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Do C2C12 myotubes retain a cellular memory of repeated testosterone dosing?

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Preface

*"Personally, I'm always ready to **learn**, although I do not always like being **taught**."*
-Sir Winston Churchill (1874-1965).

As I am submitting this thesis, I am closing a chapter in my life with mixed emotions. During my time at NIH, I have been lucky enough to meet some fantastic people with different backgrounds and interests, many of whom I can proudly call "friends" today. I will truly miss the lunch breaks, the futsal games, the volleyball games, the gym sessions, the burger-eating, the taco evenings, and so much more! And who can forget **that** trip to Germany! I will hold these memories dear for the rest of my life. To all you fantastic people: Thank you!

First and foremost, a massive thank you to my supervisor **Dr. Adam Sharples**. Thank you for the patience shown and guidance provided in the planning and execution of this project. Your feedback was always supporting, precise, and helpful.

Secondly, I would like to thank Ph.D. student **Piotr Gorski** for the lab training and all the formal and informal chats we have had over the past year. Despite countless stupid questions, you always provided help whenever I needed it.

To my **family**, thank you for the love and support you have provided me during this year. Also, a thank you to my **Uncle Trygve** for help and useful comments during the final stages of this thesis. To the boys in Verdansk, the escape into the virtual world with you guys was truly a joy during the winter lockdown.

However, as one chapter ends, another one begins and, with an academic background from NIH, I am excited about the future.

Magnus Løkken

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Abstract

Introduction: Testosterone is a vital male sex hormone responsible for many physiological processes in the human body. In addition, it elicits a hypertrophic effect on the human skeletal muscle. Due to this effect, some explore abusing synthetic variants of testosterone to gain performance in sports or enhance their physique. Previous studies have alluded to that this effect is retained after the individuals stop using the substances, a 'muscle memory' of doping. This thesis aimed to investigate the muscle memory of repeated testosterone administration, looking at gene expression values of differentiation markers and genes that have previously retained a memory of anabolism induced by resistance exercise.

Methods: C_2C_{12} myotubes were dosed at an early (day 3) and/or late (day 7) stage of differentiation using supraphysiological doses of testosterone (100 nmol). There were four conditions during this experiment. Early testosterone (ET), late testosterone (LT), early and late testosterone (ET+LT), and controls only treated with differentiation media (DM). Morphological data analysis (myotube numbers, area and, diameter) and gene expression analysis of selected genes were used to assess the effect of this dosing.

Results: The morphological data showed that the ET+LT condition at day 10 had significantly greater myotube numbers ($P \leq 0.0001$) than all other conditions. More importantly, it was significantly greater than the LT condition suggesting that ET+LT retained a 'memory' of this first dosing at day 3 supported by the fact that ET alone did not evoke any morphological difference vs. DM control. Analysis of myotube size (area and myotube) observed a similar ET+LT > LT > ET and DM trend on day 10, although not all of these numbers reached significance. The gene expression analysis did not indicate that the genes selected due to their association with 'muscle memory' were involved in the observed morphological memory.

Conclusion: C_2C_{12} myotubes retain a morphological memory of testosterone-induced hypertrophy when later re-encountering testosterone at a late stage of differentiation. However, this memory was not reflected in the 'muscle memory genes' observed with resistance exercise-induced hypertrophy.

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4E-BP1	4E-binding protein 1
5caC	5-caboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5meC	5-methylcytosine
AAS	Anabolic androgenic steroids
AGO	Argonaute-2 protein
Akt	Protein Kinase B
AR	Androgen Receptor
ARKO	Androgen receptor knockout mice models
BER	Base excision repair
BICC1	BicC Family RNA Binding Protein 1
CNS	Central nervous system
CSA	Cross-sectional area
CTTN	Cortactin

DGCR8	Drosha-DiGeorge syndrome critical region 8
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DM	Differentiation media
DMEM	Dulbecco's Modified Eagle Medium
DNMT	DNA methyltransferase
EDL	Extensor digitorum longus
eEF2K	Eukaryotic elongation factor-2 kinase
EGF	Epidermal growth factor
eIF2B	Eukaryotic translation initiation factor 2B
elF4E	Eukaryotic translation initiation factor 4E
ER	Estrogen Receptors
ERK	Extracellular signal-reduced kinases
ET	Early testosterone
ET+LT	Early testosterone and late testosterone
FBS	Fetal bovine serum

FHS	Follicle-stimulating hormone
FLNA	Filamin A
FLNB	Filamin B
FOXO	Forkhead box O family
GM	Growth Media
GnRH	Gonadotropin-releasing hormone
GRIK2	Glutamate Ionotropic Receptor Kainate Type Subunit 2
GSK-3β	Glycogen synthase kinase 3β
HECT	Homologous to E6-AP carboxy-terminus
HSP	Heat shock proteins
IGF-1	Insulin-like growth factor 1
IOC	International Olympic Committee
JNK	Jun HN2-terminal kinases
KGF	Keratinocyte growth factor
LH	Luteinizing hormone
LT	Late testosterone

MAFbx	Muscle atrophy F-box
MAPK	Mitogen-activated protein kinases
miRNA	Micro RNA
MPS	Muscle protein synthesis
mTOR	Mammalian target of rapamycin
MuRF1	Muscle RING-finger protein-1
MYCH1	Myosin heavy chain 1
MYCH4	Myosin heavy chain 4
MyoD	Myoblast determination protein 1
NBCS	Newborn Calf Serum
ncRNA	Non-coding RNA
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
Pen-Strep	Penicillin-Streptomycin
PI3K	Phosphoinositide 3-kinases
PPARδ	Peroxisome proliferator-activated receptor δ

PPP	Picropodophyllin
RE	Resistance exercise
RING	Really Interesting New Gene
RISC	RNA-induced silencing complex
rpS6	Ribosomal protein s6
RRT	Randomized Response Technique
rt-qRT-PCR	Reverse transcription-quantitative real-time polymerase chain reaction
S6K1	p70 ribosomal S6 kinase 1
SAM	S-adenosyl-methionine
SHBP	Sex hormone binding protein
STAG1	Stromal Antigen 1
T	Testosterone
TET	Ten-eleven translocation
TGD	Thymine DNA glycosylase
TGF-β	Transforming growth factor-β
TNF-α	Tumor-necrosis factor α

TRAF	TNF receptor-associated factor
UPS	Ubiquitin-proteasome system

1 Introduction

Testosterone (17β-Hydroxyandrost-4-en-3-one: $C_{19}H_{28}O_2$) is a crucial male sex hormone naturally produced from cholesterol responsible for many physiological processes in the body, such as muscle maintenance, fat distribution, and bone metabolism (Hughes et al., 2012). In addition, it is well documented that testosterone elicits a hypertrophic effect on the human skeletal muscle (Bhasin et al., 1996; Bross et al., 1998; Elashoff et al., 1991). Due to this ability to enhance athletic abilities, androgenic-anabolic steroids (AAS; a synthetic variant of testosterone) and other performance-enhancing drugs (PEDs) have been banned by the International Olympic Committee (IOC) since 1974 (Dotson & Brown, 2007). Still, some are willing to risk potential bans and social backlash in search of fame and glory.

Over the last decade, several high-profile athletes have been caught using AAS or other PEDs of the exact nature. Notably, the two former 100-meter world record holders Justin Gatlin and Tyson Gay, have tested positive for anabolic steroids. However, this is not limited to athletes competing in power-sensitive sports. For example, cyclist Alberto Contador and cross-country skier Therese Johaug have also tested positive for clenbuterol in recent times (Slater, 2012)

In accordance with World Anti-Doping Agency (WADA) policies, all of these athletes were given competition suspensions of 18 months to 4 years, dependent on the severity and intent of use. However, some argue that a lifetime ban should be implemented in the most extreme cases. The argument for this is that the advantages gained by doping are longer lasting than the suspension. Some even suggest that the benefits might be lifelong or last for decades (Egner et al., 2013). Notably, the case of Justin Gatlin has been used as an argument for longer suspensions. The former world champion returned to the sport in 2010 and performed at a surprisingly high level, despite having served multiple years of suspensions.

This proposed phenomenon of cellular memory of previous anabolic (or catabolic) stimuli, when re-encountered, is often referred to as "muscle memory" However, the term should not be confused with motor skill learning (e.g., riding a bike or kicking a ball), as it is also often referred to as "muscle memory." The first notion of this

phenomenon was observed in women who regained their fiber size and strength during six weeks of resistance retraining as quickly as the initial 20 weeks of training (Staron et al., 1991). The research that followed demonstrated similar observations with a varying population and timeline (Bickel et al., 2011; Correa et al., 2013; Ivey et al., 2000). Collectively, this data demonstrates the potential for a cellular mechanism in human skeletal muscles responsible for the rapid re-acquisition of muscle strength and mass. However, the distinct mechanism is still to this day unclear. Over the last decade, data from animal studies have attributed this to myonuclei retentions (Bruusgaard et al., 2010), while data from human studies suggests that it originates at an epigenetic level (Seaborne et al., 2018).

A better understanding of the mechanisms involved in the observed muscle memory could provide a better foundation for sentencing in future doping cases. Also, it could aid in the rehabilitation of sarcopenia related to age, injuries, or sickness in a clinical context.

2 Theory

This chapter will provide the theoretical framework for this thesis. It will include the topics of testosterone, how it is synthesized, and its role in the human skeletal muscles. Further, it will expand on the current literature regarding the phenomenon of 'muscle memory.' Lastly, it will provide the mechanisms of epigenetics and its link to muscle memory.

2.1 The history and use of androgenic-anabolic steroids

The role of testosterone in the human body has been acknowledged since ancient times. Practical use of this knowledge was used by the Romans, who castrated young males to create obedient servants or for the preservation of their prepubertal soprano voice. Furthermore, ancient Greek athletes allegedly used testicular extract as performance-enhancing drugs, foreshadowing future applications and theories (Fink et al., 2019; Nieschlag & Nieschlag, 2019).

In the modern era, acclaimed endocrinologist and scientist Charles Edouard Brown-Séquard (1817-1894) injected himself with testicular substances extracted from guinea pigs and dogs, the result of which was published in 1889. The 72-year-old reported increased strength, better appetite, and higher arc and length of this urine stream. This mixture became known as the 'elixir of Brown-Séquard.' However, fellow scientists were skeptical of this effect due to the low sample (n=1) and lack of controls. (Dotson & Brown, 2007). The first sex hormone was not isolated until 1928 when the German scientist Adolf Butenandt (1903–1995) isolated 15 mg of androsterone from 15,000 L of urine from local policemen (Dotson & Brown, 2007)

It was not until the 1930s that the term «testosterone» *("testo"* = testes, *"ster"* = sterole, *"one"* =keton) was coined when researchers isolated 10 mg of testosterone from 100 kg of bull testes (Nieschlag & Nieschlag, 2019). This finding and the reachers that followed paved the way for developing and using synthetic variants of testosterone. During the 1940s and 1950s, bodybuilders and athletes started experimenting with testosterone preparations combined with training regimes. This led to nations like the USSR and East Germany systematically doping their athletes before representing their respective countries on the international stage (Hoberman & Yesalis, 1995). During the

1970s, the use of testosterone and testosterone derivatives was banned by the IOC, and anti-doping testing programs were developed to suppress the use of steroids in sports. Today, possession or sale of steroids without a valid prescription is illegal in countries like the USA, Canada, the UK, and Norway (Piacentino et al., 2015).

2.1.1 Use of AAS amongst elite athletes

Due to the illegal nature of doping, the prevalence of doping in elite sports is unknown, and few studies have addressed this. However, based on surveys utilizing the Randomized Response Technique (RRT), it is believed that the prevalence of steroid abuse dramatically exceeds the number provided by doping controls estimated to be 1-2% annually (de Hon et al., 2015). For instance, Striegel et al. (2010) questioned 1606 German elite athletes, who were members of their respective national teams, across 43 sports regarding doping. 6.8% of the athletes confessed to having practiced doping previously, but >1% of the tests performed in the same period by the German Ani-Doping Agency were positive for illegal substances. This supports the notion that the actual prevalence of doping in elite sports is undermined in official doping control numbers. de Hon et al. (2015) even suggests that the actual number is between 14-39% of all elite athletes, with variation between sub-groups of athletes.

2.1.2 Use of AAS amongst non-athletes

Empirical data suggests that the majority of AAS users are not competitive athletes in elite or amateur sports but rather recreational users who want to "enhance" their appearance (Christou et al., 2017). It is estimated that 2.9% - 4% of Americans have tried anabolic steroids at some point in their lives (Pope et al., 2014). On a global basis, this number is estimated to be at 3.3%, with a prevalence rate of 6.4% for males and 1.6% for females (Sagoe et al., 2014). Furthermore, this trend is reportedly increasing in the UK (Mullen et al., 2020).

In general, the normal testosterone level range in adult males is ≈ 9.8 to 32.9 nmol, with peak levels in their mid-to-early 20s and slowly declining (Bhasin et al., 2010). However, testosterone levels are elevated post-resistance training but do not exceed 25 nmol in young males (Kraemer et al., 1999). However, a survey from 500 AAS users in the bodybuilding community showed that 50% of the users used weekly AAS dosed in excess of 1000 mg (Parkinson & Evans, 2006). These numbers demonstrate that the

amount of AAS used by AAS users greatly exceeds the physiological testosterone levels from *in-vivo* synthesis or evoked by resistance training.

2.2 Testosterone synthesis in-vivo

In humans, testosterone (T) is mainly produced in the Leydig cells located in the testes of males. Additionally, smaller amounts are secreted from the adrenal cortex and ovaries in females (Widmaier et al., 2016). The regulation of T production in the gonads is orchestrated via the hypothalamic-pituitary-gonadal axis (Stamatiades & Kaiser, 2018). The process of T production is initiated in the hypothalamus (Fig. 1a). Motor pulses from the central nervous system (CNS) lead to the secretion of Gonadotropin-releasing hormone (GnRH). The GnRH will, in turn, be secreted into the hypophyseal portal circulation and act on the anterior pituitary gland. In the anterior pituitary, GnRH will activate the production of Luteinizing hormone (LH) and Follicle-stimulating hormone (FSH) that will circulate via the bloodstream to the gonads (Hughes et al., 2012; Stamatiades & Kaiser, 2018)

Once at the gonads, LH binds to the LH receptor located on the Leydig cells in men and theca calls in women. These G-coupled receptors initiate a cascade of events that include converting cholesterol to pregnenolone by the enzyme *P450scc*. This is the first rate-limiting step in steroidogenesis. The production of pregnenolone is followed by a series of reactions via the $\Delta 4$ or $\Delta 5$ pathways converting pregnenolone to T (Fig. 1b). From there, T diffuses into the peripheral circulation, where the majority of T is bound to the sex hormone-binding protein (SHBP) or albumin. While the bond between T and SHBP is relatively strong, the bond between T and albumin is week. This strong bond between SHBP and T prevents T from entering cells. Therefore the T bound to albumin plus the T free fraction is termed as bioavailable T (Chin & Ima-Nirwana, 2012).

This process of T production is regulated by negative feedback, as T levels in the blood circulation suppress the secretion of GnRH, thereby regulating the production of T. In addition, other androgens are produced in the adrenal cortex of males and females. However, these are much less potent and can not maintain male reproductive function independently (Widmaier et al., 2011).

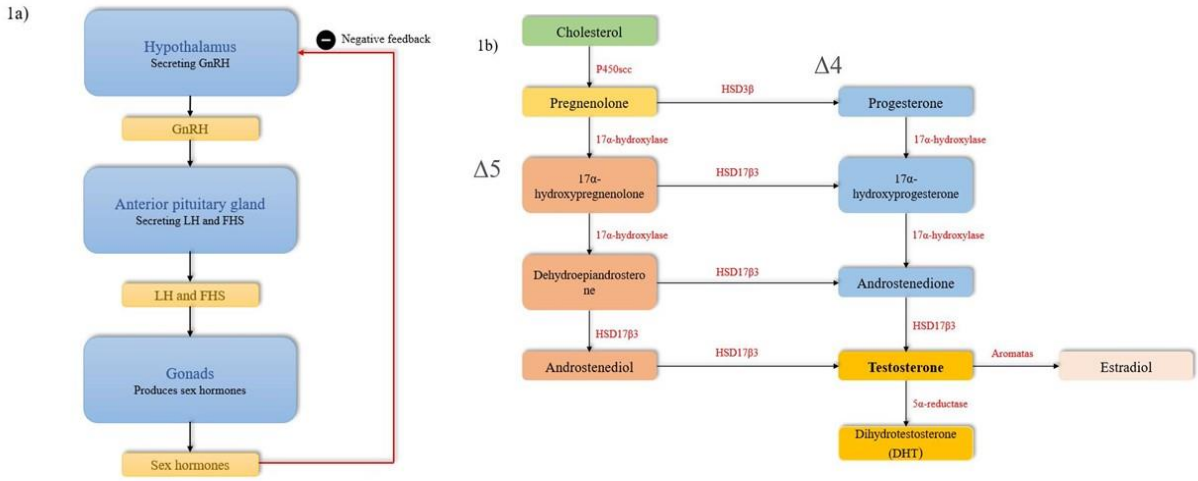

Figure 1: a) The hypothalamic-pituitary-gonadal axis initiating the production of testosterone. Figure adapted from Hughes et al. (2012). b) The conversion of cholesterol to testosterone via the Δ4 or Δ5 pathways. Enzymes responsible for each conversion marked in red. Figure adapted from Ayaz and Howlett (2015).

2.3 Androgens in peripheral tissues

Although most androgens are produced in the gonads, androgens can also be synthesized in other peripheral tissues. For example, adrenals can produce dehydroepiandrosterone (DHEA), an sex steroid precursor converted into androstenedione (Sato & Iemitsu, 2018). In the skeletal muscle, the enzyme 5α-reductase converts T to the more potent androgen dihydrotestosterone (DHT), with a higher affinity to androgen receptors within the cell (Hughes et al., 2012). Furthermore, T is metabolized to Estradiol in bone tissue via the enzyme aromatase (Mohamad et al., 2016). This aromatization is vital for bone maturation as cases of mutations of estrogen receptors (ER) or the aromatase gene (CYP19A1) have resulted in severe osteoporosis in men (Carani et al., 1997).

2.4 Testosterone in the skeletal muscle

Testosterone elicits a hypertrophic effect on skeletal muscle through a process of protein synthesis, reduced protein degradation, and reutilization of amino acids that culminate in increased fat-free mass and muscle strength (Bhasin et al., 1996). However, the

molecular mechanisms of which T elicits its anabolic effect on skeletal muscle is, despite the utilization of cell culture, animal- and human models, still inconclusive (Hughes et al., 2012).

2.4.1 Genomic actions of testosterone

The two main pathways to a hypertrophic response of T are through the androgen receptor (AR) mediate and independent pathways (Fig. 2). The AR, a nuclear and steroid receptor superfamily member, is primarily located in the cytoplasm associated with heat shock proteins (HSP), cytoskeletal proteins, and other chaperones. Once testosterone is bound to the AR, the AR undergoes a conformational change dislocating it from the HSP complex, enabling AR to interact with co-activators such as ARA70, filament A, and importin-α, leading to a translocation of the AR to the nucleus (Bennett et al., 2010). Once in the nucleus, the AR interacts with androgen-responsive elements (ARE), allowing transcription of genes such as insulin-like growth factor 1 (IGF-1), myoblast determination protein 1 (MyoD), and myosin heavy chains (MYHC) (Bennett et al., 2010; Hughes et al., 2012). However, T also exerts a non-genomic effect through growth factors such as IGF-1, keratinocyte growth factor (KGF), and epidermal growth factor (EGF) independent of androgens via mitogen-activated protein kinases (MAPK) and PI3K/Akt pathways. This non-genomic signaling is characterized by speed, with response time being seconds and minutes (Bennett et al., 2010).

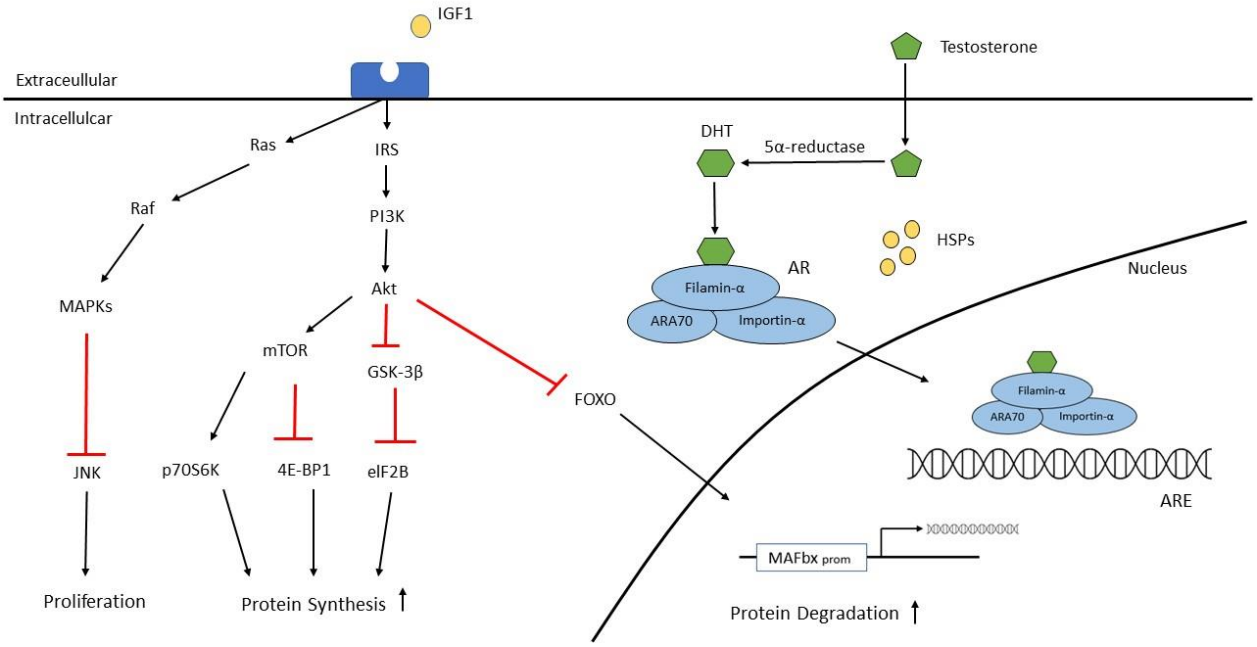

Figure 2: The potential pathways testosterone might exert its anabolic effect on skeletal muscle cells. The direct pathway via the AR on the right and the indirect path through IGF-1 and its downstream effectors on the left. Figure adapted from Bennett. et al. (2010).

2.4.2 Testosterone effect on signaling pathways

Although cells are surrounded by a membrane separating their internal environment from their outer surroundings, they can respond to extracellular stimuli. This cascade is initiated by extracellular ligands interacting with a specific transmembranal receptor, which subsequently initiates a signaling cascade involving a complex number of proteins (Shaul & Seger, 2007). This section will look at some of these signaling pathways and how T alters their behavior.

2.4.2.1 Testosterones and IGF-1/mTOR/Akt crosstalk

The binding of the hormone IGF-1 to its receptor initiates a cascade of signaling involving mediators regulating protein synthesis and degradation (Fig. 2). It starts with the phosphorylation of the phosphoinositide 3-kinase (PI3K). This will further phosphorylate and activate Ser/Thr Akt kinase (Akt, also known as Protein Kinase B) and the subsequent mammalian target of rapamycin (mTOR) (Dubois et al., 2012; White et al., 2013). Through mTOR, protein synthesis can be initiated its downstream effectors, the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and

the p70 ribosomal S6 kinase 1 (S6K1). The phosphorylation of S6K1 can regulate further processes by phosphorylation of other downstream effectors such as eEF2K (translational elongation), rpS6 (cell growth), and SKAR (mRNA processing) (Magnuson et al., 2011). In addition, the phosphorylation of 4E-BP1 prevents its binding to elF4E, allowing it to initiate cap-dependent translation (Laplante & Sabatini, 2009). The glycogen synthase kinase 3β (GSK-3β), a downstream substrate of Akt, can also contribute to protein synthesis. Once Akt is phosphorylated, GSk-3β also becomes phosphorylated and inhibited. This inhibition leads to the activation of eukaryotic translation factor eIF-2B and further protein synthesis (Hughes et al., 2012). In addition to enhancing protein synthesis, Akt can also inhibit protein degradation by inhibiting the phosphorylation of the forkhead box O family (FOXO), transcription factors strongly associated with muscle atrophy (White et al., 2013). If unphosphorylated, the FOXO proteins translocate from the cytosol to the nucleus and promote atrophy genes such as muscle RING-finger protein-1 (MuRF1) and muscle atrophy F-box (MAFbx, also known as Atrogin-1).

Some studies have indicated an association between the IGF-1/mTOR/Akt pathway, and T. White et al. (2013) demonstrated a reduction in phosphorylation of S6K1(42%) and 4E-BP1 (59%)in T depleted (castrated at week 8) mice, compared with sham control. Also, the gene expression of MAFbx mRNA and MuRF1 mRNA increased by 70% ($p=0.02$) and 85% ($p=0.001$), respectively. The same study also treated cultured C_2C_{12} myoblasts with incremental doses of T. Their results demonstrated that the mTOR and its downstream targets were sensitive to T administrations as low as 5nM.T's effect on the IGF-1/mTOR/Akt pathway can be explained by the synergetic relationship between IGF-1 and T. However; studies indicate that IGF-1 is not the predominant pathway for mediating Ts anabolic effects (Serra et al., 2011). Indeed, Hughes et al. (2015) demonstrated this by blocking IGF-1R and AR in the presence of exogenous T. Blocking of the IGF-1R, using picropodophyllin (PPP), cells significantly decreased myotube numbers compared with DM control after 72h of incubation. However, PPP co-incubated with T induced myotube hypertrophy demonstrated with increases in myotube numbers and mean nuclei pr, myotube. Furthermore, cells treated with AR inhibitors (flutamide) showed inhibition of Ts hypertrophic effect, suggesting that AR plays a more dominant role in myotube hypertrophy induced by T, compared with IGF-1R (Hughes et al., 2016)

2.4.2.2 Testosterone and MAPK/Notch signaling crosstalk

MAPK's are a family of serine/threonine kinases which IGF-1 can also activate. The MAPK family includes the Jun HN2-terminal kinases (JNKs), the extracellular signal-reduced kinases (ERKs), and the p38 MAP kinases (MAPKs). ERK1/2, members of the ERK subfamily, are activated during growth stimuli and involved in meiosis, mitosis, and post-mitotic function (Johnson & Lapadat, 2002).

The JNK signaling pathway is considered a critical component of apoptotic signaling in the skeletal muscle during aging. Mouse models have demonstrated that, when supplemented with T, p38 is triggered, and JNK is inhibited (Brown et al., 2009). Therefore, by suppressing JNK, testosterone can promote cell proliferation and subsequent muscle growth (Kovacheva et al., 2010). Furthermore, T-induced fiber hypotrophy observed in Brown et al. (2009) was accompanied by increased activation of Notch signaling, as evident by an increase in the active Notch 1 and Notch 2. This was subsequently followed by increased proliferating cell nuclear antigen (PCNA) levels, supporting the notion that Notch signal activation enhances proliferation. Indeed, similar results were found when treating older adults with 300 mg T doses (Sinha-Hikim et al., 2006).

2.4.2.3 Testosterone and TGF-β/myostatin crosstalk

Testosterones anabolic effect positive regulators of muscle protein synthesis (MPS) are one aspect of T's elicit effect in the skeletal muscle. However, T also affects negative regulators of MPS leading to reduced protein degradation, growth inhibition, and cell apoptosis (Hughes et al., 2012).

Myostatin, a member of the transforming growth factor-β (TGF-β) superfamily, is a potent negative regulator of muscle growth. Numerous studies have demonstrated this in human and animal studies *in-vivo* and *in-vitro* (McFarlane et al., 2006; Rodriguez et al., 2014). Myostatin exerts this catabolic effect by suppressing the Akt/mTOR signaling pathway (Suryawan et al., 2006), reduce the translation efficiency of muscle hypertrophy genes (Rodriguez et al., 2011), and up-regulate mediators of the ubiquitin-proteasome system (Seiliez et al., 2013). Furthermore, myostatin also suppresses muscle development by inhibiting cell proliferation (McCroskery et al., 2003).

Testosterone-deprived adult rats demonstrated muscle atrophy and significantly up-regulated myostatin proteins, while the subsequent T-treatment reduced myostatin levels back to normal levels (Mendler et al., 2007). Testosterone exerts this effect on myogenin via AR/βCatenin crosstalk and up-regulating the myogenin antagonist, follistatin (Braga et al., 2012).

2.4.3 Testosterone effect on satellite cells

Muscle growth induced by overload activates satellite cells embedded in the basal lamina (but outside the sarcolemma). These satellite cells will proliferate and migrate to the damaged site, at which point they are referred to as myoblasts (Hughes et al., 2012). These myoblasts eventually differentiate into post-mitotic myotubes and fuse with the myofiber, thus providing new myonuclei for the adult myofiber (Blaauw & Reggiani, 2014). Satellite cells are considered targets of androgens as elevated satellite cell numbers have been observed following T-induced hypertrophy in young and older men (Sinha-Hikim et al., 2003). More importantly, AR protein levels are also increased following T-treatment (Doumit et al., 1996), supporting this notion further. Thus, as these satellite cells undergo proliferation and differentiation, they provide new focal points for AR expression and maintain the nuclear-to-cytoplasm balance. However, it is not clear whether the observed increase of myonuclei is due to enhanced proliferation or differentiation. Doumit et al. (1996) observed a T-induced suppression in satellite cell differentiation of day 2-4 after treatment. Furthermore, data from androgen receptor knockout mice models (ARKO) demonstrated increases in Cdkn1c (a protein coupling differentiation and cell cycle exit in myoblasts), causing premature differentiation and reduced muscle mass in ARKO (MacLean et al., 2008). Based on these findings, MacLean et al. (2008) conclude that T's effect on satellite cells is mediated by maintaining myoblasts in the proliferative state, thereby delaying differentiation.

2.5 Muscle memory

Memory is a process of information learning, storage, and retrieval. In the modern view, the process is strongly associated with the field of psychology. However, gym and training studio folklore tell the tale of a 'muscle memory, that is, the re-acquisition of strength and muscle mass after muscle atrophy (Gundersen, 2016). This phenomenon was previously limited to motor learning only (Rutherford & Jones, 1986), but animal

and human models suggest that cellular mechanisms can also attribute to the observed memory (Egner et al., 2013; Seaborne et al., 2018; Sharples et al., 2016a).

2.5.1 Evidence of muscle memory in animal models

The textbook model for muscle size regulation has been that myonuclei number changes in proportion to muscle size, meaning that myonuclei are gained from satellite cells during muscle hypertrophy and subsequently eliminated during muscle atrophy via apoptosis (Gundersen, 2016). However, new evidence from animal models suggests that myonuclei are retained during atrophy. Most notably, findings by Egner et al. (2013) demonstrated that T-administrated mice had a 66% increase in the number of myonuclei and a 77% increase in fiber cross-sectional area after 14 days of T administration. Three weeks after removing T, the CSA was back to baseline, but the number of myonuclei remained elevated for three months (> 10% of the mouse lifespan). The T-administered mice were also more sensitive to the following overload exercise (31% CSA increase), likely explained by the retained myonuclei. Similar findings of nuclei retention were also found during severe denervation-induced atrophy in mice for an abundant period of the mice's lifespan (Bruusgaard et al., 2010). Furthermore, the training-detraining-retraining-model using physiological relevant resistance exercise in rats also reported no loss of nuclei during muscle atrophy during the detraining (Lee et al., 2018).

However, this shift in paradigm remains highly debated. Several animal studies have demonstrated a reduction in myonuclear numbers in atrophy models, such as denervation (Aravamudan et al., 2006) and mechanical unloading (Allen et al., 1997). Furthermore, Psilander et al. (2019) did not observe a loss of myonuclei in humans during atrophy following resistance exercise-induced hypertrophy, questioning the application of this model in humans.

2.5.2 Evidence of muscle memory from human models

To investigate the phenomenon of muscle memory in humans, several researchers have utilized the model of resistance exercise-induced hypertrophy, followed by a period of atrophy and subsequent retraining with varying timelines, subjects, parameters, and training protocols (Table 1). Based on these studies, the degree of strength preservation depends on the length of the detraining period. Some reports preserved strength after 12

weeks (Blocquiaux et al., 2020) and 32 weeks (Staron et al., 1991) of detraining, while 1-year of detraining reverses strength gains completely (Correa et al., 2016). However, these models rarely investigated the possibility of a memory mechanism residing within the muscle cells. Therefore, the observed preservation of strength could be attributed to motor learning only. Althougth, Seaborne et al. (2018) alludes to an epigenetic mechanism involved in the observed memory (discussed later in this chapter).

In-vitro studies of human tissue have also been utilized to examine this phenomenon. For example, human muscle cells have also demonstrated the ability to remember their fetal environment with lasting effects into later life. Myotubes biopsies taken from severely obese (BMI >50) individuals demonstrated metabolic abnormalities such as insulin resistance and reduced fatty acid oxidation compared to their lean counterparts. These abnormalities were apparent when satellite cells from the cultured muscle biopsies demonstrated a "memory" of their original *in-vivo* environment (Houmard et al., 2012). Similar results were found when investigating metabolic differences in physical activity levels (Green et al., 2013).

Table 1: Table of different studies investigative muscle memory in humans using the training-detraining-retraining model.

Authors	Subjects	Target muscle(s)	Intervention	Training protocol	Effect
Correa et al. (2016)	12 sedentary elderly women	Knee extensors, elbow flexors, and rectus femoris	Twelve weeks (wk) of training, 1-year detraining, 12 weeks of retraining.	Training 2x week with 48h intervals. Progressive increase of intensity and volume	↑ Muscle mass and 1RM tests following training. ↓ To baseline following detraining. No significant ↑ in retraining compared to the training period
Blocquiaux et al. (2020)	30 older men and ten controls	Knee extensors	12 wk training, 12 wk of detraining, and 12 wk of retraining	Whole-body RT program. 3x sessions pr. wk. Sets ↑ and repetition ↓ progress with increasing load.	1RM knee extension ↑ following training. A modest loss of strength and power following detraining. Retraining restored strength in 1RM knee extension, but no additional gains. 1RM dynamic tests demonstrated additional gain.
Staron et al. (1991)	Six previously trained females + seven untrained women who only participated in the retraining phase.	Lower extremities (squats, leg press, leg extension)	20 wk strength training, 32 wk detraining, and 6 wk retraining.	3x 6-8 (reps to failure) of squats, leg press, leg extensions, and leg curls 2d/wk	Initial training led to ↑ dynamic strength and hypertrophy of all three fiber types. Detraining had little effect on fiber size. Dynamic strength ↓ but not to pre-training levels. Retraining ↑

					1RM to similar levels seen post-training.
Seaborn et al. (2018)	Eight healthy, previously untrained men.	Whole-body training	7 wks of training. 7 wks of detraining and 7 wks of retaining.	Training 3d/wk (2d lower body + 1d upper body). 3-4x 10 RM (with adjusted load) reps. 90-120s between sets and 3 min between exercises.	Lean mass ↑ following training and ↓ after detraining. Even greater ↑ in lean mass following retraining compared to post-training.
Henwood & Taaffe (2008)	Thirty-eight older adults associated with loss of mobility.	Whole-body training (chest press, supported row, biceps curls, leg press, leg curls, and leg extensions)	24 wk training and 24 wk detraining. 12 wk retraining of either high-velocity training (HV) or strength training (ST).	Training 2d/wk. Min. 48h between each session. ST group: 3x8 reps of 75% 1RM (3-sec movement speed). HV group: 1 set of 45%, 65% and 75% 1RM 9-11 reps (explosive concentric movement)	1RM ↑ in leg press and chest press following training and ↓ post-detraining. Retraining ↑ 1RM back to post-training, but no further gains.
Psilander et al.. (2019)	Ten women and nine men. All healthy, young, and untrained	Lower extremities (leg press and leg extension)	Bilateral training for one leg for 10 wk. 20 wk detraining and 5 wk unilateral retraining.	Training 3d/wk. Two sessions of 3x 10-12 1RM and one session of 3x 5-7 80-85% 1RM. 1-2 min rest between sets and 3-4min between each exercise.	CSA, muscle thickness (MT), and strength ↑ in trained-leg following training MT and CSA ↓ during detraining, but strength remained elevated. MT and strength ↑ during retraining. CSA, fiber volume, and the myonuclear number was unaffected
Bickel et al. (2011)	Seven young adults and nine older adults	Lower extremities (knee extension, squat, and leg press)	16 wk training and 16 wk detraining.	Training 3d/wk. 3x8-12 reps at 75-80% 1RM. 90s rest between sets and exercises.	Lean leg mass ↑ post-training and ↓ to baseline after 8 wk of DT. Strength ↓ 7% after 8 wk DT, but 23% ↑ baseline.

2.6 Epigenetics

One of the most recent chapters in the short history of genetics is epigenetics. Epigenetics (*epi*: on top, *genetics:* gene) refers to the study of modifications that directly affect the expression of genes but are not associated with changes in the DNA sequence (Gayon, 2016). The mechanisms behind epigenetic regulation of gene expression usually involve methylation of the DNA or histone modification to alter the chromatin structure, but also include expression of non-coding RNA (ncRNA), e.g., micro-RNA (miRNA). These epigenetic changes can be quite stable and can be passed on to daughter cells, which means that a pre-existing epigenetic signature can be passed on to an offspring and determine how that individual reacts to a specific stimulus, e.g., exercise or nutrition. Indeed, it has been demonstrated in mammalian models that reduced nutritional availability during gestation negatively affects skeletal muscle

morphology in the offspring (Sharples et al., 2016b). This modification of epigenetics passed on to offspring is referred to as transgenerational epigenetics.

Three epigenetic mechanisms are thought to play a role in muscular adaptations: (1) DNA methylation, (2) histone modification, and (3) expression of ncRNA, mainly miRNA (Jacques et al., 2019).

2.6.1 DNA methylation

DNA methylation is a central epigenetic process that suppresses gene expression by inhibiting access of the transcription machinery to bind with the chromatin (Barres et al., 2012). The process starts with adding a methyl group from S-adenosyl-methionine (SAM) to the 5-carbon position of a cytosine base. The cytosine bases most susceptible to methylation are found in the 5' CpG' 3 ('cytosine-phosphate-guanine') nucleotides. When the CpG base pairs are greater than 200, and the CpG% is greater than 50%, they are referred to as CpG islands. They are often found in promoter regions of the DNA (McGee & Hargreaves, 2019).

The conversion of cytosine to its methylated form, 5-methylcytosine (5meC), is initiated by the enzyme family, DNA methyltransferase (DNMTs). DNMT1 is the primary "maintenance" methyl enzyme. It can identify hemimethylated DNA (duplex DNA in which the only one of the two strands are methylated) and copy the pattern to the daughter strand. The DNMT3a and DNMT3b are classified as "de novo" transferases as they are vital for the DNA methylation patterns. Although the DMNTs are classified as "maintenance" and "de novo," they are not absolute, and they serve the same roles to some extent (Meier & Recillas-Targa, 2017).

The process of methylation is not static and can be reversed actively or passively. The active DNA demethylation is an enzymic process that removes the methyl group of 5meC by breaking the carbon bond. By contrast, passive DNA methylation is inhibition or absence of DNMT1 during successive rounds of DNA replication. While the mechanisms behind passive DNA demethylation are broadly understood and accepted, the mechanisms behind active DNA demethylation are more controversial due to the numerous enzymes and pathways involved (Kohli & Zhang, 2013). The simplest way to achieve DNA demethylation is through the removal of the methyl group in 5mC

enzymatically. However, this would require an enzyme so catalytically powerful that it would be thermodynamically unfavorable (Wu & Zhang, 2010).

The identification of the Ten-eleven translocation (TET) enzymes unveiled that active DNA demethylation can be initiated by nucleotide excision repair. The TET enzyme family (TET1, TET2, and TET3) catalyze the conversion of 5meC to 5-hydroxymethylcytosine (5hmC) by oxidative demethylation (Meier & Recillas-Targa, 2017; Wu & Zhang, 2010). Further oxidation of 5hmC by the TET enzymes leads to intermediate products such as 5-formylcytosine (5fC) and 5-caboxylcytosine (5caC), resulting in the removal and replacement of "new" cytosine via thymine DNA glycosylase (TDG) mediated base excision repair (BER) (Fig.3) However, this process is one of several proposed mechanisms by which DNA demethylation can occur (Wu & Zhang, 2010).

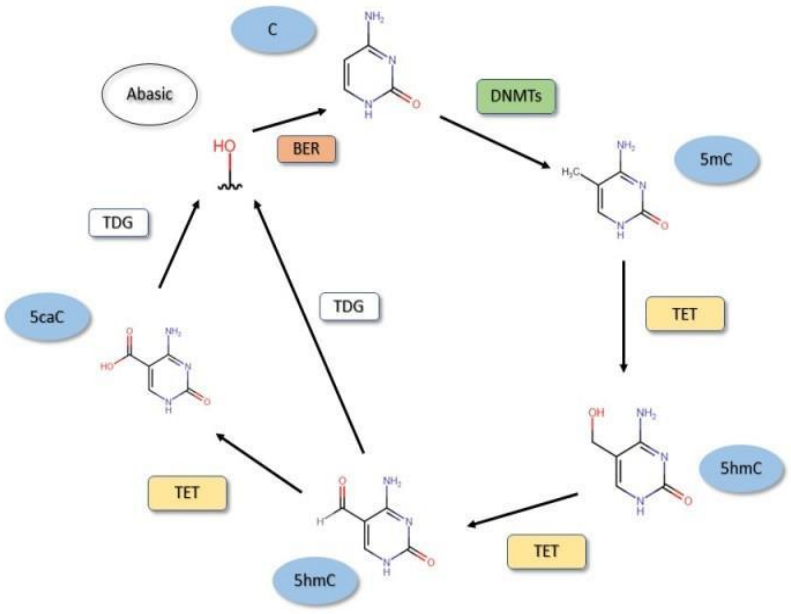

Figure 3: Cycle of active DNA methylation and demethylation. Figure inspired by Xu and Walsh (2014).

2.6.2 Histone modification

Chromatin, a complex 3D structure, is folded to allow extensive lengths of DNA to be packaged in the nucleus. This tightly packed chromatin will inhibit the transcriptional machinery and transcription factors' access to the promotors and gene regions from

initiating transcription in a repressed state. However, modifications of the histone terminal (N) tail (acetylation, phosphorylation, methylation, and ubiquitylation) can alter the permissive state of the chromatin, thus regulating gene expression (Bannister & Kouzarides, 2011; Jacques et al., 2019; McGee & Hargreaves, 2019). The process of methylation, phosphorylation, and acetylation is regulated by the balance of enzymes that either deposit the histone mark, called "writers," and enzymes that remove histone modification, called "erasers" (Gates et al., 2017). These modifications' subsequent effect depends on histone protein, amino acid residue, and type/level of modification (Seaborne & Sharples, 2020). For example, methylation on H3 lysine 4 is associated with histone acetylation and subsequent transcriptional activation, while methylation on H3K36 is associated with transcriptional repression (McGee & Hargreaves, 2011).

2.6.3 MicroRNAs

miRNAs are small non-coding molecules that silence messenger RNAs (mRNA) in a post-translational manner. In mammals, it is predicted that the most conserved miRNAs regulate 50% of the gene protein-coding gene (Polakovičová et al., 2016). To date, over 2500 miRNAs have been identified in humans, with most of them associated with cancer, cardiovascular disease, diabetes, inflammation, and neurological disorder. However, miRNAs have also emerged in control over metabolic pathways (Poy et al., 2007). Interestingly, miRNAs have also been identified as intracellular modulators of mitochondrial metabolism, muscle recovery, and muscle atrophy. For example, miR-1 is up-regulated during induced muscle atrophy and targets HSP70, upregulation of MuRF1, Atriogin-1, and miR-133 is associated with enhanced myoblast proliferation (Sharma et al., 2014; Wei et al., 2017).

The cellular biogenesis of miRNA is a multi-step process that starts with transcription from a specific sequence of DNA by the RNA polymerase II, resulting in a primary miRNA (pri-miRNA). The pri-miRNA is recognized by the protein Drosha-DiGeorge syndrome critical region 8 (DGCR8). The enzyme Drosha associated with DGCR8 to form the microprocessor complex. This complex cuts the pri-miRNA to a smaller pre-miRNA, 60-70 nucleotides long and shaped like a hairpin. The pre-miRNA is then transported out of the nucleus, via pores, by the transport protein exportin 5, into the cytoplasm. In the cytoplasm, the pre-miRNA is further cleaved by the Dicer enzyme and binds to the Argonaute-2 protein (AGO), forming the RNA-induced silencing

complex (RISC). Finally, the RISC complex will reach the specific target via complementary base pairing and gene silence by different mechanisms, degradation of target mRNA, and inhibition of translation by blocking the ribosome subunit from binding (Silva et al., 2017; Widmann et al., 2019).

2.6.4 Epigenetic memory

To the author's knowledge, only a handful of studies have investigated the concept of epigenetic memory. Sharples et al. (2016a) demonstrated that C_2C_{12} myoblasts retain an 'epi-memory' of early life tumor-necrosis factor α (TNF-α) exposure following 30 population doublings in the absence of TNF-α and subsequent low serum media in absence or presence of TNF-α. More specifically, the cells that displayed hypermethylation patterns during the early life TNF-α showed the same pattern following the 30 population doublings.

Also, Maples et al. (2015) investigated the DNA methylation signatures of severely obese women as the underpinning mechanism to their impaired ability to increase gene expression related to fatty acid oxidation upon lipide exposure. Human skeletal muscle cell biopsies demonstrated, when exposed to lipid oversupply, increased DNA methylation of peroxisome proliferator-activated receptor δ (PPARδ) corresponding with gene suppression in the severe obese vs. lean controls. This finding implies that the cell's epigenetic signature was retained when isolated from their metabolic environment and isolated in culture (Maples et al., 2015).

However, one of the most recent studies by Seaborne et al. (2018) investigated genome-wide DNA methylation patterns after seven weeks of resistance training, followed by seven weeks of cessation of resistance exercise (detraining) and subsequently, seven weeks of retraining. They found many of the CpG sites to be significantly modified following the initial seven weeks of training, with a large number being hypomethylated. Interestingly, these hypomethylations were retained during the detraining period where muscle mass returned to baseline. The initial hypomethylation led to an enhanced gene expression that also persisted during the detraining period. Some modified genes demonstrated an even greater hypomethylation and gene expression during the subsequent retraining period because of the initial modification during the training and detraining period. The second trend observed in this study was

that following the initial CpG modification during the training period, the hypomethylation and gene expression returned baseline during the detraining period. However, during the retraining period, hypomethylation and gene expression respond to an even greater extent than the initial training period. Of all the genes analyzed, the E3 ubiquitin ligase UBR5 demonstrated this distinct inverted relationship between hypomethylation and gene expression during all the phases most clearly. This maintenance of hypomethylation and subsequent increase during the retraining period suggests that the muscle "remember" the epigenetic modifications, demonstrating an 'epi-memory' from previous bouts of hypertrophy.

2.6.5 UBR5

UBR5 is a relatively unstudied HECT (homologous to E6-AP carboxy-terminus) domain E3 ligase. The E3 ligase is a component of the ubiquitin-proteasome system (UPS), along with E1 and E2 enzymes. The UPS mediates the intracellular proteolysis and is an established pathway, pivotal in the increased protein degradation seen in muscle atrophy (Cao et al., 2005). Furthermore, the UPS works in such a way that ubiquitin tags proteins, marking them for "destruction." The roles of the E1, E2, and E3 enzymes are to activate (E1), carry (E2), and couple the activated ubiquitin to the protein substrate (E3) and subsequent degradation on the 26S proteasome. (Cao et al., 2005).

In general, the E3 ligases fall into two categories: HECT domains or RING (Really Interesting New Gene) finger domain. Most of the known E3 ligases to date fall under the RING finger category. Among these are the well-characterized MuRF1 and MAFbx ligases strongly associated with muscle atrophy (Seaborne et al., 2019). However, the results from Seaborne et al. (2018) demonstrated that UBR5 might be related to muscle recovery. Indeed, this was further investigated in a follow-up study by the same group investigating the DNA methylation pattern and gene expression during muscle remodeling and muscle hypertrophy. The latter was investigated using acute loading of bioengineered 3D mouse muscle *in-vitro* and chronic intermittent electrical stimulation in rats *in-vivo.* Results from these experiments showed that UBR5 hypomethylation occurred during chronic stimuli but not acute loading. However, gene expression was elevated during both conditions. Results from hindlimb suspension induced-atrophy *in-vivo* and tetrodotoxin-induced atrophy *in-vivo* showed increased UBR5 gene expression

in the early recovery phases. This hypothesis of UBR5 involvement in anabolism is now confirmed as silencing of UBR5 *in-vivo* leads to muscle atrophy and disturbances in critical protein synthetic signaling (Hughes et al., 2021).

2.7 Aim of this study

Seaborne et al. (2018) demonstrated that specific genes possessed the memory of anabolic stimuli evoked by resistance exercise (RE). However, these genes have not been studied using T to mediate this anabolic effect. Therefore, his study aimed to investigate the potential "muscle memory" by dosing supraphysiological doses of exogenous T on C_2C_{12} myotubes during the early and/or late stages of differentiation, respectively. Morphological analysis and gene expression analysis was used to measure the effectiveness of the testosterone dosing. The genes selected for analysis were chosen due to their involvement in muscle differentiation and muscle hypertrophy or their association with "muscle memory" (Seaborne et al., 2018).

2.7.1 Scientific questions

Based on the aims of this study, these scientific questions were formed:

I) Do C_2C_{12} myotubes retain a morphological muscle memory of repeated testosterone dosing at early and late stages of differentiation?

II) Do the "muscle memory genes" observed in Seaborne et al. (2018) retain memory when subjected to repeated testosterone at early and late stages of differentiation?

We hypothesize that the myotubes would retain a memory of their previous bout of dosing, enabling a greater hypertrophic upon the second dose. These changes would, in turn, be reflected in the gene expression and morphological analysis.

3 Methods and materials

All research and experiments took place at The Norwegian School of Sport Sciences at the Department of Physical Performance at the Cell Biology & Biochemistry Laboratory during spring 2021.

3.1 General cell culture

C_2C_{12} murine myoblasts (P12; Sigma-Aldrich, ECACC, UK) were brought up from liquid nitrogen, defrosted at room temperature, and subsequently grown in T75 flasks (Thermo Fischer Scientific, USA) that had been pre-gelatinized (0,2% Gelatin; Merck Millipore, USA) for a minimum of 10 min. The flasks were kept in an incubator at 37˚C and 5% CO_2 atmosphere and seeded at 1 x 10^6 with 13 ml of growth media (GM), composed of DMEM (Gibco, Waltham, MA; USA), 10% heat-inactivated fetal bovine serum (HI-FBS; Gibco, USA), 10% heat-inactivated Newborn Calf Serum (HI-NBCS; Gibco, USA), 1% Penicillin-Streptomycin (PenStep; Gibco, USA) and 1% L-glutamine (Gibco, USA). The cells were incubated for 48-72h until ~80% confluence was reached. Then, the media was removed, and the cells were washed with phosphate-buffered saline (PBS, HyClone, UK) twice and trypsinized (Gibco, Waltham, MA, USA) with 1,5 ml of trypsin. The trypsin was neutralized by the addition of 3 ml of GM. The following content was removed from the T75 flasks and homogenized with a 19G 10 ml plastic syringe (Fischer Scientific, USA) to avoid cell clumping. The cell suspension was counted using 10 µl trypan blue solution (Gibco, USA). The cell suspension was diluted 1:1 and pipetted into a hemocytometer. The 8 grids were counted, and the average was used in the following equation:

$$(average\ of\ 8\ grids\ \times 2)\ \times\ 10^4\ cells\ pr.ml^{-1}$$

After the cells were counted, they were transferred to pre-gelatinized 6-well plates at a concentration of 70 000 cells per ml. in 2 ml GM per well and incubated in 37˚C and 5% CO_2 atmosphere for 24-48h until confluent. Once confluent, the media was aspirated, the cells were washed twice with PBS, and differentiation media (DM) was added. DM was composed of DMEM, 2 % horse serum (Gibco, Waltham, MA, USA), 1% PenStrep (Gibco, USA), and 1% L-Glutamine (Gibco, USA). This time point was noted as 0h and marked the beginning of the experiment.

3.2 Testosterone dilution

7,2 mg of testosterone (Tokyo Chemical Industry, Oxford, UK) were diluted in 5 ml of DMSO (Fischer BioReagents, Pittsburgh, PA) and aliquoted in 5 mmol stocks. 1 µm of these 5 mmol stocks were diluted in 10 ml of fresh DM, creating a stock solution. The stock solution was diluted at 1:5 with fresh DM, creating a working solution of 100 nmol. 2 ml of working stock was applied to the cells for dosing.

3.3 Cell treatment

There was a total of 4 conditions during the experiment. Early testosterone (ET); exposure to testosterone on day 3 for 240 min (4 hours), and fresh DM was added on day 7 and left to incubate till day 10. The late testosterone condition (LT) received fresh DM at day 3 and testosterone at day 7 and left to incubate till day 10. ET+LT treated cells were exposed to testosterone for 240 min on day 3 and testosterone for 30 min on day 7 and left to incubate till day 10. On day 10, all remaining wells were RNA isolated and fixed (Fig. 4). DM control only received DM throughout the experiment. All cells were washed with PBS twice before receiving testosterone dosing or fresh DM at all timepoints. DMSO was used as the solvent for the testosterone at a concentration of 0.01%. DMSO in such a small concentration would not affect the differentiation as trail runs have demonstrated (data not shown). RNA isolation and cell fixation were done for all the conditions and all the timepoints.

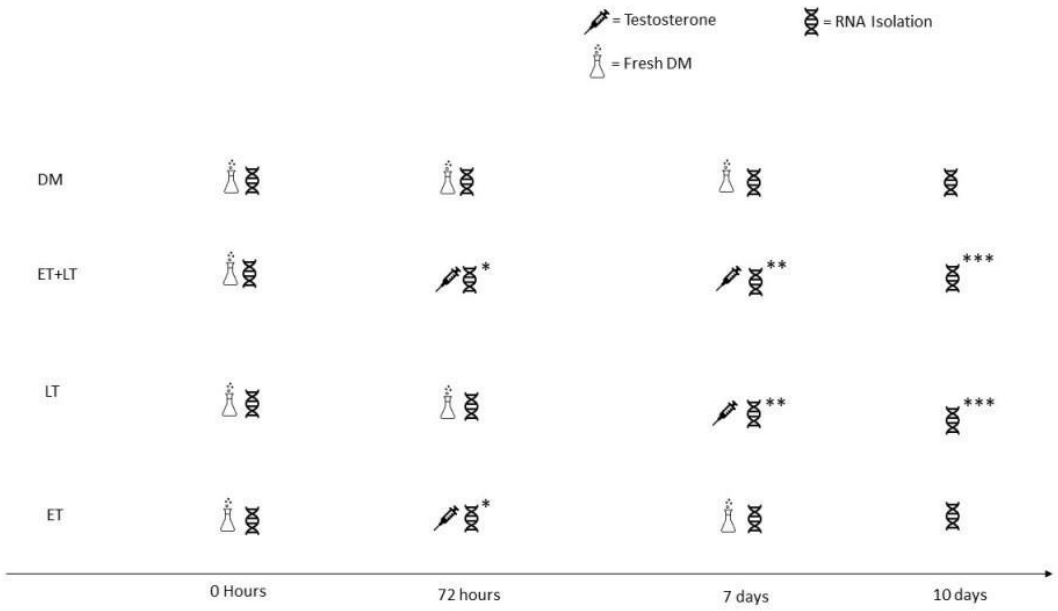

*Figure 4: A schematic view of the experiment demonstrating treatment for each time point and condition. *cells T-dosed for 4 hours before isolation **cells T-dosed for 30 min before isolation ***cells T-dosed for 3 days before isolation.*

3.4 RNA treatment

3.4.1 Cell extraction

Media was aspirated from each well before the cells were washed with PBS twice, and RNA was extracted using 250 µl Trizol Reagent (Invitrogen, Life Technologies, USA) per well. Each well was scraped with a disposable cell scraper (Fischer Scientific, USA). The content was collected into a 1,5 ml RNase tube (Eppendorf, Germany) and stored at -4˚C until further processing.

3.4.2 RNA isolation

Cells were vortexed and incubated at room temperature for 10 min. to thaw out the samples before 200 ml of chloroform per 1 ml of Trizol of the experimental sample was added and centrifuged at 12 000g for 15 min at 4˚C using a refrigerated microcentrifuge (Heraeus Fresco 17, Thermo Fischer, USA). After that, the transparent RNA layer was pipetted into a separate 1,5 ml RNase-free tube, and the rest was discarded. Then, 500 µl of isopropanol per 1 ml of Trizol was added to the sample before being vortexed, incubated at room temperature for 10 min, and centrifuged at 12 000g for 10 min at 4˚C. Following the centrifugation, an RNA pellet was visible, and the supernatant was

removed without disturbing the pellet. The RNA pellet was washed in 1 ml 75% ethanol (VWR Chemicals, USA) per 1 ml of Trizol and centrifuged at 7 500g for 8 min at 4˚C. Next, the remaining ethanol was pipetted, and the pellet was air-dried. Once dry, the pellet was re-suspended in 21 µl of DEPC H_2O.

3.4.3 RNA quantity assessment

The RNA quantity was assessed using a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies, USA). A Qubit working solution was made up of diluting Qubit RNA BR Reagent 1:200 in Qubit RNA BR Buffer. Both the RNA reagent and buffer were from the Qubit RNA BR Assay Kit (Invitrogen, Life Technologies, USA). 195 µl of the working solution were added in a Qubit tube (Thermo Fischer, USA) along with 5 µl of the RNA sample and placed in the Qubit 2.0 Fluorometer, and the concentration of the sample was recorded.

3.5 Reverse transcription-quantitative real-time polymerase chain reaction (rt-qRT-PCR)

The rt-qRT-PCR was carried out using Rotorgene 3000Q (Qiagen, UK) and facilitated via Rotogene software (Hercules, USA). The master mix solution was composed of 5 µl QuantifastTM SYBR Green, 0,075 µl Forward Primer, 0,075 µl Reverse Primer, and 0,1 µl of RT mix per experimental sample. The RNA was prediluted to a concentration of 7,37 ng/µl. The content of the final 0,01 ml Strip Tube (Qiagen, Hilden, Germany) was 4,75 µl prediluted RNA (35ng of RNA, concentration = 7.37ng/µl), and 5,25 µl Master Mix. All samples were run in duplicates. The rt-qRT-PCR was performed as follows: 10 min at 50 °C (reverse transcription), 5 min at 95 °C (transcriptase inactivation and initial denaturation) followed by: 10 s at 95 °C (denaturation), 30 s at 60 °C (annealing and extension) for 40 cycles. Following completion, a melt curve analysis was performed to ensure only genes of interest were amplified and exclude primer-dimer formation. All melt curve analyses in this study presented single peaks. Relative mRNA expression was quantified for UBR5, MYHC1, MYHC4, IGF-1, myogenin, MyoD, GRIK2, and STAG1 (Table 2) using the Ct ($^{\Delta\Delta}$ct) method (Livak & Schmittgen, 2001) against a stable reference gene RPIIB (mean Ct value = 18.17 ± 0.18) and calibrator of CON treatment at 0h. The average PCR efficiency of amplification for the genes of interest were as follows: UBR5 90.41 ± 2.9%, MYHC1 90.5 ± 3.1%, MYHC4 89 ± 4.0%, MyoD 90 ± 2.2%, Myogenin 89.9 ± 4.2%, IGF-1 91.4 ± 5.5%, GRIK2 90.3 ± 4.1%,

STAG1 91 ± 7.6%. All genes were comparable to the housekeeping gene RPIIB (90,04 ± 1.6%).

Table 2: Primers sequences (5'-3'), sequence number, and amplicon length for the genes of interest in this study.

Gene	Primer Sequence (5'-3')	Ref. sequence number	Amplicon length (bp)
RPIIB	F:GCATCAATTAAATGGAGTAGCGTC R:GGTCAGAAGGGAACTTGTGGTAT	NM_153798.1	197
UBR5	F:GTCTGCTGGAGCTCGGTGATT R:TGCTGGAATAACTGGCTGGG	NM_001081359.3	106
MYHC1	F:CGGTCGAAGTTGCATCCCTA R:TTCTGAGCCTCGATTCGCTC	NM_030679.1	149
MYHC4	F:AGGAGGCTGAGGAACAATCC R:TTCTCCTGTCACCTCTGAACA	NM_010855.3	192
Myogenin	F: CCAACTGAGATTGTCTGTC R: GGTGTTAGCCTTATGTGAAT	NM_031189	173
MyoD	F: CATTCCAACCCACAGAAC R: GGCGATAGAAGCTCCATA	NM_010866	125
IGF-1	F: GCTTGCTCACCTTTACCAGC R: TTGGGCATGTCAGTGTGG	NM_010512.5	280
GRIK2	F: CACATACAGACCCGCTGGAA R: GGTCTAAAATGGCACGGCTG	NM_001166247.1	110
STAG1	F: GCATTTTTAGCAACTTCTACCAGC R: AACTTGAATTTGGCAGGGCA	NM_005862.2	115

3.6 Morphological assessment

Following media aspiration and 2x PBS wash, cells were fixed by adding 2 ml of methanol/acetone/TBS solution (ratio: 1:1:2) per well and incubated on ice for 10 min. The solution was aspirated, and 2 ml of methanol and acetone (ratio 1:1) was added to each well and incubated on ice for a further 10 min. Following the aspiration of the solution, wells were washed with PBS, and 2 ml of PBS was added per well to prevent samples from drying out, and the plate was stored at 4˚C for further analysis.

Three images (magnification x20) were taken using a microscope camera (Moticam x3, MoticEurope, Spain) attached to an inverted microscope (Motic AE2000, MoticEurope, Spain) of each condition at each time point from each experiment (a total of 9 images)

creating a mean ± SD of myotube numbers, myotube area, and myotube diameter. Morphology was analyzed using the ImageJ software (Java software, National Institutes of Health, USA). Morphology was assessed by the number of myotubes per view, myotube area (μm^2), and myotube diameter (μm). A myotube was defined as containing 3+ nuclei encapsulated within a cellular structure. This would avoid counting any cells undergoing mitosis. Myotube area was measured using the "freehand selection" tool on ImageJ, outlining the myotube structure. Myotube diameter was calculated using the average of the myotube at 25, 50, and 75% length. Pixels were converted to μm using the scalebar attached to each image via the Motic software (Motic Images Plus 3.0, MoticEurope, Spain).

3.7 Statistical analysis

Experiments were performed in triplicate (n=3) apart from gene expression analysis for STAG1 and GRIK2 (n=2). Data are presented as mean ± SD unless stated otherwise. Statistical significance for interaction between the condition (DM, ET, LT, ET+LT) and time points (72h, 7d, and 10d) were assessed using a two-way mixed ANOVA test (GraphPad Software, Inc., USA) for the mRNA expression (Table 2) and morphology (myotube numbers, area, diameter). Tukey's post hoc tests were performed for pairwise comparisons. Statistical significance was set at $P \leq 0.05$.

4 Results

4.1 Morphological analysis

4.1.1 Myotube numbers

Examples of images collected and used for morphological analysis are displayed in figure 5. There was a significant main effect for time ($P \leq 0.0001$) on myotube numbers, demonstrating an increase in myotube number over the 72h, day 7, and day 10 time points of differentiation/myotube formation (Fig. 6a). The cells only treated with differentiation media (DM) significantly increased myotube numbers from 72h to day 7 (72h 5.22 ± 1.64 vs. 7d 8.08 ± 1.52; $P \leq 000.1$) and from day 7 to day 10 (7d vs. 10d 9.55 ± 1.94; $P \leq 0.0001$). There was also a significant main effect for condition ($P \leq 0.0001$). Cells treated with early testosterone for 4 hours showed no significant difference at 72h compared to basal differentiation (DM control 5.22 ± 1.64 vs. ET 6.33 ± 1.6; P = NS). However, this was expected as testosterone had only been applied for 4 hours before the images used for morphological analysis were taken. This dosing for 4 hours at 72h alone did not induce sufficient myotube formation at day 7 either (DM vs. ET 8.88 ± 1.02; P = NS), but the combination of early dosing and late, acute dosing for 30 min at day 7 resulted in significantly higher levels of myotube numbers (DM vs. ET+LT 8.78 ± 3.49; $P \leq 0.0001$). Surprisingly, cells only dosed late for 30 min on day 7 (LT) produced significantly higher myotube numbers than DM controls (DM vs. LT 8.44 ± 3.80; $P \leq 0.05$).

Finally, day 10 is where the effect of the first and second dosing can be assessed. On day 10, it is clear that the early dosing of testosterone at 72h was not enough to increase myotube numbers above numbers seen in the DM controls at day 10 (ET 9.33 ± 1.66 vs. DM 9.55 ± 1.94; P = NS). A single dose of late testosterone (LT) between day 7 and day 10 increased myotube number above DM conditions (DM vs. LT 10.66 ± 1.24; $P \leq 0.05$). Importantly, by administering both an early (first) dose of testosterone for 4 hours at 72h and the late (second) dose of testosterone between 7 and 10 days, was able to increase myotube number above all conditions (DM and ET) and importantly above those in LT conditions that had also received the same testosterone dose between 7 to 10 days (LT vs. ET+LT 12.88 ± 2.71; $P \leq 0.0001$).

4.1.2 Myotube area

There was a significant main effect for time ($P \leq 0.0001$) on myotube area, demonstrating an increase in myotube hypertrophy/size during basal differentiation from 72h to day 7 (72h 3422.68 ± 1744.73 μm^2 vs. 7d 6009.81 ± 2424.73 μm^2; $P \leq 0.0001$; Fig. 6b) and from day 7 to day 10 (7d vs. 10d 9082.64 ± 885.16 μm^2; $P \leq 0.0001$). There was also a main effect for condition ($P \leq 0.0001$). Like the myotube count, there were no significant difference between the testosterone dosed cells and the DM treated cells at 72h (DM 3422.68 ± 1744.73 μm^2 vs. ET 3685.91 ± 1311.21 μm^2; P = NS). This dosing for 4 hours at 72h alone did not induce sufficient myotube hypertrophy at day 7 either (DM 6009.81 ± 2424.73 μm^2 vs. ET 6140.23 ± 2137.53 μm^2: P = NS). Still, the combination of early dosing for 4 hours and late, acute dosing for 30 min at day 7 created a significantly greater myotube size than the DM-only treated cells (DM vs. ET+LT 6298.61 ± 1063.37 μm^2; $P \leq 0.0001$). As expected, the LT only condition showed no difference to DM 7 days (DM vs. LT 5904.36 ± 3512.29; P =NS) because, at this point, the late dose of testosterone (LT) had only been applied for 30 minutes.

On day 10, the early dosing for 4 hours was not enough to significantly increase myotube size (DM 9082.64 ± 885.16 μm^2 vs. ET 8906.67 ± 1549.53 μm^2; P = NS). The late, chronic dosing from day 7 to day 10 promoted none-significant myotube hypertrophy and increased myotube size (DM vs. LT 9958.33 ± 2209.23 μm^2; P = NS) at day 10. However, early dosing for 4 hours, combined with chronic, late dosing from day 7 to day 10, resulted in significant myotube hypertrophy (DM vs. ET+LT 12090.89 ± 2342.62 μm^2; $P \leq 0.0001$). More interestingly, this early dosing at 72h combined with late, chronic dosing from day 7 to day 10 resulted in a greater hypertrophic response than the cells that only received testosterone from day 7 to day 10 (ET+LT vs. LT; $P \leq 0.0001$).

4.1.3 Myotube diameter

The ANOVA analysis shows that time had a significant main effect ($P \leq 0.0001$) on myotube diameter. Cells only treated with DM increased myotube diameter

significantly from 72h to day 10 (72h 13.24 ± 5.35 µm vs. 10d 25.23 ± 10.69 µm; P ≤ 0.0001; Fig. 6c).

The condition also had a main effect on myotube diameter (P ≤ 0.0001). Following the overall trend, no significant difference between the DM treated and cells dosed for 4 hours (DM vs. ET 12.48 ± 3.89 µm; P = NS) was observed at 72h. After 7 days, the early dosing at 72h for 4 hours had a slight increase in myotube diameter. However, it did not significantly increase (DM 17.94 ± 4.48 µm vs. ET 18.43 ± 7.59; P =NS). As expected, there was no significant difference between the DM controls and the cells testosterone administered for 30 min before the morphological images were taken (DM vs. LT 17.34 ± 7.43 µm; P = NS). Nonetheless, dosing cells early at 72h and acute, late for 30 min at day 7 was enough to evoke significantly wider myotubes at this time point (DM vs. ET+LT 19.69 ± 6.12 µm; P ≤ 0.0001).

On day 10, where the hypertrophic effect of testosterone is most apparent, the early testosterone dosing at 72h (ET) was not enough to evoke a significantly greater myotube diameter (DM 25.23 ± 10.69 µm vs. ET 25.72 ± 16.22 µm). That also applies to the cells that experienced late, chronic testosterone administration between day 7 and day 10 (DM vs. LT 30.94 ± 9.32 µm; P =NS). However, the mean myotube diameter for the cells in the LT condition was wider than the ones under the DM condition and ET condition, but this number did not reach significance. The combination of early testosterone dosing at 72h and late, chronic dosing between day 7 and day 10 induced a significantly greater myotube diameter than the DM condition (DM vs. ET+LT 33.55 ± 12.73 µm; P ≤ 0.0001) but were not significantly wider compared to the ET- and LT conditions. However, despite lacking statistical power, the mean myotube diameter was still greater for ET+LT condition vs. LT condition and therefore follows the same trend seen in myotube formation and myotube size.

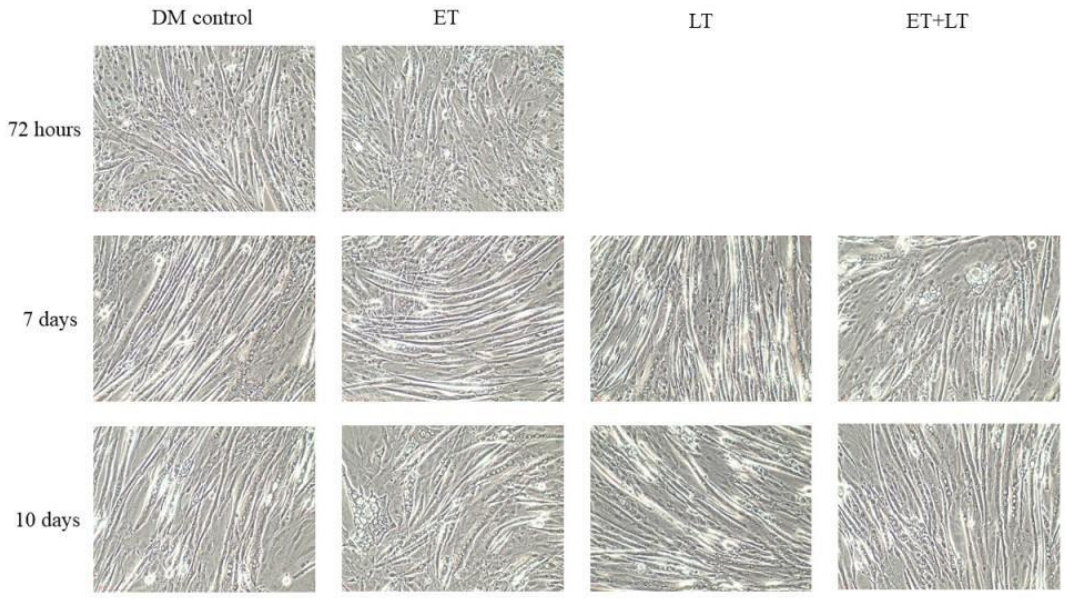

Figure 5: Examples of morphological images (magnified x20) used to analyze myotube numbers, myotube area (µm^2), and myotube diameter (µm) for the different conditions at different time points.

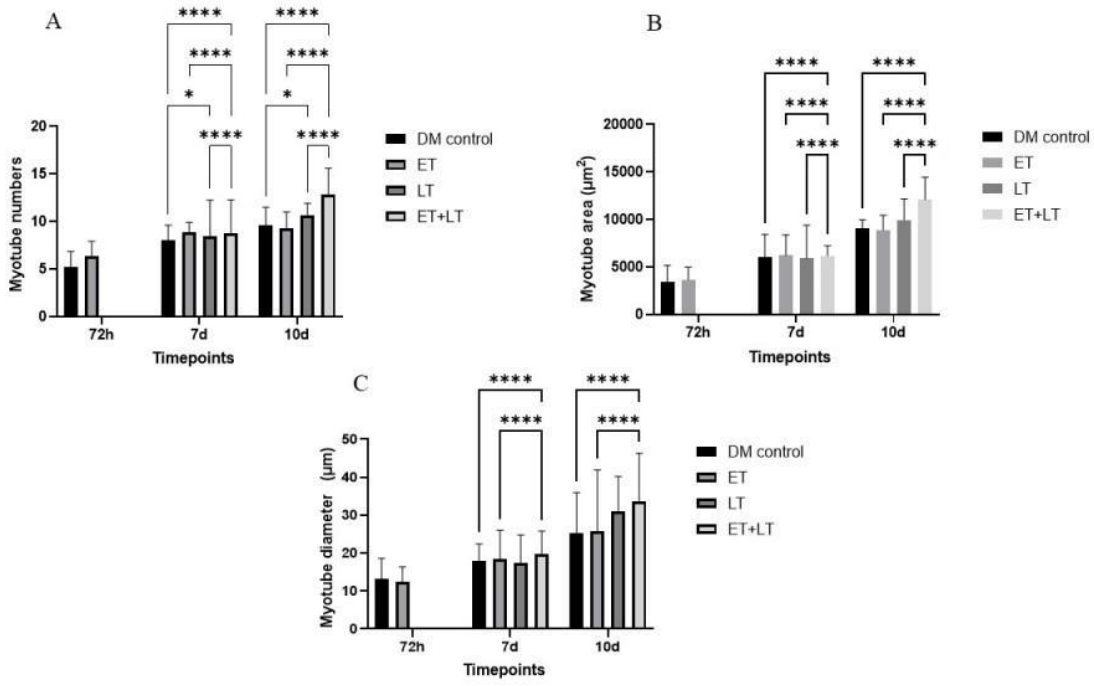

*Figure 6: a) Myotube numbers, b) Myotube area (µm^2), and c) Myotube diameter (µm) for the DM control, early testosterone (ET), late testosterone (LT), and early and late testosterone (ET+LT) at 72h, day 7 and day 10. *P ≤ 0.05, ****P ≤ 0.0001. Mean ± SD, n=3.*

4.2 Relative fold gene expression

4.2.1 Myogenin, MyoD, and IGF-1

Looking at the expression for myogenic regulatory factors (MyoD and myogenin) and IGF-I, all involved in early differentiation and myotube formation, neither time nor condition had a significant main effect.

As expected, given the morphological increase in myotube number between 72h and day 7 in DM conditions, and MyoD and myogenin being involved in early differentiation (myoD) and fusion (myogenin), the expression of these genes increased from 72hrs to day 7 (MyoD 72h 1.92 ± 0.56 vs. 7d 2.24 ± 0.46; myogenin 72h 3.44 ± 1.88 vs. 7d 11.95 ± 13.81; Fig. 7a and 7b) in DM condition and returned at day 10. IGF-1 also demonstrated average increases in expression of a large magnitude in DM conditions across the time course of differentiation/myotube formation (e.g., 7, 40, 92 expression values at 72, 7d, 10 d, respectively; Fig. 7c), which has been frequently observed before (Watanabe et al., 2020). However, due to large variation, none of these increases in gene expression were significant. Despite myotube numbers/area being largest in ET+LT at 10 days, there were also no increases/significant changes in the expression of these between dosing conditions.

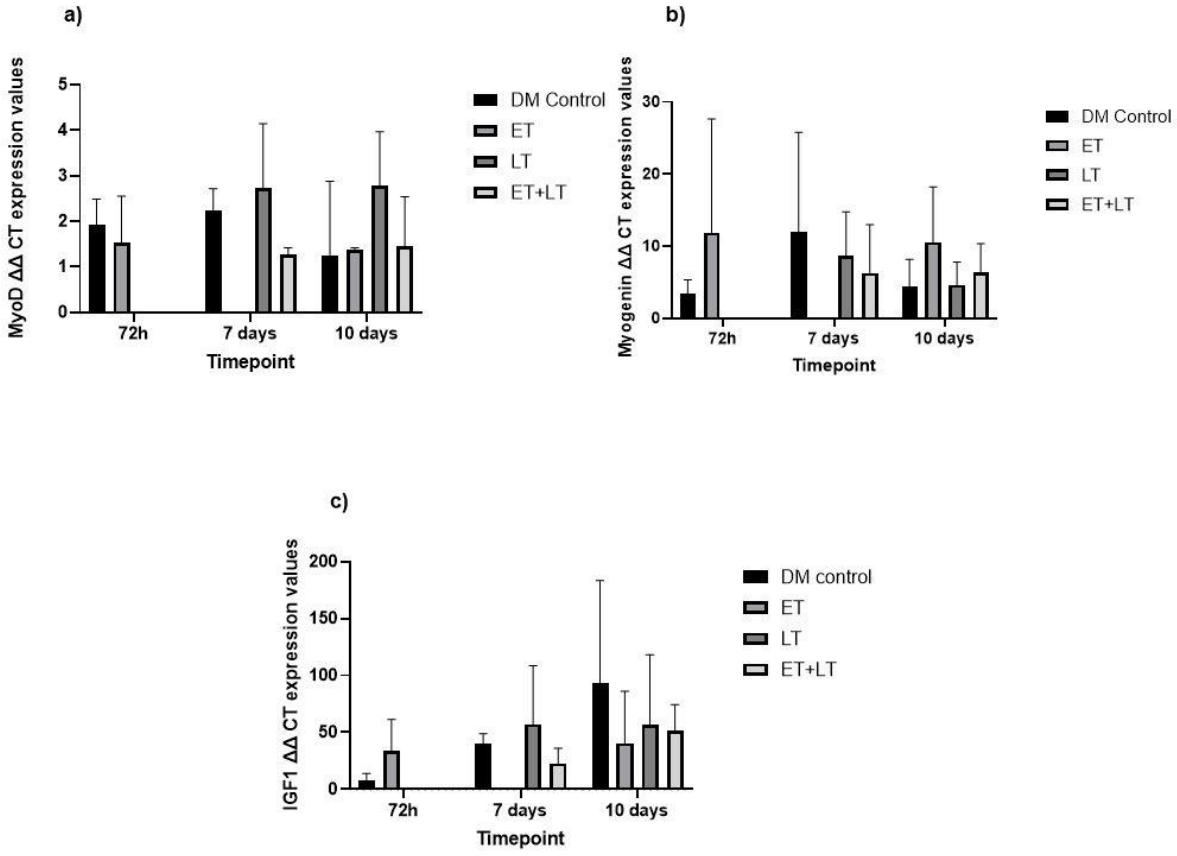

Figure 7: Fold gene expression for a) MyoD b) myogenin, and c) IGF-1 over all time points (72h, 7d, and 10d). Mean ± SD, n=3.

4.2.2 MYHC1 and MYHC4

Myosin heavy chains (MYHC) are considered markers of myotube differentiation/formation, particularly in the later stages. The fold gene expression for MYHC1 in the DM condition had an average increase from 72h to day 7 (72h 241.15 ± 114.85, day 7 552,50 ± 624.65; P = NS; Fig. 8b). Concerning MYHC1, fold gene expression demonstrated a gradual, average increased fold gene expression across the myotube differentiation (e.g., 36, 89, and 105 at 72h, 7d, and 10d, respectively; Fig. 8a). These increases in fold gene expression adhere with the myotube hypertrophy seen in the DM controls during differentiation/maturation (Wannenes et al. 2008). However, our data was not significant. At the same time, there is no significance in the testosterone dosed conditions (ET, LT, and ET+LT) that suggests these genes were involved in the effect that repeated testosterone administration had on myotube size.

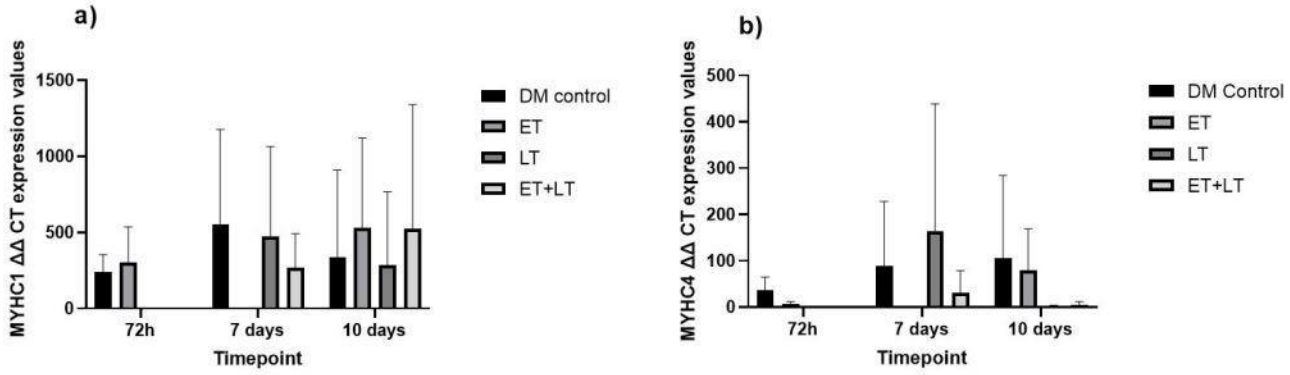

Figure 8: Fold gene expression for a) MYHC1 b) MYHC4 over all time points (72h, 7d, and 10d). Mean ± SD, n=3

4.2.3 UBR5, GRIK2, and STAG1

UBR5, GRIK2, and STAG1 are genes with distinctly different functions. The GRIK2 kainate receptor, encoded by the GRIK2 gene, is heavily associated with excitatory neurotransmission (Dutta et al., 2007). Meanwhile, STAG1 encodes a subunit of the core cohesin complex associated with sister chromatin cohesion, while UBR5 is an E3 ubiquitin ligase involved in skeletal muscle hypertrophy (Lehalle et al., 2017; Seaborne et al., 2019). However, all three genes have also been linked to skeletal muscle memory following resistance exercise (Seaborne et al., 2018). We, therefore, wanted to investigate if these were involved in testosterone dosing in muscle cells.

At basal differentiation, there are some temporal changes from 72h to day 7 and day 10. GRIK2 demonstrating increased fold gene expression at all time points (72h 0.05 ± 0.02, 7d 0.33 ± 0.34, 10d 0.45 ± 0.53; P = NS; Fig. 9b), while STAG experienced elevated fold gene expression from 72h to day 7 (STAG1 72h 0.14 ± 0.03, 7d 0.66; Fig. 9c). UBR5 had a 2-fold increase in fold gene expression from 72h to day 7 (72h 1.9 ± 1.01, 7d 3.97 ± 3.34; P = NS; Fig. 9a). Similar fold changes for UBR5 during differentiation have been observed in human skeletal muscle cells (Seaborne et al., 2019). However, data from the testosterone dosed conditions (ET, LT, and ET+LT) did not show any changes related to the observed morphological data.

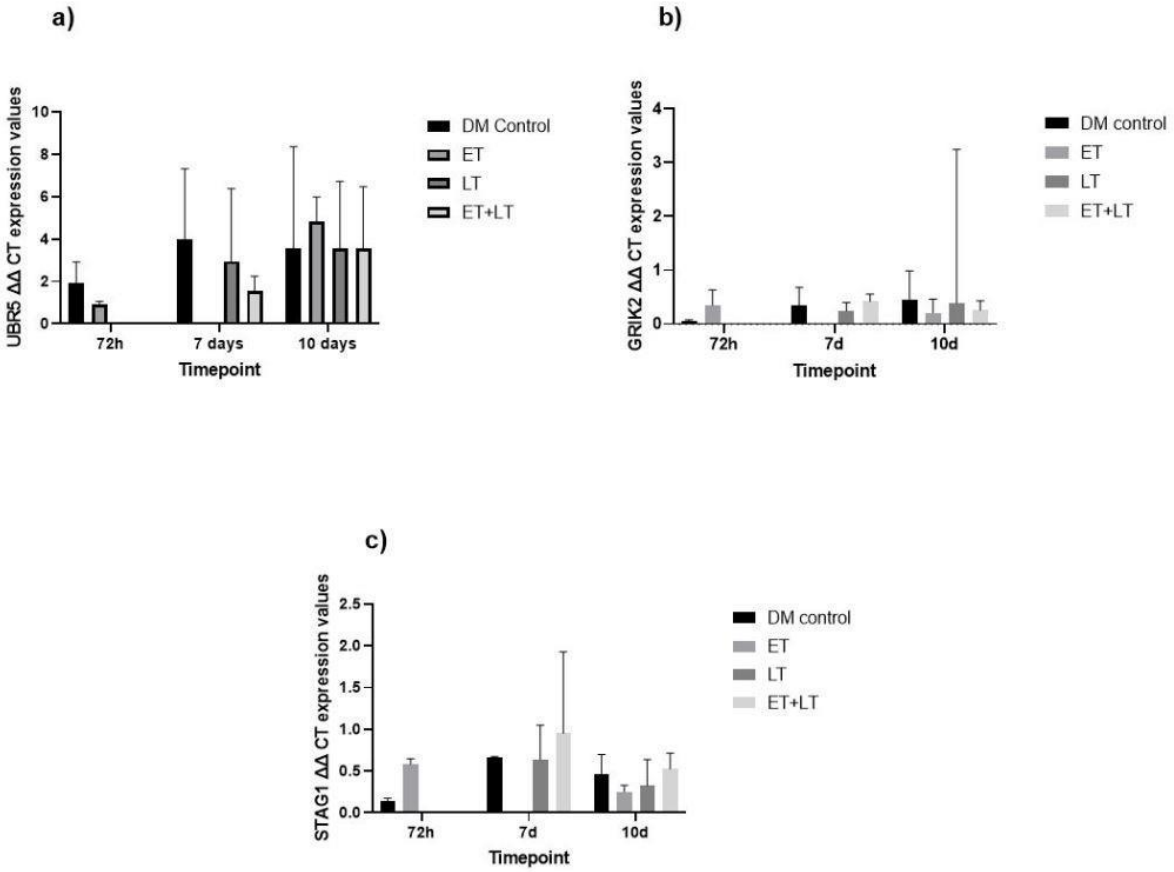

Figure 9: Fold gene expression for a) UBR5 b) GRIK2 and c) STAG1 over all time points (72h, 7d, and 10d). Mean ± SD, UBR5 n=3, GRIK2 and STAG1 n=2.

5 Discussion

This master thesis aimed to investigate the potential muscle memory of repeated testosterone administration on C_2C_{12} myotubes by dosing supraphysiological doses at the early and/or late stages of differentiation, respectively. Morphological analysis and relative fold gene expression of differentiation markers were used to measure the effectiveness of the testosterone administration.

5.1 Main findings

Results from the morphological analysis at day 10 show that early and late testosterone (ET+LT) dosing resulted in greater myotube numbers and myotube hypertrophy/size compared to myotube only dosed with late (LT). This LT condition, in turn, resulted in greater myotube numbers and hypertrophy compared to the untreated cells (DM) and cells dosed at an earlier time point (72 h) for 4 hours (ET). However, there were no significant differences in the relative fold gene expression data on the differentiation markers or the genes related to “muscle memory” in this experiment.

5.2 The effect of testosterone on myotube morphology and muscle memory

It is well established that testosterone dosing promotes/enhances cellular events (differentiation, myotube fusion, and proliferation) responsible for myogenesis (Herbst & Bhasin, 2004; Hughes et al., 2012). It is, therefore, no surprise that the cells dosed from day 7 to day 10 (LT and ET+LT) demonstrated elevated levels of myotube number and hypertrophy (size and diameter) similar to observations in other testosterone studies (Deane et al., 2013; Hughes et al., 2016).

The morphological data from this experiment show that administration of testosterone for 4 hours, which was undertaken in an attempt to mimic peak serum testosterone after oral consumption of anabolic steroids *in-vivo* (Leder et al., 2000) at the early stages of differentiation, was enough to evoke a greater response at T dosing during the later stages of differentiation. The fact that the early dosing for 4 hours was not enough to promote significant changes on its own highlights that the cells maintained 'memory' of the first encounter with testosterone (at least identified at the morphological level in this study). A morphological cellular memory has been reported previously by Sharples et

al. (2016a). Not of muscle hypertrophy but muscle atrophy, where C_2C_{12} myoblasts were cultured with TNF-α (a pleiotropic cytokine heavily associated with reduced strength and muscle loss), followed by 30 population doublings in the absence of TNF-α, and reintroduced to TNF-α in low serum media. The cells that experienced TNF-α before and after 30 population doublings demonstrated lower myotube numbers and creatine kinase activity (a biochemical marker of differentiation) vs. the cells only treated with TNF-α after 30 population doublings. Although this study did not include any population doubling, the premises were the same.

The work done by Bruusgaard et al. (2010) and Egner et al. (2013) attributes a cellular muscle memory to the retention of myonuclei. Due to time constraints caused by available time in the labs due to COVID-19, this study did not investigate the myonuclei content. Therefore, one cannot evaluate whether or not retained myonuclei played a role in the observed morphological muscle memory in this study. However, based on the data reported by Gundersen et al. (2010), myonuclei numbers increased 6 days post extensor digitorum longus (EDL) overload and stabilized at day 11. This preceded the rise in CSA after 9 days and stabilized after 14 days. Therefore, it is perhaps unlikely that retained myonuclei for ET+LT explain the myotube hypertrophy at day 10. The potential memory of T dosing at 72h would not be transparent until perhaps after the 10-day mark. Existing satellite cells can support fiber hypertrophy to a certain level without adding new satellite cells by enhancing their protein synthesis. However, fiber area increases above $\approx$26% require new myonuclei (Kadi, 2008).

Although not directly measured in this project, as alluded to earlier, one can not rule out epigenetic modifications as a possible mechanism behind the morphological memory observed. Shaples et al. (2016) demonstrated hypermethylated regions of MyoD following early proliferative life TNF-α administrations that were retained for 30 rounds of population doublings (cellular divisions), highlighting the stability of certain epigenetic modifications over a considerable time course. Although this study investigated the effects of catabolic stress, the model is still comparable to the hypertrophic effect of T in this research. Potential epigenetic modifications are discussed in more detail later in this chapter.

5.3 The effect of testosterone on fold gene expression

The genes analyzed in this study were chosen due to their involvement in differentiation and myotube formation (MyoD, myogenin, IGF-I) and myotube hypertrophy (MYHC1 and MYHC4) and skeletal muscle memory (UBR5, STAG1, and GRIK2).

5.3.1 Genes involved in myogenesis and hypertrophy

This study shows increases for MyoD and myogenin from 72h to day 7 and subsequent drop at day 10 on a basal differentiation. With MyoD and myogenin being vital regulators of early differentiation and myoblast fusion, which corresponded with increased myotube number across the timecourse, these changes were expected (Sabourin & Rudnicki, 2000). Also, the drop in myoD and myogenin by day 10 came as no surprise. These myogenic regulatory factors are essential in early differentiation but not later myotube maturation. In a study by Deane et al. (2013), a similar amount of testosterone (100 nmol) was dosed at 72h and further supplemented with 1 ml of T on day 7 to investigate improved hypertrophy in impaired myoblasts. At the 72h time point, there were significantly higher myogenin expression values in the T-treated cells vs. untreated CON, but no difference at day 7. Unlike the study by Deane et al. (2013), our cells had at this time point only been dosed for 4 hours and not from day 0. However, it was still sufficient to raise gene expression levels above those observed in the untreated cells. Furthermore, in line with previous findings, the T treated cells at the day 10 mark did not show significant expression values that would reflect the morphological data (apart from elevated gene expression values for ET that can likely be explained by sizeable statistical variation in the samples) (Deane et al., 2013; Hughes et al., 2016). Thus, based on this study and previous literature, myogenin is likely not the primary mechanism of action on T-induced hypertrophy and muscle memory.

Further comparisons to Hughes et al. (2016) display no effect of T dosing on MyoD expression at the 72h and 7-day time points. This study did not demonstrate any impact on gene expression of T dosing at any time (72, 7d, and 10d) either. Although the LT-only condition experienced some increased gene expression on day 7 and day 10 compared to all other conditions (DM, ET, and ET+LT), that is likely due to the statistical variation amongst the samples.

As expected, IGF-1 demonstrated increased expression values at basal differentiation throughout the 10 days due to its role in promoting proliferation and differentiation (Scimè & Rudnicki, 2006). There is evidence that T mediates its hypertrophic effect through IGF-1 and its downstream effectors (Serra et al., 2011); it is no surprise that T-treated cells displayed higher IGF-1 expression than DM-treated cells at 72h (Fig 3C). However, this pattern was not necessarily repeated on day 7 and day 10, with DM expression values being higher than ET+LT condition on day 7 and higher than all T treated conditions on day 10. Previous observations from T dosing over a 72h period show elevated IGF-1 expression, although at an earlier stage of differentiation (Serra et al., 2011). Once again, our findings can be attributed to sizeable statistical variation between the samples. Particularly one sample in the DM condition at day 10 demonstrated abnormal low expression values compared to the other samples (18 vs. 196 and 64).

The MYHCs are widely used as markers used to evaluate myotube morphology. For example, Serra et al. (2011) found a 4 fold up-regulation of MYHC consistent with increases in morphological data when subjected to 50 nmol T dosing. Wannenes et al. (2008) also reported elevating MYHC1 and MYHC4 (expressed in fast-twitch fibers) expression when subjected to smaller doses (10 nmol) of T treatment. Considering the relatively low T doses used by Serra et al. (2011) and Wannenes et al. (2008), one would expect even greater expression values when subjected to supraphysiological amounts as 100 nmol. Our data demonstrated a gradual gene expression increase in the experiment's basal level (Fig 4a and 4b). Still, there are conflicting data on the effect T had on the MYHCs gene expression. Data from MYHC1 on day 10 demonstrated some upregulated gene expression for the ET and ET+LT conditions, but not the LT condition. Otherwise, there is no data here that reflect the myotube hypertrophy or elevated myotube numbers observed.

5.3.2 Genes involved in muscle memory

Although STAG1 and GRIK2s role in skeletal muscle hypertrophy are relatively unknown, these were two of the genes identified to possess a memory of resistance exercise induced-hypertrophy in Seaborne et al. (2018). In that study, both STAG1 and GRIK2 demonstrated increased gene expression after chronic resistance exercise (7 weeks). Furthermore, this elevated gene expression continued through the seven weeks

of detraining compared to baseline and further was enhanced in the following reloading period. Also, these genes were hypomethylated following only a single acute resistance exercise session, and this was retained even during detraining (following training), resulting in larger hypomethylation and with enhanced gene expression in the later retraining, supporting the notion of an epigenetically regulated memory gene (Seaborne et al., 2018). Indeed, GRIK2 also demonstrated hypomethylation *in-vitro* when subjected to mechanical loading (Turner et al., 2021). Additionally, hypomethylation of GRIK3 and GRIK4 genes in myonuclei has been observed in the plantaris muscle of mice when overloaded (Von Walden et al., 2020), supporting this notion further.

Based on this, we wanted to investigate if the same would occur when subjected to supraphysiological T dosing. Although our data demonstrated increased gene expression on a basal level throughout the 10 day experiment, no data in the T treated conditions would suggest that STAG1 or GRIK2 are involved in the morphological increases seen in myotube number and hypertrophy, nor the morphological muscle memory observed at day 10 for the ET+LT condition.

UBR5s is an E3 ubiquitin ligase. Increases in E3 ligases to date have been associated more commonly with muscle atrophy due to their role in tagging proteins for degradation in the proteome. However, UBR5 has been recently associated with opposite roles too well-characterized muscle-specific E3 ligases (e.g., MuRF1/MAFbx) where the genes increases and protein levels are associated with recovery from atrophy and hypertrophy, rather than atrophy per se (Hughes et al., 2021; Seaborne et al., 2019). Indeed, UBR5 was first characterized to be hypomethylated and upregulated after resistance training in humans with even greater hypomethylation and enhanced gene expression following later retraining (Seaborne et al., 2018). Genotypes associated with increased expression are also more frequent in humans with larger type II fibers and strength/power athletes (Seaborne et al., 2019). Also, various hypertrophy stimuli across species and models evoke robust increases in gene expression and protein levels. Recovery from atrophy also evokes increases in UBR5, where MuRF1 and MAFbx are unchanged. Finally, *in-vivo* knockout of UBR5 evokes atrophy by dysregulation of protein synthesis and protein synthetic signaling in mice (Hughes et al., 2021). Again further suggesting that adequate/increase levels of UBR5 are anabolic and hypertrophic, and its loss evokes atrophy. In the present study, UBR5 increases in gene expression

across the timecourse of basal differentiation when cells are formed into myotubes, similar to that seen at the protein level in human-derived cells as they differentiate (Seaborne et al., 2019). In this study, the hypothesized increase in gene expression with testosterone and repeated testosterone did not occur, where testosterone evokes a decrease in UBR5 gene expression. For each time point when the respective conditions were T dosed (ET for 72h, LT and ET+LT for 7d and 10d), gene expression fold changes were lower than the DM controls. It could be feasible that when testosterone is supraphysiological high and able to signal via its receptor (AR) that this anabolic signal is adequate to result in a smaller requirement for increased UBR5 in muscle mass regulation. However, this link between UBR5 and testosterone-AR requires further investigation.

5.4 Limitations in this study

This thesis aimed to investigate the underlying mechanisms of the phenomenon of muscle memory. However, we did not directly analyze the methylation pattern since there were notable changes in gene expression in the muscle memory genes, and these analyses are expensive. Therefore, one can not say if hypomethylation was retained (STAG, GRIK2) or enhanced (UBR5) with repeated T-dosing as observed in repeated resistance exercise (Seaborne et al., 2018). A future direction from this limitation could be investigating other genes associated with "muscle memory." Namely, the genes TRAF1 and BICC1 also demonstrated retained hypomethylation for seven weeks following acute RE, resulting in enhanced gene expression 22 weeks later (Seaborne et al., 2018). The TNF receptor-associated factor 1 (TRAF1) and TRAF2 forms the heterodimeric complex necessary for TNFα activation of MAPK pathways (Edilova et al., 2018). Furthermore, *in-vitro* studies demonstrated up-regulation of negative regulators of MPS (atrogin1/MAFbx) when exposed to TNFα (Li et al., 2005). Indeed, TNFα levels are significantly elevated following strenuous exercise, likely caused by an inflammatory response (van de Vyver & Myburgh, 2012). BICC1 is an RNA-binding protein used as a prognostic biomarker in gastric cancer (Zhao et al., 2020). However, BICC1s role in the skeletal muscle is unknown and warrants further investigation.

Furthermore, a whole transcriptome analysis of the same dataset found the genes FLNB and CTTN hypomethylated with corresponding gene expression following acute and chronic RE training (with retained methylation during detraining and retraining) (Turner

et al., 2019). Cortactin (CTTN) is an actin-binding protein regulating cell migration, actomyosin contractility, and GTPase signaling (Schnoor et al., 2018).

Filament B (FLNB) is a member of the filament family. Its role in RE-induced hypertrophy or muscle memory is limited, but mutations of FLNB are associated with skeletal deformities (Xu et al., 2017). Furthermore, Filamin A (FLNA) is phosphorylated in response to endurance exercise (Deshmukh et al., 2006), and Filamin C (FLNC) depleted mice demonstrated reduced morphology suggesting inhibition of myogenesis (Dalkilic et al., 2006), suggesting that the complete role of FLNB in skeletal muscles is perhaps yet to be discovered. Therefore, future studies should investigate the methylation and gene expression of these selected genes to understand better their respective roles in the muscle memory of anabolism.

Furthermore, we looked at the muscle memory genes associated with retained hypomethylation following RE-induced hypertrophy. Given that we found a morphological memory but no changes in mRNA gene expression in these candidate genes, it could be that changes to these genes are limited to mechanical/metabolic hypertrophy and perhaps not responsive to hormonal induced hypertrophy, as pathways might be different. A further direction from this limitation would be to run a genome/methylome-wide methylation analysis to see which pathways and genes are involved in T-induced hypertrophy.

The lack of statistical power played had an impact on this project. As a consequence of the COVID-19 pandemic, the shipment of critical equipment and C_2C_{12} cells was delayed, delaying the start of the project by several months. This delay left no time for reanalyzing samples or do more repeats of cell culture. The subsequent lockdown that followed further restricted the timetable allowing little time for the margin of errors that would inevitably occur.

In this study, C_2C_{12} mouse cells were used to investigate the effect of repeated T dosing due to easy accessibility, rapid growth rate, and relatively low cost. However, this thesis aimed to examine the possible mechanisms behind muscle memory of doping in sports. Therefore, in this context, the use of C_2C_{12} can be questioned since C_2C_{12} cells exhibit different metabolic behavior and transcriptomic profiles compared to human skeletal

muscle cells (Abdelmoez et al., 2020). Notably, rodents' skeletal muscles consist predominantly of fast-twitch fibers (type IIa and IIx), while human skeletal muscles consist mainly of slow-twitch fibers (type I). Due to the rodent origins of C_2C_{12}, the level of MYHC1 and MYHC4, which are associated with fast-twitch fibers, could demonstrate higher gene expression values than what would be observed in human skeletal muscle, as has been previously reported (Abdelmoez et al., 2020).

5.5 Conclusion

This thesis aimed to investigate the concept of "muscle memory" of doping previously observed (Egner et al., 2013; Seaborne et al., 2018) by dosing C_2C_{12} myotubes with supraphysiological doses of T (100 nmol) at an early- and late stage of differentiation. As hypothesized, at the 10-day mark, the cells dosed early and late (ET+LT) demonstrated significantly greater myotube numbers than the cells dosed only at a late stage (LT). At the same time, the early dosing alone (ET) did not elevate myotube numbers significantly. A similar trend was observed for myotube hypertrophy and size. These findings suggest that the muscle cells retain a morphological memory of the first T bout, enhancing Ts hypertrophic effect to a greater extent upon second dosing. However, the fold gene expression from the differentiation and myotube hypertrophy markers (MyoD, myogenin, IGF-1, MYHC1, and MYHC4) and the "muscle memory genes" (GRIK2, STAG1, UBR5) identified in response to repeated exercise training in Seaborne et al. (2018) do not reflect the changes in morphological induced memory with repeated T-dosing. Therefore it is unlikely that these were involved in the mechanism behind muscle memory. Future studies, however, should look to identify epigenetically regulated genes of repeated T-induced hypertrophy across the genome, as these may be different from exercise-induced memory genes.

5.5.1 Implication for public health and sport

Resistance exercise is widely used to increase muscle mass and strength for aesthetical purposes or improve physical performance related to sports. Still, it is also an effective treatment for sarcopenia and the subsequent loss of muscle mass and function (Phu et al., 2015). As a consequence increased lifespan of the world's population, age-related sarcopenia is a major threat to achieving a healthy lifestyle (Granic et al., 2019). Our morphological data, along with the findings of Egner et al. (2013) and Seaborne et al. (2018), suggest that skeletal muscle cells possess a memory of previous anabolic stimuli

and periods of training-induced muscle hypertrophy. Although the mechanisms behind this memory are not entirely understood, further research into this field could enable scientists to create a more potent, long-lasting memory in older adults preventing age-related sarcopenia. Furthermore, the same principle can be used on athletes when recovering from long-term injuries in a sports context.

In an anti-doping context, this muscle memory could make a case for stricter or even indefinite bans for athletes who purposely use performance-enhancing drugs. Under the current regulations, the World Anti-Doping calls for a 2-year suspension when abusing steroids (WADA, 2009). Based on existing literature, this should be reconsidered.

Additionally, suppose a methylation profile of AAS abuse can be identified. In that case, we could potentially use this to detect previous abuse (e.g., people who cycle on and off steroids) and influence the length of suspension until the methylation profile is "normal" again.

This subject also raises questions surrounding the current criteria on which transgender women can compete in the female category in elite sports. The current criteria set by the IOC require transgender females to suppress serum testosterone levels below 5 nmol at least 12 months before and during competition in an attempt to abolish the gender-related performance gap. The concept of muscle memory could attenuate the loss of muscle mass and strength imposed by these restrictions and facilitate strength gains upon retraining (Hilton & Lundberg, 2021). However, this is a matter of ethics and biology and needs to be further understood before any conclusions can be made.

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