a two-way mixed design ANOVA, with the factors 'time' and 'treatment'. Significant main effects or interactions were assessed using Bonferroni adjustment *post hoc* analysis.

4.4 Results

Cellular Metabolic Activity

After 10 days of differentiation, repeat testosterone treatment in young cells significantly increased cellular metabolic activity compared to control (TEXP 166.4% vs CON 100%; *P* = 0.002) and single dose treatments (TEXP 166.4% vs TCON 120.4%; *P* = 0.014) (**Fig 4.2A**). There was no significant effect of testosterone treatment on cellular metabolic activity in the multiple population doubled (MPD) cells (*P* = N.S) (**Fig 4.2B**).

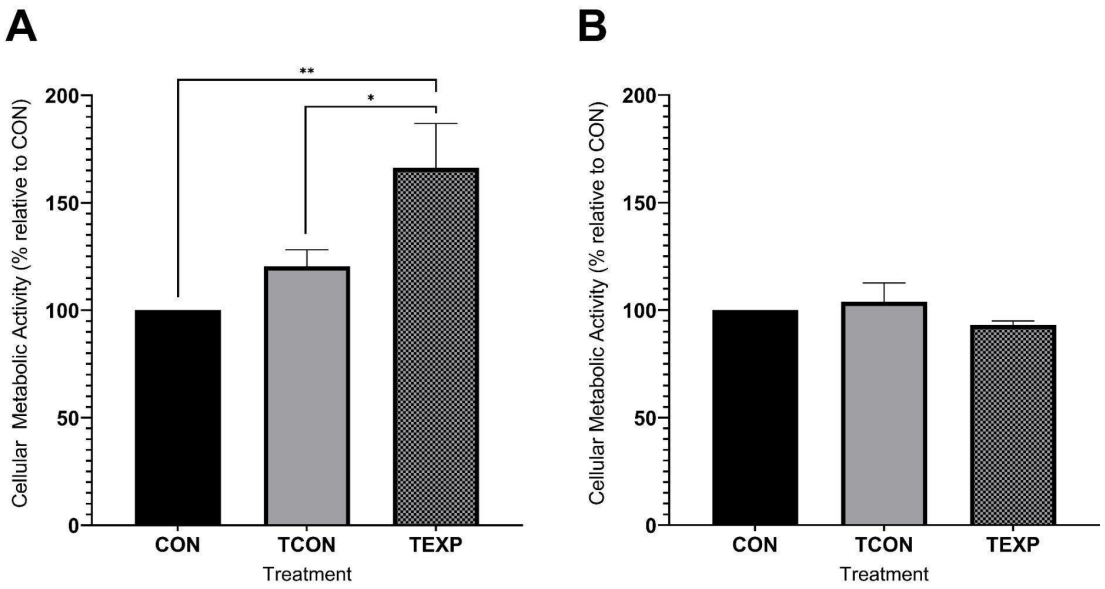

Fig 4.2. The effects of single and repeat 100 nM testosterone treatment on cellular metabolic activity after 10 days differentiation in **A)** young cells and **B)** replicatively aged cells. n = 3. * $P \leq 0.05$, ** $P \leq 0.01$. n = 3.

Myotube Morphology

The effects of testosterone treatment on myotube number

Myotube number increased significantly between each timepoint in the young cells ($F_{3,45}$ = 158.29, P < 0.001), apart from between 72 hours and 5 days (P = 0.065) (72h 44.7 ± 5.9 myotubes vs 5 days 50.5 ± 7.5 myotubes vs 7 days 61.7 ± 6.2 myotubes vs 10 days 80.2 ± 8.7 myotubes) (**Fig 4.3A**). There was no significant main effect of testosterone treatment on myotube number in the young cells, however there was a significant interaction between time and treatment, where after 10 days myotube number was significantly greater in both testosterone treated conditions compared to control treated cells ($F_{6,45}$ = 6.9, P < 0.001) (TCON 83 ± 3 myotubes vs CON 70 ± 3 myotubes; P = 0.001) (TEXP 87 ± 7 myotubes vs CON 70 3 ± 3 myotubes; P < 0.001) (**Fig 4.3A**).

There was a significant main effect for time on percentage change in myotube number in the young cells ($F_{2,30}$ = 11.55, P < 0.001) where the change in myotube number increased significantly between 72 hours and 10 days (72 hours 5.8 ± 8.5% vs 10 days 18.6 ± 7.8%) (P = 0.001) (**Fig 4.3C**). Repeat testosterone treatment resulted in a significantly greater percentage increase between timepoints compared to control treatments ($F_{2,15}$ = 187.56, P = 0.002) (TEXP 44.8 ± 6.9% vs CON 25.5 ± 6.5%) (**Fig 4.3C**). There was also a significant interaction between time and testosterone treatment ($F_{4,30}$ = 3.302, P = 0.023) where between 5 and 7 days TEXP cells displayed a significantly greater percentage increase compared to CON (TEXP 42 ± 23% vs CON 8 ± 9%; P = 0.004) (**Fig 4.3C**). TCON and TEXP cells also displayed a significantly

greater percentage increase compared to CON between 7 and 10 days (TCON 35 ± 5% vs CON 21 ± 16%; $P = 0.025$) (TEXP 36 ± 12% vs CON 21 ± 16%; $P = 0.014$) (**Fig 4.3C**).

Neither testosterone treatment had a significant effect on myotube number or the percentage increase in myotube number between timepoints in the MPD cells (P = N.S) (**Fig 4.3B, 4.3D**). There was a significant main effect for time on myotube number in the MPD cells ($F_{3,27} = 245.175$, $P < 0.001$), with a significant increase in myotube number per field between 72 hours and 5 days (72 hours 32 ± 1.9 myotubes vs 5 days 105 ± 2.9 myotubes) ($P < 0.001$) and a significant decrease between 7 days and 10 days (7 days 116 ± 4.3 myotubes vs 10 days 45 ± 3.4 myotubes) ($P < 0.001$) (**Fig 4.3B**).

There was also a significant main effect for time on percentage change in myotube number in the MPD cells ($F_{2,18} = 371.5$, $P < 0.001$) where the percentage increase in myotube number significantly decreased from each previous time point (5 days 73.42 ± 9.8% vs 7 days 10.75 ± 14.4% vs 10 days -70 ± 14.25%; $P < 0.001$) (**Fig 4.3D**).

There was a significant main effect for time on branched myotube number in young cells ($F_{3,45} = 32.64$, $P < 0.001$) where branched myotube number increased significantly between 72h and 5 days (72h 1 ± 0.8 branched myotubes vs 5 days 6 ± 2 branched myotubes) ($P < 0.001$) (**Fig 4.3E**). Repeat dose testosterone treatment significantly increased the number of branched myotubes in the young cells compared to the control treatment (TEXP 6 ± 0.6 branched myotubes vs CON 4 ± 0.6 branched myotubes) ($P = 0.023$) (**Fig 4.3E**). There was also a significant interaction between time and treatment, ($F_{6,45} = 2.35$, $P = 0.047$), where TEXP treated cells increased in branched myotube number at each timepoint, whereas CON and TCON treated cells peaked at 5 and 7 days respectively and decreased between 7 and 10 days (**Fig 4.3E**).

There was a significant main effect for time on branched myotube number in the MPD cells ($F1_{.79,16.1} = 5.93$, $P = 0.014$) where the number of branched myotubes increased between 72 hours and 7 days, but

then reduced to 0 after 10 days (72h 0 ± 0 branched myotubes vs 7 days 1 ± 0.3 branched myotubes vs 10 days 0 ± 0 branched myotubes) (**Fig 4.3F**). Neither testosterone treatment had a significant effect on branched myotube number in the MPD cells (*P* = N.S) (**Fig 4.3F**).

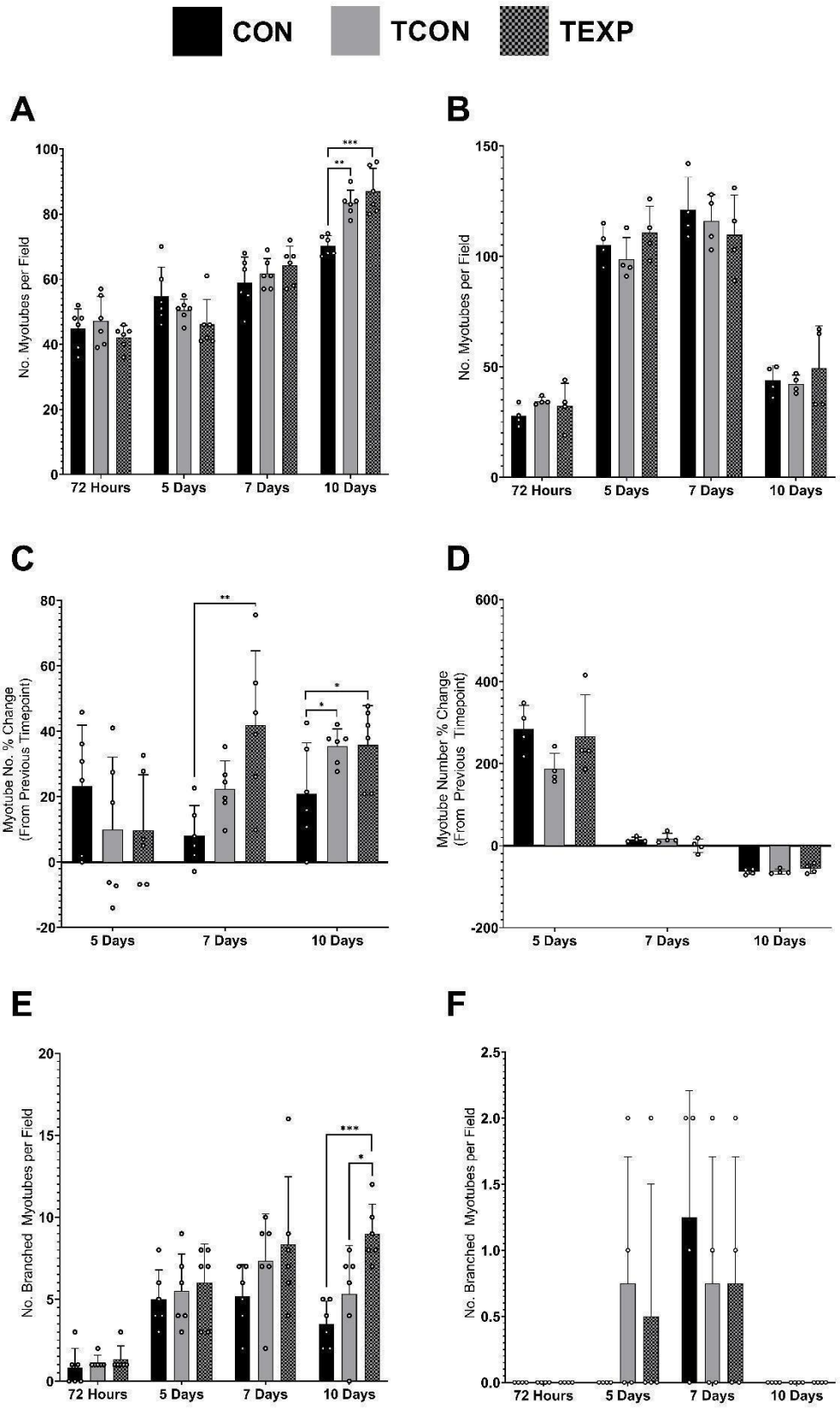
CON
TCON
TEXP
A
No. Myotubes per Field
72 Hours
5 Days
7 Days
10 Days
B
No. Myotubes per Field
C
Myotube No. % Change (From Previous Timepoint)
D
Myotube Number % Change (From Previous Timepoint)
E
No. Branched Myotubes per Field
F
No. Branched Myotubes per Field

Fig 4.3. The effects of single and repeat 100 nM testosterone treatment on **A)** Myotube number in young cells **B)** Myotube number in MPD cells **C)** the percentage increase in myotube number between timepoints in young cells **D)** the percentage increase in myotube number between timepoints in MPD cells **E)** branched myotube number in young cells **D)** branched myotube number in MPD cells. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. n = 3.

The effects of testosterone treatment on myotube diameter

There was a significant main effect for time on myotube diameter in young cells ($F_{3,45} = 186$, $P < 0.001$), with a significant increase in myotube diameter between 72 hours and 7 days followed by a significant decrease in diameter between 7 and 10 days (72 hours 3.6 ± 0.2µm vs 7 days 5.2 ± 0.3µm vs 10 days 4.7 ± 0.3µm) ($P < 0.001$) (**Fig 4.4A, Fig 4.5**). Testosterone treatment had no significant main effect on myotube diameter in the young cells, however there was a significant interaction between time and treatment where after 72h TCON and TEXP cells showed a greater myotube diameter compared to CON cells (CON 3.4 ± 0.2µm vs TCON 3.7 ± 0.1µm vs TEXP 3.7 ± 0.3µm; $P = 0.006$ for TCON vs CON, $P = 0.004$ for TEXP vs CON) (**Fig 4.4A**). After 5 days TCON and TEXP cells maintained a greater myotube diameter compared to CON (CON 4.5 ± 0.3µm vs TCON 5.1 ± 0.3µm vs TEXP 5.2 ± 0.4µm; P=0.011 for TCON vs CON, $P < 0.001$ for TEXP vs CON) (**Fig 4.4A, Fig 4.5**). At 5 days TEXP cells also showed a significantly greater myotube diameter compared to TCON cells (TEXP 5.2 ± 0.4µm vs TCON 5.1 ± 0.3µm; $P = 0.047$) (**Fig 4.4A**). After 10 days CON cells were significantly greater in myotube diameter compared to TEXP cells (CON 4.9 ± 0.4µm vs TEXP 4.6 ± 0.3µm; $P = 0.025$)(**Fig 4.4A, Fig 4.5**).

There was a significant main effect for time on percentage change in myotube diameter in young cells ($F_{2,30} = 132.5$, $P < 0.001$) where the percentage increase in diameter decreased significantly between each time point until cells were actually reducing in myotube diameter (5 days 1.3 ± 0.06% vs 7 days 0.3

± 0.06% vs 10 days -0.5 ± 0.08%) (P < 0.001) (**Fig 4.4C**). Repeat testosterone treatment resulted in a significantly greater percentage change in myotube diameter between timepoints compared to control treated cells ($F_{2,15}$ = 5.47, P = 0.016) (TEXP 14.9 ± 1% vs CON 8.5 ± 1%) (P = 0.018) (**Fig 4.4C**). There was also a significant interaction between time and treatment ($F_{4,30}$ = 5.85, P = 0.001) where despite TEXP cells showing a significantly greater increase in myotube diameter between 72 hours and 5 days compared to CON (TEXP 32.8 ± 5.8% vs CON 11.1 ± 30.9%) (P = 0.001), after 7 days myotube diameter in TEXP cells decreased at a rate significantly greater than that of CON cells (TEXP -15.1 ± 6.8% vs CON -5.1 ± 4.6%) (P = 0.021) (**Fig 4.4C**).

Single dose testosterone treatment resulted in a significantly greater myotube diameter compared to repeat dose treated cells in the MPD cells ($F_{2,9}$ = 4.4, P = 0.048) (TCON 3.4 ± 0.5µm vs TEXP 3.2 ± 0.5µm) (**Fig 4.4B, Fig 4.6**). There was a significant main effect of time on myotube diameter in the MPD cells ($F_{2.23,20.11}$ = 100.9, P < 0.001) where myotube diameter significantly decreased between 72 hours and 10 days differentiation (72 hours 3.7 ± 0.7 µm vs 10 days 2.6 ± 0.5 µm) (**Fig 4.4B, Fig 4.6**).

There was no significant effect of testosterone treatment on the percentage change in myotube diameter (P = N.S) (**Fig 4.4D**). Although, there was a significant main effect for time on percentage change in myotube diameter in the MPD cells ($F_{2,18}$ = 25.33, P < 0.001) where the percentage decrease in myotube diameter increased between 5 and 10 days (5 days -0.1 ± 0.9% vs 10 days -0.9 ± 0.7%) (**Fig 4.4D**).

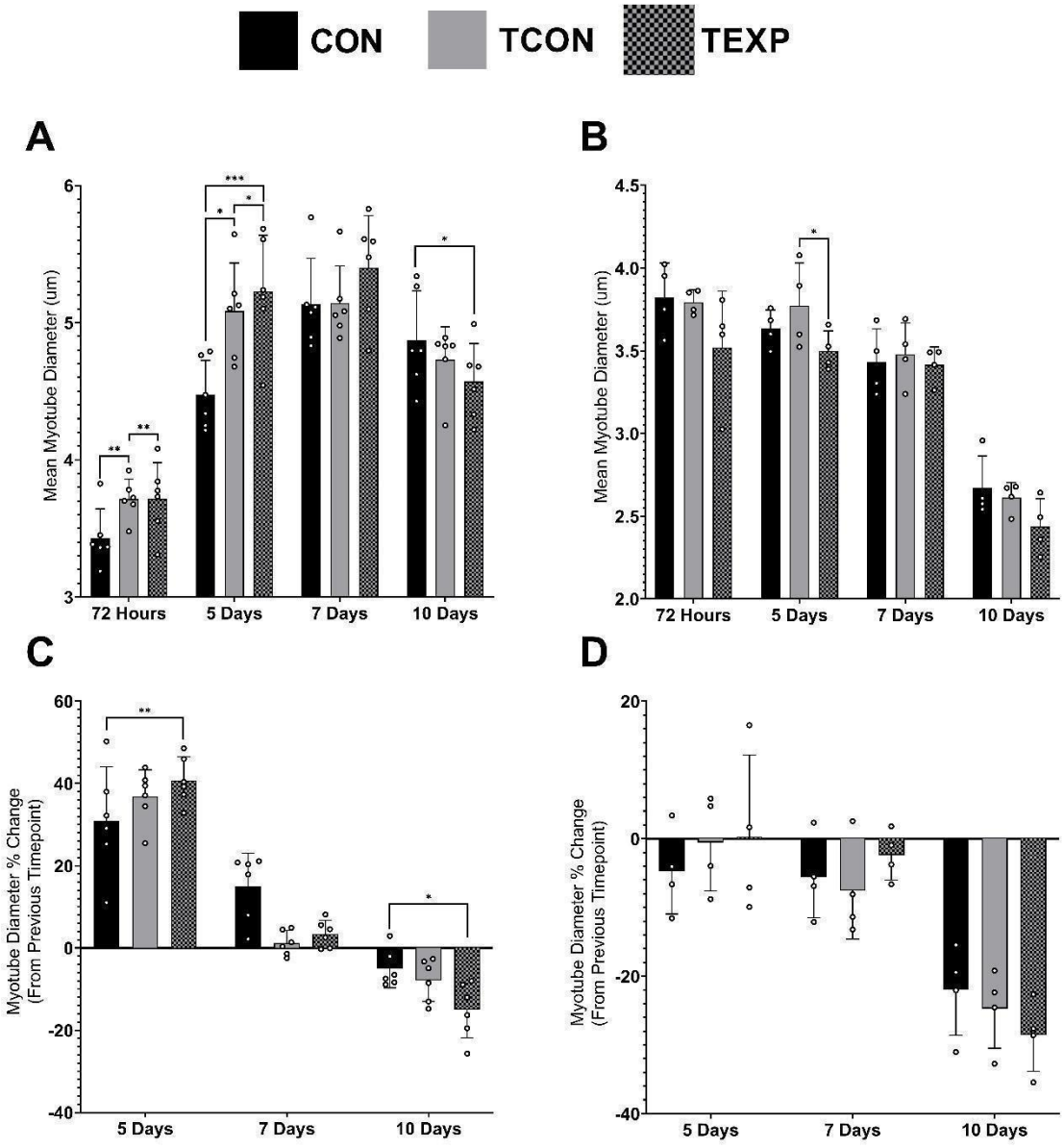

Fig 4.4. The effects of single and repeat 100 nM testosterone treatment on **A)** myotube diameter in young cells **B)** myotube diameter in MPD cells **C)** the percentage increase in myotube diameter between timepoints in young cells **D)** the percentage increase in myotube diameter between timepoints in MPD cells. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. n = 3.

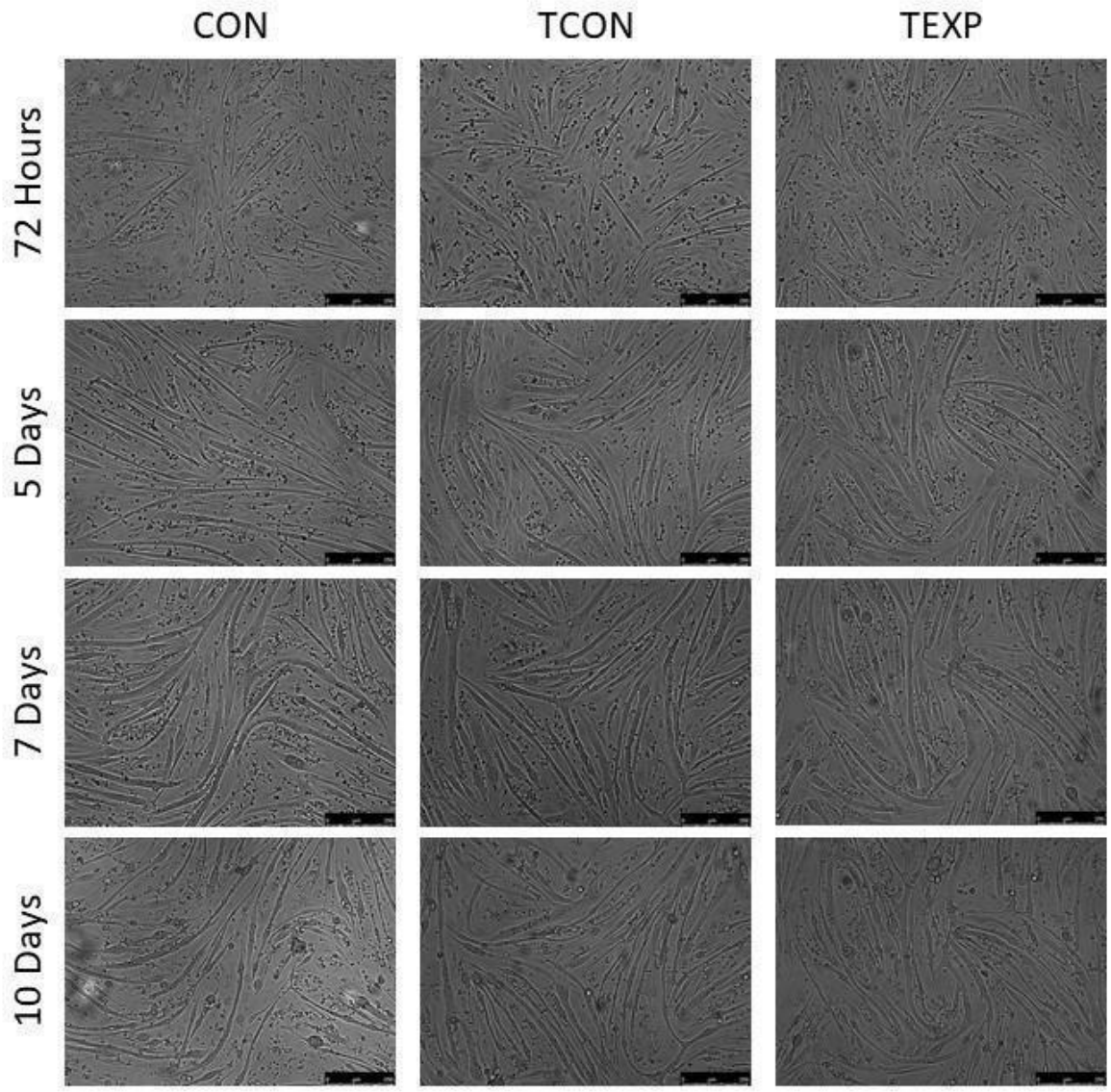

Fig 4.5. 10x objective brightfield images of young C2C12 skeletal muscle myoblasts treated with 100 nM DMSO (**CON**), a single 100 nM testosterone dose (**TCON**) and repeated 100 nM testosterone doses every 48 hours (**TEXP**).

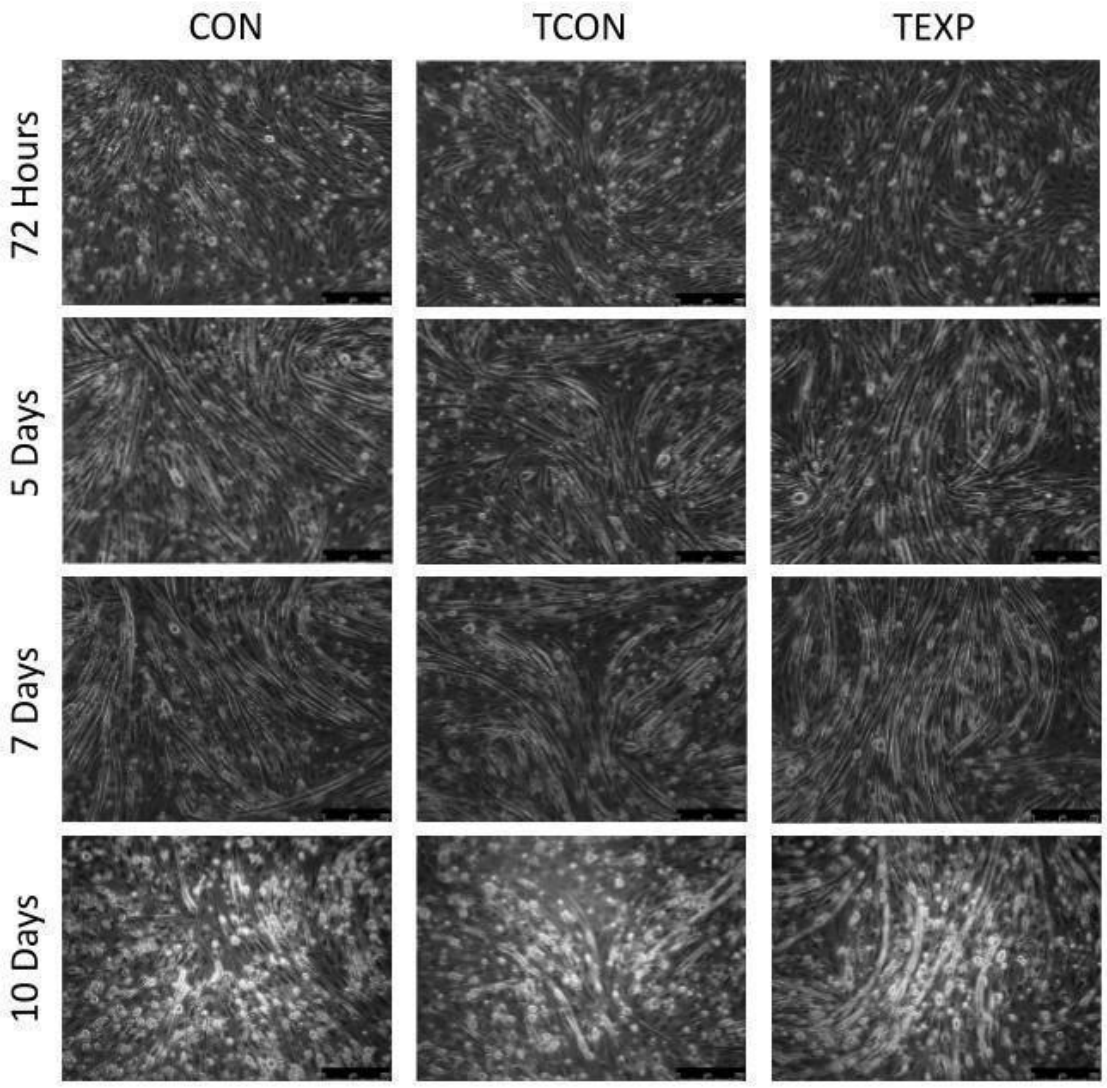

Fig 4.6. 10x objective brightfield images of replicatively aged C2C12 skeletal muscle myoblasts treated with 100 nM DMSO (**CON**), a single 100 nM testosterone dose (**TCON**) and repeated 100 nM testosterone doses every 48 hours (**TEXP**).

Myonuclear Accretion

After 7 days of differentiation, there was no significant effect of testosterone treatment on nuclear fusion index, or the average number of nuclei incorporated per myotube in young or MPD cells (*P* = N.S for all) (**Fig 4.7A-D, Fig 4.8)**.

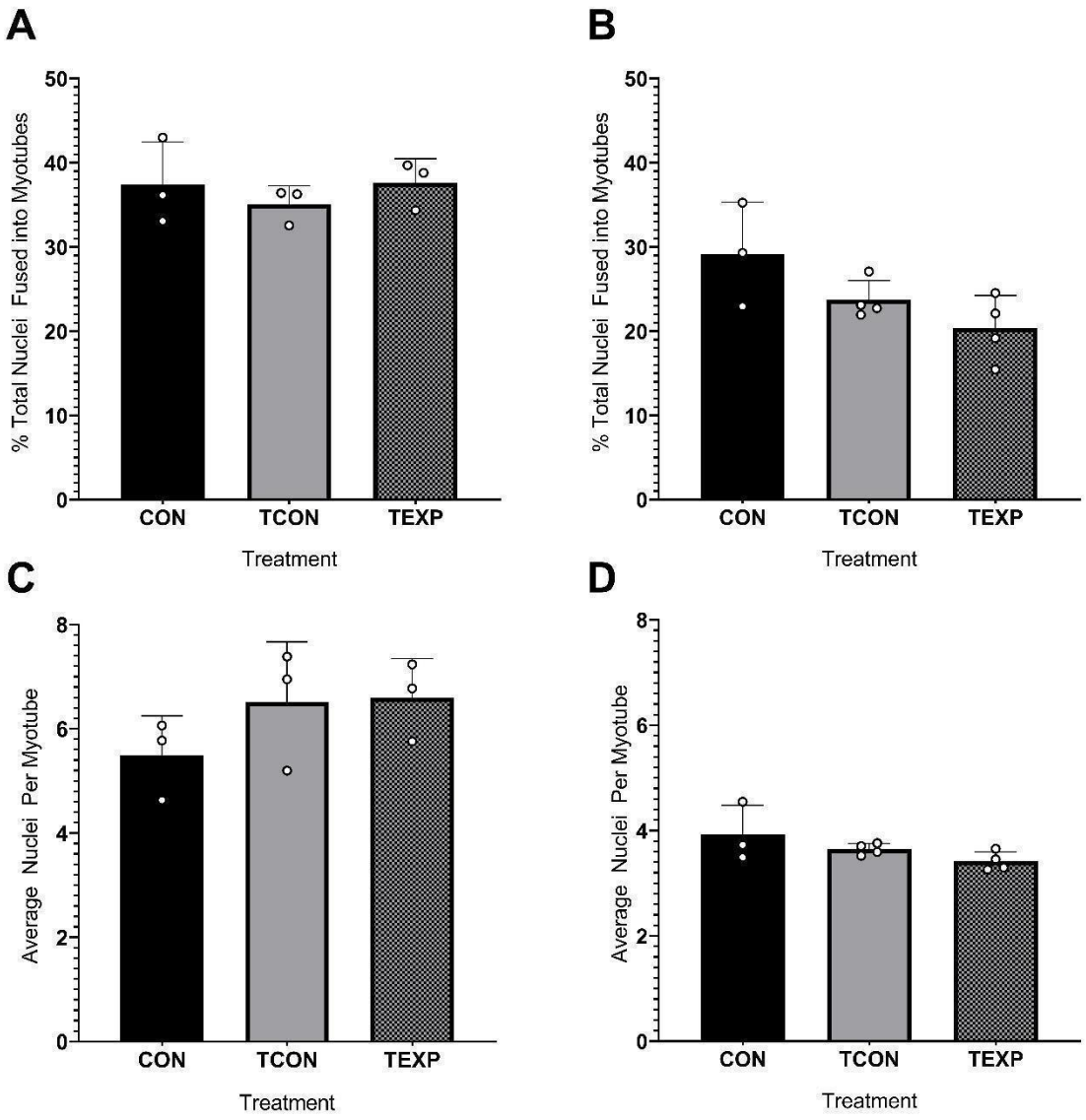

Fig 4.7. The effects of single and repeat 100 nM testosterone treatment after 7 days differentiation on **A)** nuclear fusion in young cells **B)** nuclear fusion in MPD cells **C)** the average number of nuclei per myotube in young cells **D)** the average number of nuclei per myotube in MPD cells. n = 3.

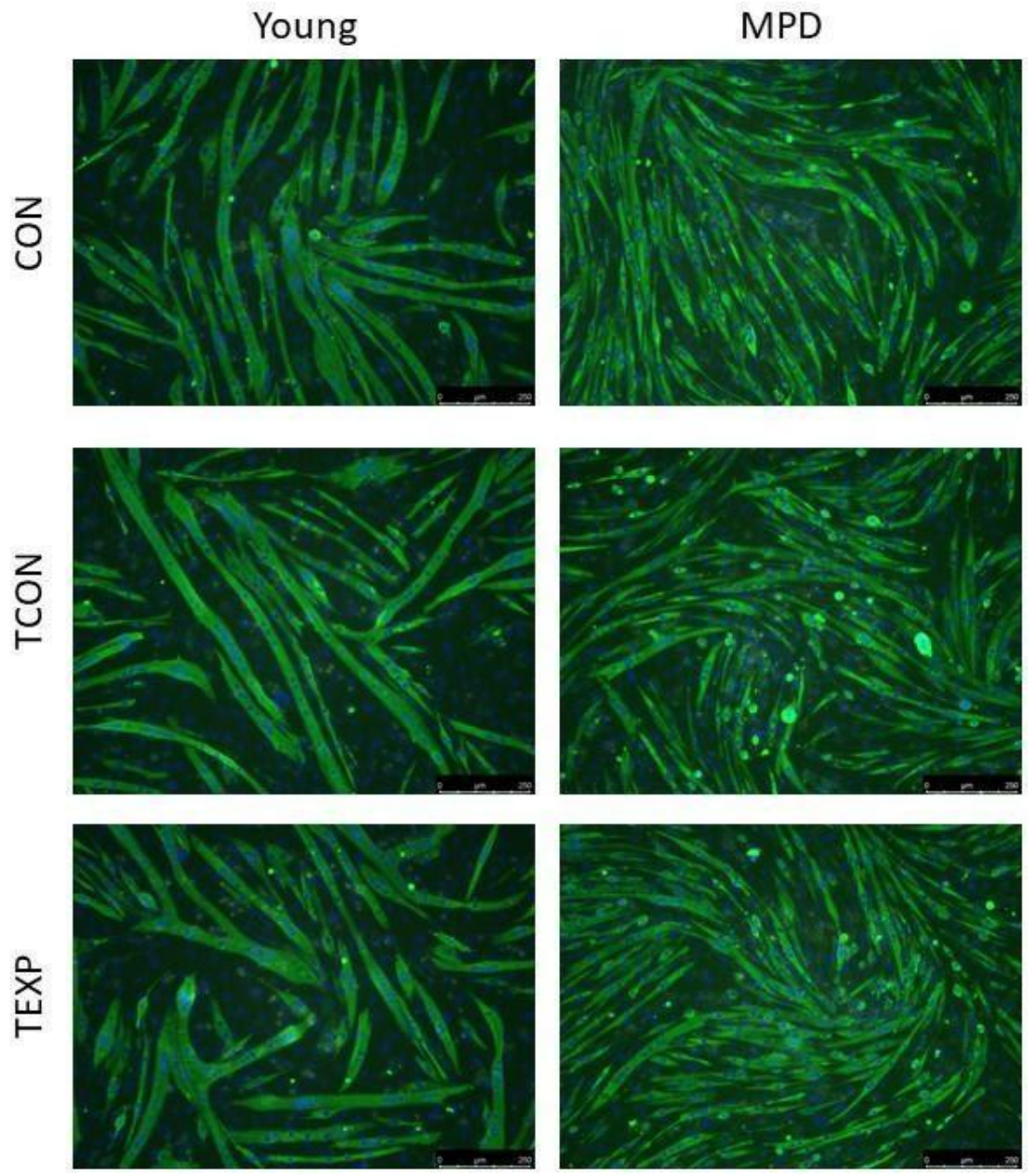

Fig 4.8. Immunostained 10x objective images for nuclei (DAPI; blue) and MHC (MF20; green) of young and replicatively aged cells (**MPD**) after 7 days differentiation following treatment with 100nM DMSO (**CON**), 100nM testosterone (**TCON**) and repeated doses of 100 nM testosterone (**TEXP**).

mRNA Expression

The effects of testosterone treatment on AR expression

There was a significant main effect of time on AR gene expression in young cells (F 2,8 = 5.633, *P* = 0.03) where AR gene expression decreased significantly between 72 hours and 7 days differentiation (72 hours 5.57 ± 2.44 vs 7 days 2.01 ± 1.28, *P* = 0.46) (**Fig 4.9A**). There was no significant effect of testosterone treatment on AR gene expression in the young cells, however there was a potential trend of testosterone treatment increasing AR gene expression (*P* = 0.067) (**Fig 4.9A**). There was no significant effect of testosterone treatment in the MPD cells (*P* = N.S) (**Fig 4.9B**).

The effects of testosterone treatment on MyoG expression

There was no significant main effect of time on MyoG gene expression in young cells, however there was a trend of MyoG gene expression decreasing over time (*P* = 0.051) (**Fig 4.9C**). There was no significant main effect of testosterone treatment on MyoG expression in the young or MPD cells (*P* = N.S), however, there was an interaction effect in the MPD cells where MyoG expression in control and repeat dose treated cells increased between 72 hours and 5 days differentiation, whilst cells treated with a single dose of testosterone decreased ($F_{2.69,\ 4.01}$ = 6.95, *P* = 0.046) (**Fig 4.9D**). There was also a significant main effect of time in the MPD cells, where MyoG expression decreased significantly between 72 hours and 7 days differentiation (72 hours 131.29 ± 67.91 vs 7 days 76.91 ± 9.25) (*P* = 0.039) (**Fig 4.9D**).

The effects of testosterone treatment on MyoD expression

There was no significant effect of testosterone treatment or time on MyoD expression in the young or MPD cells (*P* = N.S) (**Fig 4.9E-F**).

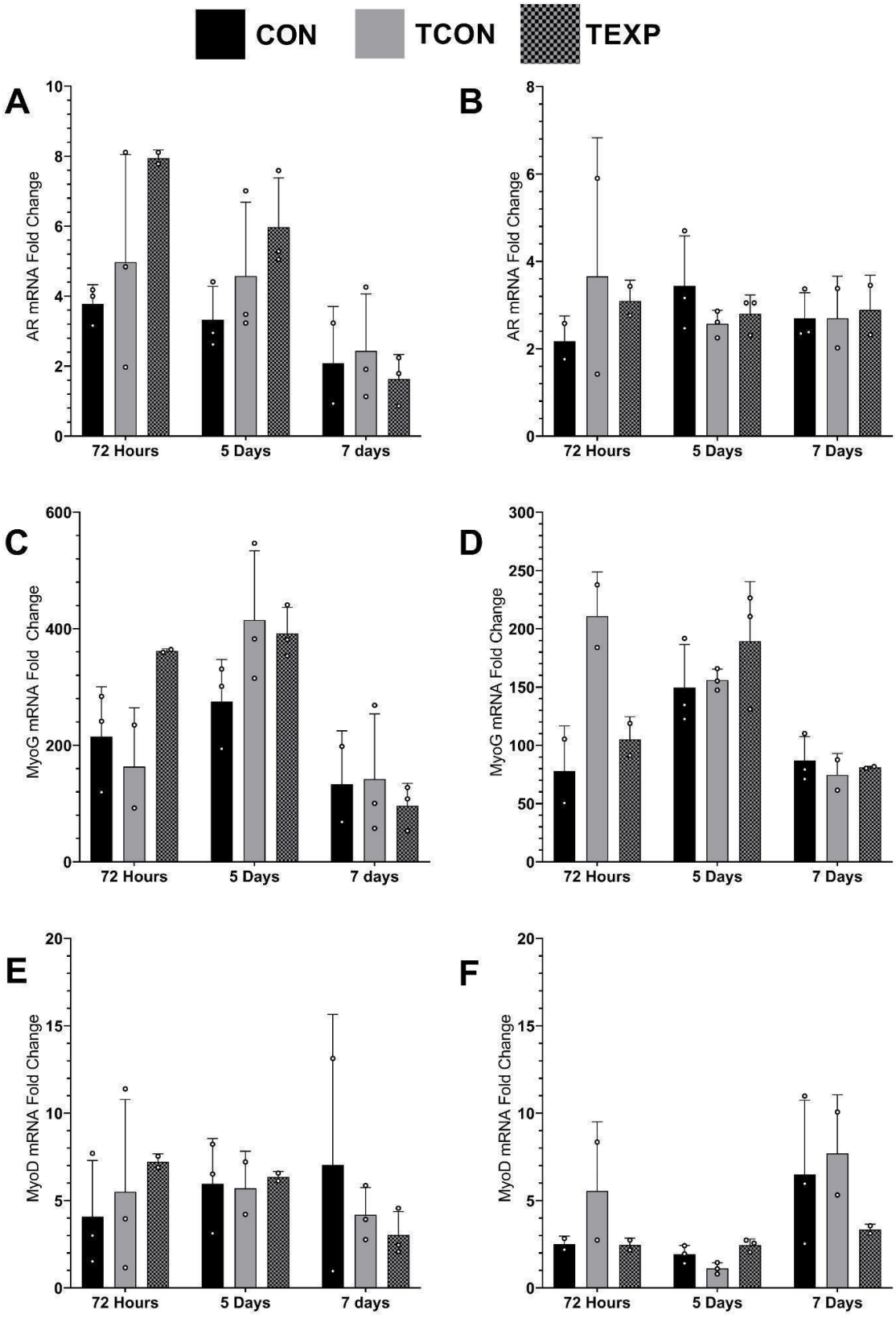
CON
TCON
TEXP
A
AR mRNA Fold Change
72 Hours
5 Days
7 days
B
AR mRNA Fold Change
72 Hours
5 Days
7 Days
C
MyoG mRNA Fold Change
72 Hours
5 Days
7 days
D
MyoG mRNA Fold Change
72 Hours
5 Days
7 Days
E
MyoD mRNA Fold Change
72 Hours
5 Days
7 days
F
MyoD mRNA Fold Change
72 Hours
5 Days
7 Days

Fig 4.9. The effects of single and repeat 100 nM testosterone treatment on **A)** AR expression in young cells **B)** AR expression in MPD cells **C)** MyoG expression in young cells **D)** MyoG expression in MPD cells **E)** MyoD expression in young cells **F)** MyoD expression in MPD cells. n = 3. All values relative to 0h control.

4.5 Discussion

This study aimed to determine the morphological, transcriptional and metabolic effects of single and repeated testosterone administration in young and old cells. Testosterone administration induced earlier increases in myotube diameter and increased the total number of myotubes and branched myotubes after 10 days differentiation in young cells, with repeated doses of testosterone incurring greater effects. Furthermore, there was a trend of testosterone administration increasing AR expression in young cells, along with a significant increase in cellular metabolic activity. There was no significant effect of testosterone administration on myonuclear accretion, MyoD expression or MyoG expression, and testosterone administration had no significant effect on any measures in the aged cells.

Testosterone treatment increases myotube number in young cells but not in replicatively aged cells, with repeat dosing incurring earlier increases in myotube number

Both single and repeat dose testosterone treatments caused a significant increase in myotube number in young cells, however this increase did not occur until the later stages of differentiation. Young cells treated with repeat doses of testosterone started to show significantly greater increases in myotube number compared to control cells after 5 days differentiation, whilst cells treated with a single dose only exhibited significant differences after 7 days differentiation. This suggests that repeated testosterone treatment may induce earlier differentiation compared to control and single dose testosterone treatments. Furthermore, this acceleration of differentiation is sustained with redosing throughout the

early stages of differentiation. Prior research has demonstrated a myostatin induced inhibition of myoblast differentiation therefore, as chapter 3 has already shown the capability of testosterone to inhibit myostatin transcription, the reported increase in myotube number following testosterone treatment may be a result of this inhibition (Langley et al., 2002). Although, myostatin typically inhibits myoblast differentiation via downregulating MyoD expression, and this study found neither testosterone treatment had a significant effect on MyoD expression.

Neither testosterone treatment had a significant effect on myotube number in the aged cells. Whilst prior studies have shown that testosterone can improve hypertrophy in fusion impaired myoblasts, they report similar findings to this study, demonstrating no effect of 50nM or 100nM testosterone treatment on myotube number after 72 hours or 7 days differentiation (Deane et al., 2013; Hughes et al., 2016). Prior research by Sharples et al. (2011) suggests that fusion impairment in population doubled myoblasts is in part due to a reduction in MyoG and MyoD expression, therefore the lack of testosterone induced change in MyoG and MyoD expression in the replicatively aged cells may explain the lack of change in myotube number. Whilst prior research has demonstrated testosterone induced increases in MyoG expression in population doubled cells with no corresponding increase in myotube number, testosterone has been shown to only restore MyoG expression to half that of the expression in young, healthy cells (Hughes et al., 2016).

As far as the author is aware, this is the first *in vitro* study comparing the effects of single and repeat doses of testosterone on MPD myoblasts. These results support and build on prior research by Hughes et al. (2016) and Deane et al. (2013), confirming that single dose 100nM testosterone treatment has no effect on myotube number in population doubled cells, and reporting novel findings showing that repeated doses do not rescue the effects of replicative aging on myotube number. Testosterone has

previously been shown to increase skeletal muscle size and strength in aged muscle, these results and prior findings suggest that this is not a result of increased myoblast differentiation and instead may be due to greater myotube hypertrophy (Sinha Hikim et al., 2002; 2006). Further work is required to determine if testosterone is capable of rescuing myoblast fusion in replicatively aged cells. Future trials should investigate if testosterone is capable of restoring MyoG expression to control levels in cells having undergone fewer population doublings, or by using greater doses of testosterone in older cells, and if a restoration of MyoG expression can rescue the effects of replicative aging on myotube number *in vitro*.

Testosterone treatment accelerates differentiation in young cells but not replicatively aged cells

Both single and repeat dose testosterone treatments caused an initial increase in myotube diameter in young cells, with testosterone treated cells displaying a significantly greater myotube diameter after 72 hours and 5 days compared to control treated cells. These results support previous findings from Hughes et al. (2016) and Deane et al. (2013) demonstrating early increases in myotube diameter following testosterone administration. Repeat doses of testosterone also appeared to incur further increases in myotube diameter between 72 hours and 5 days, whilst the single dose of testosterone had no effect on myotube diameter between these two timepoints. Between 5- and 7- days there was little change in myotube diameter in young testosterone treated cells suggesting they had reached peak differentiation. Conversely control treated cells continued to increase in diameter until they reached the same size as testosterone treated cells at 7 days differentiation. Whilst prior studies have reported that testosterone administration increases myotube diameter, these results suggest that testosterone instead increases the speed at which cells differentiate into myotubes and grow, resulting in larger myotubes at earlier time points but not once cells have reached peak differentiation (Deane et al., 2013; Hughes et al., 2016). Prior research by Lee (2002) has demonstrated the role of testosterone-AR signaling in enhancing

myogenin expression, resulting in accelerated myoblast cell differentiation and suppression of myoblast cell growth. An acceleration of differentiation potentially allows for the development of larger muscle fibres *in vitro*, as more myotubes would be formed within a set time period without the limitations of the size of a six well plate. Between 7- and 10-days differentiation all three conditions began to atrophy, this is likely due to myotubes reaching peak differentiation and exhausting fuel sources in the six-well plates. Despite prior research, and previous findings from chapter 3, demonstrating that testosterone treatment in the later stages of differentiation can inhibit atrophy, the greatest reductions in myotube diameter occurred in the testosterone treated cells (Roy et al., 2016; Serra et al., 2013; Zhao et al., 2008). This may be due to the greater number of myotubes and earlier peak differentiation exhausting fuel sources quicker than the control cells, meaning that at 10 days differentiation the testosterone treated cells were in a later stage of atrophy than the control cells.

This atrophy is a clear limitation of the 2D muscle culture model used in this study, as in physiological conditions myotubes would not atrophy upon reaching peak differentiation and there would not be an exhaustion of fuel sources at this stage. Recent research has demonstrated the use of a basement membrane matrix to culture primary mouse myoblasts into more mature myofibers, which could be used in future studies to investigate the effects of testosterone treatment past peak differentiation (Mackey & Kjaer, 2017; Pimentel et al., 2017). Despite myotube diameter decreasing after 7 days differentiation in the young cells, myotube number continued to increase in all three conditions. This may be due to myotubes fragmenting as they atrophied or potentially due to myoblasts continuing to fuse and differentiate, as unfused myoblasts and small developing myocytes can be seen in the brightfield and Immunostained images at 7- and 10-days differentiation. These findings provide further evidence for repeat treatments of testosterone increasing the speed at which myoblasts differentiate and form

myotubes, and the rate at which these myotubes grow, as well as demonstrating potential limitations of the 2D cell culture model for future research.

Testosterone treatment had no significant effect on myotube diameter in the aged cells, contrary to prior research by Deane et al. (2013) and Hughes et al. (2016) who demonstrated that a single 100 nM dose of testosterone in MPD cells could increase myotube diameter. However, the cells used in this study are likely to have undergone significantly more population doublings than those used by Deane et al. (2013) and Hughes et al. (2016), therefore it may be that testosterone's effects on MPD cells diminishes with subsequent doublings. In contrast to the young cells, myotube diameter consistently decreased throughout the time course in the aged cells, providing further evidence for the cells having undergone too many population doublings to grow properly. Surprisingly, after 5 days differentiation myotube diameter was significantly greater in aged cells treated with a single dose of testosterone compared to those treated with repeat doses, although there was no significant difference in the percentage change in diameter between timepoints between the two treatments, therefore it is unlikely that the single dose of testosterone resulted in any form of inhibition of atrophy compared to the repeat dose treated cells.

After 7 days differentiation there was no significant effect of either testosterone treatment on myonuclear accretion in young or replicatively aged cells, contrary to prior findings by Deane et al. (2013) and Hughes et al. (2016) who demonstrated significant increases in the average number of nuclei per myotube following single dose testosterone treatment in young and MPD cells. Increases in myonuclear accretion may have occurred at an earlier time point as a result of testosterones acceleration of differentiation. Despite these results, it is likely that testosterone does increase myonuclear accretion in young cells as numerous studies have demonstrated increases *in vitro* and *in*

vivo, using both single and repeat dose testosterone treatments (Deane et al., 2013; Hughes et al., 2016; Kadi et al., 1999; Sinha Hikim et al., 2002;).

There was no significant effect of testosterone treatment on branched myotube number until after 10 days differentiation in the young cells, when myotubes were atrophying. At 10 days differentiation, repeat dose testosterone treated cells had a significantly greater number of branched myotubes compared to single dose and control treated cells. This appears to be due to repeated doses of testosterone preventing a decrease in branched myotube number between 7- and 10-days differentiation. This lack of reduction in branched myotubes may be due to a greater number of myotubes as a result of testosterone induced differentiation of myoblasts in the later time points, leading to greater myotube contact giving the appearance of branched tubes. As previously mentioned in chapter 3, these results currently have little transfer to physiological muscle and there is a need for further research to determine if myotube branching occurs *in vivo* before future experiments investigating myotube branching in 2D cell culture models can be deemed necessary.

Further work should utilise 3D bioengineered muscle models to better represent the effects of testosterone administration on myotubes as recent research suggests that the use of 2D cell culture on plastic surfaces, whilst optimised for tissue culture, is not representative of the complex microenvironment of *in vitro* tissue and contributes to the low success rate of many drug treatment trials (Langhans et al., 2018). This can be seen in this study, as myotubes began to atrophy after 7 days differentiation, likely due to an exhaustion of fuel sources as previously described. Numerous studies have demonstrated the effectiveness of bioengineered 3D skeletal muscle constructs in forming multi-layered bundles of aligned myofibers from myoblasts, therefore the use of these 3D constructs could provide more extensive data on how testosterone influences myoblast fusion, myotube growth

and even myofiber alignment (Gholobova et al., 2018; Kim et al., 2020; Urciuolo et al., 2020). The use of a 3D construct would also allow for the effects of testosterone on skeletal muscle differentiation to be investigated over a much longer time course. As previously discussed, further research is also required to determine the effects of different testosterone doses in rescuing the effects of replicative aging in cells undergoing various numbers of population doublings, as prior studies have demonstrated that testosterone is capable of increasing myotube size in cells exposed to fewer cycles of doubling.

Testosterone treatment has no significant effect on AR expression in young or replicatively aged cells

Consistent with prior findings by Hughes (2014) and Hughes et al., (2016), there was no significant main effect of testosterone treatment on AR expression over the course of 7 days differentiation in the young or replicatively aged cells, however, there did appear to be a trend of testosterone treatments increasing AR expression in the earlier stages of differentiation. This trend may support findings previously reported in chapter 3, where single dose testosterone treatment initially increased AR gene expression. Prior *in vivo* studies have demonstrated testosterone induced increases in AR expression, therefore, as myotubes reached peak differentiation they may have become limited by the 2D cell culture model, as previously discussed, resulting in a reduced AR expression. Further research using the previously described 3D bioengineered muscle and basement membrane matrix models may show results more consistent with *in vivo* findings. Prior studies have also demonstrated further effects of repeat dose treatments on AR expression, consistent with the initial trends displayed in this study, although these studies were all in hypogonadal, elderly patients (Ferrando et al., 2007; Sinha-Hikim et al., 2004). If repeated, or greater, doses of testosterone do in fact appear to cause greater increases in AR expression as suggested to be the trend in these results and prior *in vivo* research, then AR expression may increase in response to the specific level of testosterone being administered, in order to prevent receptor saturation and an excess

of unbound testosterone. Prior work has demonstrated testosterone influences AR content in vivo, with castration in rats causing significant decreases in AR content, which were then rescued following administration of testosterone pellets, suggesting a strong relationship between androgen receptor content and androgen availability (Michel and Baulieu, 1980; Dahlberg, Snochowski and Gustafsson, 1981; Antonio et al., 1999). Although, it is unknown whether testosterone is capable of increasing AR content above normal physiological levels, and if there is a significant dose response relationship. The impact of testosterone increasing androgen receptor content has been previously discussed in chapter 3, along with the need for future work to determine if testosterone can incur increases in androgen receptor content beyond normal physiological levels.

Testosterone treatment has no significant effect on MyoG expression in young or replicatively aged cells

Neither testosterone treatment had a significant effect on MyoG expression in young or aged cells at any point throughout differentiation, contrary to prior research in C2C12s demonstrating significant testosterone induced increases in MyoG expression in the early stages of differentiation in young and MPD cells (Deane et al., 2013; Hughes, 2016; Wannenes, 2008). This is likely due to the significantly greater number of population doublings the cells used in this study underwent resulting in a resistance to the effects of testosterone. Although, these findings do agree with the majority of research showing testosterone treatment has no significant effect on MyoG expression in the later stages of differentiation, as MyoG is primarily expressed during the inducement of differentiation. Prior research has demonstrated that testosterone increases MyoG expression, resulting in an acceleration of differentiation (Lee, 2002). Therefore, it would be expected that this study would show an increase in MyoG expression in the young repeat dose treated myotubes to coincide with the increases in myotube

number and diameter. Whilst there was not a significant increase in MyoG expression, there was a noticeable increase at 72 hours in the young repeat dose treated cells. As there was no testosterone induced increase in myotube number in the aged cells, a similar MyoG expression between conditions was to be expected. Although, there was a surprising interaction effect where MyoG expression peaked in the single dose testosterone treated cells at 72 hours then decreased, whilst repeat dose and control treated cells peaked later after 5 days differentiation. This was unexpected as there were no significant increases in myotube number at 72 hours differentiation in the single dose cells. This presents a potential need to increase the n value of this study in order to negate the effects of biological variability or erroneous samples on the statistical analysis. Further work should aim to determine if differing doses of testosterone are capable of rescuing MyoG expression, or at least reductions in myotube number, following various numbers of population doublings, as previously described in the morphology discussion.

Testosterone treatment has no significant effect on MyoD expression in young or replicatively aged cells

In agreement with Hughes et al. 's (2016) prior research, this study found testosterone treatment to have no significant effect on MyoD expression in the young or aged cells. These findings are in contrast to prior work by Singh et al., (2003) who found that testosterone supplementation increased MyoD protein levels in a dose dependent manner after 12 days, with significant increases occurring with as little as 30 nM testosterone. However, Singh et als research was conducted in mouse 10T1/2 cells, which have been shown to behave differently throughout differentiation to C2C12s (Kubo, 1991). As previously shown, testosterone's effects can be highly tissue specific, which is likely to explain the differing results. Furthermore, Singh et al. examined protein content rather than mRNA expression, which this study

currently does not have access to. Further work will enable better comparisons between the two studies once protein data is available to be analysed. As MyoD functions to remove cells from the cell cycle and induce differentiation via an upregulation of MyoG, an increase in MyoD expression may potentially accelerate differentiation and further increase myotube number (Blais et al., 2005).

Repeat dose testosterone treatment increases cellular metabolic activity in young cells but not replicatively aged cells

Repeated testosterone treatment significantly increased metabolic activity in the young cells compared to single dose and control treatments, further supporting previous findings from chapter 3 and recent studies suggesting that testosterone is capable of increasing cellular viability (Gorgey et al., 2020; Pongkan et al., 2015; Pronstato et al., 2020). In contrast to the findings from chapter EDT, single dose testosterone treatment did not appear to significantly increase metabolic activity, however the 20% increase that did occur was incredibly similar to the results shown in chapter EDT, therefore the larger standard deviation as a result of the significantly greater metabolic activity in the repeat dose sample is likely to have affected the statistical analysis. Whilst some studies have demonstrated that testosterone treatment has no or a negative effect on cell viability, the majority of these were performed in different tissues to this study (Estrada et al., 2006; Kanda et al., 1996; Zhao et al., 2013). The tissue specific effects of testosterone have been discussed in chapter 3, showing that the findings from this study and prior research in skeletal muscle tissue supports testosterone increasing cell viability in skeletal muscle myotubes.

To the authors knowledge this is the first study to compare the effects of single and repeat dosing of testosterone on skeletal muscle cell metabolic activity. Findings from chapter 3 of this thesis suggest that testosterone is able to increase metabolic activity regardless of what stage of differentiation cells are at,

therefore it is reasonable that repeated treatment throughout differentiation would yield greater increases in cell viability. These results suggest that the mechanisms by which testosterone may increase cell viability are not easily saturated by subsequent doses. Whilst these mechanisms are not identifiable from the current findings alone, a detailed discussion of how testosterone may potentially increase cell viability can be found in chapter 3, with the most likely conclusion being that testosterone influences mitochondrial dynamics and remodeling in an AR-independent manner.

Testosterone treatment had no significant effect on cellular metabolic activity in the replicatively aged cells, suggesting that if decrements in cellular metabolic activity occur with multiple population doublings, then testosterone is unable to rescue these effects. Although, prior research has demonstrated testosterone's ability to rescue the effects of smaller numbers of population doublings on morphological and transcriptional measures, therefore it may be that whilst the cells used in this study were too damaged to be affected by testosterone administration, younger cells may still experience increases in cellular metabolic activity (Hughes et al., 2016). Furthermore, testosterone may have incurred increases in metabolic activity at earlier stages of differentiation, however as metabolic activity was only measured after 10 days of differentiation these changes may no longer have been visible, due to the myotubes undergoing significant atrophy. Further work is required to assess the effects of testosterone on cellular viability in population doubled cells, using samples throughout differentiation and in cells undergoing different numbers of population doublings. As previously discussed in chapter 3, a more comprehensive array of cellular viability assays is required to confirm that testosterone does in fact increase cellular viability. Furthermore, a dose response experiment may be beneficial to determine if the greater increase in cell viability incurred by repeated doses of testosterone is due to the repeated exposure to testosterone or the greater total concentration of testosterone cells were exposed to. Future experiments should also investigate the effects of various numbers of population doublings on cell

viability in C2C12s, this could begin by simply repeating an MTT assay on samples of skeletal muscle cells having undergone various numbers of population doublings to assess if there are significant differences in cellular metabolic activity.

Chapter Summary

This chapter has demonstrated testosterone's ability to increase cellular metabolic activity and accelerate differentiation in myotubes, with repeated doses of testosterone incurring greater effects. Furthermore, this chapter builds on prior research by Deane et al. (2013) and Hughes et al., (2016) by demonstrating that whilst testosterone may be able to rescue the effects of replicative aging in skeletal muscle cells there is a limit to the number of population doublings myoblasts can undergo before becoming irreparable and immune to the effects of testosterone administration. Further work is required to determine exactly how many population doublings myoblasts can undergo before becoming impervious to testosterone treatment. Understanding the number of population doublings myoblasts can undergo before becoming immune to the effects of drug administration is likely to be crucial in the future use of this model to replicate the effects of aging. Similarly, significant work is required to determine if the reported increases in cellular metabolic activity represent an increase in cellular viability.

Chapter 5

Thesis Synthesis

5.1 Realisation of Aims

Aim 1 - To determine the effect of testosterone administration on skeletal muscle androgen receptors

The effect of single and repeat dose testosterone administration on androgen receptor mRNA expression is presented in chapters 3 and 4. Chapter 3 describes a significant increase in AR expression after 72 hours differentiation following testosterone administration vs no treatment in undifferentiated myoblasts, whilst in chapter 4 a non-significant trend towards increased AR mRNA expression following repeat testosterone administration was observed after 72 hours and 5 days differentiation.

The hypothesis for chapter 3 was that testosterone would have no effect on AR expression and therefore this hypothesis was rejected. In chapter 4 it was hypothesised that repeated doses would induce increases in AR mRNA expression versus non treated cells and thus this hypothesis was also rejected. To confirm these findings, it is necessary to quantify AR protein content. Whilst protein samples were collected during both studies, these could not be analysed due to time (COVID-19 delay) and financial constraints as described in section 5.2, therefore aim 1 was only partly addressed in the current thesis.

Aim 2 - To determine the effects of testosterone administration at different timepoints throughout differentiation

In chapter 3, testosterone was administered to undifferentiated myoblasts at 0 hours differentiation and to myotubes nearing peak differentiation at 7 days differentiation. Morphological and transcriptional effects were analysed after 72 hours and 10 days differentiation. Live-imaging demonstrated that testosterone administration at the onset of differentiation encourages greater commitment to terminal

differentiation in myoblasts resulting in an increased number of myotubes, whilst administration in differentiating myotubes either induces further increases in myotube size or inhibits atrophy. This aim was partly realised in chapter 3, however due to the previously mentioned lack of protein data this aim was not fully addressed in the current thesis.

The hypothesis that testosterone administration in undifferentiated myoblasts would increase myotube number, whilst administration in differentiating myotubes would prioritise an increase in myotube diameter was accepted. RT-qPCR suggested that treatment in undifferentiated myoblasts caused increases in AR expression and reductions in myostatin expression not present in myotubes treated later with testosterone. Therefore, the hypothesis that testosterone administration in undifferentiated myoblasts would reduce myostatin expression was accepted, whilst the hypothesis that testosterone administration would have no effect on AR expression was rejected.

Aim 3 - To determine whether aging affects skeletal muscle androgen receptor content and the anabolic effect of testosterone

In chapter 4, the morphological and transcriptional effects of testosterone administration were compared between young and replicatively aged cells that had undergone 30 passages. The two populations were analysed using live-imaging, immunocytochemistry and RT-qPCR. The effect of testosterone on cellular metabolic activity was also compared between the young and aged cells. Whilst testosterone significantly increased myotube diameter, number and cellular metabolic activity in the young cells, none of these effects were present in the aged cells. Resultantly, the hypothesis that testosterone would rescue the negative effects of replicative ageing on myotube size and myonuclear accretion was rejected. Unfortunately, due to limitations mentioned below, further work is required to

fully address the aim of this experiment as the effects of varying numbers of population doublings on skeletal muscle androgen receptors was not addressed.

5.2 Limitations

COVID-19

The most obvious limitation affecting this thesis was the occurrence of COVID-19. Due to the pandemic the laboratory was closed for extended periods of time, preventing any long-term experiments from being performed as there was no guarantee the laboratory would remain open throughout the entire experiment. This meant that the experiments originally planned for this thesis had to be replaced by multiple shorter experiments. The closure of the laboratory also extended the length of this MPhil project, resulting in a number of financial and time constraints. This reduced the amount of time available to complete data collection once the laboratory did reopen.

Protein Data

Despite protein samples being collected at each timepoint, they could not be analysed as a result of the previously mentioned financial and time constraints. Whilst gene expression data does provide a view of what is happening to specific genes, increased gene expression does not always translate to increased protein content (Koussounadis et al., 2015). Furthermore, protein content data would have demonstrated if any increases in gene expression incurred by testosterone administration had lasting effects. This data would have also allowed for much better comparisons to previous *in vivo* research, especially regarding the effects of testosterone and aging on the androgen receptor (Ferrando et al., 2002; Poole et al., 2011; Ratamess et al., 2005).

2D Cell Culture Model

As discussed in chapter 4, the 2D cell culture model used resulted in myotubes atrophying after 5-7 days differentiation. As larger and more numerous myotubes are likely to be more metabolically active, the samples experiencing the greatest growth may atrophy earlier, as seen in the cells treated with repeated doses of testosterone. This is not an accurate model of muscle growth as *in vivo* myotubes would not atrophy upon reaching peak size provided adequate stimuli are present to support the size of the muscle including nutrients and mechanical input. The use of 3D bioengineered muscle models would be beneficial to investigate the effects of testosterone administration beyond 7 days differentiation, as discussed in chapter 4.

Sample timepoints for immunostaining and MTT

As previously mentioned, it was unknown that the myotubes would reach peak differentiation and atrophy by 7 days differentiation, therefore when the immunostained and MTT samples were taken after 7- and 10-days differentiation the myotubes had atrophied significantly. Furthermore, if testosterone did accelerate differentiation then there were likely changes in myonuclear accretion in the earlier stages of differentiation that were no longer visible by the time the immunostained images were taken. Further work will investigate the effects of testosterone on myonuclear accretion and cellular viability throughout the course of differentiation by utilizing more sampling points.

Use of MTT assay for cell viability

The MTT assay alone is not a sufficient measure of cellular viability. As discussed in chapter 3, an MTT assay is a measure of cellular metabolic activity, not specifically cellular viability. Therefore, an increase in metabolic activity cannot be attributed to greater cellular viability without further evidence. Future experiments could utilise a propidium iodide assay in order to measure the occurrence of cell death within samples, this will provide further evidence for if the increase in cellular metabolic activity

reported in this study represents an increased cellular viability. The propidium iodide assay is used to detect dead cells within a population by binding propidium iodide to the DNA of dead cells, increasing the dyes fluorescence 20-30-fold. As propidium iodide is not permanent to live cells, only dead cells within a population appear stained. The dyed samples are then commonly analysed using fluorescence microscopy, flow cytometry and fluorometry (Riccardi and Nicoletti, 2006).

Age of population doubled cells

As a result of the aforementioned time-constraints, cells that had previously undergone multiple passages had to be used as the replicatively aged samples, rather than accurately exposing the cells to multiple population doublings at the start of the study. This meant that the number of population doublings the cells underwent was unknown, however judging by the passage numbers it was likely to be significantly more than the cells used in prior research. Whilst this prevented direct comparisons to prior work by Hughes (2014) and Sharples et al. (2011), it did provide evidence for greater population doublings preventing testosterone from rescuing the effects of replicative aging on C2C12 skeletal muscle cells. Further work will aim to determine how C2C12 myoblasts are affected with increasing numbers of population doublings, using similar measures to those used in this thesis, as briefly described in section 5.4.

5.3 Conclusions and Implications

The primary findings from this thesis are that testosterone administration accelerates aspects of myoblast differentiation and this effect was amplified following repeated testosterone administration. Whilst prior studies have demonstrated an increase in myotube diameter following testosterone administration, this thesis' use of coordinate-based live imaging to analyse increases in myotube

diameter within samples suggests that testosterone administration does not increase myotube diameter *in vitro*, and instead increases the speed at which myotubes reach their peak diameter (Deane et al., 2013; Hughes et al., 2016). In the context of physiological human muscle, these findings suggest that testosterone administration may increase skeletal muscle size by increasing the rate at which myotubes regenerate and grow. However, as previously discussed in chapter 4, further work is required using 3D cell culture models to investigate the acceleration of differentiation without the restrictions imposed by the 2D model used in this thesis.

Testosterone was also shown to increase AR mRNA expression, with a trend of repeated doses incurring greater increases. Prior studies have suggested that AR content may be the limiting factor in skeletal muscle growth, therefore testosterone induced increases in AR content may be a key mechanism of action for the associated increases in skeletal muscle hypertrophy. Furthermore, if testosterone is able to elicit greater hypertrophy through an increase in AR content, then testosterone treatment protocols in sarcopenic patients should be designed with an individual's relative AR content in mind. This thesis also expanded on prior research by Hughes et al. (2016) and Deane et al. (2013) by demonstrating that whilst testosterone is capable of rescuing the effects of multiple population doublings on skeletal muscle cells, there is a limit to the number of doublings a population can undergo before testosterone ceases to have any effect. Therefore, future studies utilising a model of multiple population doublings need to be exact with the number of doublings cell populations undergo, furthermore, research should aim to determine the number of population doublings that is best representative of aged skeletal muscle. As well as these novel findings, this thesis provided further evidence for testosterone inhibiting skeletal muscle atrophy, likely via an inhibition of myostatin expression.

An interesting and somewhat unplanned finding of this thesis was that testosterone induced increases in cellular metabolic activity, suggesting a potential increase in cell viability, with greater increases in metabolic activity occurring following repeated exposure to testosterone. In combination with prior studies, these results provide further evidence for testosterone potentially improving mitochondrial function in skeletal muscle cells and form the basis of a preliminary study investigating testosterone's effects on cell viability. As discussed in chapter 3, significant work is still required to confirm the effects of testosterone administration on cell viability.

Future work should aim to determine the effects of testosterone administration on skeletal muscle cell viability and mitochondrial function, as well as investigating the effects of testosterone and aging on androgen receptor content *in vivo* and *in vitro*. Whilst prior research has focused on the immediate effects of testosterone and aging on skeletal muscle, it may be that the key to treating severe hormonal induced sarcopenia is the long-term regulation of androgen receptor content.

5.4 Proposed epigenetics study

Due to the previously mentioned limitations of COVID-19, the primary study planned for this thesis had to be postponed. Below is an initial literature review and an outline of the experimental design to provide an insight into some of the original plans for this thesis and theoretical work completed prior to the pandemic.

5.4.1 Brief Literature Review

Epigenetics

Epigenetics is the study of heritable changes in gene expression as a result of non-genetic DNA and histone modifications (Bollati and Baccarelli, 2010). Epigenetic changes can be gained from acute

exposures, chronic exposures or inherited from parent cells (Hansen et al., 2008; Petruk et al., 2012). The most researched mechanism of epigenetic modification is DNA methylation. In mammals, nearly all DNA methylation occurs on CpG islands (high density CpG dinucleotide regions) at gene promoter regions associated with gene inactivation (Goldberg, Allis and Bernstein, 2007). DNA methylation and histone modification causes alterations in gene transcription. DNA methylation is catalysed by DNA methyltransferase enzymes, which convert cytosine to 5-methylcytosine (5mC) (Nevin and Carroll, 2015). 5mC then attach to CpG islands, blocking RNA polymerase access to bases, thereby repressing transcription (Bogdanovic and Veenstra, 2009). CpG methylation represses gene expression, whilst a lack of methylation promotes transcription. Whilst CpG islands are the most common targets for DNA methylation, 5mC can also attach to CpA, CpT and CpC sites (Patil, Ward and Hesson, 2014).

Skeletal Muscle Memory of Exercise

Research has shown that skeletal muscle retains a memory of early life resistance training, and that this memory leads to greater increases in skeletal muscle mass following later life resistance training (Seaborne et al., 2018). Further DNA methylation analysis has also demonstrated that following resistance training, areas with the greatest hypomethylation display the greatest increase in lean mass, suggesting that epigenetics is likely to underpin skeletal muscle memory (Seaborne et al., 2018; Sharples et al., 2016). Therefore, skeletal muscle memory retaining a memory of exercise is likely due to hypomethylation of genes relating to hypertrophy. Recent research by Seaborne et al. demonstrated a hypomethylation of the AR gene following acute RE and following retraining after a 7-week unloading period. However, Seaborne found no significant change in AR methylation following chronic RE training, therefore it may be that acute hypomethylation of the AR gene contributes to an increase in AR content at the onset of training or following a return to exercise after a detraining period. This could contribute

to the idea of 'newbie gains' experienced by individuals just starting RE. Seaborne also found no change in methylation following 7 weeks detraining, suggesting that hypomethylation occurring as a result of acute RE at the onset of training or following a return to training is not lost during detraining, although as the study only utilised a 7-week detraining period it is unknown whether this hypomethylation persists over longer time periods.

Epigenetic Effect of Testosterone Administration

Prior research has shown that skeletal muscle maintains elevated methylation from some drug administrations throughout its proliferative lifespan, therefore it may be possible for skeletal muscle cells to retain the benefits of acute testosterone administration in later life (Sharples et al., 2016). Research in rodents demonstrated that early life testosterone administration led to an increase in myonuclei, preceding an increase in skeletal muscle CSA. Once testosterone had been withdrawn muscle mass returned to baseline levels, however the additional myonuclei were retained, suggesting greater potential for skeletal muscle growth in the future due to an increased capacity for protein synthesis (Bruusgaard et al., 2010).

Testosterone administration can cause DNA methylation, as testosterone administration in castrated rats has been found to increase ER-a methylation in the brain, thereby decreasing ER expression (Auger et al., 2011). It is unknown whether this epigenetic effect of testosterone in the rodent's brains remained long after treatment. Similar research in rodents found that testosterone induced long lasting DNA methylation effects in gene promotors in the liver, including the demethylation of genes associated with encoding the AR-coactivator dopa decarboxylase, although there was no significant change in the DNA methylation status of the AR promoter (Dkhil et al., 2014; Wafa et al., 2012). Conversely, similar studies

have shown that the AR promoter gene in the brain is susceptible to testosterone mediated epigenetic modification, therefore it may be that testosterone induced AR methylation is tissue specific (Imamura, 2011). Testosterone has been shown to act epigenetically not only via DNA methylation, but also likely via histone modification. Research in C. elegans found that testosterone epigenetically modified pharyngeal pumping rate by binding to nuclear receptors expressed by nhr-69 (a putative ortholog of the human AR gene) (Gamez-Del-Estal et al., 2014). The effects of testosterone remained for four generations following administration, suggesting that testosterone epigenetically impaired nervous system function via nhr-69. These changes could not be due to DNA methylation as C. elegans does not possess a predictable DNA methyltransferase in its genome or 5mC in its DNA (Bird, 2002). A lack of 5mC prevents DNA methylation, as 5mC is responsible for modulating the binding of transcription factors, although, recent research has found other base modifications with potential for epigenetic modifications, including N6-methyladenine (6mA) in C. elegans (Greer et al., 2015).

Based on the current literature, testosterone appears to have long-lasting effects in skeletal muscle and a variety of other tissues, most notably increasing the potential for skeletal muscle growth. Whether these potential increases in skeletal muscle growth are via an increase in myonuclei, changes in androgen receptor expression or a variety of other factors remains to be determined. Similarly, further research is required to determine what mechanism, or mechanisms, cause these epigenetic changes.

Epigenetics and Sarcopenia

With age the number of myonuclei depletes. As testosterone has been shown to potentially cause a permanent increase in myonuclei, enabling greater skeletal muscle mass, testosterone administration during earlier life may help to prevent sarcopenia in older adults (Bruusgaard et al., 2010). Testosterone

may also increase the effectiveness of resistance exercise as a treatment for sarcopenia and reduce the effects of disuse atrophy associated with retirement in older adults. Insulin resistance has been shown to increase methylation in CpG islands of the AR promoter, leading to decreased AR mRNA expression and protein (Kim et al., 2013). As aging has been associated with increased insulin resistance, it may be possible that reduced AR expression as a result of increased insulin resistance plays a role in the development of sarcopenia. (Fink et al., 1983). Age-related increases in insulin resistance are often a result of increases in body fat and reductions in fat-free mass due to changes in diet and significant reductions in physical activity commonly experienced by retired adults, both of which factors are associated with the onset of sarcopenia (Ryan, 2000).

Summary

This preliminary literature review has demonstrated that skeletal muscle can undergo epigenetic changes resulting in greater increases in size and strength in later life. Furthermore, this review has provided mechanisms of action for a potential epigenetic effect of testosterone administration in skeletal muscle cells via DNA methylation effects. This lasting effect of testosterone however is yet to be demonstrated *in vitro* at a cellular level in skeletal muscle cells and it is unknown if these effects diminish with repeated proliferative cycles. Regardless, the current literature suggests that early life testosterone treatment by aiding in the prevention of sarcopenia via increasing the effectiveness of exercise, reducing muscular dystrophy and increasing androgen receptor content.

5.4.2 Aims

This study aimed to determine if testosterone administration in young skeletal muscle myoblasts would retain its effects following multiple proliferative cycles, induced via multiple population doublings. This

study also aimed to investigate the morphological and transcriptional effects of various numbers of population doublings on skeletal muscle myoblasts, for use in future studies.

5.4.3 Experimental Design

Young C2C12 myoblasts (as low a passage number as possible to ensure accurate estimation of population doubling numbers) were to be seeded at $1x10^6$ cells in T75 flasks in GM in the presence of 100 nM testosterone or 100nM DMSO and incubated until approximately 80% confluent. Cells would then be trypsinized and seeded onto new T75s at $1x10^6$ for 48 hours, with doubling time and cell numbers being recorded throughout. This cycle would be repeated, with stocks of cells being frozen and stored every 20 population doublings upto 80 population doublings. Cells would then be thawed, grown to 80% confluency in T75s and seeded on sixwell plates, where they would be induced to differentiate in DM. At each timepoint (72 hours, 5 days and 7 days differentiation) morphological measures (myotube number, diameter and myonuclear accretion) would be taken using live imaging, specimen fixation and immunocytochemistry. Cells would also be isolated for RNA and protein at each timepoint for analysis of gene expression and protein content via PCR and western blotting. After 7 days of differentiation, cells would undergo an MTT assay to measure cellular metabolic activity, as well as a propidium iodide assay to measure cellular viability. The effects of multiple population doublings would be compared between samples having undergone various numbers of doublings, as well as the effects of early life testosterone administration in these cells.

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