The Role of Vitamin D in Skeletal Muscle Function and Regeneration

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A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of Doctor of Philosophy

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Authors Declaration

I declare that the work in this thesis was carried out in accordance with the regulations of Liverpool John Moores University. Apart from the help and advice acknowledged, the work within was solely completed and carried out by the author.

Any views expressed in this thesis are those of the author and in no way represent those of Liverpool John Moores University and the School of Sport and Exercise Science.

This thesis has not been presented to any other University for examination either in the United Kingdom or overseas. No portion of the work referred to in this research project has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

Signed

Date

I. Abstract

Skeletal muscle is a direct target for the group of seco-steroids collectively termed Vitamin D. Skeletal muscle expresses both CYP27A1 and CYP27B1 encoding for the hydroxylases required to convert Vitamin D to 25[OH]D and subsequently the bioactive 1α-25-dihydroxyvitamin D_3 (1α-25$[OH]_2D_3$) (Girgis et al., 2014b). Crucially, the Vitamin D receptor (VDR) is also present in skeletal muscle (Srikuea, Zhang, Park-Sarge, & Esser, 2012) and upon exposure, binds to its ligand 1α-25$[OH]_2D_3$ and initiates genomic and non-genomic rapid signalling responses. At present there is a global prevalence of low serum Vitamin D (25[OH]D) concentrations due to a lack of sun exposure (the primary route for Vitamin D synthesis) as a function of latitude and/or an indoor lifestyle coupled with few dietary sources of Vitamin D (Chen et al., 2007). Accumulating data are now suggestive that low 25[OH]D may be associated with perturbations in contractile activity and the regeneration of human skeletal muscle (Owens, Fraser, & Close, 2014), although a definitive causal relationship is yet to be established. Therefore, this thesis aimed to establish a more precise role for Vitamin D in human skeletal muscle function and regeneration. There were four overarching aims:

1. Explore the role of Vitamin D in human skeletal muscle contractile properties in humans *in vivo.*
2. Identify the role of Vitamin D in human skeletal muscle contractile properties *ex vivo.*
3. Investigate the role of Vitamin D in skeletal muscle regeneration following eccentric exercise induced muscle damage *in vivo.*
4. Elucidate cellular mechanisms of the muscle regeneration process that are responsive to Vitamin D *in vitro.*

The main findings from this work imply that serum 25[OH]D concentrations across a broad range from 18->100 $nmol.L^{-1}$ do not affect skeletal muscle contractile properties. Conversely

elevating serum 25[OH]D from <50 nmol.L^{-1} to >75 nmol.L^{-1} resulted in significant improvements in the recovery of maximal voluntary contraction force following a bout of eccentric exercise. Implementing an *in vitro* model of muscle regeneration also identified potential cellular mechanisms for these observations: Muscle derived cells treated with a total amount of 10 nmol 1α-25$[OH]_2D_3$ following a mechanical scrape improved migration dynamics and fusion capability of skeletal muscle derived cells.

Taken together, low Vitamin D concentrations are highly prevalent but can be easily corrected with supplementation of Vitamin D_3. This may have the advantage of optimising the regenerative capacity of skeletal muscle amongst other health benefits previously characterised by others.

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I. Acknowledgements

I would like to thank my supervisors Graeme Close and James Morton for giving me countless opportunities to develop as a scientist over the course of my PhD. Both have given me invaluable advice and kept me on the right track. Most of all I value the friendship I have with them, which I hope will continue for a long time.

I would also like to thank Claire Stewart and Adam Sharples who have offered considerable time and effort to teach me new skills and direct what has become an excellent research project. Their enthusiasm has had a profound effect on me and is a primary reason that I have developed a strong desire to pursue a career in systems biology.

I wish to acknowledge Prof. Bill Fraser and Jonathan Tang from the University of East Anglia for performing the LC-MS/MS analysis described in this thesis.

Finally, the department of sport and exercise science at LJMU has a unique set of individuals who volunteer their time and willingly participate in physically demanding protocols with no incentive or reward. You are the reason that we can push the boundaries to understand human physiology and without you we have no research! A massive thanks goes to all those who volunteered for the studies in this thesis, you made it all possible.§

II. Dedication

For my parents, who have supported me in many ways throughout the entirety of my education. I hope that I can become the type of person that both of you are.

III. Abbreviations

1α,25[OH]2D3 - 1α,25-dihydroxyvitamin D3

24,25[OH]D – 24,25-hydroxyvitamin D

25[OH]D – 25-hydroxyvitamin D

ADP – adenosine diphosphate

AMP – adenosine monophosphate

AP – action potential

ATP – adenosine triphosphate

BCA – bicinchoninic acid

CSA – cross sectional area

CI – confidence interval

CK – creatine kinase

Ct – cycle threshold

CV% - co-efficient of variation (%)

DAPI - 4',6-diamidino-2-phenylindole

DBP – vitamin D binding protein

DHPR – dihydropyridine receptor

DMEM – dulbeccos modified eagles medium

DNA – deoxyribonucleic acid

dNTP - nucleoside triphosphates

ECM – extracellular matrix

EDTA - Ethylenediaminetetraacetic acid

EMS - electromyostimulation

EtOH - ethanol

FBS –fetal bovine serum

GM – growth media

GS – goat seurm

Hz - hertz

ICC - immunocytochemistry

IGF – insulin like growth factor

IKD – isokinetic dynamometry

IU – international units

LC-MS/MS – liquid chromatography tandem mass spectrometry

MAPK – mitogen activated protein kinase

MDC – muscle derived cell

MDM – murine myoblast differentiation medium

MGM – murine myoblast growth medium

MHC – myosin heavy chain

MRF – myogenic regulatory factor

mRNA – messenger ribonucleic acid

mTOR – mammalian target of rapamycin

Myf – myogenic factor

MYOG – myogenin

MVC – maximum voluntary contraction

OD – optical density

PAR-Q – physical activity readiness questionnaire

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PCr - phosphocreatine

PTH – parathyroid hormone

QM – quiescent media

Rad - radian

RCF – relative centrifugal force

RCT – randomized controlled trial

RNA – ribonucleic acid

ROM – range of motion

RPL13a – ribosomal protein L13 a

SC – satellite cell

SD – standard deviation

SL – sarcomere length

SR – sarcoplasmic reticulum

TBS – tris buffered saline

TM – transfer media

TMT – tris-mes-triton

TnC – troponin C

TRITC - Tetramethylrhodamine

UV – ultraviolet

VDR – vitamin D receptor

VEGF – vascular endothelial growth factor

VL – vastus lateralis

VM – vastus medialis

WT – wild type

Chapter 1

General Introduction

This chapter gives a broad background into the environmental challenges that account for the prevalence of Vitamin D deficiency and introduces the field of Vitamin D research.

2.1 General Introduction

The monogenesis model of human evolution dictates that early modern man (*Homo sapiens*) emerged from sub-Saharan Africa and thus developed its biochemical mechanisms for maintaining life on the rich organic molecules available in its immediate milieu. Macro- and micronutrients could be obtained from animal and plant life and as such, mechanisms to digest and metabolize them evolved. Since sunlight was also in abundance, a photosynthetic reaction in the dermal layer of skin developed to make use of solar radiation (ultraviolet), which is now understood to be the main pathway for the synthesis of Vitamin D, a group of pleiotropic seco-steroid hormones. The evolution of these efficient phenotypic adaptations allowed man to flourish as an intelligent species.

However, fossil evidence implies that at some point around 35-40,000 years ago, *Homo sapiens* migrated away from Africa and colonized areas of Europe (Stringer, 2003), a dramatic change to the sun-rich climate of sub-equatorial Africa. Consequentially, new environmental challenges to homeostasis were placed on man. One such challenge was a drastic difference in the annual pattern of sunlight exposure. Phenotypic changes have emerged to account for this altered exposure. Indeed, a degree of skin depigmentation occurred in migrants that can be readily observed today between the races of northern Europe and Africa. A simple explanation for this is that as man's exposure to solar radiation lessened, so did the need for melanin, which absorbs ultraviolet radiation competing with the steroid precursor that converts to pre-Vitamin D under thermal isomerization. However, the significance of this phenotypic change in skin pigmentation is further reaching than photo-protection. It is probably an evolutionary attempt to redefine the balance between protection against ultraviolet (UV) radiation and to increase the UV-B dependent photosynthetic production of Vitamin D from 7-dehydrocholesterol in the skins dermis. This environmental challenge has however, occurred too recently in evolutionary history and as such, depigmentation has not been an effective mechanism to compensate. This is evidenced by population-based data from Northerly latitudes that describe a high prevalence of Vitamin D

deficiency. Moreover, even in sun rich climates close to the equator there is a high prevalence of Vitamin D deficiency due to a predominantly indoor lifestyle, the covering of large amounts of skin with clothing and sunscreen application.

Until the identification of the fat soluble Vitamin D by Elmer McCollum in 1913 our understanding of the physiological significance of Vitamin D was undefined. Since then, particularly in the past 20 years, the scientific literature has witnessed a renaissance in Vitamin D research and our knowledge base has increased exponentially (Figure 1.1). It is now understood that Vitamin D regulates numerous biological processes in man.

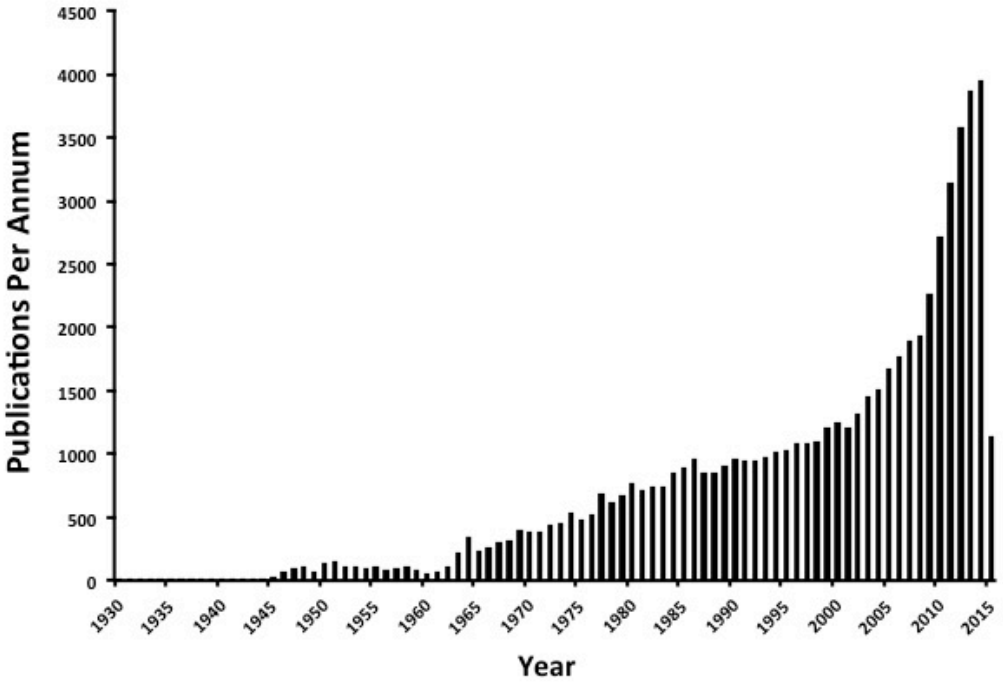


Figure 1.1 Research interest in Vitamin D based on articles published per annum as reported by PubMed (National Centre for Biotechnology Information).

Consequently, there has been a search for the 'optimal' Vitamin D concentration for all health outcomes. Unfortunately, as with other steroids, different tissues respond differently

to the same stimulus, thus it is unlikely that there is an optimal Vitamin D concentration for global health benefits.

Forming the basis of this thesis is the research interest in Vitamin D and skeletal muscle. As will be discussed there are preliminary data that support a role for Vitamin D in skeletal muscle function and regeneration, however definitive causal relationships are yet to be established. Understanding how Vitamin D may impact upon skeletal muscle will undoubtedly provide insights into the potential of manipulating Vitamin D status to promote muscle health throughout the lifespan, which is of interest from developmental, athletic and ageing perspectives. This thesis aims to examine in more detail the role of Vitamin D in skeletal muscle function and regeneration by employing a systems biology approach to the primary research question.

Chapter 2

Review of the Literature

This chapter gives a synthesis of research findings that describe human skeletal muscle function and repair with reference to factors that mediate both processes. A review of the literature on Vitamin D in relation to muscle function and repair is then provided culminating in the scientific rationale for this thesis.

Publications resulted from this chapter; Owens, D.J., Fraser, W.D. and Close, G.L. (2014). Vitamin D and the Athlete: Emerging Insights. European Journal of Sport Science, 18:1-12.

2.1 Human Skeletal Muscle Form and Function

2.1.1 Background: In humans, skeletal muscle provides a mechanism of locomotion, comprises ~40% of body mass and plays a critical role in metabolic homeostasis accounting for ~30% of the resting metabolic rate (RMR), is the predominant storage tissue for glycogen in addition to being the predominant site of insulin stimulated glucose disposal (Thiebaud et al., 1982). Physical *inactivity* is therefore unsurprisingly a major risk factor for a host of chronic disease states including type II diabetes mellitus, heart disease, obesity and age related loss of muscle or sarcopenia (Booth, Roberts, & Laye, 2012). Exercise training however partially reverses such disease processes and is in fact the most effective medicine in the treatment and prevention of type II diabetes and sarcopenia (Borst, 2004; Colberg et al., 2010). Further, the type of exercise training may be manipulated in terms of modality, duration, frequency and intensity such that subsequent local and systemic factors produce differential transcriptional responses ultimately remodelling the muscle proteome to suit the profile of innervation that it repeatedly experiences. This highlights the extraordinary plasticity of skeletal muscle, demonstrating its ability to respond positively and negatively to different stimuli and signifies the importance of maintaining skeletal muscle health to promote global health benefits, prevent disease and optimize physical performance. However, skeletal muscle is also prone to perturbations in form and function as a consequence of dietary deficiencies, disease and injury. Understanding the aetiology of skeletal muscle dysfunction in these contexts will undoubtedly lead to the development of strategies to maintain muscle and optimize musculoskeletal performance.

2.1.2 Overview of Skeletal Muscle Structure: The structure of skeletal muscle is organised at a number of levels making it highly adapted to its primary function: the generation and transmission of force. Whole muscle structures are enveloped by the epimysium, connective tissue that contains bundles of skeletal muscle fibres. These fibre bundles are encased by perimysium and within consist of myofibres, which are surrounded by connective tissue

termed endomysium. The units of skeletal muscle that contain the necessary components for force production are myofibres. These syncytial tubes range in length based on anatomical location and in cross sectional area (CSA) based on fibre type (Rivero, Talmadge, & Edgerton, 1998). There are several fibre types in mammalian skeletal muscle, however human skeletal muscle is comprised of the pure type I, type IIa and type IIx (Harridge et al., 1996) with some hybrid fibres also reported (Sant'ana Pereira, Wessels, Nijtmans, Moorman, & Sargeant, 1995). The classification of fibres has developed considerably on the basis of correlated histochemical and physiological studies of individual motor units, electron microscopy of fast and slow skeletal muscles, novel procedures for myosin ATPase histochemistry and biochemical studies on oxidative and glycolytic enzymes in different muscles (Schiaffino & Reggiani, 2011). Table 2.1 provides a succinct overview of human fibre type properties.

Table 2.1 Human skeletal muscle fibre contractile, metabolic and morphological properties.

	Type I	**Type IIa**	**Type IIx**
General Properties			
Myosin heavy chain isoform	MHC1	MHC2A	MHC2X
[b]Relative myosin heavy chain %	33.9 ± 11.4	46.3 ± 3	19.8 ± 11.5
Metabolic Characteristics	Highly oxidative	Oxidative/glycolytic	Glycolytic
Power output	Weak	Intermediate	Strong
Endurance capacity	High	Moderate	Low
Recruitment threshold	Low	Moderate	High
Morphological Properties			
Capillaries per fibre (density)	4.2	4.0	3.2
Mitochondrial Density	High	Intermediate	Low
[b]Fibre Size (CSA - μm^2)	4844 ± 1286	6174 ± 1587	5160 ± 1324
[c]Myonuclear domain size (μm^3)	24.9± 1.1	32.9 ± 2.1	42.7 ±4.9

Table adapted from Saltin and Gollnick (1983).

[b]Data from Staron et al. (2000); Ninety-five young healthy males aged 21.5 ± 2.4 yrs. Fibres isolated from *Vastus Lateralis*.

[c]Data from Liu et al. (2009); values from 460 μm sections of single muscle fibres from *Vastus Lateralis* expressing different MHC isoforms. Data expressed as mean and s.e.m.

2.1.3 Skeletal Muscle Microstructure: Each myofibre contains the contractile filaments that allow movement, the proteins actin and myosin, in a highly organised array to allow both longitudinal and lateral force transduction within and between fibres (Bloch et al., 2004). This arrangement forms the contractile element termed a myofibril. Within each myofibril exist a number of sarcomeres in series and parallel, which can be identified in an electron micrograph as the unit between Z-lines (where actin is held in a square array). Figure 2.1 provides simplistic schema and micrograph images of the arrangement of the contractile apparatus.

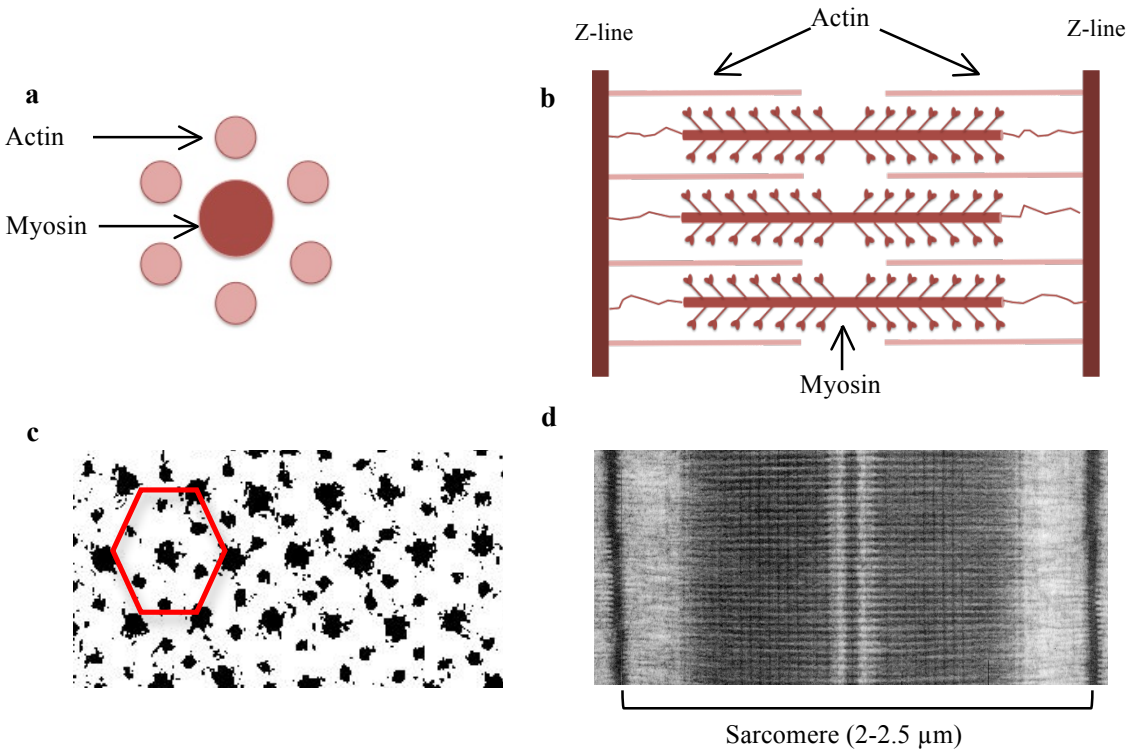


Figure 2.1. Schema that describe the arrangement of actin and myosin filaments.**a**) Each myosin filament is surrounded by six actin filaments such that the myosin filament may interact with any of the six actin filaments. Each actin filament is also capable of binding to any of three myosin filaments **b**) A fragment of an electron micrograph of a transverse section of a fibre from rabbit psoas muscle in the low temperature, relaxed state. Thick and thin spots correspond to myosin and actin filaments, respectively (Bershitsky et al., 2010) **c**) This assembly allows actin filaments to 'slide' between myosin filaments. This unit from Z-line to Z-line is termed a sarcomere **d**) An electron micrograph of a sarcomere typically 2-2.5µm in length in mammalian muscle (obtained from sacromere.org on 10/09/2014).

2.1.4 Skeletal Muscle Function: The purpose of skeletal muscle in all animals including humans is to generate tension. The generation of tension occurs in the myofibre by interaction of the contractile filaments achieved via electrochemical signalling. This is brought about by sarcolemmal depolarization accomplished by a nerve impulse derived from the motor cortex of the brain and transmitted by branched terminal axons of motor neurons innervating the fibre. When a nerve impulse reaches the neuromuscular junction (NMJ), the neurotransmitter acetylcholine (ACh) is released from intracellular vesicles into the synaptic junction. The amount of ACh released from a single synaptic vesicle is termed a quantum. On the post-synaptic membrane of the muscle fibre, ACh can bind to its receptor (AChR) and produce a miniature membrane potential or MEPP. A single MEPP depolarizes the sarcolemma but only when sufficient quanta are released into the synaptic junction at the same time are enough MEPP's generated to produce an action potential (AP). The AP generated at the NMJ is brought about through an influx of Na^+ and efflux of K^+ resulting in a change in membrane potential.

The AP is propagated along the sarcolemma and travels down invaginations into the fibre termed T-tubules. As the T-tubules are depolarized, the surface membrane near the NMJ is repolarized by active transport of Na^+ out of the cell and K^+ into the cell via Na^+K^+ ATPase pumps. It is the depolarization of the T-tubular system that allows the electrical signal generated at the sarcolemma to be transmitted to the interior of the fibre by converting the electrical signal into a chemical signal. This phenomenon occurs through voltage dependent Ca^{2+} channel (or L-type) release and dihydropyridine (DHPR) and ryanodine receptor (RyR) interaction. In the latter the two receptors are not linked with contact but lie in close proximity to allow efficient interaction. This arrangement permits a signal coupling mechanism for calcium release from the sarcoplasmic reticulum (SR) into the sarcoplasm of the fibre. The SR surrounds groups of approximately 200 actin and myosin filaments and lies close to the T-tubular system. These areas of close contact known as terminal cisternae provide an intimate and efficient mechanism of calcium signalling. Intracellular Ca^{2+}

concentrations differ between fibre types as slow fibres have resting concentrations of 40-50 nmol whereas fast twitch fibres are lower at 30 nmol (Fraysse et al., 2003; Fraysse et al., 2006). It is due to fluxes in intracellular Ca^{2+} concentration that cross bridge formation is permitted, subsequently allowing myosin heads to exert force on actin monomers through a change in myosin head angle powered by adenosine triphosphate (ATP) hydrolysis.

Ca^{+2} is released from the SR when the voltage change in the DHPR causes opening of the ryanodine channel on the SR membrane. The calcium released can stimulate further calcium release from adjacent sites but most importantly binds to the troponin isoform C (TnC) causing a conformational change to the protein tropomyosin that surrounds actin, thus allowing myosin to interact with actin. Once the myosin head has bound an actin monomer, the myosin head rotates and stretches a compliant portion of the myosin filament and generates force that is exerted onto the actin filament allowing actin to 'slide' between myosin filaments. Once contraction has occurred, calcium is sequestered in the SR via calcium ATPase. This process requires Mg^{2+} and transports two Ca^{2+} ions in exchange for one H^{+} ion at the expense of an ATP molecule. Calcium is then sequestered in the terminal cisternae of the SR by the calcium binding protein calsequestrin at a concentration of ~1 $mmol.L^{-1}$. A number of factors can however affect the release and re-uptake of calcium in skeletal muscle, ultimately modulating the ability to generate force. Examples of such factors include exercise-induced fatigue (Li et al., 2002; Westerblad, Dahlstedt, & Lannergren, 1998) generation of reactive oxygen species (Andersson et al., 2011) and dietary factors such as caffeine (Allen & Westerblad, 1995).

As each sarcomere is linked in series at the Z line of actin filaments, the force produced during actin-myosin cross bridge cycling is transmitted through the length of the muscle permitting a whole muscle contraction. Importantly, costameric proteins that are arranged in a rectilinear manner, parallel the underlying contractile apparatus allowing force to be transmitted laterally to the extracellular matrix (ECM) and provide a two way signalling pathway between Z-line and ECM transducing mechanical stress signals from the outer cell

to intracellular signalling pathways (Bloch et al., 2002; Peter, Cheng, Ross, Knowlton, & Chen, 2011).

As eluded to in the previous section, different skeletal muscle fibre types exhibit differences in their contractile properties. There are a number of features that characterize the term 'contractile properties', including the shape of the twitch, relaxation time from an isometric tetanus, fusion frequency and fatigability. A summary of the twitch and fatigue profiles of the major human motor unit types is found in Figure 2.2.

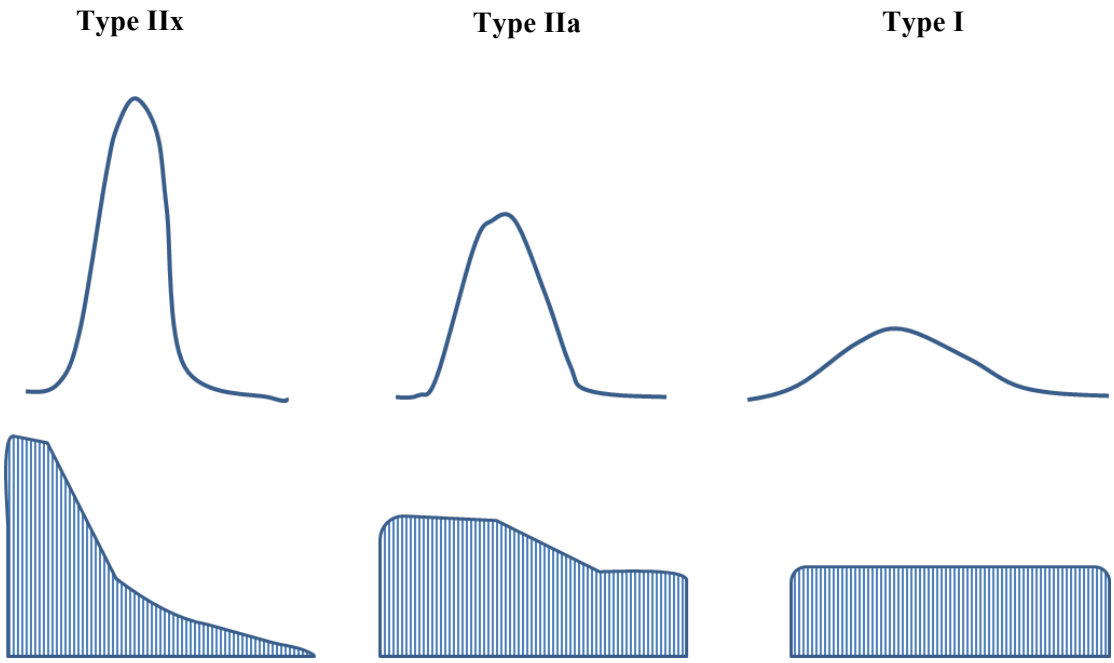


Figure 2.2. Contractile characteristics of the main human motor units. Upper curves are forces generated from a single 1Hz twitch. Lower traces represent force fatigue curves during tetanic stimulation (e,g. 100Hz).

Investigation of these profiles allows for the detection of subtle changes in muscle plasticity. Indeed, force profiles for single twitches, force frequency profiles and fatigue profiles at both the whole muscle and single fibre level have demonstrated that changes can be detected as a consequence of physiological stress such as hypoxia (Degens et al., 2010), disuse (D'Antona et al., 2003), muscular dystrophies (Williams, Head, Lynch, & Stephenson, 1993)

and exercise training (Harber, Gallagher, Creer, Minchev, & Trappe, 2004; Malisoux, Francaux, Nielens, & Theisen, 2006; Trappe et al., 2000). Coupling single fibre studies with whole muscle measures therefore provides a powerful translational tool for investigating the functional characteristics of striated muscle.

2.2 Skeletal Muscle Damage and Repair

2.2.1 Background: As a consequence of episodic bouts of the differential muscle contractions described in the previous section, skeletal muscle undergoes functional adaptation and remodelling. Minor day-to-day damage caused by such contractions is effectively repaired by simple recruitment of intracellular vesicles (Steinhardt, Bi, & Alderton, 1994). However, since mature myofibres are terminally differentiated, severe damage requiring degeneration of damaged necrotic tissue and subsequent regeneration is reliant on the timely activation, proliferation and differentiation of the resident muscle stem cell population termed 'satellite cells' (SCs).

2.2.2 **Mechanisms and Nature of Muscle Damage:** Skeletal muscle damage may be defined as the loss of muscle function caused by physical disruption of muscle structures involved in producing or transmitting force. Injury may be caused by a physical insult such as a contusion or tear, may be drug induced or more commonly, as a consequence of unaccustomed or eccentric exercise. For the purpose of this thesis, only the latter will be discussed. It is noteworthy however that following the primary damaging event, the process of degeneration and regeneration is similar between drug and exercise induced muscle damage (Ciciliot & Schiaffino, 2010).

Prior to considering events subsequent to damaging contractions, it is appropriate to explore the type of contraction that most commonly induces injury and mechanical factors that can explain this. Early research utilised mouse models in which muscle was stimulated to

contract whilst length and shortening velocity were controlled for. Using such a model, it was feasible to induce concentric, eccentric and isometric contractions in the mice, take maximal force measures following the bout of contractions and then isolate the muscle to assess histological changes in muscle architecture. This pioneering work demonstrated that only after a bout of eccentric contractions was there a significant loss in force and substantial histological damage (McCully & Faulkner, 1985). Such findings have been replicated in other animal studies and indeed in humans using different eccentric exercise modalities such as downhill running and isokinetic dynamometry (Close et al., 2006; Paulsen et al., 2007).

Following this early work, investigations have sought to understand the mechanical factors associated with different contractions to elucidate mechanisms for the observed damage. Muscle tension, lengthening and lengthening velocity were initially investigated in rodents (Warren, Hayes, Lowe, & Armstrong, 1993). The investigators determined that force loss following eccentric contractions was most strongly correlated to peak tension. Similarly Brooks, Zerba, and Faulkner (1995) demonstrated that peak tension and work done both strongly correlated to the degree of damage but importantly also showed that lengthening velocity and tension during lengthening were important determinants of the degree of damage. The latter findings have been supported by further data on the length-tension-damage relationship (Talbot & Morgan, 1998) and in summary state that slow velocity high tension eccentric contractions result in greatest muscle damage.

Logically, it is important to also ask what the morphological consequences of skeletal muscle damage are. A hallmark of eccentric contraction induced injury is focal damage to the sarcomere. Newham, McPhail, Mills, and Edwards (1983) first demonstrated this with the use of electron microscopy, providing evidence of single and half disrupted sarcomeres and Z-line streaming, all evident immediately post damage and persisting and increasing up to three days post damage (see Figure 2.3).

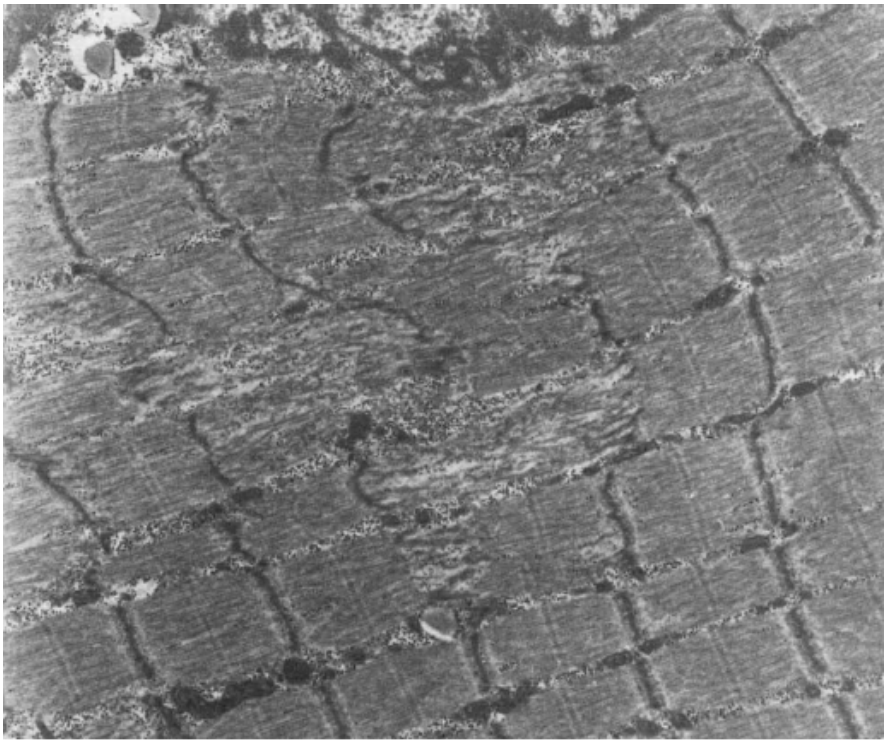


Figure 2.3. An area of extensive focal sarcomere damage resulting from eccentric contraction induced muscle damage (Newham et al., 1983).

Aside from sarcomeric disruption, cytoskeletal integrity is compromised and may be a cause of force loss following eccentric contraction induced injury. Identifying changes in cytoskeletal integrity is achieved by examining localization and abundance of cytoskeletal proteins, first eloquently demonstrated by Lieber, Schmitz, Mishra, and Friden (1994). The authors confirmed a 20% loss of the cytoskeletal protein Desmin 1-day post eccentric induced damage by immunohistochemistry. Further studies have observed similar cytoskeletal protein changes in Titin and Dystrophin (Lieber, Thornell, & Friden, 1996; Lovering & De Deyne, 2004). It is likely that this loss of protein content is due to the increased activity of Ca^{2+} dependent, cysteine protease Calpain following eccentric contractions (Zhang et al., 2012).

Numerous findings have supported the notion that in order to effectively repair focal damage to the myofibre, the resident muscle satellite cell (SC) is vitally important. Hyldahl, Olson, Welling, Groscost, and Parcell (2014) employed eccentric contractions to cause muscle

injury in humans showing that there is a significant expansion of the SC pool which correlates with histological signs of damage and this SC expansion is needed to proceed to regenerate the damaged fibre.

2.2.3 Satellite Cells: SCs are so termed due to their sublaminal residence, lying intimately to the myofibre sarcolemma (see Figure 2.4). Upon discovery SCs were thus immediately postulated as a cell pool for muscle hypertrophy and repair, owed to the post mitotically quiescent nature of mature myofibres (Mauro, 1961). Indeed, data later confirmed an absolute requirement for SCs in the regeneration process, as muscle does not regenerate when SCs are ablated (Reviewed by Relaix & Zammit, 2012).

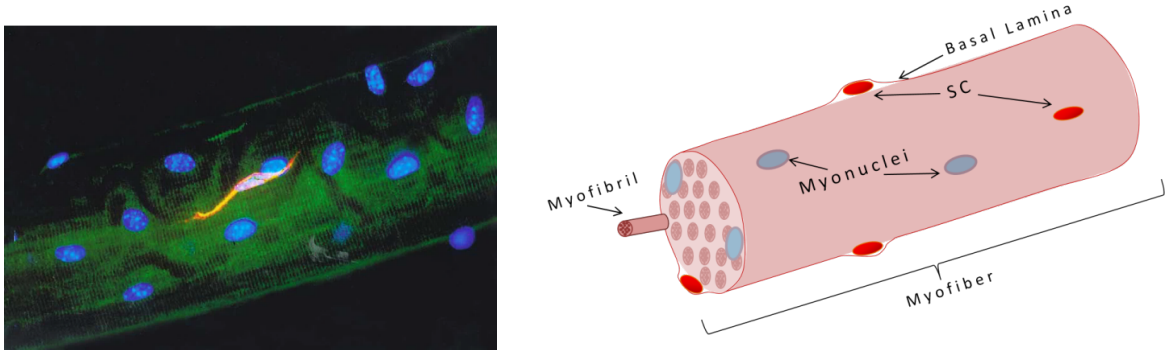


Figure 2.4. Left: A satellite cell on an isolated muscle fibre. The fibre has been stained with an antibody to M-cadherin (red) and desmin (green) and counterstained with DAPI (blue) to show the nuclei. M-cadherin is present on the satellite cell. Desmin is present in the striations of the muscle fibre and in the satellite cell (image from Morgan & Partridge, 2003). **Right**: a graphical representation of the location of satellite cells in relation to the single muscle fibre.

2.2.4 Overview of Factors Controlling SC Activation & Function: In intact muscle, SCs like other somatic stem cells are highly reliant on their microenvironment, termed a stem cell niche (Jones & Wagers, 2008). This niche allows SCs to remain in G_0 (quiescence) phase until exposure to appropriate stimuli that signal exit from quiescence and entry to the cell

cycle (Scadden, 2006). Myogenic commitment of SCs in postnatal muscle remodelling is determined by the activity of myogenic regulatory factors (MRFs), which act with pleiotropic transcription factors to control muscle gene expression patterns that initiate differentiation (Braun & Gautel, 2011). The committed muscle progenitor is termed an adult myoblast. The behaviour of SCs is dictated by multiple structural and biochemical factors within their immediate niche, from the local milieu and from the systemic milieu as depicted in Figure 2.5 (adapted from Yin, Price, & Rudnicki, 2013).

Within the SC niche, there are a number of factors that are reported to be vital to the activation of SCs following muscle damage. Regulatory signalling pathways that appear to be important in this process include Wnt/Notch signalling pathways and sphingolipid signalling.

- **Wnt signalling** is implicated in numerous processes involved in the muscle regeneration process such as cell adhesion, cell polarity, cell proliferation and cell morphology. Wnt glycoproteins bind to Frizzled receptors and low density lipoprotein receptor related proteins (LRP) to initiate intracellular effects. These downstream effects appear to be mediated by either β-catenin or planar cell polarization (PCP) pathways. The exact function of the Wnt/β-catenin pathway in muscle regeneration remains a topic of debate, however it is likely that different activating and inhibitory Wnt ligands function to control β-catenin localization and thus quiescence and proliferation in the muscle regeneration process (Otto et al., 2008). As for the Wnt/PCP pathway, signalling through Frizzled and LRP receptors activates Rho/Rac small GTPase and Jun NH_2-terminal kinase (JNK) to regulate cytoskeleton organization and gene expression. Of interest, Wnt7a has been demonstrated to act through this pathway and mediate symmetric division of SCs but also stimulate the migration of SCs resulting in improved muscle regeneration and strength (Bentzinger et al., 2014).

- **Notch signalling** is an important effector of proliferation, differentiation and cell fate determination. The Notch transmembrane receptor is activated by its ligands, Delta and Jagged, following which the receptor undergoes enzymatic cleavage to form an active shortened form of Notch. Activated Notch may then interact with the suppressor of Hairless and Lag-1 (CBF1) leading to transcriptional activity of target genes. In muscle injury, Delta is up regulated in SCs and myofibres leading to a consequential increase in the notch intracellular domain (NICD; cleaved form of Notch). This allows for the expansion of SCs and their progenitors. Importantly, blunting this pathway inhibits SC activation and attenuates muscle regeneration (Conboy & Rando, 2002).
- **Sphingolipid signalling** is implicated in cell proliferation, migration, death and senescence. Of prime importance in regenerating skeletal muscle appears to be sphingomyelin which is an important mediator of mitogenic S1P synthesis. It is the synthesis of S1P that signals SC entry to the cell cycle and permits normal muscle regeneration (Nagata, Partridge, Matsuda, & Zammit, 2006). Inversely, when the sphingomyelin to S1P synthetic pathway is inhibited, there is a marked decrease in activated SC number and overall impairment in muscle regeneration (Calise et al., 2012). These observations are likely due to the proliferative, pro-inflammatory and anti-apoptotic functions of the S1P pathway.

Aside from these key intracellular pathways are extracellular matrix interactions and other related factors that control SC activation and function. As previously discussed, SCs reside between the apical surface of the myofibre and the basal lamina. As a result, there is considerable binding between the SC and the ECM through integrins (specifically $\alpha 7\beta 1$). This is crucial for the anchoring of SCs and for the transduction of mechanical force that stimulates intracellular signalling responses within the SC (Boppart, Burkin, & Kaufman, 2006). Such binding also exists with the myofibre, whereby integrins on the SC membrane associate with muscle specific laminin. This intricate architecture not only allows SCs to be

anchored at a given site and respond to mechanical stimuli, but also allows a mechanism of migration through integrin binding and recycling at polarized edges of the activated SC/myogenic progenitor (Huttenlocher & Horwitz, 2011). In addition, SCs also display surface receptors that allow interaction with growth factor precursors that are vitally important to their activation and function.

- **Hepatocyte Growth Factor (HGF or Scatter Factor)** is one such precursor that is released following muscle damage (Tatsumi, Anderson, Nevoret, Halevy, & Allen, 1998). This is brought about by the synthesis of nitric oxide (NO) by NO synthase (NOS) in response to muscle damage. NO stimulates the release of HGF that interacts with its receptor, c-MET, on the surface of SC's to control various downstream processes. Indeed, Tatsumi et al. (2006) and Tatsumi, Sheehan, Iwasaki, Hattori, and Allen (2001) have provided both *in vivo* and *in vitro* evidence for the important role of NO-HGF signalling in satellite cell activation, demonstrating that stretching muscle liberates HGF in a NO dependent fashion leading to SC activation to remodel the stretched muscle.
- **Insulin-like Growth Factors (IGF)** 1 and 2 demonstrate important roles in SC activation and function. IGF1 mediates cell migration, proliferation and differentiation whilst IGF2 has a more dedicated role in signalling myoblast differentiation (Coolican, Samuel, Ewton, McWade, & Florini, 1997). The pleiotropic functions of IGF1 are achieved through its binding protein, IGF1R, which functions as a tyrosine kinase to initiate signal transduction pathways that include the phosphatidinositol-3-kinase (PI3K)/Akt/mTOR and PI3K/Akt/P38-MAPK, key effectors of myoblast differentiation and hypertrophy. Importantly, IGF1R signalling also activates the Ras/Raf/ERK pathway, which is crucial for SC expansion (Coolican et al., 1997). The importance of IGF signalling in myogenesis has been evidenced through the generation of mice carrying null mutations of components of either the IGF1 or IGF2 pathway. $IGF1R^{-/-}$ mutants are born with a dystrophic phenotype and typically die shortly after birth (Liu,

Baker, Perkins, Robertson, & Efstratiadis, 1993) and IGF2$^{-/-}$ mutants are born lighter (~40%) than WT mice (DeChiara, Efstratiadis, & Robertson, 1990).

- **Fibroblast Growth Factors (FGFs)** are comprised of a family of mitogenic factors that are implicated in SC function. FGFs function through dimerization with their receptors (FGFR), which display tyrosine kinase activity and activate the Ras/MAPK pathway. Activation of this pathway by FGF/FGFR leads to pro-proliferative and anti-differentiation effects allowing normal skeletal muscle regeneration. In addition, FGF displays pro-angiogenic properties that enable muscle regeneration (Lefaucheur, Gjata, Lafont, & Sebille, 1996).
- **Matrix Metalloproteinases (MMPs)** in skeletal muscle play an important role in the homeostasis and maintenance of myofibre integrity by breaking down ECM components and regulating skeletal muscle cell migration, differentiation and regeneration (Chen & Li, 2009). Relating to migration, it is important that myogenic progenitors can degrade the ECM to migrate efficiently toward the site of damage to fuse to and repair the Myofibre. Investigations have demonstrated that MMPs are important to this process *in vitro* and *in vivo* (El Fahime, Torrente, Caron, Bresolin, & Tremblay, 2000; Zimowska, Brzoska, Swierczynska, Streminska, & Moraczewski, 2008). Indeed, the inhibition of MMPs results in markedly impaired migration capacity causing attenuated repair (Nishimura et al., 2008), whilst overexpression increases migration speed (Allen, Teitelbaum, & Kurachi, 2003). Consequently MMPs are crucial for successful migration of skeletal muscle progenitors and thus an important factor in efficient muscle regeneration. MMPs also act as important regulatory molecules in the activation of SCs. Yamada et al. (2006) isolated SCs from 9-month-old male Sprague-Dawley rats and subjected them to mechanical stretch. The authors report that MMPs released as a result of mechanical stretch are involved in the NO dependent release of HGF which, as previously discussed, is a key regulator of SC activation.

Outside of the immediate niche, within the muscle fascicle lie a number of factors that have the ability to mediate SC function and ultimately muscle regeneration.

- **Neurons** play a crucial role in maintaining muscle health through electrical activity permitting contraction and remodelling. Thus, when denervation of muscle occurs there is a marked atrophy of the muscle and a significant reduction in SC number (Rodrigues Ade & Schmalbruch, 1995). The reduction in SC content is likely due to the absence of neurotrophic factors such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF), although mechanisms involved are far from being established.
- **Interstitial cells** such as fibroblasts secrete growth factors (such as FGF) and ECM remodelling factors (collagen, laminin, fibronectin, tenascin and heparin sulphate protoeglycans) that are crucial for SC homeostasis. Recent data also propose that fibroblasts influence muscle progenitor differentiation and alignment in contact dependent and independent manners *in vitro* (Rao et al., 2013).
- **Microvasculature** provides an important pathway for the delivery and removal of substances to skeletal muscle. Interestingly, genetically engineered mice allowing direct muscle SC visualization indicate that, in addition to being located beneath myofibre basal laminae, SCs are patently close to capillaries (Christov et al., 2007). Indeed, endothelial cell cultures enhanced SC growth, through IGF-1, HGF, bFGF, PDGF-BB, and VEGF. Satellite cells and myoblasts also produce vascular endothelial growth factor (VEGF) promoting angiogenesis in muscle repair. Thus, SCs, myoblasts and endothelial cells interact and cooperate to facilitate angiogenesis and myogenesis.

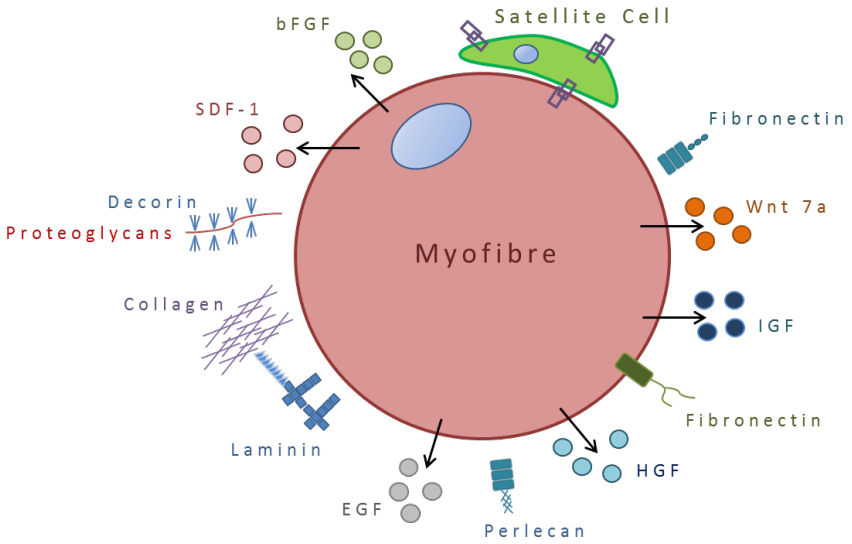
A final point of discussion around factors implicated in SC function is the systemic milieu. Circulating factors are important both in the stimulation of SC activation but also indirectly in the muscle degeneration/regeneration process.

- **Immune cells** are key players in the muscle regeneration process and have a dual role in dissolution of the damaged fibre and in the activation of SCs. Following an initial infiltration of neutrophils, two macrophage populations namely $CD68^{+}/CD163^{-}$ and $CD68^{-}/CD163^{+}$, are implicated in pro and anti-inflammatory signalling. The $CD68^{-}/CD163^{+}$ population have been reported to activate SC, which is in keeping with the

highly orchestrated nature of muscle regeneration since this population of macrophages are implicated in the last phase of fibre degeneration prior to regeneration by SCs (Tidball & Villalta, 2010). Recent evidence implies that the two macrophage populations may not in fact be distinct but undergo a phenotypic switch from a pro to anti-inflammatory phenotype (Arnold et al., 2007).

- **Androgens** such as testosterone bind to the androgen receptor (AR) present in SCs, which are responsive to androgen signalling (Doumit, Cook, & Merkel, 1996). Testosterone has been demonstrated to activate rat SCs to proliferate (Joubert & Tobin, 1995) and increase myonuclear number (Sinha-Hikim, Cornford, Gaytan, Lee, & Bhasin, 2006) and myofibre/whole muscle size in human adult males (Sinha-Hikim et al., 2002).
- **Nitric oxide** (NO) can be produced by numerous cells types by NO synthases (NOS) and has a strong function in both healthy and regenerating muscle. Macrophage and myofibrillar production of NO during muscle regenerating aids the lysis of the damaged fibre and reduces other inflammatory induced damage suggestive of facilitative and protective role of NO in muscle regeneration. The inhibition of NO production has demonstrated impaired SC activation during regeneration as a consequence of reduced HGF-c-Met interaction. Furthermore, NO may stimulate SC migration permitting fast regeneration of the focal damage site (Otto, Collins-Hooper, Patel, Dash, & Patel, 2011) and facilitate long-term regeneration by regulating SC fate (Buono et al., 2012).

a



b

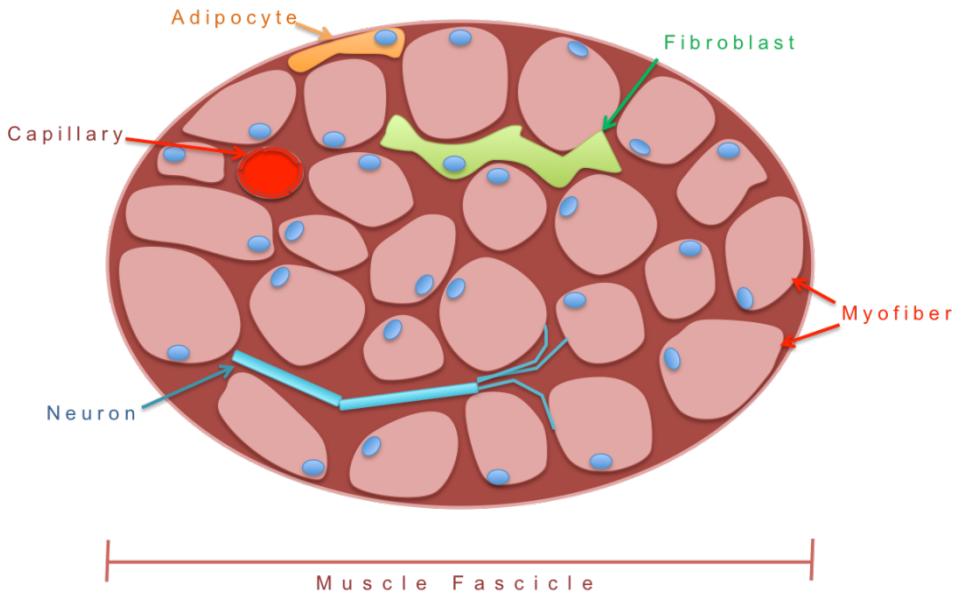


Figure 2.5. a) Niche and **b)** fasicle factors that influence satellite cell behaviour. Abbreviations: bFGF – fibroblast growth factor. SDF-1 – stromal cell derived factor 1. Wnt 7a – integration/Wingless 1. IGF – insulin like growth factor. HGF – hepatocyte growth factor. EGF – epidermal growth factor

2.2.5 Skeletal Muscle Regeneration Steps: The skeletal muscle regeneration process occurs in three well-orchestrated, sequential but overlapping stages: **1**) the inflammatory response; **2**) the activation, migration, differentiation, and fusion of SCs; and **3**) the maturation and remodelling of newly formed myofibres (Yin et al., 2013). Preceding the regeneration process however, is an important phase of degeneration, driven by calcium-dependent proteolysis, a consequence of increased calcium influx due to sarcolemma dissolution and calcium release from the sarcoplasmic reticulum (Armstrong, 1990). The necrosis of the myofibre importantly activates the complement cascade and subsequently inflammatory responses, the first process in muscle regeneration. Firstly, a population of neutrophils infiltrate the damaged muscle followed by two populations of macrophages ($CD68^{+}/CD163^{-}$ and $CD68^{-}/CD163^{+}$) that secrete pro-inflammatory cytokines in the early stages of inflammation and anti-inflammatory cytokines in later stages preceding the termination of the inflammatory response. Recent data are suggestive that injured skeletal muscle recruits monocytes exhibiting inflammatory profiles that operate phagocytosis and rapidly convert to anti-inflammatory macrophages that stimulate myogenesis and fiber growth (Arnold et al., 2007). Indeed, the importance of the inflammatory response has been demonstrated by selectively depleting monocytes/macrophages by dipethria toxin injection in mice, which completely impairs regeneration following muscle damage (Arnold et al., 2007). Importantly, the $CD68^{-}/CD163^{+}$ sub population are known to aid in the activation of SCs (Tidball & Villalta, 2010).

Upon appropriate cues discussed in section 2.2.3, SCs will be activated and undergo stochastic or asymmetric division as represented below in Figure 2.6. This allows for replenishment of the SC pool whilst also providing a myogenic progeny for muscle repair and remodelling.

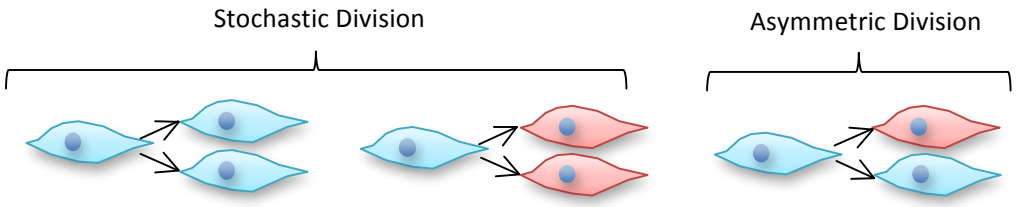


Figure 2.6. Fates of the activated satellite cell. Stochastic division involves the generation of two daughter SCs or two myogenic progenitor cells whereas asymmetric division involves the generation of one daughter cell and one myogenic progenitor.

The commitment of satellite cells to a myogenic fate is orchestrated by the temporal expression of myogenic regulatory factors (MRFs), a group of transcription factors that target myogenic loci. This expression profile is characterized by an early increase of Myf5 and MyoD, which precedes increases in Myogenin and Mrf4 expression and a decrease in the early differentiation MRFs, Myf5 and MyoD.

2.2.6 Myoblast Migration: In order to efficiently repair damaged tissue following activation myoblasts must migrate toward the site of damage to fuse to the area of focal injury to complete the resolution of the myofibre. Myoblasts are capable of migration between myofibres, muscles, across basal lamina and connective tissue during development and regeneration (Hughes & Blau, 1990; Jockusch & Voigt, 2003). Briefly, this process involves key steps that include: polarisation of the cell (establishing a front and rear), formation of stable adhesions with the extracellular substrate, formation of cell protrusions at the leading cell edge, cytoskeletal contraction and rear edge detachment. Recent insights also point to alternative mechanisms of SC movement *in vivo* via cytoplasmic changes in hydrostatic pressure and membrane blebbing (Charras & Paluch, 2008).

- **Cell polarisation** is poorly understood but is vitally important to allow the cell to have a 'sense' of direction. Migration will only be effective if the cell moves towards the target

area, in the case of muscle damage this is the focal injury site. Polarisation is characterised by asymmetric localization or activation of specific signalling proteins, creating a morphological asymmetry. This can be conceptualised by the formation of lamelipodia (broad, flat structures) and filapodia (thin, long strictures) at the leading edge of the cell. The establishment of these structures is brought about by localized actin polymerization at the leading edge of the cell (Mogilner & Oster, 1996).

- **Creation of tension** through focal adhesion formation is necessary to create traction between the cell and the substratum and to organize signalling networks that regulate migration. Via integrin-mediated linkages with the ECM a link is formed between the cell and the substratum. Upon binding to the substratum, integrin clusters are formed creating multi-protein complexes that create intracellular signalling pathways and coupling to the actin cytoskeleton. The process of integrin binding dynamically forming and 'recycling' coupled with the polarized assembly and disassembly of adhesions are essential for peak migration velocity and directionality (Bridgewater, Norman, & Caswell, 2012)
- **Cytoskeletal contraction** is achieved through traction signals transmitted via integrin adhesion complexes and provides a feedback loop between extracellular tension and cytoskeletal tension to mediate cytoskeletal organization and migration (Yamaguchi & Condeelis, 2007).
- **Rear edge detachment** is equally as important as the events at the cells anterior because in the absence of co-ordinated disassembly of adhesion complexes, cells do not move (Palecek, Schmidt, Lauffenburger, & Horwitz, 1996). The process of rear edge detachment appears to be strongly mediated by the function of the proteolytic enzyme Calpain. A number of studies provide evidence of a necessity for Calpain in attachment and migration (Dedieu et al., 2004; Mazeres, Leloup, Daury, Cottin, & Brustis, 2006; Van Ba & Inho, 2013).

- **Blebbing or ameboid-based movement** has been demonstrated to be an important mechanism of SC movement *in vivo,* functionally distinct from canonical lamellipodial migration and involves the creation of membrane blebs via hydrostatic pressure created by the sarcoplasm under the control of the actomyosin cortex (Charras & Paluch, 2008).
- **Cell-cell signalling** is known to occur between many cells through secreting and releasing proteins and vesicles. However, until a recent investigation by Ma et al. (2015) mechanistic understanding as to how this occurs during migration was limited. The authors provide evidence of 'migrasomes' – exosome like organelles secreted by leading migrating cells through retraction fibres, that can be taken up by trailing cells and may potentially provide guidance cues between cells.

Many regulatory molecules have been implicated in myoblast migration such as MAPKs, phoshpholipases, Ser/Thr & Tyr kinases. However, key regulatory proteins in myoblast migration believed to be most influential are the Rho family of GTPases that link cell surface receptors to a variety of intracellular responses connected to cell migration (Nobes & Hall, 1999). Three Rho GTPases are implicated in cell migration; Rho, Rac and Cdc42. Rho regulates the assembly of contractile filaments whilst Rac and Cdc42 regulate actin polymerization to form lamelipodia and filapodia, respectively. All three Rho GTPases can regulate the assembly of integrin based adhesion complexes and can affect the microtubule cytoskeleton and gene transcription.

During cell migration, important upstream signalling of the Rho GTPases are the PI3-K and P130Cas/CrkII/Dock180 pathways. Dimchev, Al-Shanti, and Stewart (2013) demonstrate that inhibition of the natural PI3-K inhibitor phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN) results in improved myoblast motility. Additionally, when PI3-K/Akt are inhibited this improved migration returns to control levels. Similarly, the receptor tyrosine kinase c-MET is also required for normal myoblast migration. Mutant c-MET null mice do not regenerate muscle efficiently following acute injury as a consequence of

defective lamellipodia formation, shorter progenitor migration distances and slower migration compared to control myoblasts (Webster & Fan, 2013).

2.2.7 Myoblast Fusion: To successfully finish the regeneration process, myoblasts fuse with the damaged myofibre contributing new myonuclei. This process is highly orchestrated and crucial for regeneration, for which the underpinning molecular mechanisms are still largely undefined. At present it is understood that cell-cell recognition, adhesion and membrane merging are three mechanistically linked phases of myoblast fusion.

More protracted efforts have been made to study these processes and their regulators in *Drosphilia*, however only recently has research efforts focussed on mammalian models. Przewozniak et al. (2013) denervated and crushed the *soleus* muscle of 3-month-old mice to identify the role of adhesion proteins in skeletal muscle fusion following injury. The authors first demonstrate that integrin α3, integrin β1, ADAM12, CD9, CD81, M-cadherin and VCAM-1 all increase their expression profile during fusion and myotube formation following injury *in vivo*. Subsequently, the researchers show the complexity and importance of individual members of multi-protein adhesion complexes by silencing the integrin α3 subunit via RNA interference. This *in vitro* experiment provided evidence that under expression of integrin α3 leads to an impaired fusion capability of myoblasts, an observation that was not related to modifications in expression of other proteins (those previously mentioned). This highlights that the under expression of a single member of a multi-protein adhesion complex can impair the capability of the complex to orchestrate cell-cell adherence and fusion. Recently a novel membrane protein termed Myomaker, required for the activation and fusion of myoblasts was identified (Millay et al., 2013). Over expression of the protein significantly enhances fusion capability and abolition results in perinatal death due to a failure to form myotubes. Similarly, but to a lesser extent than Myomaker, normal myoblast fusion requires Myoferlin (Doherty et al., 2005). As described earlier, myoblast fusion requires the alignment and fusion of two phospholipid bilayers (sarcolemma) and Myoferlin appears important for binding phospholipids in a calcium-sensitive manner.

Myoferlin -/- mice still form myotubes however show an impaired regenerative capacity and develop smaller myotubes than WT controls, instead displaying a dystrophic phenotype. Taken together, a number of interacting systems control myoblast fusion and impairments even in single proteins can significantly alter normal myoblast fusion.

2.3 Vitamin D

2.3.1 Background: Vitamin D was first discovered, like other vitamins, through investigation into diseases attributable to dietary deficiencies (McCollum, 1914). Since then, there has been a considerable increase in research attention toward Vitamin D. This is in part due to the re-emergence of the preventable bone disorder rickets and a well-documented prevalence of worldwide Vitamin D deficiency (discussed in further detail section 2.3.9). There have been a number of key research findings which have advanced the field considerably, particularly the identification of the Vitamin D receptor (VDR) in many tissues through which Vitamin D exerts the majority of its effects (Demay, 2006). Moreover, the generation of the VDR knock out mouse (Li et al., 1997) has provided great insight into the multiplicity of biological actions of Vitamin D. It is now understood that aspects of innate and acquired immunity (Chun, Liu, Modlin, Adams, & Hewison, 2014), bone health (Ebeling, 2014), cardiovascular health (Lavie, Dinicolantonio, Milani, & O'Keefe, 2013) and processes within skeletal muscle are just some of the physiological features thought to be regulated by Vitamin D.

2.3.2 Forms of Vitamin D: There are a number of biological forms of Vitamin D that occur in dietary sources and that are synthesized endogenously in the human body. Vitamin D_2 (also termed ergocalciferol) and D_3 (also termed cholecalciferol, both graphically presented in Figure 2.7) are the major sources of Vitamin D contributing to Vitamin D status and are both found in food sources with only the D_3 form synthesized in the body following appropriate exposure to UV irradiation.

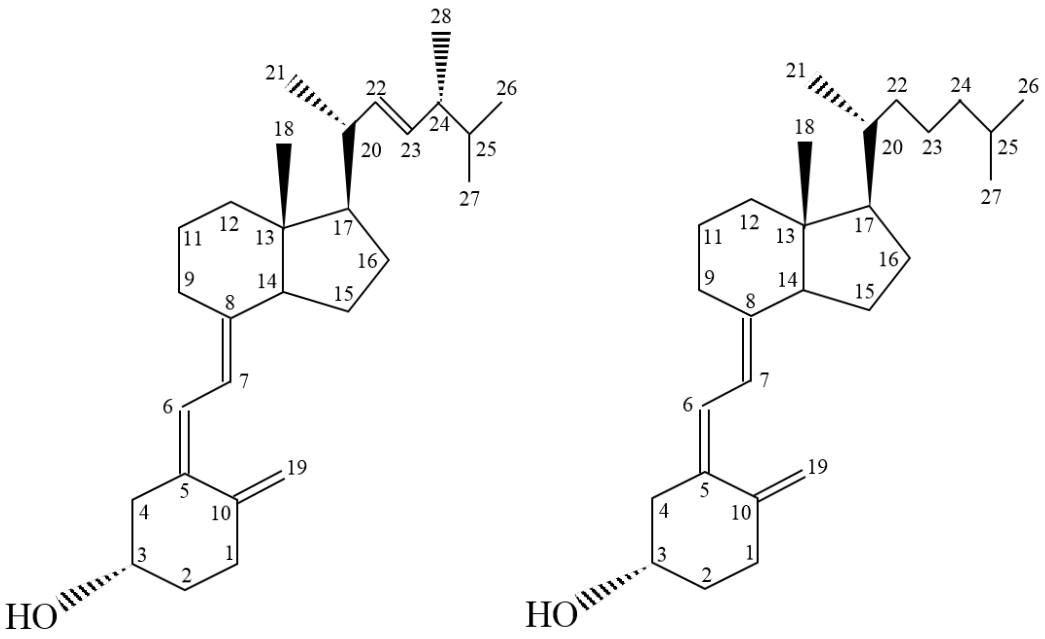


Figure 2.7. Nutritional forms of Vitamin D. Left; Vitamin D_2 and Right; Vitamin D_3. Note the broken 9,10 carbon bond of the B ring giving the Vitamin D compounds their seco-steroid classification. The structural difference between vitamin D_2 and vitamin D_3 is in their side chains. The side chain of D_2 contains a double bond between carbons 22 and 23, and a methyl group on carbon 24.

2.3.3 Vitamin D Synthesis: The human genome developed the mechanism for Vitamin D synthesis in the skins dermis that is activated by sunlight, or more specifically ultraviolet B irradiation (UVB) at a wavelength of approximately 290-315 nm (Holick, 1995). During this photosynthetic reaction, the B ring of 7-dehydrocholesterol (precursor to cholesterol and pre Vitamin D_3) is cleaved and resultantly the thermodynamically unstable pre-Vitamin D_3 (pre-cholecalciferol) is formed. Thermal isomerization following this cleavage yields Vitamin D_3, a seco-steroid hormone. In human skin, the thermal isomerisation process converts ~80 % of pre-Vitamin D_3 to Vitamin D_3 within 8 hours of exposure to UVB (Tian, Chen, Matsuoka, Wortsman, & Holick, 1993). When a sufficient quantity of Vitamin D has been synthesized in the skin (at approximately 7-10% of the original 7-dehydrocholesterol content), pre-

Vitamin D_3 is converted to the biologically inactive photoproducts tachysterol and lumisterol (Holick, MacLaughlin, & Doppelt, 1981), serving as an endogenous toxicity regulator.

There are many factors that can affect the dermal synthesis of Vitamin D. For example, the amount of radiation that actually reaches the biosphere (thus available for cutaneous Vitamin D photosynthesis) is a product of wavelength and the amount of ozone that solar radiation must pass, which itself is a function of the solar zenith angle dependent on latitude, season and time of day (Chen et al., 2007). If UVB reaches the skin, another influential factor impacting the subsequent photosynthetic reaction is skin pigmentation as melanin competes with 7-dehydrocholesterol for absorption of UVB radiation. Resultantly, those with darker skin require exposure to a stronger source of UVB or more prolonged exposure time to elicit comparable changes in circulating Vitamin D concentration seen in lighter skinned persons (Chen et al., 2007; Clemens, Adams, Henderson, & Holick, 1982).

2.3.4 Dietary Sources of Vitamin D: An obvious alternative route for obtaining vital nutrients not naturally synthesized (such as the other vitamins) is through dietary intake. Indeed there are some, but very few, dietary sources of Vitamin D although it is unlikely that humans obtain enough Vitamin D from such sources to compensate for a lack of sun exposure. Unlike dermal synthesis that solely produces Vitamin D_3, dietary intake provides both Vitamin D_2 and D_3. Dietary sources of Vitamin D include oily fish, eggs, fortified breakfast cereals, shitake mushrooms and powdered milk. However, intake of these foods appears to be limited in developed countries (Hill, O'Brien, Cashman, Flynn, & Kiely, 2004; Moore, Murphy, Keast, & Holick, 2004; Survey, 2011; Tylavsky, Cheng, Lyytikainen, Viljakainen, & Lamberg-Allardt, 2006). Large scale investigations have identified that < 2% of individuals studied met the recommended daily allowance (RDA = 600 $IU.day^{-1}$ or 15 $\mu g.day^{-1}$ for adults; 100 IU =2.5 μg) for Vitamin D intake from foods (Hill, Jonnalagadda, Albertson, Joshi, & Weaver, 2012). Adding further complexity to obtaining sufficient

Vitamin D in the diet, Chen *et al.* (2007) provide evidence that many foods stating fortification with Vitamin D may in fact contain < 80% of the Vitamin D claimed on the label whilst 14% of samples tested contained no detectable Vitamin D. Thus, when UVB exposure is lacking, it may be difficult to obtain sufficient Vitamin D from the diet.

2.3.5 Vitamin D Metabolism: Regardless of how Vitamin D is obtained (through dermal synthesis or from dietary sources), both forms will travel in the circulation bound to the Vitamin D binding protein (DBP; Cooke & Haddad, 1989). It is of importance to note at this stage that Vitamin D_2 (ergocalciferol) which enters systemic circulation from dietary food sources undergoes the same metabolic steps as Vitamin D_3, however D_3 has a relative potency to Vitamin D_2 of 9.5:1 for increasing serum 25[OH]D (Armas, Hollis, & Heaney, 2004).

Once circulating bound to DBP, Vitamin D will undergo two important hydroxylation steps occurring in the liver (via 25-hydroxylase) and in the kidney (via 1α-hydroxylase) or tissues directly expressing 1α-hydroxylase such as skin, pancreas, brain, colon (Zehnder et al., 2001), immune system cells (Moran-Auth, Penna-Martinez, Shoghi, Ramos-Lopez, & Badenhoop, 2013) and skeletal muscle (Girgis et al., 2014b; Srikuea et al., 2012). The resultant 1α,25-dihydroxyvitamin D ($1\alpha,25[OH]_2D_3$), is metabolically active and it is this form of Vitamin D that exerts its biological effects. The efficacy of tissues expressing 1α-hydroxylase to convert DBP bound 25[OH]D will be dependent upon the ability of the cell to internalize the 25[OH]D-DBP complex. In skeletal muscle, this is likely to be achieved by the expression of membrane bound Megalin that has recently been identified (Abboud et al., 2013). Following the final activating hydroxylation step, $1\alpha,25[OH]_2D$ will bind to the Vitamin D receptor (VDR) and exert genomic and non-genomic effects or may also interact with protein disulphide isomerase, family A, member 3 (Pdia3) to exert rapid cell signalling effects.

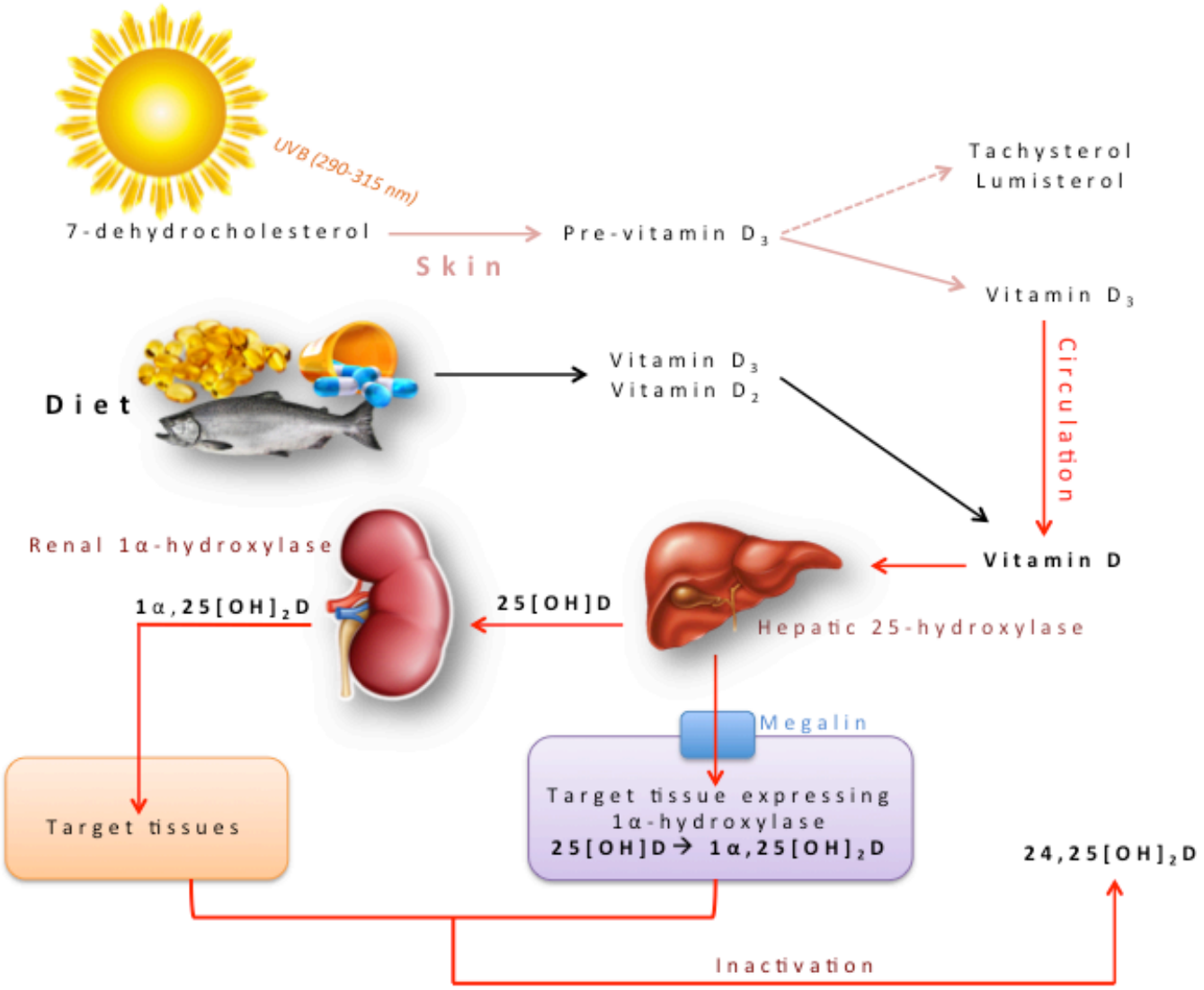


Figure 2.8. Simplistic schematic of the Vitamin D metabolic pathway. Vitamin D obtained from dermal photosynthesis or dietary exposure undergoes two hydroxylation steps leading to activation. Note that both the kidney and target tissues that express 1α-hydroxylase and are therefore capable of hydroxylating 25[OHD at the 1α carbon resulting in activation. When biological activity is no longer required, further hydroxylation at carbon 24 inactivates $1,25[OH]_2D_3$ forming 1,24,25[OH]D.

2.3.6 The Vitamin D Receptor: A significant advancement in the understanding of the VDR has developed since its cloning and recognition as a member of the nuclear receptors that regulate gene expression (McDonnell, Mangelsdorf, Pike, Haussler, & O'Malley, 1987). The receptor is constituted of 427 amino acids with its two major functional units being the N-terminal zinc finger DNA-binding domain and the C-terminal ligand-binding domain. The intricacies of the VDR structure are not in the scope of this literature review and have been eloquently discussed elsewhere (Haussler et al., 1998).

Of relevance to this thesis are the functional characteristics of the receptor. In its basal state, the VDR has been identified by fractionation and immunocytochemical localization as un-liganded in the nucleus of many cells including skeletal muscle cells (Berger et al., 1988; Buitrago & Boland, 2010; Clemens et al., 1988; Milde, Merke, Ritz, Haussler, & Rauterberg, 1989; Walters, Hunziker, & Norman, 1980; Zanello, Collins, Marinissen, Norman, & Boland, 1997). Upon exposure to $1\alpha,25[OH]_2D$ there is evidence of nuclear-sarcoplasmic translocation, with the VDR localizing in close proximity to caveolae within the sarcolemma (Buitrago & Boland, 2010). Ligand binding of $1\alpha,25[OH]_2D$ to the VDR may induce translocation of the complex to the cell nucleus where it tightly binds with 9-*cis*-retinoic acid receptor (RXR) to form a heterodimer (MacDonald et al., 1993). This VDR:RXR complex is capable of penetrating the deep groove of DNA and recognizing Vitamin D response elements (VDREs) in the sequence of Vitamin D regulated genes. In a series of immunoprecipitation experiments, Chiba, Suldan, Freedman, and Parvin (2000) first demonstrated that the liganded VDR:RXR complex binding to the Vitamin *D r*eceptor *a*ctivating *p*rotein (DRIP) co-activator complex induces interaction with RNA polymerase II to activate transcription. Data have subsequently established that upon binding to VDREs the complex can recruit other transcriptional co-activators such as steroid co-activators (SRC-1, -2 and -3) and Smad proteins that possess histone acetyl transferase (HAT) activity (MacDonald, Baudino, Tokumaru, Dowd, & Zhang, 2001). The VDR:RXR heterodimer binding to DNA appears to alter the receptor dynamics including the co-activator binding

surfaces and the sequence of the DNA response element can determine these dynamics. Further, agonist binding results in changes in the stability of the VDR binding domains (DBD), indicating that the ligand itself may play a role in DNA recognition. These data imply a mechanism by which the VDR:RXR complex expresses promoter specificity permitting differential effects on numerous Vitamin D related target genes (Zhang et al., 2011).

It is also important to recognise the established non-genomic functions of the VDR. A number of investigations have demonstrated that treatment of C_2C_{12} skeletal myoblasts and isolated primary chick myoblasts with Vitamin D stimulates tyrosine phosphorylation cascades within seconds to minutes of exposure (Nemere et al., 1998). Importantly, the use of anti-VDR antisense oligonucleotides inhibits such rapid responses lending support to a genomic role (Santillan, Baldi, Katz, Vazquez, & Boland, 2004). Further backing for this non-genomic function has been provided in an investigation in which the suppression of protein translation with cycloheximide did not inhibit the rapid effects of $1\alpha,25[OH]_2D$-VDR signalling (de Boland & Boland, 1987).

There is some conjecture as to whether the classical VDR is involved in all of the apparent rapid signalling responses that occur upon exposure to $1\alpha,25[OH]_2D_3$. Some authors have suggested that the VDR involved in the rapid activation of signal transduction pathways is a non-nuclear form of the receptor distinct from the nuclear isoform and some suggest a receptor distinct to the VDR (termed 1,25-dihydroxyvitamin D-*m*embrane-*a*ssociated, *r*apid *r*esponse *s*teroid-binding receptor or MARRS) with $1\alpha,25[OH]_2D$ binding potential and exerting cellular effects in skeletal muscle (Nemere, Garbi, & Winger, 2015). Protracted efforts to investigate the roles of these two apparently distinct receptors have revealed that Pdia3 is located in caveolae of bone and cartilage cells and is also implicated in rapid VDR signalling. Binding of $1\alpha,25[OH]_2D$ to Pdia3 establishes interaction with phospholipase A2 (PLA2) activating protein (PLAA) and caveolin-1 (Cav1) to initiate rapid signalling via Ca^2-calmodulin-dependent protein kinase II (CaMKII), phospholipase C (PLC), protein kinase C

(PKC), and in turn, the extracellular receptor mediated protein kinases (ERK) 1/2 family of the MAPKs (Boyan, Chen, & Schwartz, 2012). In some cases VDR is reported to be separate from such function and present on the sarcolemma where it is required for 1α,25$[OH]_2$D induced rapid activation of Src signalling. Although yet to be confirmed in skeletal muscle, crosstalk of signalling pathways has recently been implicated as Chen et al. (2013) demonstrate an interaction between Pdia3 and VDR in MC3T3-E1 osteoblasts, initiating rapid intracellular signalling through PLA2 and c-Src in response to1α,25$[OH]_2D_3$ exposure.

It is also interesting to consider that there are three known transcript variants of the VDR. Variants 1 and 2 encode the same protein and differ only in 92 base pairs. Variants 1 and 2 differ in the 5' UTR and coding sequence compared to variant 3 (VDRB1). The resulting isoform (VDRA) is shorter at the N-terminus compared to isoform VDRB1. As the N-terminal of the VDR is responsible for DNA binding, it is possible that isoforms 1 and 2 initiate rapid signalling whilst VDRB1 is responsible for the genomic actions of Vitamin D. Data to support this notion are not currently available and the functional differences (if any) of the two VDR isoforms remains to be established.

Supporting the role of the classical VDR in Vitamin D/skeletal muscle signalling are recent data that provide evidence for differential expression of the VDR in response to ligand availability (Garcia, King, Ferrini, Norris, & Artaza, 2011; Girgis et al., 2014b; Srikuea et al., 2012). This is in line with previous findings in other tissues such as bone and intestine, where VDR content is positively associated with an increase in biological activity following exposure to 1α,25$[OH]_2$D (Dokoh, Donaldson, & Haussler, 1984; Halloran & DeLuca, 1981). These findings strongly suggest that the VDR is important for the biological function of Vitamin D in skeletal muscle.

Although the described evidence provides potential mechanisms by which Vitamin D may exert biological effects in skeletal muscle, the presence of the VDR in skeletal muscle has in

itself recently been called into question. Many reports spanning from 1985 utilising sucrose density gradients through to 2011 where more advanced immunostaining methods have been employed have supported the presence of the VDR (summarized in Table 2.2). However, Wang, Zhu, and DeLuca (2012) recently reported that the VDR is not present in skeletal muscle as determined by immunoblotting and immunohistochemistry and suggest that antibodies previously used to detect the VDR where not specific to VDR protein. They report that the Santa Cruz VDR 9A antibody is most specific to the VDR and failed to detect the presence of the receptor in skeletal muscle, also detecting only very small levels of VDR transcripts by PCR. Conversely, Srikuea et al. (2012) provide robust evidence for the presence of the VDR in C_2C_{12} myoblasts by employing a number of techniques including immunoblotting, immunocytochemistry, PCR-based cloning and DNA sequencing, all of which successfully detected the presence of the VDR in skeletal muscle. Similar findings are described by Girgis et al. (2014b). The investigators successfully demonstrate the presence of the VDR in skeletal muscle of two *in vitro* models (C_2C_{12} cell line and primary mouse myotubes) using PCR, immunoblot and immunohistochemistry. Interestingly, Kislinger et al. (2005) implemented large-scale gel-free tandem mass spectrometry to detect proteome alterations throughout the myogenic differentiation programme in C_2C_{12} myoblasts and observed an up regulation of the VDR during early myoblast differentiation. Similar observations have been made demonstrating transient changes in VDR expression as discussed earlier. It is plausible that the time point at which the VDR protein is probed in skeletal muscle may affect the ability to identify it, as its expression appears to fluctuate based on ligand exposure and cell cycle stage.

Table 2.2. Summary of investigations directly assessing the presence of the VDR in skeletal muscle.

Authors	Model	Methodology	VDR Detected (+/-)
Simpson, Thomas, and Arnold (1985)	G8, H9c2 myoblasts	Sucrose density gradient, chromatography.	+
Costa, Blau, and Feldman (1986)	Cloned human muscle	[^{3}H]1α,25-[OH]$_2$D$_3$ binding assay (sucrose density gradient).	+
Boland, Norman, Ritz, and Hasselbach (1985)	Primary chick embryo myoblasts	Sucrose density gradient	+
Sandgren, Bronnegard, and DeLuca (1991)	Rat skeletal muscle	Immunoradiometric assay	-
Buitrago, Vazquez, De Boland, and Boland (2000)	Primary chick myoblasts	SDS-PAGE Western Blot	+
Bischoff et al. (2001)	Human skeletal muscle tissue	Immunohistochemistry	+
Boland et al. (2002)	Primary chick myoblasts	SDS-PAGE Western Blot	+
Capiati, Benassati, and Boland (2002)	Primary chick myoblasts	SDS-PAGE Western Blot	+
Endo et al. (2003)	VDR -/- mice and C2C12 cell line	Immunohistochemistry, RT-qPCR, Northern Blot	+
Wang and DeLuca (2011)	Six- to 7-wk-old C57BL/6 mice	Immunohistochemistry, RT-qPCR	-

Taken together, recent findings lend support to the notion that the majority of actions of Vitamin D in skeletal muscle are mediated through the classical VDR, which evidently is necessary for both the genomic and rapid non-genomic actions of the hormone. Furthermore, exposure to Vitamin D appears to be an important determinant of VDR signalling although

as will be discussed, many individuals lack adequate Vitamin D, compromising tissue exposure.

2.3.7 Measuring Vitamin D Concentration: Prior to discussion of worldwide Vitamin D status is the necessity to consider how and what to measure. Over 40 metabolites of Vitamin D have been identified (Bouillon, Okamura, & Norman, 1995), but most of these have a very short half-life in the circulation and so are currently of minimal interest. Although the parent sterol Vitamin D has a half-life close to 24 hours (Clemens, Adams, Nolan, & Holick, 1982) this is relatively short compared to 25[OH]D with a half–life of 21-30 days (Zehnder et al., 2001). The measurement of circulating 25[OH]D is a better indicator of Vitamin D exposure whether obtained from UVB exposure (contributing 80-90% of 25[OH]D) or dietary sources (contributing 10-20%). The most potent physiologically active circulating metabolite produced by humans is 1α,25$[OH]_2$D which has a half-life of 4-15 hours and while 25[OH]D circulates in nmol.L^{-1} concentrations, 1α,25$[OH]_2$D is present in much lower concentrations (pmol.L^{-1}).

Supplementation of foods and health products or supplemental treatment with either Vitamin D_2 or D_3 can increase the percentage derived from exogenous sources and so assay technology needs to be able to measure both D_2 and D_3 metabolites. The main rate limiting step in the pathway of Vitamin D metabolism is the 25-hydroxylation that takes place in the liver. This step is primarily dependent on the substrate concentration (Vitamin D) and is the reason why seasonal variability exists related to UVB exposure. 1α-hydroxylation mainly takes place in the kidney but can also happen in bone, skin, placenta, granuloma tissue and indeed skeletal muscle (Srikuea et al., 2012; Zehnder et al., 2001) and requires 25$[OH]D_2/D_3$ as the substrate. The rate of 1α,25$[OH]_2$D production by the kidney can be influenced by the prevailing calcium and parathyroid hormone (PTH) concentration. For these reasons, as well as the short half-life, measurement of total 1α,25[OH]2D is a poor indicator of overall Vitamin D status as total 25[OH]D needs to decrease to around 10 nmol.L^{-1} for total 1α,25$[OH]_2$D to decrease significantly (Need et al., 2008).

Isotope dilution LC-MS/MS is considered the gold standard method for measuring serum total 25[OH]D and can simultaneously quantify 25[OH]D_2 and 25[OH]D_3 with summation of the two values resulting in an accurate and precise measure of total 25[OH]D. All immunoassays evaluated appropriately have a problem with the recognition of 25[OH]D_2 and under-recover this molecule whether added in the natural/synthetic form or estimated in the circulation following ingestion. Detailed comparison of the performance of commercially available assays inclusive of assay data can be found in Snellman et al. (2010).

2.3.8 Categorization of Vitamin D Status: The categorization of Vitamin D status has been the subject of intense debate in the past decade. Conflicting views as to what constitutes deficiency and what is optimal are at the centre of this dispute. The present guidelines put forward by the US Institute of Medicine are generally adhered in worldwide medical practice (Table 2.3).

Table 2.3. US Institute of Medicine (2011) Vitamin D concentration classification and suggested optimal (Zittermann, 2003).

Serum 25[OH]D	Classification
< 12 nmol.L^{-1}	Severely deficient
12 – <30 nmol.L^{-1}	Deficient
30 – 50 nmol.L^{-1}	Inadequate
> 50 nmol.L^{-1}	Adequate

However, many have called for revision of these guidelines, claiming that they are too conservative. As examples, it has been suggested that only when serum 25[OH]D is >75 nmol.L^{-1} should this be considered adequate since perturbations to calcium and phosphate homeostasis occur below this level with intact parathyroid hormone concentrations being elevated (Heaney, 2013). Some authors have suggested that >100 nmol.L^{-1} should be

considered optimal as those living and working outdoors in sun rich environments present with concentrations in this range (Zittermann, 2003). The debate over what constitutes classification of Vitamin D limits its usefulness and therefore investigation into the role of Vitamin D in skeletal muscle function should be discussed in relation to absolute circulating concentration of serum 25[OH]D.

2.3.9 Worldwide Vitamin D Status: Vitamin D deficiency and insufficiency as classed by the US IoM is common with estimates of 1 billion people worldwide into the category, even in areas with ample sunlight exposure for dermal synthesis throughout the year. In the United States, it is reported that approximately 40% of the population has vitamin D concentrations < 50 nmol.L^{-1} (Forrest & Stuhldreher, 2011). A similar prevalence has been observed in Australia (Daly et al., 2012) and also areas in the Middle East (Elsammak, Al-Wossaibi, Al-Howeish, & Alsaeed, 2011; Hoteit et al., 2014). At Northerly latitude's (> 35^{o} N) in Europe, deficiency is also common. Large population-based studies from Ireland (Cashman et al., 2012) Denmark (Thuesen et al., 2012) France (Chapuy et al., 1997), Germany (Hintzpeter, Mensink, Thierfelder, Muller, & Scheidt-Nave, 2008) and the UK (Bates et al., 2003) report high frequency of serum 25[OH]D levels < 50 nmol.L^{-1}. In sub populations such as professional athletes, Vitamin D deficiency is also common. Our research group has previously established this in a cohort of 64 professional UK based athletes. These data are in line with other reports in Europe and the rest of the world (See table 2.4).

Table 2.4. Summary of trials assessing Vitamin D status in athletic populations.

Authors	Elite Sample Population	Test Month (Season)	Serum 25[OH]D Concentrations
Hamilton, Grantham, Racinais, and Chalabi (2010)	93 middle eastern professional and semi-professional athletes from sports including; football, athletics, squash, handball, cycling, martial arts and body building.	Apr-Oct (random testing)	91% <50 nmol.L^{-1}
Morton et al. (2012b)	20 FA Premier League soccer players	Aug and Dec (Summer vs Winter)	65% <50 nmol.L^{-1}
Close et al. (2013b)	64 Professional UK based athletes from sports including; football, rugby horse racing	Nov-Jan (Winter)	62% <50 nmol.L^{-1}
Wolman et al. (2013)	19 elite classical ballet dancers	Winter vs summer	74% 25–75 nmol.L^{-1} 26% <25 nmol.L^{-1}
Valtuena, Dominguez, Til, Gonzalez-Gross, and Drobnic (2014)	408 elite Spanish athletes across more than 30 different sports.	Winter (Jan) vs Spring (Apr) vs Summer (Ju)l vs Autumn (Oct)	82% <75-50 nmol.L^{-1} 45% <50-27.5 nmol.L^{-1} 6% <27.5 nmol.L^{-1}
Wilson et al. (2013)	37 elite professional jockeys	Jan-Apr (Winter)	31% 30-50 nmol.L^{-1} 42% 12.5-30 nmol.L^{-1} 5% <12.5 nmol.L^{-1}

As discussed previously, there are many factors that limit the cutaneous synthesis of Vitamin D and few dietary sources can provide sufficient quantity to compensate for a lack of sun exposure. These factors explain the epidemiological data above as all of the geographical locations described fall into the category of developed countries where a sun-shy lifestyle due to indoor work, excessive sun-screening and government guidance to avoid sun exposure is commonplace.

Although the data are striking, they do not describe the consequence of Vitamin D deficiency, which ultimately influences the relevance of such statistics. Indeed there are a number of physiological roles for Vitamin D and known consequences of deficiency. Classically, severe Vitamin D deficiency is understood to cause rickets in children and osteomalacia in adults, as the Vitamin D-parathyroid hormone (PTH) axis is a crucial regulator of bone resorption and turnover. Consequentially, it is reported that markers of bone resorption and turnover are elevated above the normal reference ranges in individuals with insufficient serum 25[OH]D concentrations (Dawson-Hughes et al., 1991; Sahota, Masud, San, & Hosking, 1999; Sahota et al., 2004). It is also now well established that Vitamin D is a potent regulator of aspects of innate and acquired immunity (Chun et al., 2014) and regulates cell cycle progression, potentially protecting against the development and advancement of fatal diseases such as cancer (Pilz, Gaksch, Hartaigh, Tomaschitz, & Marz, 2015).

Emerging insights from human trials suggest severe Vitamin D deficiency correlates with smaller heart size (Allison et al., 2014), impaired energy metabolism (Sinha, Hollingsworth, Ball, & Cheetham, 2013), diabetes (Dalgard, Petersen, Weihe, & Grandjean, 2011), oxidative stress (Uberti et al., 2014) and Alzheimer's diseases (Littlejohns et al., 2014). The focus of this thesis however pertains specifically to skeletal muscle for which scientific data are lacking in the context of Vitamin D.

2.3.10 Vitamin D and Skeletal Muscle: Observational Studies: Early interest in vitamin D and skeletal muscle health was stimulated by numerous observational findings. It has repeatedly been reported that severe Vitamin D deficiency is accompanied by skeletal muscle myopathy that is reversible with supplemental Vitamin D (Mingrone, Greco, Castagneto, & Gasbarrini, 1999; Prabhala, Garg, & Dandona, 2000; Rimaniol, Authier, & Chariot, 1994; Russell, 1994; Ziambaras & Dagogo-Jack, 1997). In a large meta-analysis of randomised controlled trials (Bischoff-Ferrari et al., 2009), it was reported that Vitamin D reduced the corrected odds ratio of falls by 20% (corrected OR, 0.78; 95% confidence

interval [CI], 0.64-0.92) compared with patients receiving calcium or placebo. One observational study described a two-fold greater risk in the development of sarcopenia in individuals with baseline serum 25[OH]D <25 nmol.L^{-1} (Visser, Deeg, Lips, & Longitudinal Aging Study, 2003). In another report, baseline 25[OH]D <50 nmol.L^{-1} correlated with a greater 3-year risk of physical performance decline than individuals with concentrations >50 nmol.L^{-1} (Wicherts et al., 2007).

These data are limited to clinical cohorts, however in non-clinical populations similar observations have been made. Barker et al. (2013a) provide correlative evidence that higher serum 25[OH]D associates with a faster recovery of force from eccentric exercise and subsequently have demonstrated that young individuals receiving a Vitamin D supplement recover quicker than those receiving placebo (Barker, Schneider, Dixon, Henriksen, & Weaver, 2013b), unfortunately no participants started the trial with Vitamin D concentrations <50 nmol.L^{-1} raising to question the validity of these data. The same authors also describe a quicker recovery of peak quadriceps force in individuals with higher serum 25[OH]D following anterior cruciate ligament injury (Barker, Henriksen, Rogers, & Trawick, 2015).

These findings are limited by their design. Observational data cannot describe a causal relationship between Vitamin D and muscle function/regeneration. These data may be explained by secondary problems caused by Vitamin D deficiency such as systemically altered calcium/phosphate homeostasis. Furthermore, a major confounding variable for some of these studies is the pre-existing frailty that may be present in elderly populations preventing outdoor activity thereby causing a decline in serum 25[OH]D as a function of a lack of UVB exposure. As such, randomised controlled trials (RCTs) and mechanistic investigations are needed to confirm a cause-effect role for Vitamin D and muscle health.

2.3.11 Vitamin D and Skeletal Muscle - RCT's: Very few RCT's have been conducted into the potential role of Vitamin D in muscle function and those that exist have provided conflicting results. In the elderly, pooled data analysis from 5 RCTs suggests that Vitamin D supplementation may reduce falls risk by 22% versus elderly individuals prescribed placebo (Bischoff-Ferrari et al., 2004a). As with observational studies on Vitamin D and muscle function, these data are hampered by the selectivity of the sample population being elderly (age range 70-85 years old), predominantly institutionalized individuals. It is therefore difficult to generalize such findings across a wider age range since institutionalized persons are likely to have habitually lower physical activity levels contributing to loss of muscle mass and function.

In young, otherwise healthy individuals data are scarce. In a cohort of young active males with Vitamin D concentrations <25 nmol.L^{-1} improvements in vertical jump height and 10m sprint times were observed when participants were supplemented for eight weeks with 5,000 IU.day^{-1} Vitamin D_3 resulting in significant serum 25[OH]D increases to >100 nmol.L^{-1} (Close et al., 2012). However, the placebo group in this trial also showed improvements in most of the performance measures, although not statistically significant. Since the sample population was also small, whether an actual physiological effect of the Vitamin D supplement was observed in this trial may be debated. In a similar study by the same authors, findings did not correlate to the first trial. Young males with mean serum 25[OH]D of 51 ± 24 nmol.L^{-1} performed 1-RM bench press and leg press and vertical jump height prior to and following supplementation for twelve weeks with 20,000 or 40,000 IU.$week^{-1}$ Vitamin D_3 or placebo (Close et al., 2013a). Although serum concentrations significantly improved in the Vitamin D treatment group, no changes in musculoskeletal performance were detected.

Studies from other research groups report similarly disparate results. Wyon, Koutedakis, Wolman, Nevill, and Allen (2014) assessed Vitamin D status in professional ballet dancers demonstrating high prevalence of Vitamin D deficiency. Following randomized supplementation for four months with 2,000 IU.day^{-1} Vitamin D_3 or placebo, the

supplemental Vitamin D group improved isometric strength and vertical jump height and experienced significantly fewer soft tissue injuries in comparison to the placebo control cohort. However, Barker et al. (2012) observed no strength changes in individuals supplemented with 400 or 2,000 IU.day^{-1} Vitamin D_3 for 28 days despite significant increases in serum 25[OH]D in the 2,000 IU.day^{-1} group.

The investigations described have generally examined muscle contractile function in the context of whole muscle physiology and insights for potential mechanisms are still sparse. However, in a recent investigation Sinha et al. (2013) explored the possibility that mitochondrial oxidative function may be a potential mechanism by which Vitamin D deficiency could impair muscle function. Phosphocreatine and ADP recovery kinetics following exercise were measured via ^{31}P magnetic resonance spectroscopy in individuals (age range = 18-54 years) with severe Vitamin D deficiency (< 15 nmol.L^{-1}) prior to- and following supplementation with Vitamin D (20,000 IU on alternate days for 10-12 weeks) or placebo. Despite no changes in phosphate metabolites at rest, recovery kinetics were significantly improved in the supplemental Vitamin D group indicating an improvement in mitochondrial oxidative metabolism.

A likely explanation for inconsistency in findings has been proposed by Lappe and Heaney (2012). The authors postulate that RCTs on Vitamin D sometimes fail due to heterogeneity in baseline serum 25[OH]D concentration which leads to a failure to detect functional improvements of the tissue being investigated i.e. if the participant in question is already on the ascending limb of an improvement curve then it is difficult to detect change as the curve plateaus. Furthermore, heterogeneity in outcome measures, supplemental Vitamin D doses and a lack of sensitivity in chosen muscle function tests further complicates available data.

2.3.12 Vitamin D Receptor Polymorphisms and Muscle Function: A number of studies have aimed to identify an association between VDR single nucleotide polymorphisms (SNP)

and parameters of musculoskeletal performance. The outcomes of these association studies have produced conflicting results. Windelinckx et al. (2007) explored the presence of common Vitamin D receptor polymorphisms and their apparent correlation to muscle strength in men and women. Based on the notion that the VDR is a candidate gene for inter-individual muscle strength variability, the authors' genotyped *BsmI*, *TaqI* and *FokI* VDR polymorphisms in 253 men and 240 women and report an association for quadriceps strength with *FokI* SNP in women and with *BsmI-TaqI* haplotype in men. Although these data are widely cited and generally accepted amongst the literature, there are a number of criticisms that limit the usefulness of these findings. First, although the authors aimed to establish an association between genotype and max strength, they failed to detect any association at the slower dynamic movement velocity (60 $deg.sec^{-1}$), which would be more appropriate to elicit peak torque (Baltzopoulos & Brodie, 1989). In addition, the translational potential of these data are limited by a lack of information as to how such polymorphisms may influence muscle contractile properties. Notably, the VDR polymorphisms reported to correlate with muscle strength are located at the 3' end of the VDR gene and do not influence protein structure. Thus, functional changes in the VDR are unlikely. However, SNPs in the 3' region of the gene are associated with mRNA stability and thus could influence VDR protein expression. Since it is unknown whether VDR SNPs associate with differential responses to exogenous Vitamin D, it is currently unknown how VDR SNPs could have any influence on Vitamin D signalling.

Other data provide contrasting evidence: Allelic variants of the VDR in 271 older community dwelling men were investigated for associations with bone mineral density (BMD) and biochemical markers of bone accretion and also muscle strength (Van Pottelbergh, Goemaere, De Bacquer, De Paepe, & Kaufman, 2002). Although genotype demonstrated a trend with BMD, no association with muscle strength was detected. These data are also subject to critique since muscle function in the cohort assessed may be compromised by the already underlying age related loss of function and mass (sarcopenia).

In summary, allelic variation in VDR gene and its role in muscle function and regeneration is an area of research to be approached with caution as definitive answers are yet to be provided by well-powered, mechanistic investigations that employ sensitive investigative techniques. Additionally, genetic variation in other aspects of the Vitamin D metabolic pathway such as binding protein genotype (Malik et al., 2013) should also be investigated in conjunction with VDR genotyping to provide a more complete understanding of genetic variation of the Vitamin D pathway and its influence on downstream outcomes. In contrast, a number of animal, human, cellular and molecular investigations have provided various fragments of evidence suggestive of a functional role for Vitamin D in skeletal muscle contraction and growth signalling, regardless of VDR genotype.

2.3.13 Vitamin D and Skeletal Muscle: Animal Models: Early work on rabbits with experimental uremia and consequentially impaired Ca^{2+} transport were found to show marked improvements in calcium flux and storage following treatment with Vitamin D however exact mechanisms remained to be determined and the significance of the role for Vitamin D to be explored (Matthews et al., 1977). Protracted efforts to better understand the global role of Vitamin D led to the generation of the VDR knockout (VDR -/-) mouse (Li et al., 1997). Subsequent investigations sought to isolate skeletal muscle from the VDR -/- mouse to study morphological differences when compared with wild type (WT) mice (Endo et al., 2003). Histological sections demonstrated distinct morphological changes with all fibres observed displaying a smaller and more variable phenotype than WT. Furthermore, abnormally high and persistent expression of immature muscle genes, Myf5, E2A, and early myosin heavy chain isoforms were observed. Importantly, these factors are down regulated in WT isolated myoblasts treated with 1α,25-$[OH]_2$D *in vitro* suggestive of a crucial role for Vitamin D in muscle development. This work has stimulated much of the later work undertaken in animal and cell models in the context of Vitamin D.

Indeed, mice with congenital absence of the VDR show an impaired motor ability (Burne, Johnston, McGrath, & Mackay-Sim, 2006). In a series of swimming tests, VDR -/- mice had more sinking episodes and reduced post swimming activity versus WT mice. Although these data infer a role for Vitamin D in motor function, they do not confirm a causal relationship and thus whether these abnormalities are secondary to impairments in other tissue systems is unanswered. However, in a recent study some answers have been provided for the previous observations made by Burne and are suggestive of an interactive role between the muscle and nerve. Vitamin D_3 treated mice showed improved locomotive ability (Rotarod exercise treadmill running) whereas VDR -/- and untreated mice showed an inability and impaired ability to adapt to the exercise, respectively. The data demonstrate that Vitamin D acts directly on the skeletal muscle, enhancing myogenic differentiation and also provide novel evidence that Schwann cells in peripheral nerves express VDR and respond to Vitamin D_3 exposure. Furthermore, Vitamin D treated mice up regulate IGF-1 production in the Schwann cells to regulate neuromuscular maintenance (Sakai et al., 2015). These data provide a novel insight that Vitamin D may influence muscle health through direct and indirect mechanisms.

Aside from motor function, Vitamin D may be implicated in skeletal muscle wound healing. In order to investigate this potential relationship, Stratos et al. (2013) induced a crush injury to the soleus muscle of male rats that had undergone Vitamin D depletion or Vitamin D supplemented diets from birth. The rats were then also fed diets supplemented with Vitamin D or placebo during the regeneration process over 42 days post insult. Muscle force measures and tissue samples were obtained periodically during the 42 days post injury to assess the recovery of the soleus functionally and morphologically. The authors report a significant increase in cell proliferation and a significant inhibition of apoptosis at day 4 after injury compared to control animals. SC number was not influenced by the vitamin D application, but there was an increase in prolyl-4-hydroxylase-β (P4Hβ) expression, suggestive of up regulated extracellular matrix proteins that could potentially improve ECM

stability. As a possible consequence of rapid cell proliferation and dampened apoptosis, the researchers found a significant improvement in muscle force recovery at day 48 when compared with vehicle treated rats. Deficiency may also lead to atrophy and impair the ability of muscle to hypertrophy following damage. In order for skeletal muscle to regenerate, hypertrophy of the repaired fibres is necessary which is brought about by decreased catabolic signalling and increased anabolic signalling. Intriguingly Bhat, Kalam, Qadri, Madabushi, and Ismail (2013) have provided evidence that Vitamin D deficient rats show muscle atrophy that is driven by up regulation of the ubiquitin proteasome degradation pathway in skeletal muscle highlighting the importance of maintaining Vitamin D sufficiency during muscle regeneration.

The described data provide rich evidence for a role for Vitamin D in animals, however there is a paucity of information in humans. Despite this, a number of investigations have taken to cellular and molecular based investigations to study the bioactivity of Vitamin D on various processes in skeletal muscle in order to exploit its therapeutic potential.

2.3.14 Vitamin D and Skeletal Muscle: Cell Models: The primary focus of early *in vitro* investigations into Vitamin D and skeletal muscle was centred on calcium homeostasis. Studies on sarcolemma vesicles isolated from Vitamin D-treated chicks show significant improvements in Ca^{2+} and phosphate uptake, greater activity of Ca^{2+}-ATPase and an improved ability of mitochondria to accumulate Ca^{2+} in comparison to those derived from deficient chicks. Importantly these effects are not diminished by inhibitors of protein or RNA synthesis but are however inhibited by voltage dependent calcium channel (Ca_V 1.1) blockers (de Boland & Boland, 1987). In subsequent spectrofluorometric studies that utilise Fura-2-loaded muscle cells, additional information related to the regulation of muscle intracellular Ca^{2+} homeostasis by $1\alpha,25[OH]_2D_3$ was obtained. The data demonstrated that the cytosolic Ca^{2+} response to Vitamin D was mediated by an initial rapid sterol-induced

Ca^{2+} mobilization from IP3 sensitive stores followed by cation influx from the extracellular milieu, accounting for a sustained Ca^{2+} phase, which does not return to basal concentrations as long as the cells are exposed to $1\alpha,25[OH]_2D_3$. This Ca^{2+} influx is mediated not only by the well-established VDCC controlled Ca^{2+} entry but also by a store-operated Ca^{2+} (SOC) channels (Vazquez, de Boland, & Boland, 1997; Vazquez, de Boland, & Boland, 1998). In later work, the involvement of protein kinase C (PKC) in upstream Vitamin D signalling was established (Massheimer & de Boland, 1992) and also the involvement of G-protein coupled receptors and the proto-oncogene tyrosine-protein kinase Src (Buitrago et al., 2000) are necessary for these rapid changes in $Ca^{2+}[i]$. However, $1\alpha,25[OH]_2D_3$ may also indirectly modulate Ca^{2+} homeostasis through stimulation of protein synthesis for a number of different proteins related to Ca^{2+} binding and transport (Drittanti, Boland, & de Boland, 1989). Recent data suggest that the effects on protein synthesis may not be limited to Ca related proteins by showing that treatment of C2C12 murine myoblasts with $1\alpha,25[OH]_2D_3$ enhances the growth stimulatory effect of insulin and leucine through the Akt/mTOR signalling network (Salles et al., 2013). Taken together these findings establish a strong role for Vitamin D in calcium homeostasis and a link to growth signalling that may converge to regulate many downstream cellular actions.

Indeed *in vitro* treatment with $1\alpha,25[OH]_2D_3$ regulates growth, differentiation and survival of skeletal myoblasts from both animal and human muscle through similar operational networks as in the regulation of Ca^{2+}. Likewise these responses are non-genomic in nature occurring within minutes to hours of exposure. In a series of investigations, myoblasts treated with varying concentrations $1\alpha,25[OH]_2D_3$ showed dose dependent stimulation of mitogenic signalling pathway members ERK1/2, p38 MAPK and JNK and upstream involvement of Src kinase and Akt (Buitrago, Arango, & Boland, 2012; Buitrago & Boland, 2010; Buitrago, Pardo, & Boland, 2013; Buitrago, Pardo, de Boland, & Boland, 2003; Buitrago, Ronda, de Boland, & Boland, 2006; Buitrago et al., 2000).

The biological significance of these data is contextualised by experiments that have investigated cell behaviour in the presence of Vitamin D. Buitrago et al. (2012) effectively show that treatment of C_2C_{12} myoblasts with $1\alpha,25[OH]_2D_3$ induces myoblast proliferation through a PI3K dependent mechanism and increases Src activity and Akt activation during differentiation. However, Srikuea et al. (2012) provide conflicting data suggestive of a suppressive effect on proliferation of $1\alpha,25[OH]_2D_3$ treated C_2C_{12} myoblasts but an up regulation of the VDR during muscle regeneration. Girgis, Clifton-Bligh, Mokbel, Cheng, and Gunton (2014a) demonstrate similar findings in C_2C_{12}'s and report a suppression of myotube formation but an increase in cross sectional area of myotubes (1.8 fold change). The contrasting findings between trials may be attributable to the $1\alpha,25[OH]_2D_3$ doses used; Buitrago et al. (2012) utilised a 1 nmol dose whereas Srikuea et al. (2012) and Girgis (2014) utilised 20 nmol and 100 nmol, respectively. As Vitamin D possesses great tissue specificity and large variation in response between tissues, in some cases opposing effects, it is difficult to cross compare *in vitro* data from other cell models.

Taken together, exogenous treatment of skeletal muscle progenitor cells and mature myotubes in culture results in clear effects on implemented are often supra-physiological and thus the translation to whole tissue physiology requires in depth study of the whole tissue. As yet, this link between whole muscle and cellular data has not been firmly established when considering Vitamin D in the context of muscle function and regeneration and thus the physiological significance of the data available are still open to question.

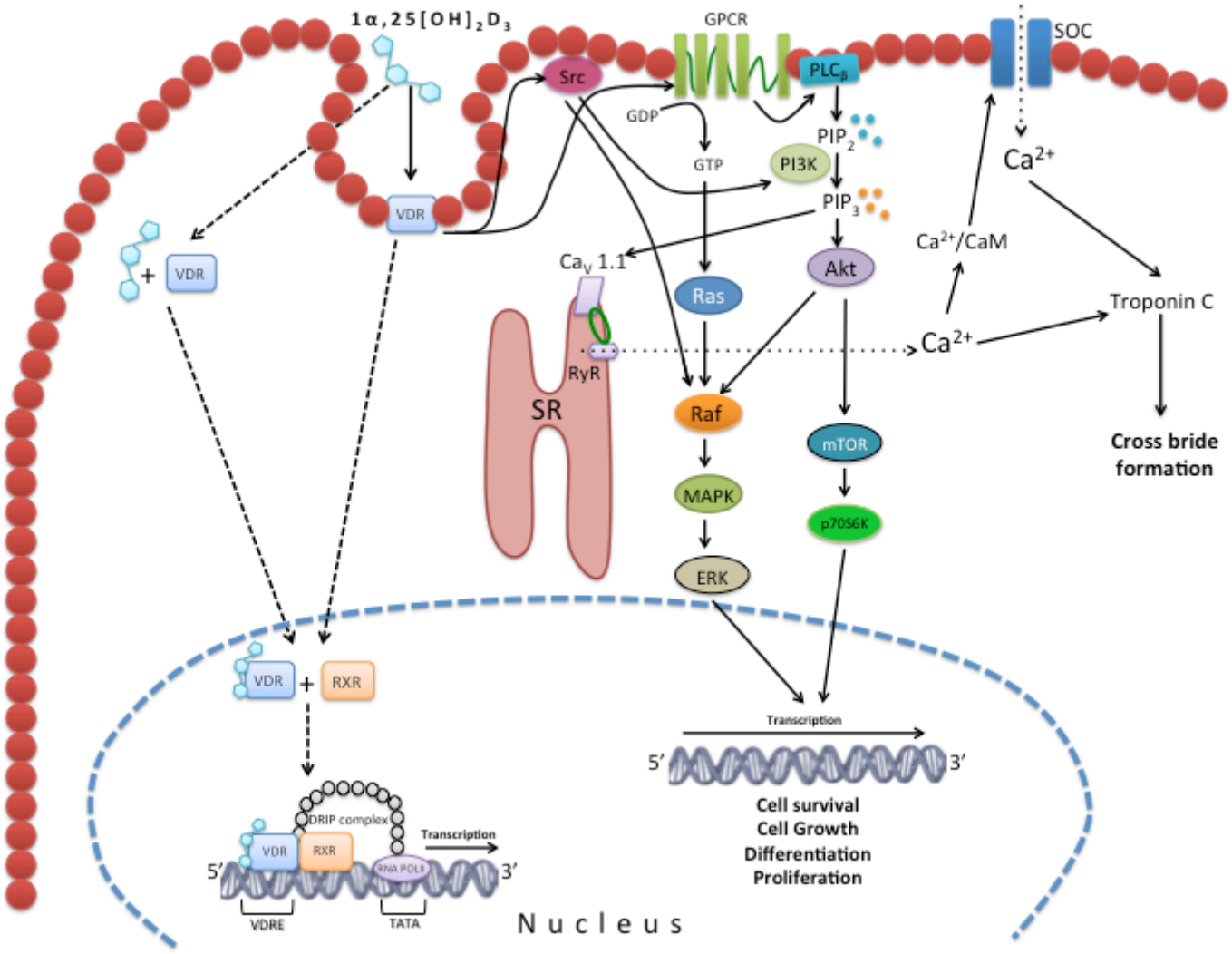


Figure 2.9. Representation of the current understanding of the role for Vitamin D in skeletal muscle. Genomic responses are highlighted to the left of the schematic and rapid signalling events mediated by VDR highlight to the right. Vitamin D binds to its receptor at caveolae or within the sarcoplasm where it may interact with its heterodimeric partner, RXR to mediate transcriptional activity of Vitamin D responsive genes. Alternatively, upon bindng at the plasma membrane, direct interaction of ligand bound VDR can occur with G protein coupled receptors or Src kinase to elicit downstream signalling responses.

2.4 Perspective

In summary, muscle contraction and regeneration are highly orchestrated processes involving input from numerous intracellular and extracellular stimuli, thus there are many potential areas for disruption to the normal functioning of skeletal muscle. Exercise plays a large role in optimizing these processes, however a number of disease states, heritable disorders and modifiable dietary deficiencies may impair contractile activity and muscle regenerative capacity. Accumulating data are suggestive that Vitamin D deficiency impairs fundamental processes related to muscle contraction such as calcium flux and the expression of calcium handling proteins. Further, the sterol may also activate intracellular signalling networks that function to stimulate satellite cell proliferation and growth, differentiation and survival of skeletal myoblasts. Research interest into Vitamin D and muscle health is therefore growing, however is still in its infancy.

The development of appropriate strategies to correct Vitamin D deficiency will depend on a greater understanding of the influence that it plays in skeletal muscle homeostasis. Therefore, the overall aim of this thesis is to investigate the role of Vitamin D in non-pathological skeletal muscle in the context of muscle contraction and regeneration to identify strategies for optimizing Vitamin D status and muscle health. To realise this aim, the following objectives will be addressed:

1. Explore the role of Vitamin D in human skeletal muscle contractile properties *in vivo.*
2. Identify the role of Vitamin D in human skeletal muscle contractile properties *ex vivo.*
3. Investigate the role of Vitamin D in skeletal muscle regeneration following eccentric exercise induced muscle damage *in vivo.*
4. Elucidate cellular mechanisms of the muscle regeneration process that are responsive to Vitamin D *in vitro.*

Chapter 3

General Methodology

This chapter describes general methodologies and theory of methodologies adopted in the investigations undertaken for this thesis. Assays specific to a methodology adopted in one chapter are described within that chapter.

3.1 Ethical Approval

Ethical approval for muscle function analyses and blood sampling procedures was granted by the Liverpool John Moores University local research ethics committee. For muscle biopsy procedures and tissue analysis, ethical approval was granted by the West Midlands National Research Ethics Committee (NREC approval number: WM/09/13). All samples and data were treated and stored in line with the Declaration of Helsinki and according to Human Tissue Act specifications.

3.2 General Screening & Inclusion Criteria

Participants who volunteered to take part in the investigations described in this thesis were first screened with a medical history questionnaire and a physical activity readiness questionnaire (PAR-Q). Inclusion criteria for all studies were:

- Male volunteers (to avoid changes in muscle function as a consequence of menstrual cycle (Sarwar, Niclos, & Rutherford, 1996) and known sex differences in Vitamin D storage (Correale, Ysrraelit, & Gaitan, 2010))
- Aged 18-30
- Free from known medical conditions
- Not taking prescribed medications
- Not taking supplemental Vitamin D_2 or D_3, fish oils or multi vitamins
- Not sun bed users
- No planned travel abroad during the study
- At the standard of readiness to exercise as indicated by PAR-Q

3.3 Blood Sampling

For the analysis of Vitamin D metabolites (D_2 and D_3) and intact parathyroid hormone (iPTH), participants provided a fasted venous blood sample collected from the antecubital

vein into two K_2EDTA plasma (10 ml) vacutainers and two serum separator ((SST) 8.5 ml) vacutainers (Beckton Dickinson. Oxford, UK). Serum samples were allowed to clot for 1 hour at room temperature whilst K_2EDTA samples were immediately stored on ice, following which all samples were centrifuged for 15 minutes at 1500 RCF at 4^{o}C. Serum and plasma were manually extracted and stored at -80^{o}C until required for analysis.

3.4 Quantification of Vitamin D Metabolites

For the analysis of total serum 25[OH]D concentration high-pressure liquid chromatography tandem mass spectrometry (LC-MS/MS) was implemented. The LC-MS/MS method of analysis has been validated against other commercially available assays and is regarded as the most valid and reliable technique for the assessment of Vitamin D metabolites (Snellman et al., 2010). Assays were performed in a Vitamin D External Quality Assurance Scheme (DEQAS) accredited laboratory.

3.4.1 LC-MS/MS Principle – LC-MS/MS combines the resolving capability of liquid chromatography with the mass analysis ability of mass spectrometry. Therefore, the process can be generally categorized into the separation step (step 1), ionization step (step 2) and the mass analysis section (step 3). In step 1 the sample (i.e. vitamins purified from serum) is forced at high pressure by a liquid (mobile phase) into a column (solid phase) to allow separation. In step 2, the sample is charged via atmospheric pressure chemical ionisation (APCI), during which a solvent reagent (to allow detection in the final step) ionizes the analyte. In step 3, the ions are separated according to their mass-to-charge ratio in an analyser by electromagnetic fields. The ions are detected by a quantitative method and the signal is then processed into mass spectra

3.4.2 LC-MS/MS Method – For the studies within this thesis, 25[OH]D_2, 25[OH]D_3 and deuterated internal standard were extracted from serum samples following zinc sulphate

protein precipitation, using Isolute C18 solid phase extraction cartridges. Potential interfering compounds were removed by initial elution with 50% methanol followed by elution of the vitamins using 10% tetrahydrofuran in acetonitrile. Dried extracts were reconstituted prior to injection into an LC-MS/MS in the multiple reaction mode. The multiple reaction mode transitions (m/z) used were 413.2 > 395.3, 401.1 > 383.3 and, 407.5 > 107.2 for 25[OH]D_2, 25[OH]D_3, and hexa-deuterated [OH]D_3 respectively. The assay was validated against published acceptance criteria (FDA, 2001). Assay sensitivity was determined by the lower limit of quantification (LLOQ): 25[OH]D_3 = 2.5nmol.L^{-1} and 25[OH]D_2 = 2.5nmol.L^{-1}. Coefficients of variation for the assay were 10% across a working range of 2.5 nmol.L^{-1} to 625 nmol.L^{-1} for both 25[OH]D_2 and 25[OH]D_3. Data validating the precision, detection and recovery of the assay are provided in Table 3.1. The assay was calibrated using a 3PLUS1 Multilevel Serum Calibrator Set and MassCheck by Chromsystems (Chromsystems Instruments and chemicals. Gräfelfing, Germany) control standards (lyophilised human serum) were used to monitor accuracy and precision of the LC-MS/MS assay. MassCheck calibration materials and controls are traceable against NIST 972 reference material.

Table 3.1. Table A) Imprecision of the LC-MS/MS assay and B) Recovery efficiency of the assay. Intra precision was assessed by running quality control materials (Chromsystems) and commercially bought material (calf serum) ten times (n=10) within a single run, and separately over 15 runs (n=15) for inter-assay assessment. Variation is expressed as standard deviation (SD) and percentage of coefficient of variation (%CV).

A

Intra-assay precision	**Chromsystem 1**		**Chromsystem 2**		**Calf serum**	
$nmol.L^{-1}$	Vitamin D3	Vitamin D2	Vitamin D3	Vitamin D2	Vitamin D3	Vitamin D2
Mean	42.9	43.4	80.2	81.1	6.9	7.8
SD	4.1	3.4	7.8	8.6	0.7	0.8
%CV	9.5	7.8	9.7	10.6	10.1	10.2
Inter-assay precision	**Chromsystem 1**		**Chromsystem 2**		**Calf serum**	
$nmol.L^{-1}$	Vitamin D3	Vitamin D2	Vitamin D3	Vitamin D2	Vitamin D3	Vitamin D2
Mean	40.4	32.4	76.8	95.3	7.7	7.8
SD	2.4	3.3	7.2	8.3	0.7	0.9
%CV	6	10	9.3	8.7	9.1	10.8

B

	Endogenous 25[OH]D_3 $nmol.L^{-1}$	**Spiked ($nmol.L^{-1}$)**	**Measured value ($nmol.L^{-1}$)**	**%Recovery**
Sample 1	5.5	50	53.7	96.4
Sample 2	10.2	100	103.4	93.8
Blank	0	500	491	98.2
	Endogenous 25[OH]D_2 $nmol.L^{-1}$	**Spiked ($nmol.L^{-1}$)**	**Measured value ($nmol.L^{-1}$)**	**%Recovery**
Sample 1	37.0	50	84	96.5
Sample 2	80.2	100	171.3	95
Blank	0	500	479	95.8

3.5 Muscle Biopsy Procedure

Participants were instructed to avoid exercise training 48 hours preceding the biopsy procedure. On arrival at the muscle biopsy suite at Liverpool John Moores University, participants were asked to relax on a hospital bed whilst the biopsy site was prepared. Briefly, the incision site (*Vastus Lateralis*) was shaved to maximise sterility and washed with an alcohol swab and Hydrex surgical scrub (ECOLAB Ltd. Leeds, UK) following which a sterile sheet was placed around the sterile site. To anaesthetise the biopsy site, bupivacaine hydrochloride (Astra Zenica. Luton, UK) was administered at a concentration of 5 $mg.ml^{-1}$ (approximately 1.5 ml administered). A sterile single use scalpel was then used to penetrate the skin and deep muscle fascia. A Bard disposable core biopsy instrument (12 g x 10cm. CR Bard Ltd. Crawley, UK) was then used to retrieve three passes of muscle tissue from within the incision site (Figure 3.1).

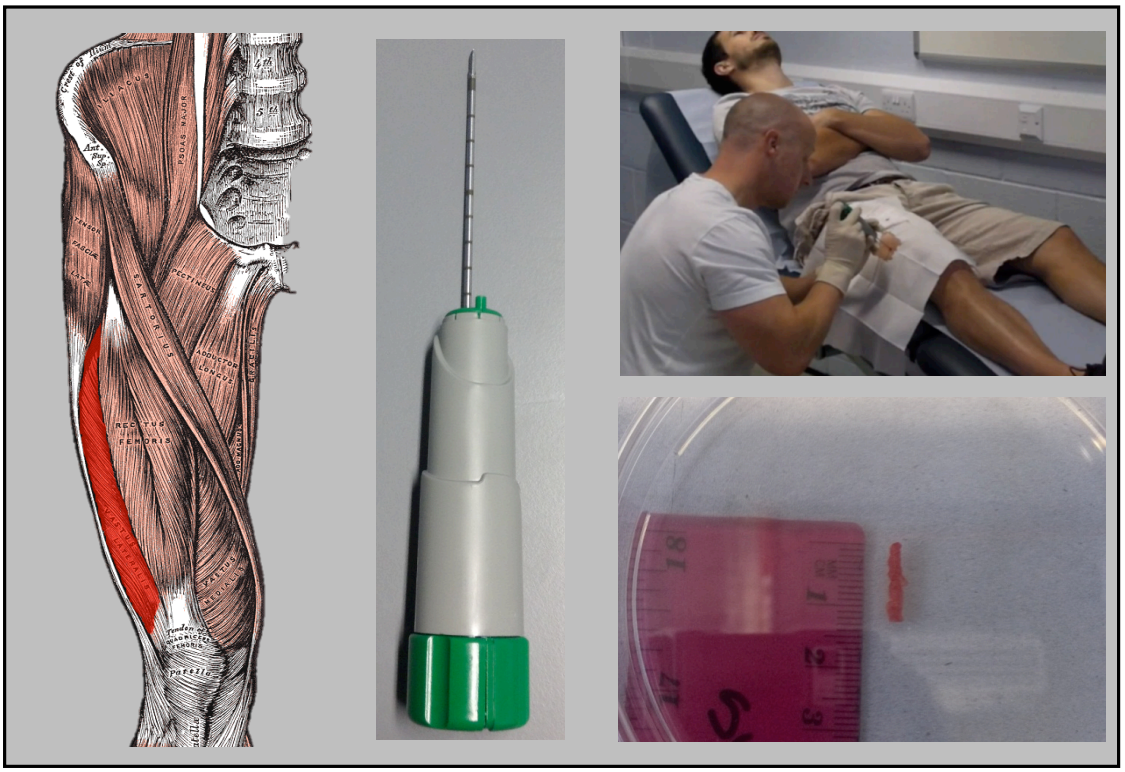


Figure 3.1. Anatomical location of the muscle biopsy procedure (*Vastus Lateralis)*, Bard biopsy needle instrument and a muscle biopsy procedure from an investigation within this thesis with an example muscle biopsy sample.

3.6 Gene Expression Analysis

For the analysis of gene expression, reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was employed.

3.6.1 Polymerase Chain Reaction Principle - The method relies on the following principle to amply mRNA isolated from a biological sample. Isolated mRNA is first synthesized into complementary DNA (cDNA) by reverse transcription using reverse transcriptase and nucleoside triphosphates (dNTPs). Complimentary DNA is then heated to denature into two single stranded DNA molecules (ssDNA). An optimal temperature for the annealing of sequence specific primers (SSPs) and transcription of the target by RNA polymerase is then achieved. The detection of amplified product is performed by labelling the cDNA with molecules emitting fluorescence, which is proportionally increased with the amplification of the cDNA molecules with each cycle of the reaction and is detected in real time (See Figure 3.2).

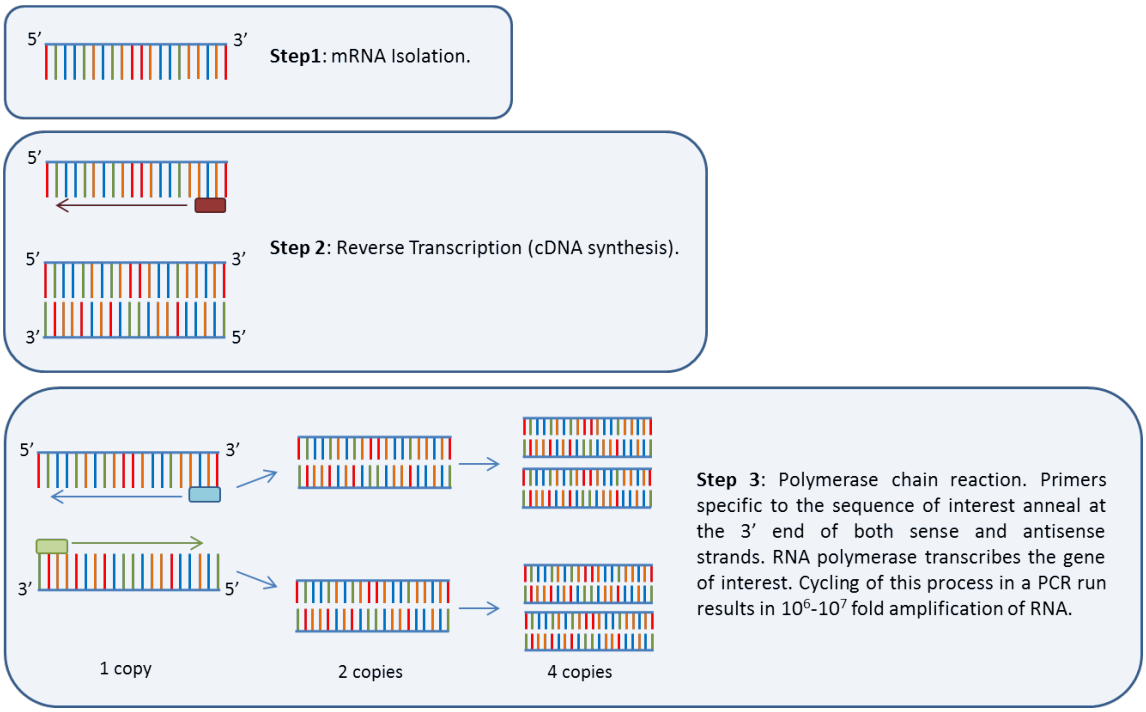


Figure 3.2. Schematic representation of the principle steps in real time quantitative polymerase chain reaction.

3.6.2 RNA Isolation Method - Single step liquid phase separation with guanidinium thiocyanate-phenol-chloroform was implemented for the isolation of RNA from cell lysates. TRI reagent contains phenol and guanidine thiocyanate that are capable of lysing cells and denaturing proteins, therefore TRI reagent also inhibits the activity of DNAse and RNAse enzymes thus maintaining RNA integrity in addition to its lysing capability. One ml of TRI reagent is ample to isolate RNA from 5-10^6 cells or 10 cm^2 of a cell culture dish of cells grown in monolayer. Since monolayers of muscle derived cells are typically seeded at a density of $8x10^5$ and grown to confluence on six well plates, a volume of 300 µl per well is sufficient to isolate total RNA. The addition of chloroform and the process of centrifugation of the lysates result in 3 phases: aqueous phase containing RNA, interphase containing DNA and a lower red/pink organic phase containing proteins (see Figure 3.3). The aqueous phase is isolated and RNA precipitated from the phase with isopropanol. The precipitated RNA is then washed with ethanol to remove residual impurities and then re-suspended in RNA storage solution for downstream application of RT-qPCR.

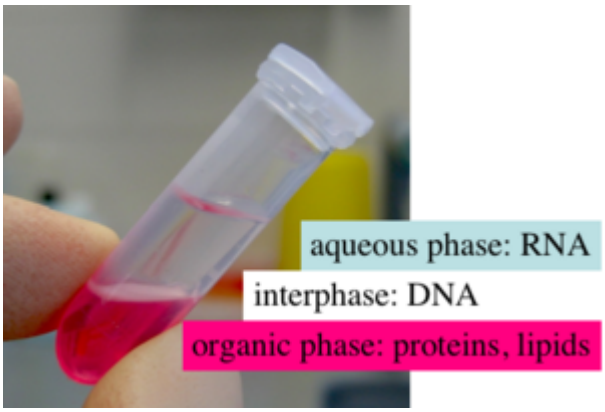


Figure 3.3. Example phase separation with TRI reagent.

To isolate RNA in the studies described in this thesis, cell monolayers were first washed twice with PBS to remove any residual media. Three hundred µl of TRI reagent was then added to each well of a 6-well plate to lyse the monolayers and isolate total RNA. The TRI reagent was left to lyse the monolayers for 5 minutes and then lysates were collected via

scraping into RNA free microfuge tubes, briefly vortexed and stored at -80^{o}C until required for analysis.

When analysis was required, 200 µl of chloroform per ml of TRI reagent was added to the sample. The mixture was then inverted 2-3 times, shaken vigorously by hand and left to stand for 5 minutes on ice before centrifugation at 12,000 RCF for 15 minutes at 4^{o}C. The RNA containing aqueous phase was then transferred to a new RNA free microfuge tube for RNA precipitation into which 500 µl of ice cold 100% isopropanol per ml of TRI reagent used was added. The sample was mixed by hand and left to stand for 10 minutes at RT before centrifugation at 12,000 RCF for 10 minutes at 4^{o}C.

Supernatant was discarded and the RNA pellet was washed with 1 ml of 75% ethanol per ml of TRI reagent used. The sample was then vortexed briefly to wash the RNA pellet and centrifuged for 5 minutes at 7,500 RCF at 4^{o}C. The wash was discarded and the wash processes repeated again. Following the second centrifugation step, the ethanol wash was discarded and the sample was left to air dry for 10-15 mins. When the sample was almost completely dry, 50 µl RNA storage solution containing 1 mM sodium citrate, pH 6.4 ± 0.2 (Ambion, Life Technologies. Waltham, MA. USA) was added and mixed by passing the solution up and down several times with the pipette tip. Samples were then heated on a heating block pre-heated to 60^{o}C for 10 minutes prior to RNA quality and quantity measurements.

3.6.3 Method for Assessment of RNA Quality - RNA purity and concentration were assessed by UV spectroscopy, using a Nanodrop UV-vis spectrophotometer (Thermo Fisher Scientific. Waltham, MA. USA). The absorbance of the diluted RNA sample was measured at 260/280 nm and 260/230 nm, known as RNA purity ratios.

- A 260/280 ratio of ~2.0 is generally accepted pure for RNA.
- The 260/230 ratio for pure nucleic acid is typically higher than that of 260/280 with common values in the range of 1.8-2.2.

An initial cleaning of the measurement surfaces of the spectrophotometer was performed with RNA free dH_2O. Following cleaning, a blank sample measurement was made by pipetting 1 µl of RNA storage solution onto the spectrophotometer for analysis. One µl of diluted sample was pipetted directly onto the measurement pedestal to allow good liquid column formation and accurate RNA assessment.

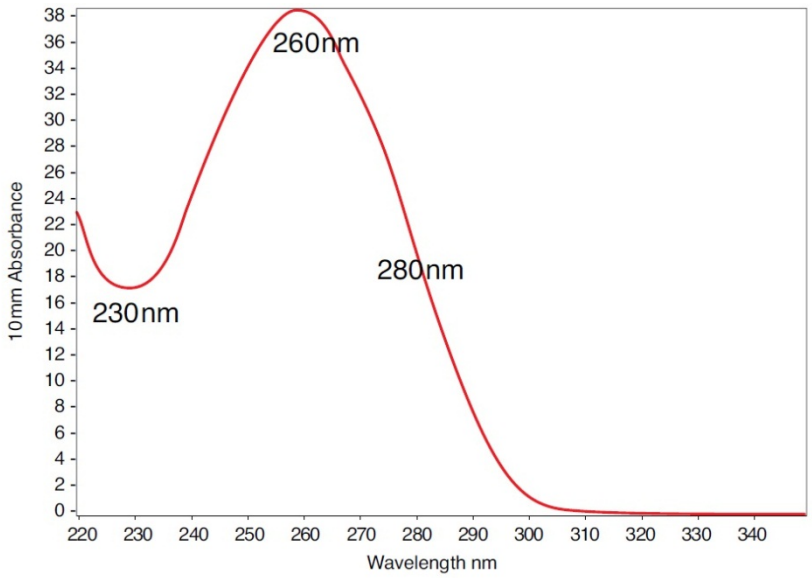


Figure 3.4. Example nucleic acid spectral image used to assess RNA sample quality.

3.6.4 Method of RNA quantification - Absorbance at 260 nm was used to determine the RNA concentration, as it is known that a 260 reading of 1.0 is equivalent of 40 µg.ml^{-1} of RNA. For the quantification of nucleic acids, a modified version of the Beer-Lambert equation was used by the Nanodrop software.

$$C = \frac{(A \times \varepsilon)}{b}$$

Where:

- C is the nucleic acid concentration (ng.µl^{-1}).
- A is the absorbance in AU.
- ε is the wavelength-dependent extinction coefficient (ng-cm.µl^{-1}). For RNA the wavelength-dependent extinction constant is 40 ng-cm.µl^{-1}.
- b is the path length in cm.

Equation 3.1. The modified Beer-Lambert equation used in the current work to determine RNA concentration via spectrophotometric analysis.

3.6.5 Polymerase Chain Reaction Method – For the experiments described in this thesis, purified RNA was diluted to 7.3 $ng.ml^{-1}$ in DNase RNase free H_2O (Sigma-Aldrich Company Ltd. Dorset, UK) and amplified with specific primer sequences in a rotor-gene Q (Qiagen) PCR machine using a one-step SYBR Green I RT-qPCR kit (Qiagen). Briefly, double stranded cDNA was first synthesized at 50^{o}C for 10 minutes with the use of dTP oligonucleotides and reverse transcriptase. Complimentary DNA was then denatured to ssDNA at 95^{o}C for 10 seconds and combined primer annealing and extension was initiated at 60^{o}C for 30 seconds. The cycle of denaturation and annealing/extension was repeated for 40 cycles. SYBR Green mix contains a green fluorescent dye (SYBR green) that binds all double stranded DNA molecules, emitting fluorescence on binding. The excitation and emission maxima for SYBR Green are 494 and 521 nm, respectively. With the use of rotor gene Q software, amplification curves for fluorescence were detected in real time throughout the PCR run (see Figure 3.5 for example). Primers sequences were designed using National Centre of Biotechnology Primer-BLAST software and highly purified salt free primer for each sequence was purchased from Sigma-Aldrich.

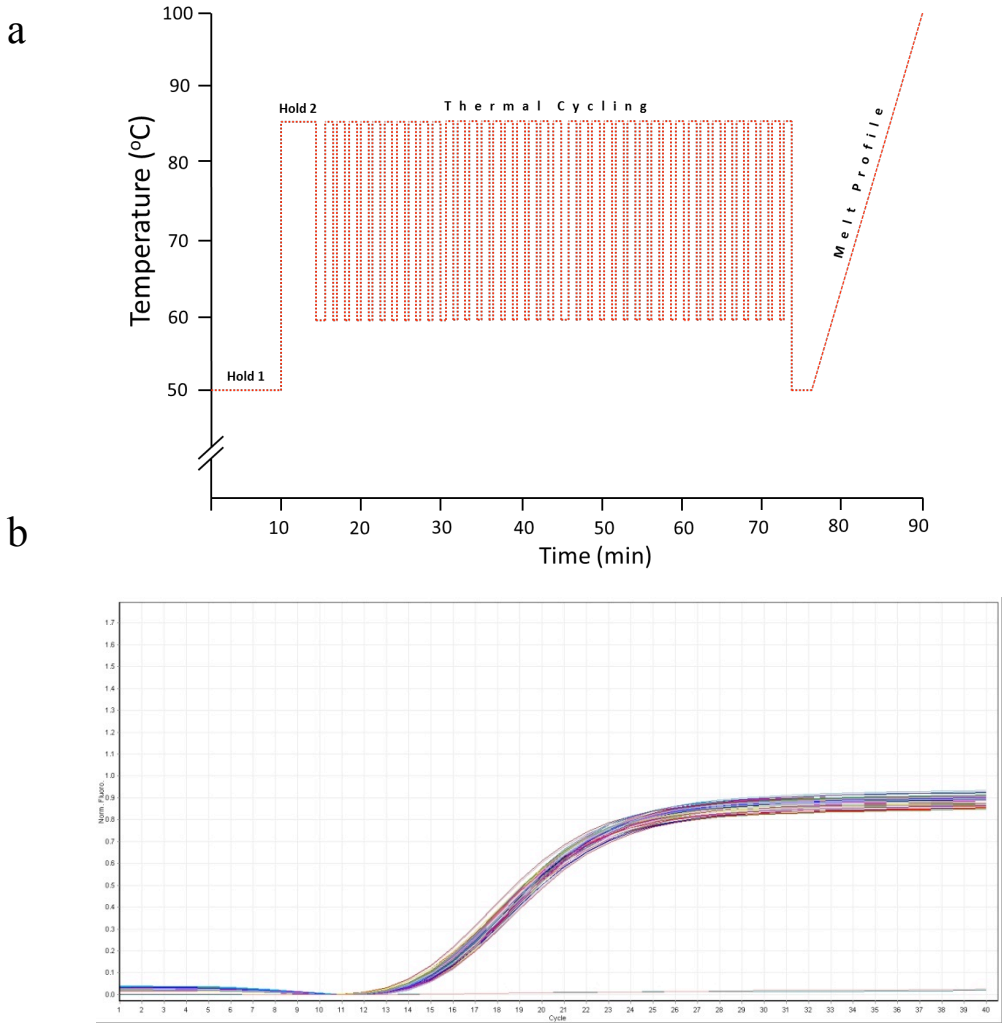


Figure 3.5. a) Design of the polymerase chain reaction protocol used in the experiments described in this thesis. **b**) examples of real time fluorescence detection curves. Cycle threshold is determined from this curve by locating the lower third of the curve below the exponential phase of amplification.

3.6.6 Quantification Method - A method of relative quantification was used to assess changes in mRNA expression. Relative quantification determines the changes in steady state mRNA levels of a target gene across multiple samples and expresses it relative to the levels of a reference gene. To determine the level of expression, the differences (Δ) between the threshold cycle (Ct) are measured. This method is known as the ΔCt method (Livak & Schmittgen, 2001). In a further step a second relative parameter termed a calibrator sample can be added such as an untreated control or a zero hour time point. The latter more complex relative quantification method is summarized as the ΔΔCt method (Livak & Schmittgen, 2001).

For the ΔΔCt calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal. Therefore, in the investigations within this thesis, samples were excluded from analysis if PCR efficiency between target and reference gene was not comparable within a CV of 10%.

3.6.7 Method for Assessment of PCR products – Specificity of each PCR was first assessed by melt curve analysis. As described earlier, the RT-qPCR protocol was designed to include a melting profile once all amplification cycles were complete. During the melt cycle, PCR products are reheated at a 1^{o}C every 5 minutes from 50^{o}C to 99^{o}C to produce a melt profile that determines whether a specific product was amplified and whether non-specific RNA has been amplified or if primer dimers have formed. A single sharp peak indicates specificity of the primers (see Figure 3.6).

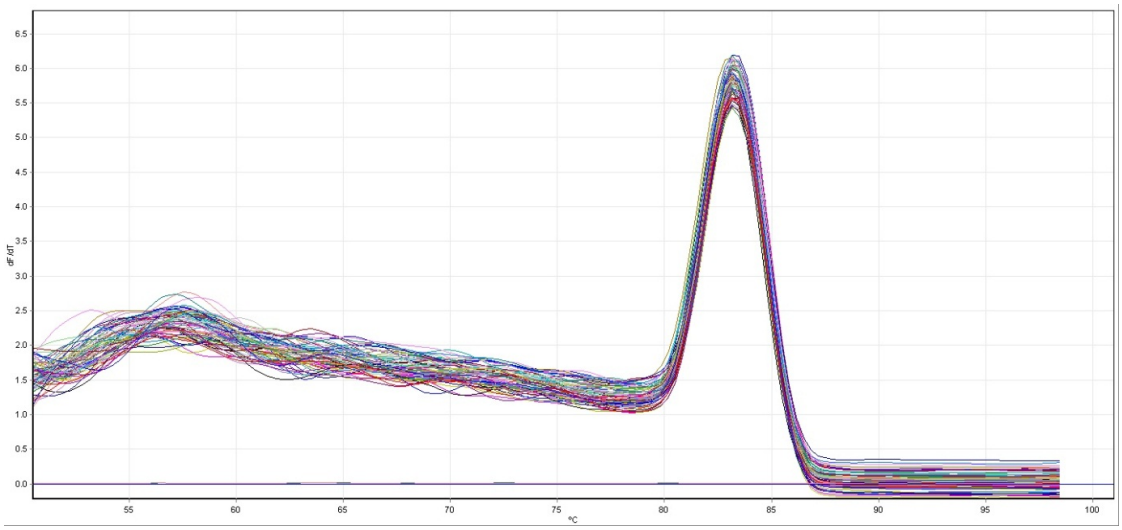


Figure 3.6. Example melt curve for the reference gene ribosomal protein L13a (*RPL13a*).

Although melt curve analysis provides an indication of primer specificity, the method cannot confirm that the product amplified is the gene of interest since information relating to amplicon size is not provided. Such information can be derived by running the PCR products through an agarose gel against a DNA ladder to determine amplicon size. In the current

work 8 µl of Norgen FullRanger 100 bp DNA ladder was loaded and 8 µl of sample was loaded after preparation with a DNA loading dye (Geneflow Ltd. Staffordshire, UK.) into an agarose gel (2% Agarose (Bioline Reagents Ltd. London,UK) in 1X Tris-acetate-EDTA buffer (Invitrogen, Life Technologies Ltd. Paisley, UK)) prepared with Midori Green nucleic acid stain (Nippon Genetics Europe GmbH. Düren, Germany) at a concentration of 1:200. Samples were electrophoretically separated by passing a 50V current through the gel for 20 minutes and a 70V current through the gel for a further 20 minutes. Samples were left to run for longer if the loading dye had not migrated ~80% through the lanes by 40 minutes. Following electrophoretic separation, gels were placed on a UV transilluminator for visualization and analysis. All PCR primer sequences and related information as well as agarose gel products can be found in Table 3.2 and Figure 3.7, respectively.

Table 3.2. Gene primer sequences for human MDC samples and amplicon lengths. All primers were used in the same PCR cycling conditions.

Gene	Accession No.		Primer Sequence	Amplicon Length (bp)	Exon Junction
Vitamin D Receptor (*VDR)*	NM_001017536	**F**	GACCTGTGGCAACCAAGACT	174	Reverse 1575/1576
		R	GGACGATCTGGGGAGACGA		
Myogenin (*MYOG*)	NM_002479.5	**F**	TCCCAGATGAAACCATGCCC	103	None
		R	AGGCCCCTGCTACAGAAGTA		
Myogenic Regulatory Factor 4 (*MRF4*)	NM_002469.2	**F**	ACCCTTCCTGGCCTAATCCT	198	None
		R	ACCCTTCCTGGCCTAATCCT		
Ribosomal Protein L13a (*RPL13A*)	NM_012423	**F**	GGCTAAACAGGTACTGCTGGG	105	Reverse: 230/231
		R	AGGAAAGCCAGGTACTTCAACTT		

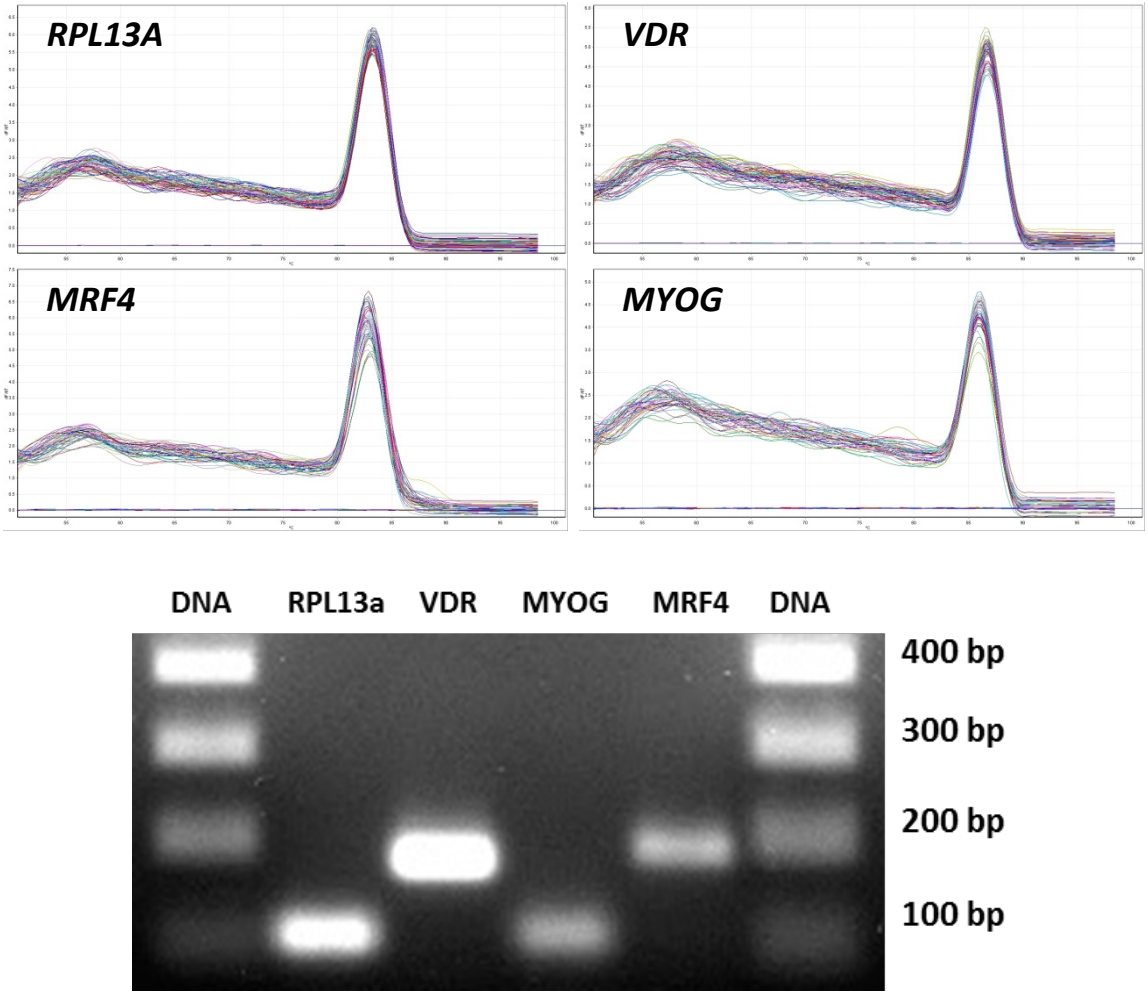
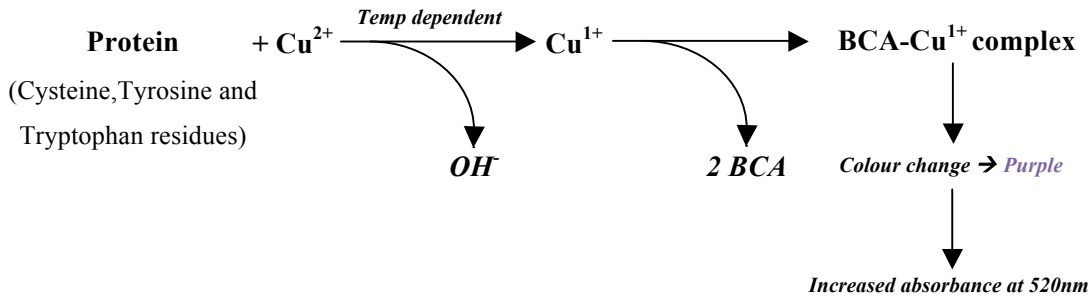


Figure 3.7. a) Melt curves for all genes assessed in this thesis. **b**) Electrophoretically separated PCR products used to determine primer specificity. Product lengths are; *RPL13a* = 105bp; *VDR* = 174 bp; *MYOG* = 103 bp; *MRF4* = 198 bp.

3.7 Protein Quantification

In order to detect changes in enzyme activity (i.e. creatine kinase enzyme activity) via enzyme linked immunosorbence assays, total protein concentration was first quantified.

3.7.1 BCA assay principle - The bicinchoninic acid (BCA) assay is a colorimetric method for the quantification of total protein in a sample. The method works on the following principle: First in the presence of peptides, a Cu^{2+} ion is reduced to form Cu^{1+}. The Curprous ion is then chelated by BCA resulting in a purple coloured reaction product. The reaction product shows strong absorbance at 520 nm that is nearly linear with increasing protein concentrations across a broad working range of 20-4000 $\mu g.ml^{-1}$



3.7.2 BCA Assay Method - Standards made with bovine serum albumin (BSA) were used to determine the concentration of protein ($mg.ml^{-1}$) based on a standard curve generated using a Thermo Multiskan Spectrum plate reader (Thermo Fisher Scientific. Waltham, MA. USA). Bovine serum albumin protein standards were prepared at 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0 $mg.ml^{-1}$ initially by diluting 4 mg BSA (in 0.9% sodium chloride and sodium azide as a preservative; Sigma) with 4.8 ml tris-mes triton (TMT; 50 µM tris-mes, 1% triton-X 100, pH 7.8) and serially 50 µl of standard with 50 µl of TMT apart from the 0 $mg.ml^{-1}$ standard which was TMT alone.

The BCA reaction materials were purchased as part of a Pierce BCA protein assay kit (Rockford, IL, USA). The kit contains two reagents; A & B. Reagent B was made up of 4% cupric sulphate and was mixed thoroughly in a multichannel trough with reagent A (sodium

carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1 M of sodium hydroxide) at a ratio of 1:50. Ten µl of standard, sample and a blank (TMT) were pipetted in duplicate into a 96 well plate. Two hundred µl of the working reagent was added to all wells using a multichannel pipette (excluding the blank), and the plate was incubated at 37^{o}C for 60 mins. Following background subtraction, the absorbance was recorded at 30 and 60 minutes at 595 nM using a Thermo Multiskan Spectrum plate reader. The standard curve was generated by plotting the average blank-corrected 595 nM measurement of each BSA standard against its pre-programmed known concentration in mg.ml^{-1}. Sample concentrations were calculated from the standard curve.

3.8 Creatine Kinase Activity

3.8.1 CK Assay Principle - Creatine kinase activity was used as a marker of myogenic differentiation in 2D muscle cell cultures as it is accepted that CK increases correlate with increases in fusion (Al-Shanti & Stewart, 2008; Foulstone, Huser, Crown, Holly, & Stewart, 2004; Foulstone, Meadows, Holly, & Stewart, 2001; Saini, Al-Shanti, Sharples, & Stewart, 2012; Sharples, Al-Shanti, Hughes, Lewis, & Stewart, 2013). The principle of the method is based upon the conversion of PCr and adenosine diphosphate to creatine and adenosine triphosphate. The resultant ATP is quantitatively determined by coupling a hexokinase reaction and glucose-6-phosphate reaction to 6-phosphogluconic acid and a concomitant reduction of NAD to NADH. The reduction of NAD results in an increased absorbency of NADH at 340 nm and is directly proportionate to CK activity:

(1) PCr + ADP → Cr + ATP

(2) ATP + Glucose → ADP + G-6-P

(3) G-6-P + NAD → 6-PG + NADH

Figure 3.8. Reactions involved in the CK assay. Reaction 1 is catalysed by creatine kinase (CK). Reaction 2 is catalysed by hexokinase (HK) and reaction 3 is catalysed by glucose-6-phosphate dehydrogenase (G-6-PDH). Abbreviations: PCr = creatine phosphate, ADP = adenosine diphosphate, Cr = creatine, ATP = adenosine triphosphate, G-6-P = glucose-6-phosphate, NAD = nicotinamide adenine dinucleotide, 6-PG = 6-phosphogluconate, NADH = reduced NAD.

3.8.2 CK Assay Method – The assay method is based on that described by the Scandinavian Committee on Enzymes and the German Society for Clinical Chemistry (Ljungdahl & Gerhardt, 1978; Oliver, 1955; Szasz, Gerhardt, & Gruber, 1977). In the current work, 10 µl of TMT cell lysate was loaded in duplicate wells on a 96 well UV plate and used for quantification of CK. The creatine kinase reaction reagent and diluent (Catachem Inc. Connecticut, US) were prepared as per the manufacturer's instructions and heated for 2

minutes at 37°C. When reconstituted, the reagent contained the following active ingredients: 30 mmol.L^{-1} PCr, 2 mmol.L^{-1} ADP, 5 mmol.L^{-1} AMP, 2 mmol.L^{-1} NAD, 20 mmol.L^{-1} N-Acetyl-L-Cystine, 3000 U.L^{-1} HK, 2000 U.L^{-1} G-6-PDH, 10 mmol.L^{-1} Mg^{2+}, 20 mmol.L^{-1} D-Glucose, 10 µmol.L^{-1} di(adenosine 5') pentaphosphate and 2 mmol.L^{-1} EDTA. The reagent mixture was then added to the samples and the change in absorbance monitored continuously over 10 minutes in a temperature controlled (37°C) spectrophotometer at a wavelength of 340 nm. Calculation of CK concentration was determined by the following equation:

$$\text{CK (U.L}^{-1}) = \frac{\Delta\ \text{OD.}\ \text{min}^{-1} \text{ x } 0.21 \text{ ml x } 1000}{6.22 \text{ x } 0.01\text{ml}}$$

Where:

- Δ OD.min^{-1} = change in absorbance per minute
- 0.21 ml = total reaction volume in ml
- 1000 = conversion of U/ml to U/L
- 6.22 = extinction coefficient
- 0.01 ml = serum sample used in assay

Equation 3.2. Equation for the calculation of CK concentration via spectrophotometric analysis. Once normalised to total protein the unit of measurement is mU.mg.ml^{-1}

3.9 Isolation and Characterization of Human Derived Muscle Cells

3.9.1 Isolation Procedure

Cells were retrieved from biopsy specimens by implementing a modified method of that previously described by Blau and Webster (1981): Biopsy samples were obtained from the *vastus lateralis* via the method described in the section 3.5 of this chapter and samples were transferred in pre-cooled transfer media (TM; containing Hams F-10, 2% hiFBS, 1% pen-strep and 1% amphotericin-B) to the laboratory. This process is unlikely to have affected the yield and variability of myoblasts retrieved as previous work has demonstrated that postnatal muscle samples can be stored at 4^{o}C in Hams F-10 culture media for up to 24 hours following sample isolation with no adverse effects on yield and variability (Blau & Webster, 1981). To dissociate the tissue, biopsy samples were carefully dissected with a sterile scalpel in petri dishes to remove connective tissue whilst still in Hams F-10 TM. Following three washes with ice cold PBS + antibiotics (1% pen-strep and 1% amphotericin-B), 5ml Trypsin EDTA was added and the samples were scissor minced to fragments <1 mm^3. The dissected sample was then triturated on a magnetic stirring platform at 37^{o}C. The trypsinization process was repeated 2 times in succession and supernatant derived following each treatment was collected and pooled with HS at a concentration of 10% of the total volume to inhibit further protease activity. Once Trypsin treatments were complete, pooled cell supernatant was centrifuged at 1300 rpm for 5 minutes to produce a cell pellet. The supernatant was then discarded and the cell pellet re-suspended in GM and plated on a T25 cm^2 culture flask for cell population expansion.

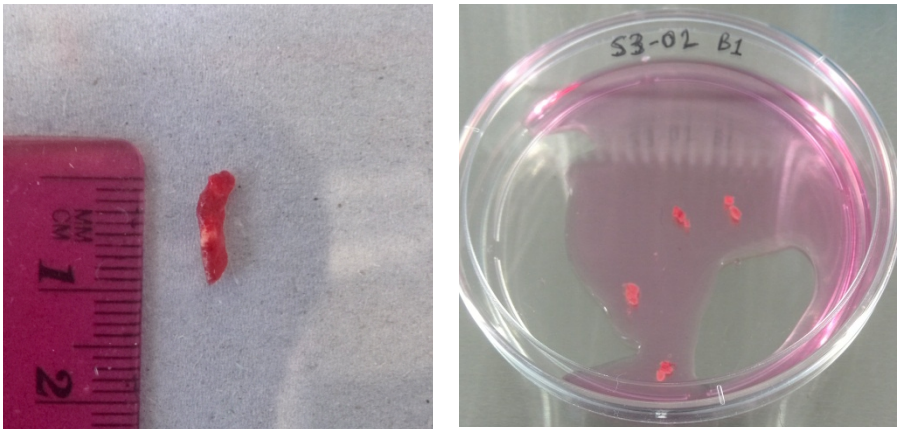


Figure 3.9. Left: fresh biopsy specimen immediately following biopsy procedure. Right: biopsy specimen in Tyrpsin EDTA following initial removal of fat and connective tissue, prior to mechanical dissociation to 1mm^3 fragments.

3.9.2. Cell Population Expansion

Following approximately 10 days in culture, T25 cm^2 culture flasks reached ~80% confluence and were passaged via trypsinization. Cells were then counted by Trypan Blue exclusion and frozen in GM with 10% dimethyl sulfoxide (DMSO) as a cryopreservant or re-plated to expand the population. For expansion, cells were grown in GM, which was changed every 48 hours following 2 washes with PBS + antibiotics.

3.9.3. Immunocytochemistry

Immunocytochemistry (ICC) Principle: ICC is a technique that allows the detection of a specific antigen (i.e. a protein such as desmin, expressed only in skeletal muscle cells) via the use of an antibody. An antibody is a protein complex produced by B cells of the immune system. The organization of an antibody includes two functional domains that, resemble the letter Y. The antigen binding fragment (Fab) domain makes up the arms of the Y, and at the end of each arm is a variable region responsible for antigen binding, called the antigen-binding site. The Fc domain comprises the tail of the Y, which other antibodies recognize primarily. This structure allows direct detection of antigens using a single fluorophore-labelled antibody or indirect detection through binding of a fluorophore labelled secondary

antibody raised against the Fc domain of an unlabelled primary antibody (see Figure 3.10). Because the Fc domain is conserved within a species, the labelled secondary antibody can be used to detect any primary antibody raised from a single species (Odell & Cook, 2013).

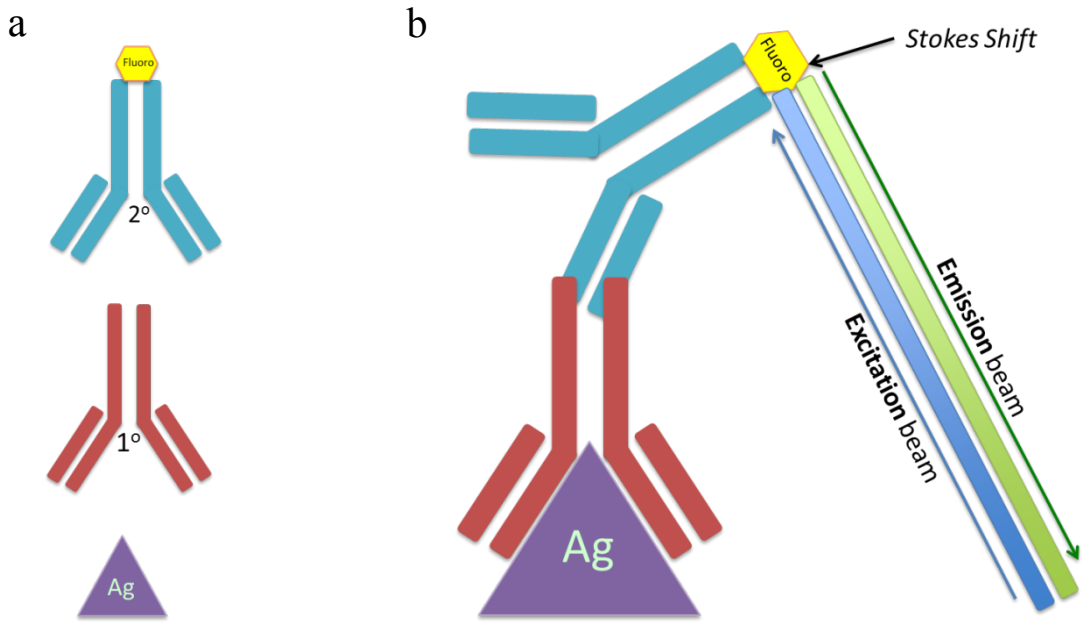


Figure 3.10. Schematic representation of the principle of immunolabelling. **a**) the basic components needed for immunolabelling including the primary antibody (1^{o}) which is an immunoglobulin specific to a particular antigen depicted as a triangle with the abbreviation Ag and the secondary antibody (2^{o}) which is raised against the Fc domain of the primary antibody. **b**) The primary antibody is first bound to the antigen following which the secondary antibody will bind to the primary antibody. The fluoroschrome molecule bound to the secondary antibody will be excited by specific wavelengths of light that excite the fluorschrome causing it to excite in its energy state and emit a light of a different wavelength.

When exposed to specific light wavelengths, the secondary antibody containing a fluorochrome will emit fluorescence. Fluorescence is a member of the luminescence family of processes in which susceptible molecules emit light from electronically excited states created by the absorption of light. Briefly, in fluorescence microscopy a wavelength of light is focus on the antibody labelled specimen to excite the electrons of the fluorochrome molecules to a higher energy state (excitation), known as Stokes shift. Subsequently, the excited electron relaxes to a lower energy level and emits light in the form of a lower-energy photon in the visible light region. The fluorescence light is then filtered through a dichroic mirror and visualised through a microscope (Figure 3.12).

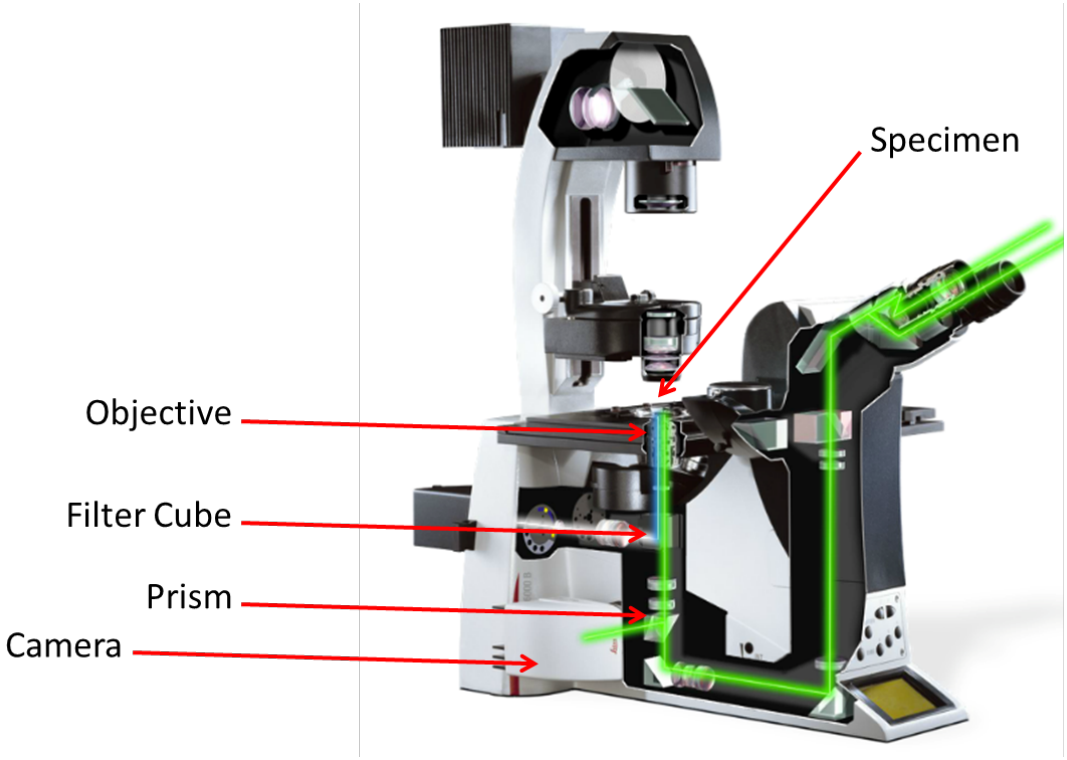


Figure 3.11. Schematic representation depicting the path of excitation and emission light in a fluorescence microscope. Excitation light (blue) is directed onto the sample via the objective. The fluorophore is then excited and emits fluorescence (green). The green light is passed through a prism filtering out light of other wavelengths and produces an image that can be seen through the eyepiece of camera of the microscope.

Fixation: Cell monolayers were fixed at passage 1 for determination of myogenicity (% Desmin^{+} cells). For the fixation of cell monolayers, methanol was used in a graded fashion i.e. 25%, 50%, 100% (v/v) in 1x tris buffered saline (TBS) to reduce the morphological distortion of nuclear detail and cytoplasm shrinkage that occurs with using absolute alcohol. Briefly, GM was aspirated from cell monolayers and the cells were washed twice with PBS following which 500µl of 25% methanol solution was added to each well of a six well plate for 5 minutes at room temperature. Following the first incubation, methanol solution was removed and the process repeated for 50 and 100 %. After the final incubation in 100% methanol, 1ml of 1x TBS solution was added to each well until immunocytochemical staining was performed.

Staining Procedure: Following fixation, monolayers were stained for Desmin, an intermediate filament protein specific to skeletal muscle, to quantify the percentage of myogenic cells in the population. Monolayers were first blocked and permeabilized for one hour with 500µl of 0.2% Triton-X, 5% Goat Serum in 1x TBS. Following three washes with 1x TBS, monolayers were incubated overnight at 5^{o}C with 1^{o} Desmin antibody (1:200). After overnight incubation, 1^{o} antibody was removed and monolayers washed three times with 1x TBS. Secondary antibody TRITC antibody (1:200) was then applied and left for two hours at 5^{o}C. Finally, following removal of 2^{o} antibody and three 1x TBS washes, a nuclear counterstain (Sytox-Green; 1:5000) was applied and monolayers incubated for 1 hour before a final three 1x TBS washes. Monolayers were stored wet in 1x TBS for fluorescent imaging.

Cell Counting: $Desmin^{+}$ cells and total nuclei were counted using ImageJ (NIH) *cell counter* plug-in. Six images were taken per well with an average of 107 (± 46) nuclei counted per image. All counting procedures were conducted in duplicate over two wells of a 6 well plate. The percentage of myogenic cells retrieved from each biopsy specimen can be observed in Figure 3.12.

Participant No.	% Desmin^{+} Cells
1	45
2	43
3	23
4	23
5	51
6	60
7	63
8	44
9	51
10	53
11	20
12	22
13	34
14	56

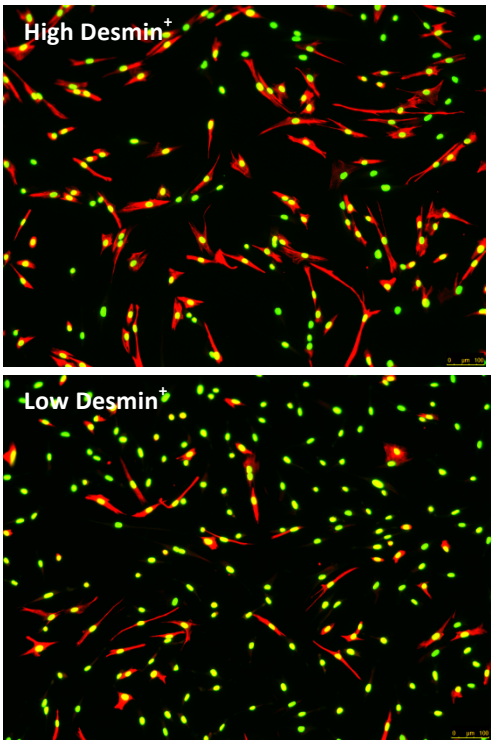


Figure 3.12. Representative images showing high (>60%) and low (<25%) Desmin^{+} cell populations retrieved from two different human muscle biopsies. Desmin stained red (TRITC) and nuclear counterstain is green (Sytox Green). Images taken with a 10x objective and scale bar is 100 µm.

3.10. Cell Culture

All cell culture experiments were performed under a Kojair Biowizard Silverline class II hood (Kojair. Vippula, Finland). Cells were incubated in a HERAcell 150i CO_2 Incubator (Thermo Scientific Inc. Cheshire, UK). All liquids, waste media and supernatant were discarded via an extraction pump (Charles Austen Pumps Ltd. Surrey, UK). Solutions were prepared using dH_2O from a MilliQ water purification system (Merck KGaA. Darmstadt, Germany). Cell populations were cultured on T75 cm^2 (Nunc. Roskilde, Denmark) and T25 cm^2 (Corning Inc., Life Sciences. Massachusetts, US) culture flasks and experiments were performed on cell monolayers seeded on sterile six well plates (Nunc. Roskilde, Denmark). Culture flasks and six well plates were coated with a 2 $mg.L^{-1}$ porcine gelatin solution (~90-110 g Bloom; Sigma-Aldrich Company Ltd. Dorset, UK) to allow cell adhesion via integrin binding.

3.11. Reagents

Dulbecco's modified eagles medium (DMEM) with added L-Glutamine (2.5 mM) and high glucose (4,500 $mg.L^{-1}$) was purchased from Sigma-Aldrich (Sigma-Aldrich Co. Dorset, UK). Sera (goat (GS), horse (HS), heat inactivated fetal bovine (hiFBS) and heat inactivated new born calf serum (NBCS)) were purchased from Gibco (Life Technologies. California, US). Hams F-10 and MEM-alpha for primary human cell culture were purchased from Lonza (Lonza, Basel, Switzerland). Antibiotics (penicillin-streptomycin (PS) and amphotericin-B) were purchased from Gibco. Phosphate buffered saline (PBS) for cell monolayer washes was purchased in tablet form from Sigma-Aldrich and reconstituted to give a working concentration of 0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl, pH 7.4 in dH_2O. We also purchased 1α,25-dihydroxyvitamin D_3 from Sigma-Aldrich and reconstituted as per the manufacturers guidelines in 100% ethanol.

Myoblast growth media (MGM) for murine C_2C_{12} cells (used in pilot studies for optimization of an *in vitro* model of muscle regeneration, see Chapter 6) consisted of DMEM, 10% FBS, 10% NBCS and 1% penicillin streptomycin (50 units penicillin/50 µg streptomycin). C_2C_{12} differentiation media (MDM) consisted of DMEM, 1% FBS, 1% NBCS and 1% penicillin streptomycin.

Human growth media (GM) used for the expansion of human muscle derived cell populations consisted of Hams F-10 growth media with added L-Glutamine (2.5 mM), 10% hiFBS, 10% NBCS, 1% PS (50 units penicillin/50 µg streptomycin) and 1% amphotericin B (2.5 $\mu g.ml^{-1}$). Human differentiation media (DM) consisted of MEM-alpha, 1% hiFBS, 1% NBCS, 1% PS (50 units penicillin/50 µg streptomycin) and 1% amphotericin B (2.5 $\mu g.ml^{-1}$). A quiescent media (QM) used in human MDC pre-treatments consisted of the same components as DM however hiFBS and NBCS were added at a concentration of 0.1% each

(0.2% serum total). The QM was also used as a transfer media (TM) to transport freshly collected biopsy specimens from the biopsy site to the cell culture facility.

3.12. Chemicals and Solvents

All chemicals and solvents were purchased from Sigma Aldrich and Fisher Scientific. Desmin polyclonal rabbit anti-human antibody (Ab 15200) was purchased from Abcam (Abcam Plc. Cambridge, UK) and anti-fibroblast monoclonal mouse anti-human antibody (clone TE) from Merck Millipore (Merck KGaA. Darmstadt, Germany). Secondary fluorophores (Alexa Fluor-488 and TRITC) and nuclear counterstains (DAPI and Sytox-Green) were purchased from Life Technologies and Sigma-Aldrich.

3.13. Microscopy and Live Imaging

All cell imaging and photography was performed on a Leica DMI 6000B inverted research microscope (Leica Biosystems GmbH. Nussloch, Germany) with recording capability (Leica DCF365 FX). For live cell imaging, the microscope was equipped with a heat controller and Pecon incubator and CO_2 controller (PeCon GmbH. Erbach, Germany) to permit a humidified environment of 37^{o}C, 5% CO_2 (Figure 3.13). Images were captured with a 10x objective and 0.5 magnification c-mount fitted to a camera.

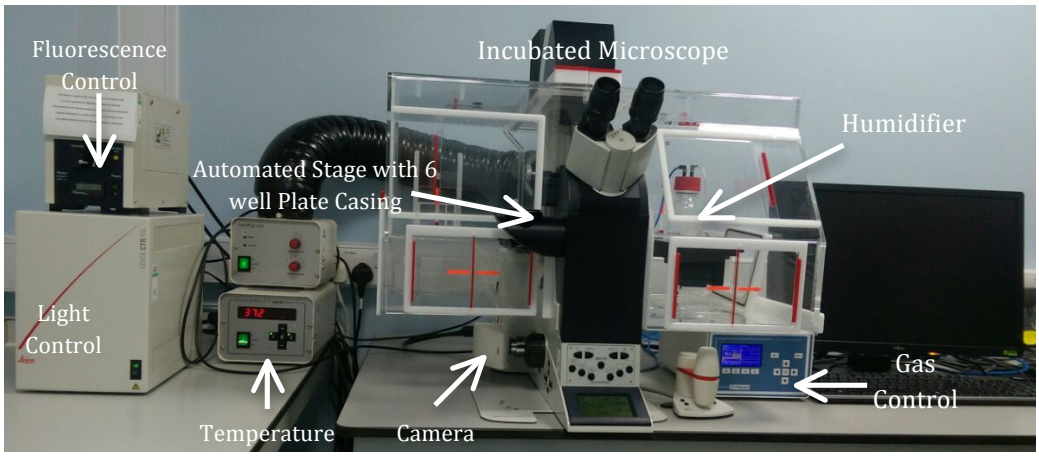


Figure 3.13. Annotated image of live imaging hardware used to perform live imaging experiments and fluorescence microscopy. Images are visualized via Leica Application Suite linked to the microscope.

3.14. Cell Migration Analysis

To assess the number of cells migrating into the wound space of the model described in Chapter 6, images were split into three equal segments (two outer segments termed segment 1 and the inner wound area termed segment 2) in the 0 hours wound area as shown in Figure 3.14 and counted at 48 hours post damage in the experiments described in Chapters 6 & 7.

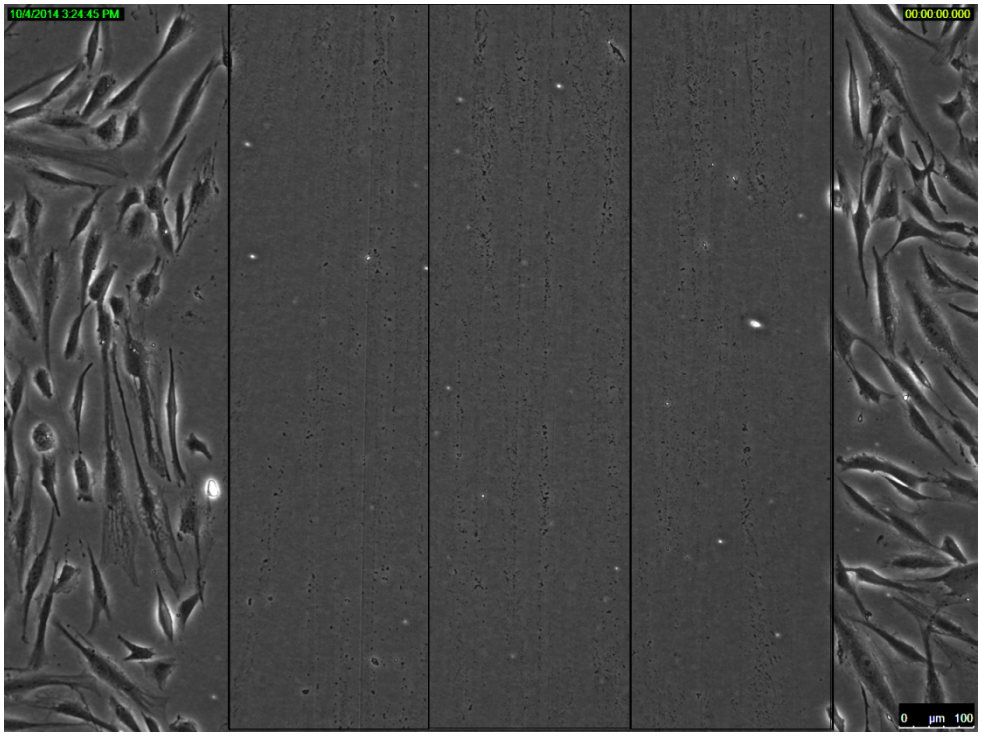


Figure 3.14. Representative image of a wound inflicted with a 1ml pipette tip. The wound area is 900 µm in width and split into 3 x 300 µm segments. Magnification is 10.5x and scale bar is 100 µm. The out most segments are referred to as segment 1 and thr single inner segment is referred to as segment 2.

3.15. Statistical Analysis and Graphical Representations

All statistical analyses were performed using SPSS Predictive Analytics Software (v.20, IBM Corporation. New York, US). For the comparison of two group means, a t-test was used and where comparison of multiple groups of means was required an analysis of variance (ANOVA) was used. Data sets were first checked for normal distribution and where data violated the assumption of normality, an appropriate correction factor was used. If data violated the assumption of sphericity Greenhouse-Geiser or Huyn-Feldt correction factors were used. Where significant main effect and interactions were present, the Bonferroni post-hoc pairwise comparisons test was used to detect where significances lay between paired comparisons, an analysis that includes correction for an ANOVA's multiple comparisons. Significance was assumed when α reached ≤ 0.05. All data are presented as mean ± standard deviation (SD). All figures were designed in Microsoft Excel v.2011.

Chapter 4

Vitamin D Status and Skeletal Muscle Contractile Properties *in vivo* and *ex vivo*

This chapter explores the influence of Vitamin D concentration on muscle function in vivo and ex vivo. Findings suggest that mild Vitamin D deficiency does not impair skeletal muscle performance and opens up further questions as to what degree of deficiency is a threat to muscle function.

Publications resulted from this chapter; Owens D.J., Webber D., Impey S.G., Tang J., Donovan T.F., Fraser W.D., Morton J.P. and Close G.L. (2014). Vitamin D supplementation does not improve human skeletal muscle contractile properties in insufficient young males. European Journal of Applied Physiology. 114(6):1309-20.

4.1 Abstract

Purpose – Vitamin D may be a regulator of skeletal muscle function although human trials investigating this notion are limited to predominantly elderly populations. The aim of the current study was to assess the effect of oral Vitamin D_3 in healthy young males upon skeletal muscle function.

Methods - Participants (n = 29) received either an oral dose of 10,000 $IU.day^{-1}$ vitamin D3 (VITD) or a visually identical placebo (PLB) for three months. Serum 25[OH]D and intact parathyroid hormone (iPTH) were measured at baseline and then at week 4, 8 and 12. Muscle function was assessed in n = 22 participants by isokinetic dynamometry and percutaneous isometric electromyostimulation at baseline and then at week 6 and 12.

Results - Baseline mean total serum 25[OH]D was 40.4 ± 16.6 $nmol.L^{-1}$ and 41 ± 21 $nmol.L^{-1}$ for PLB and VITD, respectively. VITD showed a significant improvement in total 25[OH]D at week 4 (150 ± 31 $nmol.L^{-1}$) that remained elevated throughout the trial ($P < 0.005$). Contrastingly, PLB showed a significant fall in 25[OH]D at week 12 (25 ± 15 $nmol.L^{-1}$) compared with baseline. Despite marked increases in total serum 25[OH]D in VITD and a fall in PLB, there were no significant changes in any of the muscle function outcome measures at week 6 or 12 for either group ($P > 0.05$).

Conclusions - Elevating total serum 25[OH]D to concentrations > 120 $nmol.L^{-1}$ has no effect on skeletal muscle function. It is postulated that skeletal muscle function is only perturbed in conditions of severe deficiency (<12.5 $nmol.L^{-1}$).

4.2 Introduction

Vitamin D is classically regarded as a vital endocrine regulator of bone health via its role in calcium and phosphate homeostasis (Anderson, Turner, & Morris, 2012). However, a myriad of non-skeletal effects of the steroid hormone are now known and are predominantly attributable to the identification of the VDR in virtually all tissues (Rosen et al., 2012) including skeletal muscle (Srikuea et al., 2012). Genome wide analyses suggest that any improvement in vitamin D status significantly affects the expression of a large number of genes that have many biological functions (Hossein-nezhad, Spira, & Holick, 2013). Specifically regarding skeletal muscle, it has long been understood that patients with osteomalacia resultant from inadequate dietary Vitamin D intake often display an accompanying skeletal muscle myopathy presenting as a proximal muscle weakness, that is responsive to Vitamin D supplementation (Al-Said, Al-Rached, Al-Qahtani, & Jan, 2009; Irani, 1976; Smith & Stern, 1967; Ziambaras & Dagogo-Jack, 1997). Emerging data now suggest at a cellular level that skeletal muscle is indeed a tissue that responds functionally to Vitamin D. Recent data have demonstrated *in vivo,* improvements in mitochondrial oxidative function when severe Vitamin D deficiency is corrected in humans (Sinha et al., 2013). Moreover, studies in chick embryo myoblast cultures have provided robust evidence that Vitamin D is a regulator of intracellular Ca^{2+} concentration (Capiati, Vazquez, Tellez Inon, & Boland, 2000; Morelli, de Boland, & Boland, 1993), which has also been observed *in vivo* in chicks (Vazquez, Boland, & de Boland, 1995) and *in vitro* in fully differentiated soleus muscle (de Boland, Massheimer, & Fernandez, 1988). Vitamin D may therefore be implicated in skeletal muscle contractility, which early reports from Vitamin D depleted rats have alluded to (Rodman & Baker, 1978). Aside from these observations, reports suggest a role for the bioactive 1α,25[OH]D in signalling pathways involved in myoblast proliferation and differentiation indicating a key role for Vitamin D in muscle cell development and survival (Garcia, Ferrini, Norris, & Artaza, 2013; Srikuea et al., 2012; Stratos et al., 2013). Despite such evidence, Vitamin D deficiency is still widespread around the globe (van

Schoor & Lips, 2011). This observation is consistent in sub-populations of professional athletes and otherwise-healthy, young athletic cohorts (Close et al., 2013a; Close et al., 2013b; Morton et al., 2012a)

A number of trials have sought to assess the effect of Vitamin D status on muscle function at a macroscopic scale. Insights from these investigations have established a link between Vitamin D concentration and falls and various other physical performance measures such as handgrip, lower limb strength, balance and gait speed (Bischoff-Ferrari et al., 2004b; Gerdhem, Ringsberg, Obrant, & Akesson, 2005; Houston et al., 2007; Marantes et al., 2011). However, such trials are limited to predominantly geriatric or clinical populations and it is difficult to generalise such findings to young otherwise healthy populations, due to age-related skeletal muscle disease (i.e. sarcopenia). The few trials that have been conducted in young otherwise healthy cohorts have resulted in equivocal findings, with some investigations reporting beneficial effects of Vitamin D supplementation in muscle (Close et al., 2013b; Sinha et al., 2013) and others observing no perceptible changes following supplementation (Close et al., 2013a). Hampering such findings further is the large heterogeneity in outcome measures implemented to assess muscle function, low participant numbers, heterogeneity in supplementation protocols and varying baseline Vitamin D status of the sample population making cross comparison between findings difficult.

Adding further complexity to this issue is that the daily requirement for vitamin D needed to elevate total serum Vitamin D concentrations to a level necessary for optimal physiological function is a point of debate. With regard to dose, the United States Institute of Medicine (US IoM) has set the RDI for Vitamin D at 600 IU.day^{-1} for young adults and tolerable upper intake at 4,000 IU.day^{-1} (IoM, 2011), although the US IoM set the 'no observed adverse effect limit' (NOAEL) at 10,000 IU·day^{-1} (IoM, 2011). In relation to total serum 25-hydroxyvitamin D (or 25[OH]D) concentration, the US IoM suggest > 50 nmol.L^{-1} as adequate whilst suggesting that concentrations exceeding 125 nmol·L^{-1} may be detrimental to health. Others have suggested these guidelines are too conservative and claim values

exhibited in those living outdoors in sun-rich climates (~100 nmol.L^{-1}) to be adequate, since they reflect the values present when the human genome evolved in such rich environments around equatorial Africa (Heaney, 2011; Vieth, 2011). An '*optimal*' 25[OH]D concentration for muscle function is currently still elusive and no study to date has tested the hypothesis that elevating serum 25[OH]D concentrations to >120 nmol.L^{-1} is optimal for muscle function in healthy recreationally active individuals, as previously suggested (Heaney, 2011). Our group has previously implemented doses of 20,000 IU.week^{-1} and 40,000 IU.week^{-1} oral Vitamin D_3, but failed to elevate serum concentrations > 100 nmo.L^{-1} (Close et al., 2013a). Thus higher doses may be necessary to achieve a total serum 25[OH]D concentration > 100 nmol.L^{-1}.

Given the available evidence and a clear lack of data in the area, the main objective of the current investigation was to explore the impact of Vitamin D concentration on lower limb muscle function using valid macroscopic techniques including percutaneous isometric electromyostimulation (EMS) and isokinetic dynamometry (IKD) in otherwise healthy, recreationally active young men. It was hypothesized that supplementing with high dose oral Vitamin D_3 (cholecalciferol) to elevate total serum 25[OH]D concentrations > 100 nmol.L^{-1} (suggested optimal Heaney, 2011; Vieth, 2011; Zittermann, 2003) would have a beneficial effect on our chosen outcome measures of muscle function.

Vitamin D and Skeletal Muscle Contractile Properties *In Vivo*

4.3 Aims and Hypotheses

The primary aim of this investigation was to examine whether improving Vitamin D concentration (serum 25[OH]D) with supplemental Vitamin D_3 improves parameters of muscle function in healthy young males. It was hypothesized that improving Vitamin D concentration will result in a positive change in skeletal muscle function.

4.4 Methodology

4.4.1 Participants

Twenty-nine male participants (age = 22.7 ± 3 years; height = 179.3 ± 5.9 cm; weight = 76.1 ± 16.2 kg) volunteered to partake in the current trial. Participants were screened for inclusion as described in the General Methods chapter of this thesis. After meeting this initial inclusion criteria, participants provided a venous blood sample that was analysed for total 25[OH]D via LC-MS/MS as described in detail in section 3.4 of the General Methods chapter of this thesis. Those presenting with total serum 25[OH]D concentrations ≥ 100 $nmol.L^{-1}$ were excluded from the trial. This was based on suggestions that > 100 $nmol.L^{-1}$ is necessary for optimal health and associated with serum concentrations of individuals regularly exposed to sunlight near the equator (Zittermann, 2003). Ethical approval was granted by the local ethics committee of Liverpool John Moores University and all data were collected in accordance with the Declaration of Helsinki.

Participants were initially block randomized on their baseline 25[OH]D concentration and electrically evoked 20 Hz isometric knee extensor force into two experimental groups. Of the 29 initial participants, 22 completed all test procedures whilst the remaining 6 only took part in supplementation and blood sampling and one dropped out completely. This was due to an inability on the participants' behalf to commit sufficient time for each testing point.

Fourteen participants were assigned to an oral Vitamin D treatment group (VITD) and 15 participants were initially assigned to a visually identical placebo control group (PLB (see Table 4.1. below).

Table 4.1. Block randomization data including mean total serum 25[OH]D concentration presented as nmol.L^{-1} and mean isometric force from a single stimulation at a frequency of 20 Hz and stimulation intensity of 130 mA.

	PLB (n = 11)	**VITD (n = 11)**
Mean total 25[OH]D (nmol.L^{-1})	40	41
±SD	17	21
t-test	t = 0.867, P = 0.876	
Mean Isometric Force (N)	267	306
±SD	69	67
t-test	t = -1.086, P = 0.303	

4.4.2 Vitamin D Supplementation

Participants allocated to VITD received 10,000 IU·day^{-1} of vitamin D_3 (Bio-Tech Pharmacal. Arkansas, US) taken as two 5,000 IU gelatin capsules. Those allocated to PLB received two visually identical placebo capsules each containing 90 mg cellulose (Bio-Tech Pharmacal. Arkansas, US). The supplementation period lasted 12 weeks between the months of January and April in order to achieve a plateau in serum 25[OH]D response, as previously shown in a dose response trial with 10,000 IU.day^{-1} vitamin D_3 cholecalciferol (Heaney, Davies, Chen, Holick, & Barger-Lux, 2003). Both researcher and participant were blinded until completion of the study procedures.

4.4.3 Whole Muscle Force Measurements

A number of outcome measures were employed to validly assess muscle function of the lower limb. Participants completed both an isokinetic dynamometry (IKD) protocol and

electromyostimulation (EMS) protocol. All tests were completed at baseline, week 6 and week 12 of supplementation apart from IKD, which was only completed at baseline and week 12.

Isokinetic Dynamometry (IKD)

Isokinetic torque was assessed on a Biodex isokinetic dynamometer (Biodex Medical Systems Inc. Shirley, NY), previously validated for its use in reliable assessment of muscle function variables related to force production (Drouin et al. 2004). Participants were seated as per the manufacturer's guidelines with a 90-degree flexion of the hip and non-extendable straps crossing the chest and abdomen and across the quadriceps to maximise isolation of the target muscle group (Figure 4.1). The test protocol consisted of four consecutive maximal extension and flexion movements of the right quadriceps and hamstrings at two different fixed movement velocities, 1.05 rad.sec^{-1} (60 deg.sec^{-1}) and 3.14 rad·sec^{-1} (180 deg.sec^{-1}) from which peak torque (Nm) was generated, separated by a five minute rest to allow full recovery of the high energy phosphate pool (Soderlund and Hultman 1991). All participants were familiarized with the protocol until the co-efficient of variation for each participant was < 10% (Atkinson and Nevill 1998). See Table 4.2.

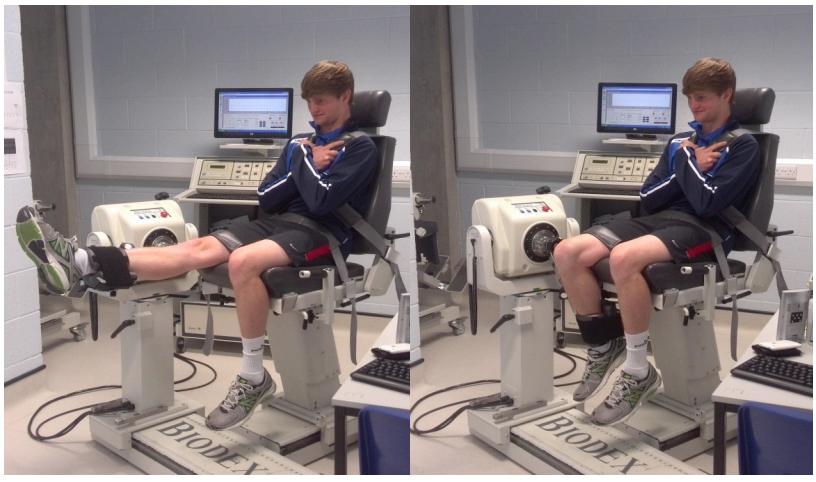


Figure 4.1. IKD experimental set up. Left: participant at full extension. Right: participant at full flexion.

Table 4.2 Co-efficient of variation for each isokinetic dynamometry variable. Data are presented as group means. 1.05 rad·sec^{-1} = 60 deg·sec^{-1}; 3.14 rad·sec^{-1} = 180 deg·sec^{-1}. Based on n = 22.

Measurement Variable	Flexion 1.05 rad·sec^{-1}	Extension 1.05 rad·sec^{-1}	Flexion 3.14 rad·sec^{-1}	Extension 3.14 rad·sec^{-1}
CV%	3.9	3.7	4.9	4.1

Electromyostimulation (EMS)

Stimulation was applied through surface electrodes, delivered via a BIOPAC systems MP100 stimulator (BIOPAC systems inc, Santa Barbara, CA) and knee extension forces were recorded with a tension compression load cell (Tedea-Huntleigh, Vishay Precision Group Inc. Malvern, PA) connected to a non-extendable strap attached to the participant's lower leg approximately 5-8 cm from the malleolus. Information collected from the force transducer was visualized and analysed using AcqKnowledge v.3.7.2 software (BIOPAC systems inc. Santa Barbara, CA). Muscles were stimulated at an intensity of 130 mA for each test procedure as described previously (Gerrits et al. 2002). For the determination of muscle contractile properties, contraction was evoked by a 1 Hz twitch. For the determination of fatigue resistance, contractions were evoked by 20 Hz, 0.2 sec square wave pulses, previously defined to be representative of normal voluntary human motor neurone firing rates (Bigland-Ritchie et al. 1979) and thus physiologically valid when used to assess the fatigue resistance of human skeletal muscle.

For every session, the position of the participant in the isometric chair (Lido Active, Loredan, Davis, CA, USA) was standardized in accordance with guidelines previously published (Morton et al. 2005). Two 3 x 5 inch (8 x 13 cm) oval self-adhesive stimulating electrodes (Chattanooga, DJO Global, CA, USA) were placed approximately 5 cm above the knee joint across the *vastus medialis* (VM) and proximal to the acetabulofemoral joint across the *vastus lateralis* (VL), as shown in Figure 4.1. Participants were required to fully extend and contract the quadriceps to make the muscle body more apparent prior to electrode placement

to minimise antagonist co-activation, although this was not monitored with electromyography during stimulation.

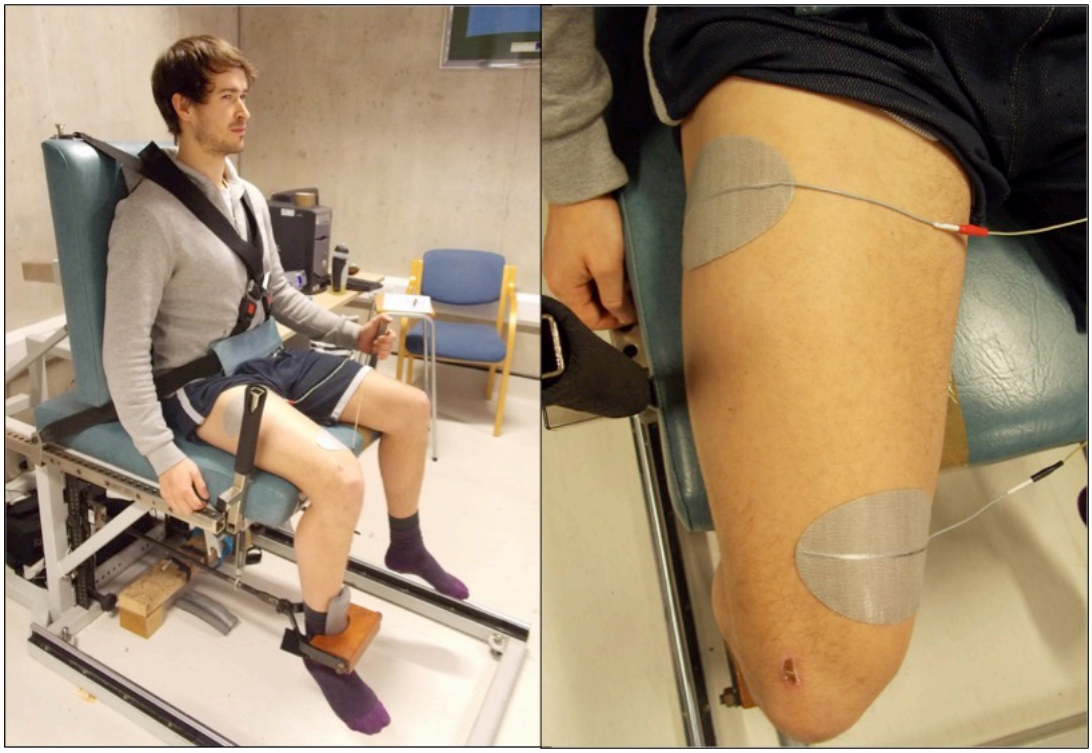
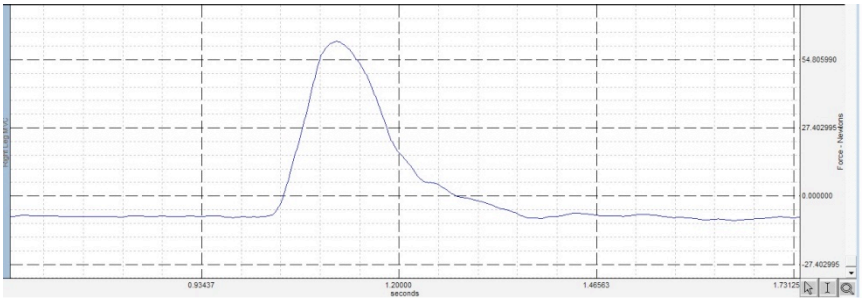


Figure 4.2. EMS experimental set up. Left: participant fully secured in the isometric chair. Right: placement of the electrodes used to deliver electrical stimuli.

To determine time to peak twitch and half relaxation time, a 1 Hz, 0.2 sec square wave pulse was administered. Time to peak twitch was measured as the time taken to reach peak isometric force from the onset of stimulation. Half relaxation time was determined as the time from peak stimulated force to reach half peak stimulated force (see Figure 4.3 a). Fatigue resistance was determined from two minutes repeated stimulation with 20 Hz, 200 msec square wave pulses every 750 msec (total of 160 evoked contractions equating to 32 secs of total stimulation). Fatigue index was calculated as the percentage force loss from the highest recorded contraction to the lowest recorded contraction (see Figure 4.3 b). Test retest reliability data revealed that the CV% for time to peak twitch and for ½ relaxation time as determined by a 1 Hz twitch at 130 mA was 4.5% and 5.7%, respectively.

a



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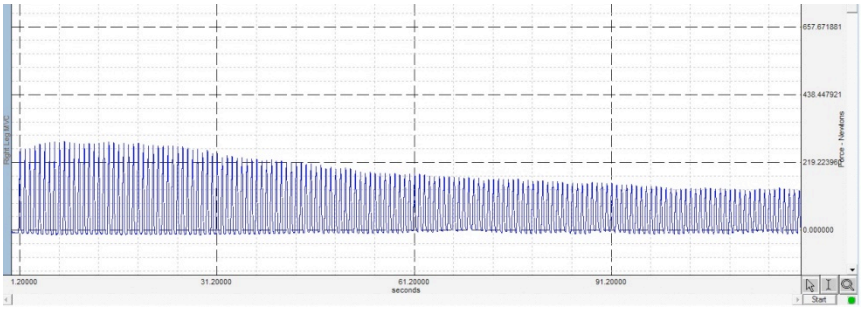


Figure 4.3. **a**) Example twitch feedback curve obtained from a 200 ms square wave pulse of 130 mA at a frequency of 1 Hz. **b**) Example fatigue data from two minutes repeated stimulation with 20 Hz, 200 msec square wave pulses every 750 msec. Representative output taken from AcqKnowledge software.

4.4.4 Statistical Methods

Statistical analyses were performed as described in the Chapter 3 of this thesis. In addition, a power calculation was performed *a priori* using Minitab software (v.16) to allow for any dropouts based on detecting a significant change in serum 25[OH]D and increased peak torque in response to Vitamin D supplementation. Pilot work from our laboratories during the winter months suggested that the standard deviation for test–retest serum 25[OH]D concentrations (taken 6 weeks apart) in young athletes is ~12 nmol.L^{-1}. Furthermore, previous work from our laboratory demonstrated that standard deviation in isokinetic torque at 1.04 rad·sec^{-1} (60 deg·sec^{-1}) is ~35 Nm. To enable the detection of a 50 nmol.L^{-1} increase in total serum 25[OH]D concentration between pre-supplementation and post-supplementation with 80% power and a 10% (21 Nm) increase in peak torque between pre-supplementation and post-supplementation with 80% power, n = 12 per experimental group was required. Therefore, initially 30 participants were recruited to allow for dropouts and maintain statistical power. Finally, a linear regression was plotted using Pearsons correlation to assess whether baseline 25[OH]D concentration was a predictor of Δ serum 25[OH]D in response to supplementation.

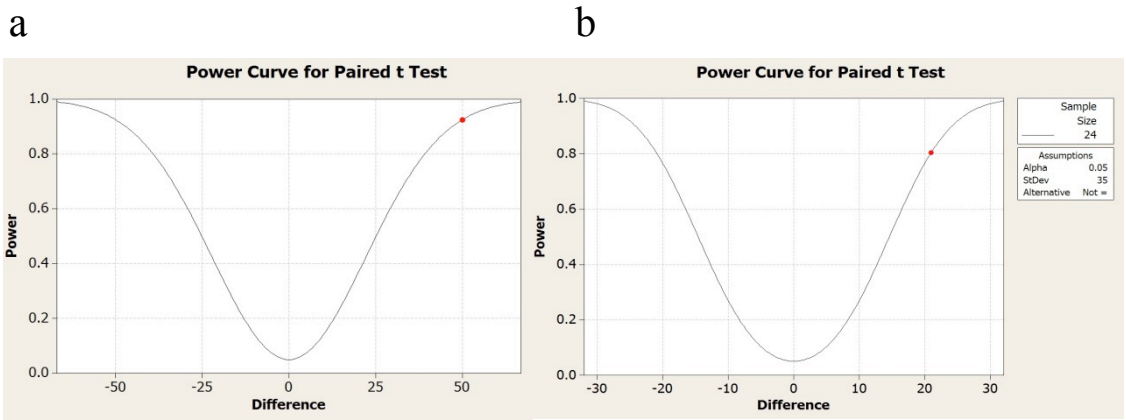


Figure 4.4. Sample size calculation based on detecting a meaningful change in **a**) total serum 25[OH]D and **b**) peak isokinetic torque.

4.5Results

Baseline Total Serum 25[OH]D is a Predictor of Response to Supplementation

Regression analysis determined that pre-treatment Vitamin D concentration was a strong predictor of 25[OH]D response to supplementation in VITD, where 83% of the variation in response to supplementation was attributable to baseline concentration (Figure 4.5.).

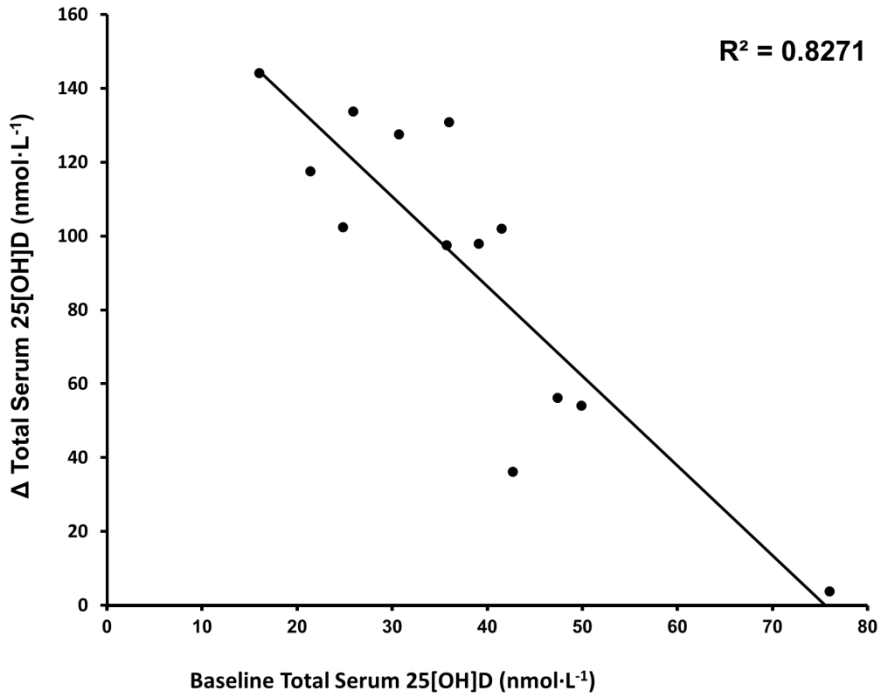
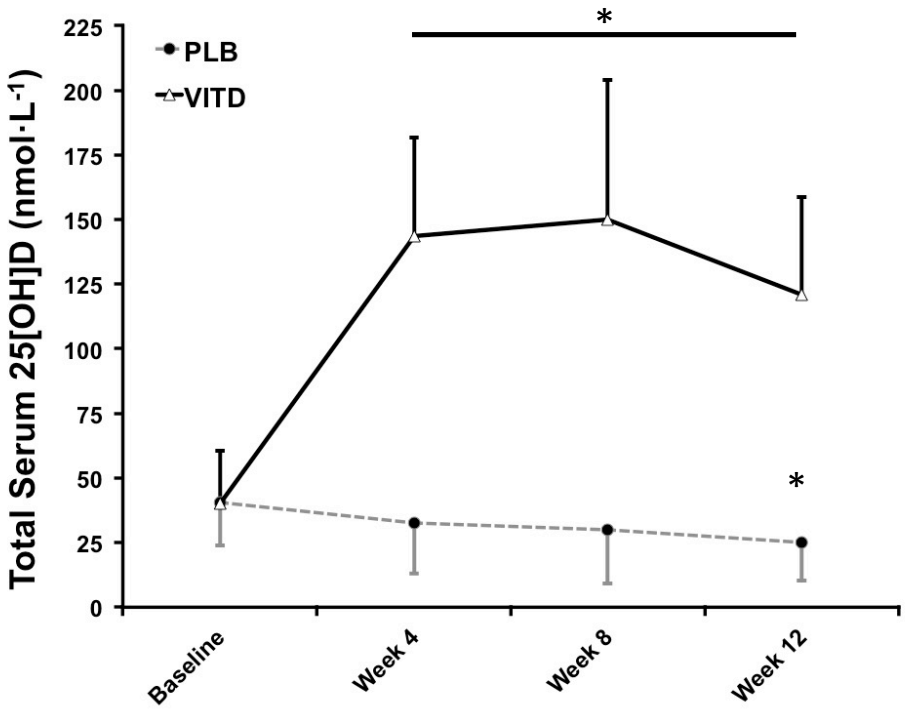


Figure 4.5. Linear regression describing the relationship between baseline Vitamin D status and response to supplementation. Baseline total 25[OH]D concentration is the predictor variable whilst change in total 25[OH]D at 12 weeks is the response variable.

Response of Total Serum 25[OH]D and iPTH to Supplemental Vitamin D_3

There was a significant interaction between treatment group and time for total serum 25[OH]D ($P < 0.005$) where there was a significant main effect for both time ($P < 0.005$) and treatment group ($P < 0.005$); Participants in VITD demonstrated significantly elevated total serum 25[OH]D concentrations at week 4 compared with baseline (pre = 41 ± 21 vs week four = 150 ± 31 $nmol.L^{-1}$) , that remained elevated throughout the trial (week twelve = 127 ± 31 $nmol.L^{-1}$). Contrastingly, participants in PLB displayed total serum 25[OH]D concentrations significantly lower at week 12 compared with baseline ((pre 40 ± 17 vs week twelve = 25 ± 14 $nmol.L^{-1}$) see Figure 4.6 a). VITD showed no significant change in iPTH concentration throughout the trial ($P = 0.322$) whereas PLB showed a significant increase in iPTH concentration at week 4 ((pre = 2.45 ± 0.61 vs week four = 5.01 ± 1.09 $pmol.L^{-1}$) $P < 0.005$), which remained elevated throughout the trial (see Figure 4.6 b.) showing an inverse relationship with serum 25[OH]D.

a



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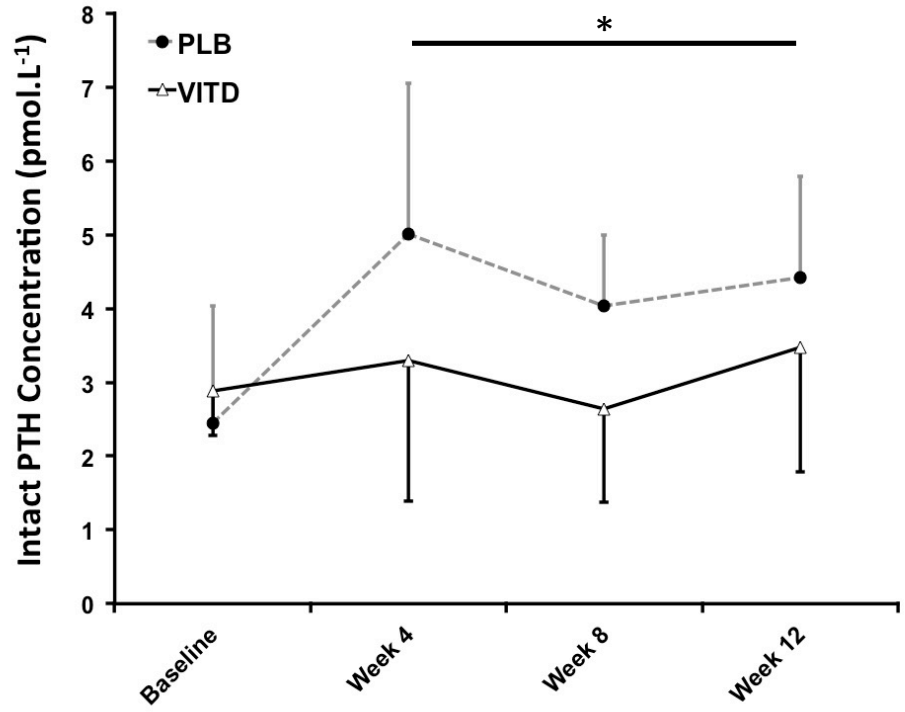


Figure 4.6. a) Response of total serum 25[OH]D to 12 weeks supplementation with 10,000 IU·day^{-1} vitamin D_3 or placebo. **b)** Response of intact parathyroid hormone to 12 weeks supplementation with 10,000 IU·day^{-1} vitamin D_3 or placebo. * denotes significance to baseline ($P < 0.05$).

Maximum Voluntary Contraction

There was no significant interaction between experimental groups for peak torque production at prior to and following the supplementation protocol at 60 deg.sec^{-1} extension (P = 0.163) and flexion (P = 0.807) or 180 deg.sec^{-1} extension (P = 0.269) or flexion (P = 0.940 (Figure 4.7)), indicating that increasing serum 25[OH]D did not affect the maximal force producing capacity of the lower limb at two dynamic movement velocities.

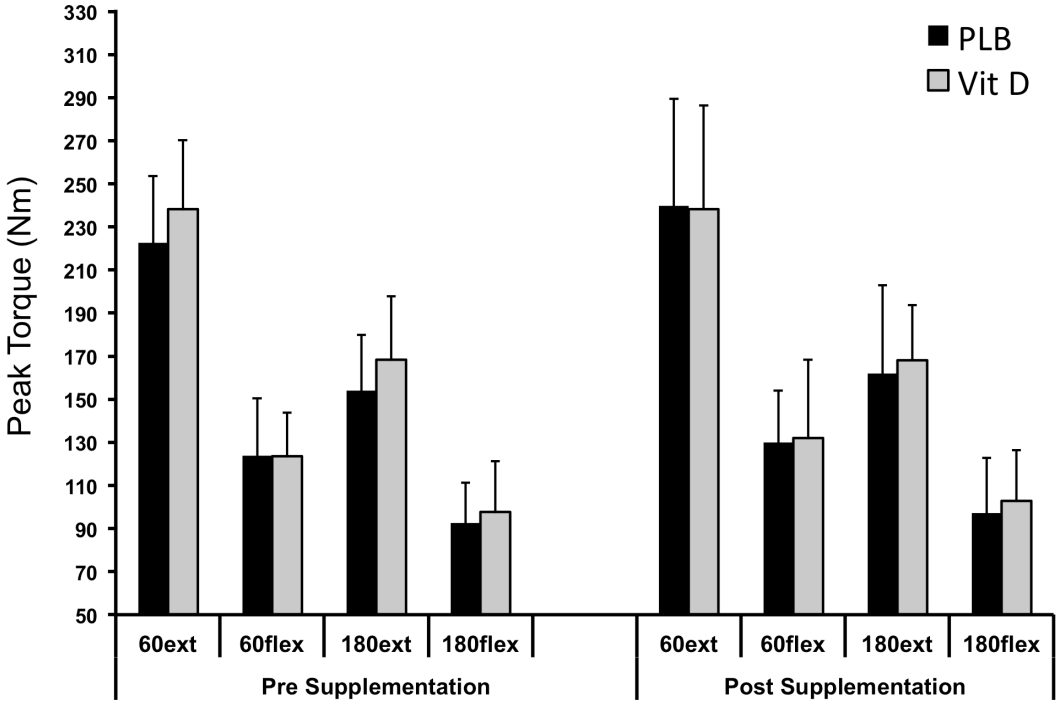


Figure 4.7. Peak torque from maximal extension (ext) and flexion (flex) of the right lower limb at two fixed velocities of 1.05 rad·sec^{-1} (60^{o}·sec^{-1}) and 3.14 rad·sec^{-1} (180^{o}·sec^{-1}) prior to and following 12 weeks supplementation with 10,000 IU·day^{-1} Vitamin D_3 (grey bars) or placebo (black bars).

Fatigue Resistance

The effects of Vitamin D supplementation on fatigue resistance of the VL & VM muscles can be seen in Figure 4.6. Data are presented as absolute maximal force (Figure 4.8 a) and percentage of maximal force (Figure 4.8 b) although statistical analysis was performed on the fatigue ratio of absolute data only. No significant interaction was detected between treatment group and time i.e. baseline, week 6 and week 12 ($P = 0.134$).

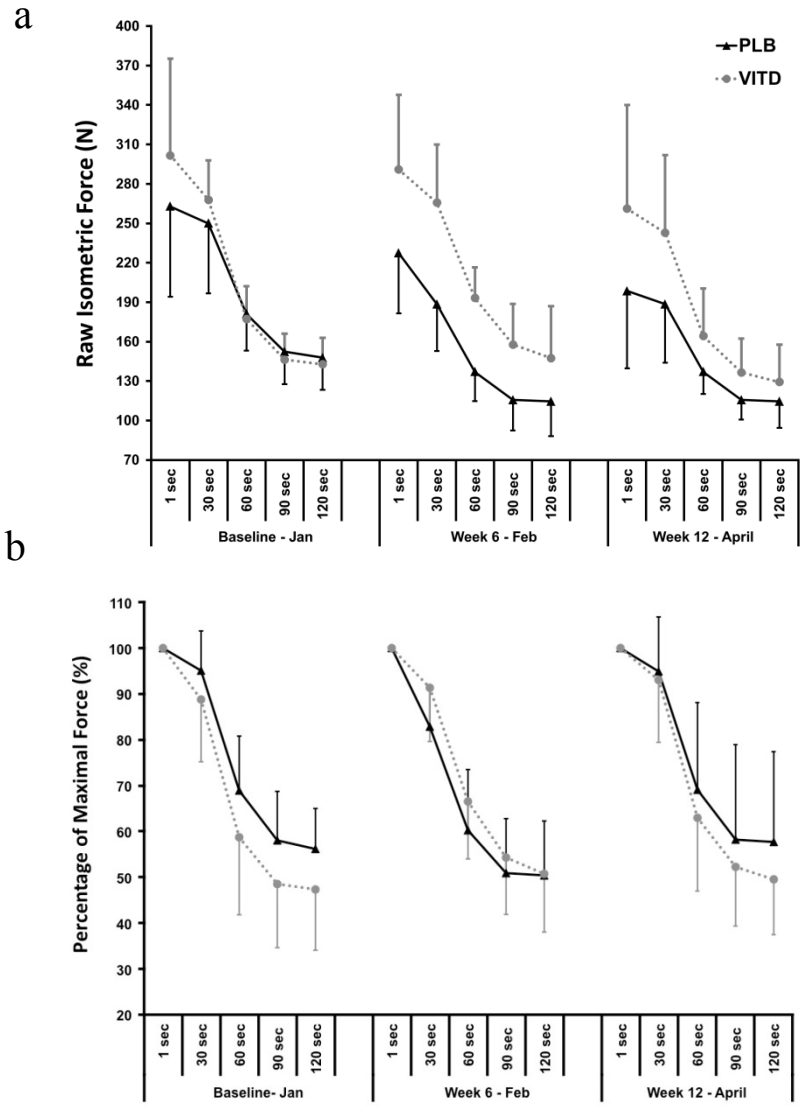


Figure 4.8. a) Absolute isometric force and **b**) percentage force loss during 120 sec of repeated unilateral lower limb stimulation from an electrically evoked square wave pulse of 0.2 msec, 20 Hz and a stimulation intensity of 130 mA, every 750 ms. Data presented prior to supplementation (baseline) and then at week 6 and 12 of supplementation.

Time to Peak Twitch and Half Relaxation Time

No significant interaction was detected between treatment group and time (baseline, week 6 and week 12) for time to peak isometric twitch force ($P = 0.968$) or half relaxation time ($P = 0.924$; Figure 4.9).

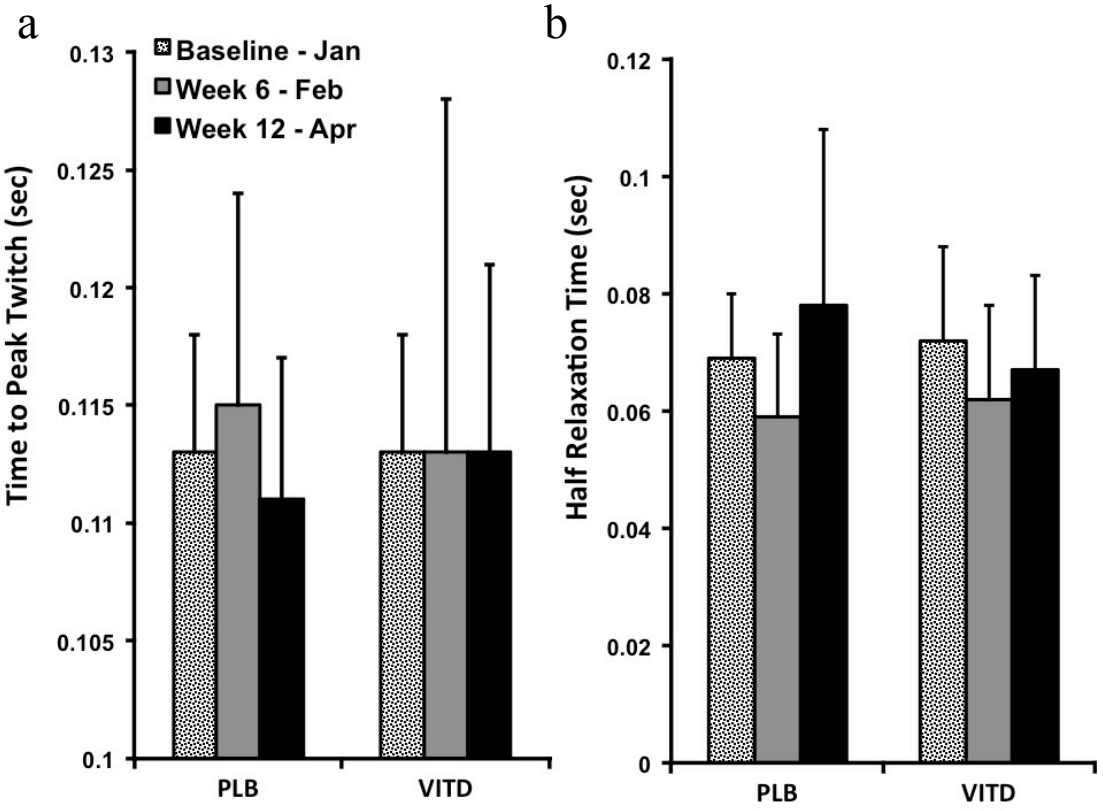


Figure 4.9. Muscle contractile properties from an electrically evoked 1 Hz twitch and a stimulation intensity of 130 mA. Data are displayed from baseline, 6 weeks and 12 weeks of supplementation with 10,000 IU.day^{-1} Vitamin D_3 or placebo.

4.6 Discussion

The aim of the current study was to investigate the impact of Vitamin D concentration on muscle function in otherwise healthy, recreationally active young men. It was hypothesized that supplementing with oral Vitamin D_3 (cholecalciferol) to elevate total serum 25[OH]D concentrations would have a beneficial effect on muscle function. However, although it was demonstrated that in a population of predominantly Vitamin D inadequate young men, supplementation with 10,000 IU.day^{-1} effectively elevated total serum 25[OH]D concentration to values > 100 nmol.L^{-1}, this had no effect (positive *or* negative) on muscle force, muscle fatigue or muscle contractile properties.

The primary findings are in agreement with other RCTs assessing the impact of Vitamin D supplementation on skeletal muscle function. Previous data have demonstrated in a sample of 30 club level athletes with a mean 25[OH]D concentration of 51 nmol.L^{-1} (SD ± 24 nmol.L^{-1}) that neither 20,000 or 40,000 IU.week^{-1} of Vitamin D_3 for 12 weeks were effective in improving 1-RM bench press, leg press or vertical jump height although both doses achieved a significant elevation of total serum 25[OH]D (20,000 & 40,000 IU.day^{-1} Vitamin D_3 = 85±10 nmol.L^{-1} & 91±24 nmol.L^{-1}, respectively) after 12 weeks (Close et al., 2013a). Similarly, others studies predominantly undertaken in elderly cohorts have failed to detect improvements in parameters of muscle strength following supplementation (Kenny, Biskup, Robbins, Marcella, & Burleson, 2003; Kukuljan et al., 2011).

The current study also found that elevating serum 25[OH]D >100 nmol.L^{-1} does not reduce the force producing capacity, fatigue resistance or contractile properties of skeletal muscle in humans, an idea that has previously been suggested. One randomized controlled trial has previously reported that annual high dose Vitamin D_3 (500,000 IU) effectively elevated serum concentrations of 25[OH]D to approximately 120 nmol.L^{-1} in community dwelling older women (Sanders et al., 2010). However, this resulted in an *increased* risk of falls and fractures compared with a placebo control group. This observation could be attributable to the dosing strategy implemented, as smaller but more frequent doses in other RCTs do not

show comparable findings. Furthermore, although Sanders *et al*. state that baseline prevalence of risk factors for falls and fractures were similar in both the supplemented and placebo groups, the physical activity profile of the two experimental groups is not reported. This is a confounding factor given that physical activity is a valuable counter measure for sarcopenia (Forbes, Little, & Candow, 2012), thus disparity in the physical activity profile of the two experimental groups is of importance when interpreting this data.

Intact parathyroid hormone concentration was also assessed in the current study and displayed an inverse relationship with serum 25[OH]D, in line with previous data (Sai, Walters, Fang, & Gallagher, 2011). The placebo group showed elevated iPTH concentration whereas the opposite was observed for VITD, which may be explained by the understanding that 1,25-dihydroxyvitamin D represses the transcription of the PTH gene and also indirectly regulates PTH secretion by regulating the expression of calcium sensing receptors (Kumar & Thompson, 2011). Thus, in addition to providing evidence that there is no negative effect of high serum 25[OH]D concentrations (> 100 $nmol.L^{-1}$) on skeletal muscle function, it is demonstrated that the supplementation protocol chosen offers a positive effect on the maintenance of iPTH concentrations. Some authors suggest that high serum 25[OH]D concentrations (> 100 $nmol.L^{-1}$) may indeed be necessary for prevention of diseases such as tuberculosis, rheumatoid arthritis, multiple sclerosis, inflammatory bowel diseases, hypertension, and specific types of cancer (Heaney, 2011; Vieth, 2011), thus the current data would imply that such concentrations needed to optimise other biological functions are unlikely to be detrimental to the normal function of skeletal muscle.

It is important to also consider the available evidence that suggests Vitamin D supplementation has a *beneficial* impact on muscle function. Case studies describe a proximal muscle weakness associated with nutritional osteomalacia that is responsive to supplementation with Vitamin D implying that improving Vitamin D status can improve musculoskeletal function (Al-Said et al., 2009; Irani, 1976; Smith & Stern, 1967; Ziambaras & Dagogo-Jack, 1997). Additionally, in a recent report and in contrast to the current findings,

an improvement in 10-m sprint times and vertical jump height following supplementation in a group of Vitamin D deficient young athletes has been observed (Close et al., 2013b). In a sample of severely deficient individuals (< 15 nmol.L^{-1}), supplementation with 20,000 IU of Vitamin D_3 on alternate days for 10-12 weeks significantly elevated serum 25[OH]D (> 100 nmol.L^{-1}) and resulted in significant improvements in phosphocreatine recovery half-time (τ½ PCr) of the soleus muscle, indicative of improved mitochondrial oxidative function as measured by ^{31}P-magnetic resonance spectroscopy (Sinha et al., 2013). The disparity between findings may be explained by a recent systematic review and meta analysis (Stockton, Mengersen, Paratz, Kandiah, & Bennell, 2011). It was found that although participants with baseline total serum 25[OH]D >25 nmol.L^{-1} show no change in muscle strength with Vitamin D supplementation, a limited number of trials demonstrate an increase in proximal muscle strength in adults with Vitamin D *deficiency* (<25 nmol.L^{-1}). It is reasonable to postulate based on such findings, that skeletal muscle function only displays perceptible perturbations in function when Vitamin D status of the sample population reaches concentrations < 25 nmol.L^{-1} as suggested by Stockton et al. (2011).

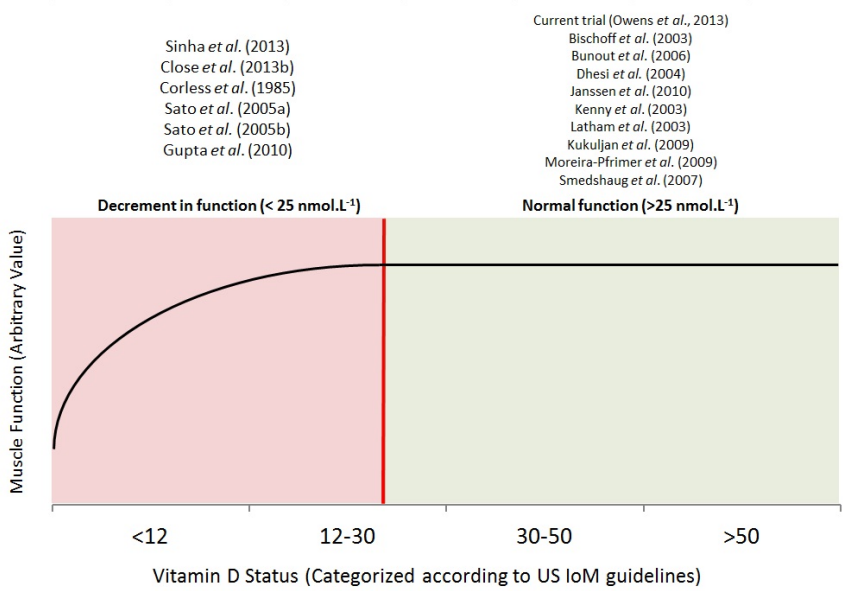


Figure 4.10. Schematic representation of a proposed theory that functional decrements occur in muscle when Vitamin D status is deficient. No additive improvements occur at concentrations > 25 nmol.L^{-1}. (Reviewed citations taken from systematic review and meta analysis by Stockton et al., 2011).

Taking the available evidence into account, it can be proposed that a possible reason that an improvement in muscle function was not detected in the current study, is because muscle function was not impaired to begin with, as only a small number (n = 4) of participants had total serum 25[OH]D concentrations < 25 nmol.L^{-1} and no participants presented with *severe* Vitamin D deficiency. If one assumes a sigmoidal physiological response of skeletal muscle to Vitamin D, it may be difficult to detect a response when nutrient status is already on the ascending limb or plateau of the response curve (Lappe & Heaney, 2012). Thus skeletal muscle may only display sharp decrements in function at serum 25[OH]D concentrations < 25 nmol.L^{-1} and thereafter show no perceptible added benefit of increasing concentrations. This could be due to a disturbance of systemic and intracellular calcium concentrations that occurs in severe deficiency, affecting both contractile activity and intracellular signalling, a notion previously put forward (Girgis, Clifton-Bligh, Hamrick, Holick, & Gunton, 2012). Although speculative, this would explain much of the human data available on Vitamin D status and muscle function.

As described, in the current study only four participants from the supplemental Vitamin D group exhibited serum 25[OH]D <25 nmol.L^{-1} (range = 16 – 25 nmol.L^{-1}). In keeping with the notion that such concentrations associate with perturbed muscle function, these data sets were extracted from the group and analysed as a sub group. However, despite all participants improving serum 25[OH]D from < 25 nmol.L^{-1} to > 75 nmol.L^{-1} no changes MVC torque at 60 deg.sec^{-1} extension (pre test = 232 ± 11.2 vs 12 weeks = 241 ± 41 Nm), 60 deg.sec^{-1} flexion (pre test = 132 ± 20 vs 12 weeks = 131 ± 22 Nm), 180 deg.sec^{-1} extension (pre test = 177 ± 34 vs 12 weeks = 186 ± 19 Nm) and 180 deg.sec^{-1} flexion (pre test = 108.7 ± 19 vs 12 weeks = 110.8 ± 19.9 Nm) were detected. Similarly, involuntary functional measures including time to peak tension (pre test = 0.114 ± 0.006 vs post test = 0.115 ± 0.007 sec) and half relaxation time (pre test = 0.069 ± 0.009 vs post test = 0.061 ± 0.004 sec) were also unchanged ($P > 0.05$). Data are presented in Figure 4.11

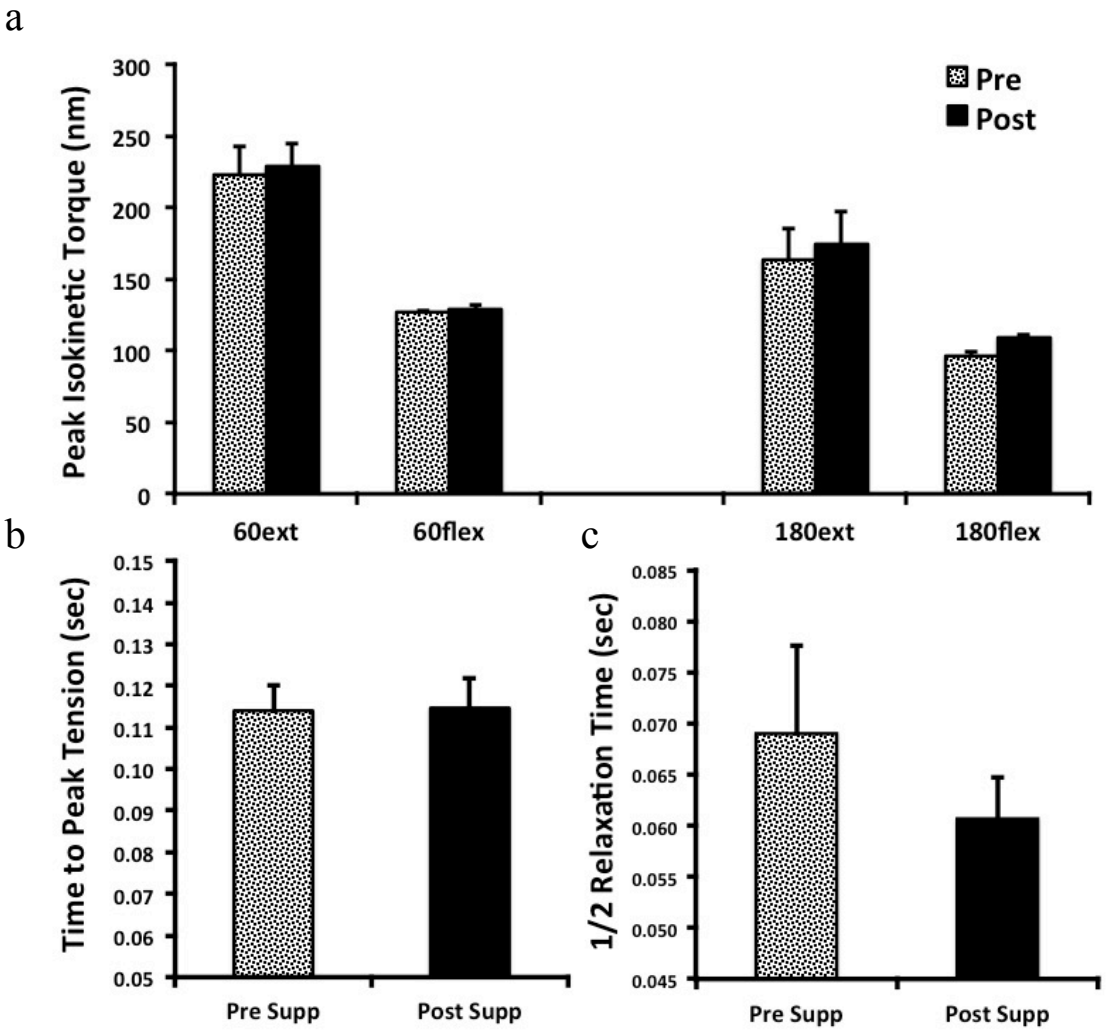


Figure 4.11. Sub group analysis of four individuals presenting with < 25 nmol.L^{-1} total serum 25[OH]D corrected to > 100 nmol.L^{-1} with twelve weeks supplementation of Vitamin D_3 (10,000 IU.day^{-1}). **a**) peak isokinetic torque of the knee extensors and flexors at 60 and 180 deg.sec^{-1}. **b**) time to peak tension and **c**) half relaxation time of the knee extensors.

In summary, the data presented from the first investigation of this determined that at the whole tissue level, supplemental Vitamin D to increase total serum 25[OH]D >100 nmol.L^{-1} offered no benefit to skeletal muscle contractile properties. Retrospective analysis of the data and available literature would suggest that this is because serum concentrations of 25[OH]D in the range of deficiency (possibly <25 nmol.L^{-1}) are necessary to hinder musculoskeletal performance. However, when a small sub group of individuals with serum 25[OH]D <25

nmol.L^{-1} were analysed, still no changes in muscle function were observed. On the other hand, the investigative outcome measures may not have been sensitive enough to detect decrements in muscle function at the whole tissue level.

4.7 Aims and Hypotheses

As a subsequent investigation to study 1, the present study aimed to examine skeletal muscle contractile properties at the single fibre level from individuals with serum 25[OH]D concentrations ≤25 nmol.L^{-1}. The single fibre technique allows for a more sensitive measure of cross bridge kinetics to be studied in the absence of other contributors to muscle specific tension. It was hypothesized that supplementation to improve serum 25[OH]D >75 nmol.L^{-1} from a level of deficiency will result in improvements in skeletal muscle contractile properties undetectable at the whole muscle level.

4.8 Method

4.8.1 Participants

Two young, healthy and physically active males met the inclusion criteria (Chapter 3, section 3.2) and were permitted in the trial (see Table 4.3). The participants were selected based on their basal 25[OH]D and willingness to provide a muscle biopsy for the isolation of single muscle fibres.

4.8.2 Vitamin D Supplementation

Participants were supplemented with 4,000 IU.day^{-1} Vitamin D_3 for eight weeks during the months February through March. Although the dose of 10,000 IU.day^{-1} was chosen in study 1, recent reports from the European Food Safety Authority (EFSA) suggest a 4,000 IU.day^{-1} upper limit for Vitamin D_3 intake (EFSA, 2012). Blood samples were drawn pre and post supplementation and analysed for total serum 25[OH]D as described in the General Methods chapter of this thesis.

Table 4.3. Participant characteristics and baseline Vitamin D status.

Participant	Age (years)	Height (cm)	Weight (kg)	Basal 25[OH]D (nmol.L^{-1})
1	29	186	95	23
2	22	183	82	25

4.8.3 Muscle Biopsy

Muscle biopsies were obtained as described in the General Methods section to this thesis. Biopsy passes were immediately transferred to ice cold relax solution containing (in mM) 4.5 MgATP, 1 free Mg^{2+}, 10 imidazole, 2 EGTA, and 100 KCL (pH 7.0 at 4^{o}C) (Degens et al., 2010). In order to preserve the biopsy passes for later analysis, biopsy specimens were transferred to 0.5 mol.L^{-1} sucrose relax, 1 mol.L^{-1} sucrose relax, 1.5 mol.L^{-1} sucrose relax

and 2.0 mol.L^{-1} sucrose relax, each for 30 min. The fibre bundles were then snap frozen in liquid nitrogen and stored in pre-cooled centrifuge tubes at -80^{o}C. For thawing, the opposite procedure was followed (Frontera & Larsson, 1997).

4.8.4 Single Fibre Preparation

Single muscle fibres were isolated from biopsy specimens and maintained in ice-cold relax solution. Fibres were treated with 50 μg.ml^{-1} saponin from Quillaja bark (Sigma Aldrich) in relax solution for 15 minutes on ice to permeabilize the fibre. Saponins selectively interact with cholesterol on the fibre sarcolemma and remove it to create pores. Fibres were then mounted onto an 802D permeabilized fibre apparatus (Aurora Scientific. Ontario, Canada) and were attached to insect pins affixed to a 403A (5mM) force transducer and 312C length controller, using ultra-fine nylon thread (see Figure 4.12 b). Fibres were maintained in relax solution throughout assembly. Sarcomere length (SL) was measured using 900B Video Sarcomere Length (VSL) software (Aurora Scientific. Ontario, Canada) and adjusted on each fibre to 2.4-2.6 μm. Fibre diameter was measured at four intervals along the fibre length, and a circular circumference assumed for the basis of cross sectional area (CSA) calculation. Fibres were maximally activated in Ca^{2+} activating solution (pCa 4.5), containing in addition to Ca^{2+}, (in mM) 5.3 MgATP, 1 free Mg^{2+}, 20 imidazole, 7 EGTA, 19.6 PCr, and 64 KCl (pH 7.0 at 4°C). Peak force was recorded and normalised to fibre CSA (Degens et al., 2010). Four fibres per participant were measured pre and post supplementation.

4.9 Results

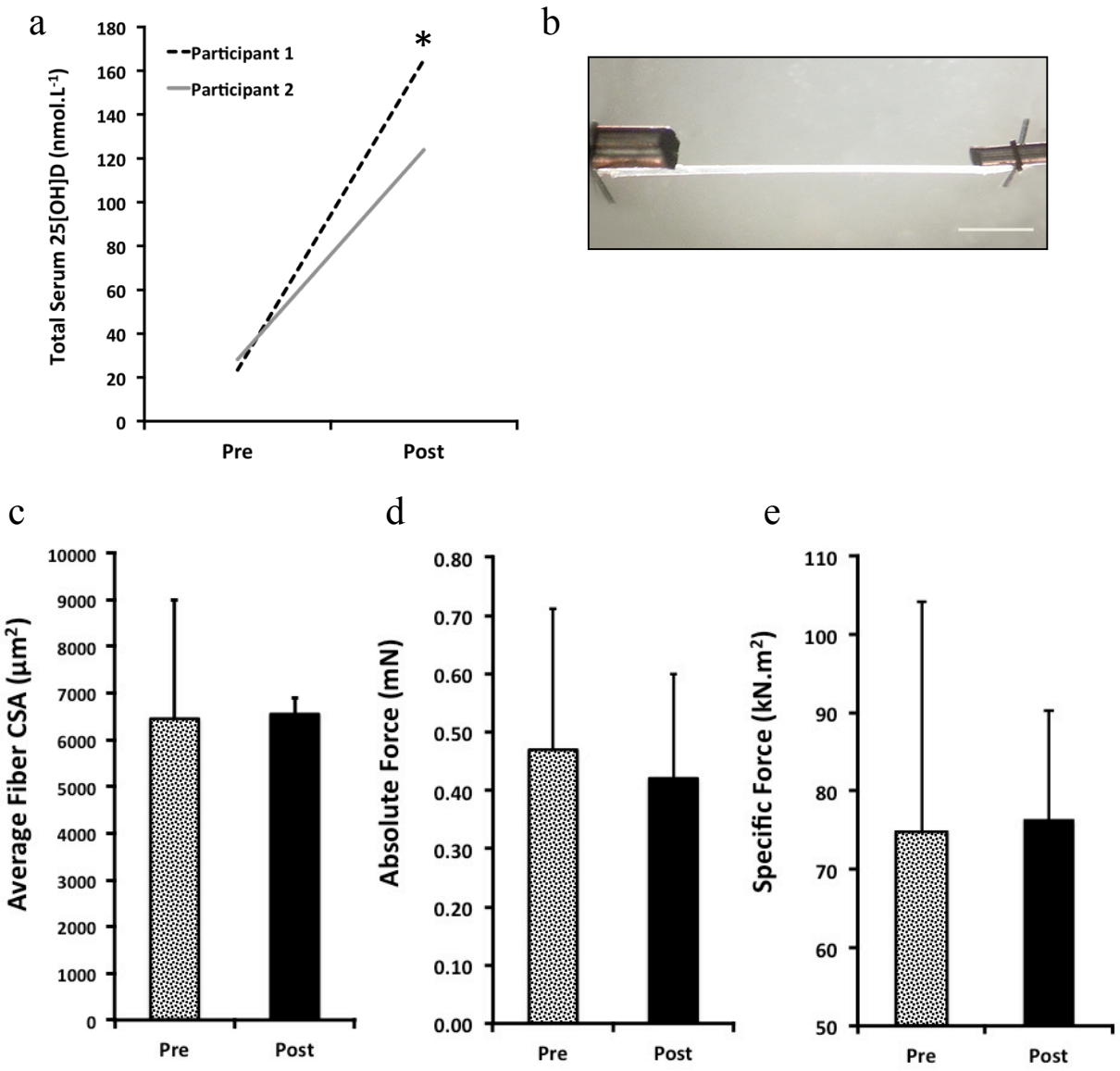


Figure 4.12. a) Response of total serum 25[OH]D (nmol.L^{-1}) of the two participants that received 4,000 IU.day^{-1} Vitamin D_3 for eight weeks. * denotes significance for both individuals versus baseline; **b**) a single skeletal muscle fibre attached to insect pins with ultra fine nylon thread; Single Fibre Characteristics prior to- and following supplementation with 4,000 IU.day^{-1} Vitamin D_3 for 8 weeks on **c**) average fibre CSA as calculated by averaging 3 fibre diameter measures and assuming a cylindrical fibre structure **d**) average fibre absolute force and **e**) average fibre specific force calculated from absolute force over fibre CSA multiplied by $1x10^6$ (for force in kN.m^2).

In a similar response to that observed in study 1, total serum 25[OH]D was elevated following daily supplemental Vitamin D_3 (4,000 IU) in both participants. However, despite significant increases in serum 25[OH]D there were no changes in fibre CSA absolute force or specific force following supplementation (Figure 4.12).

4.10 Discussion

The additional single fibre experiments undertaken aimed to determine whether changes in skeletal muscle contractile properties occur in individuals with serum concentrations ≤25 nmol.L^{-1}. It was hypothesized that with this sensitive investigative technique, positive changes in skeletal muscle contractile properties would be detected when elevating total serum 25[OH]D from ~25 nmol.L^{-1} to >75 nmol.L^{-1}.

Findings from these preliminary data do not support the hypothesis put forward and do not infer a role for Vitamin D in skeletal muscle contractile properties of single skeletal muscle fibres. Measurements were also comparable to those cited in previous investigations (Trappe et al., 2003). At this level of investigation, other contributors to muscle specific force are removed to allow investigation of cross bridge kinetics and calcium handling. Based on the data obtained, no changes in any of the observed measures could support the notion that Vitamin D regulates these processes.

It is acknowledged that these data can only be considered preliminary as only two participants were analysed. This investigation is significantly underpowered to be able to draw definitive conclusions, however the data provide the first insights at the single fibre level of the relationship between Vitamin D status and skeletal muscle function. Employing this technique across a large number of individuals and isolated fibres from those individuals in a state of Vitamin D deficiency and repletion will establish definitively whether in young otherwise healthy muscle, Vitamin D status is important for contractile function.

4.11 Chapter Synthesis

The first chapter of this thesis aimed to determine whether a relationship between Vitamin D status and skeletal muscle function exists in young non-pathological muscle. Skeletal muscle function was examined *in vivo* using IKD and EMS and *ex vivo* using the single fibre technique. Taken together, the data from these trials do not lend support to previous suggestions that Vitamin D is a regulator of muscle function as no changes in any of the chosen outcome measures of muscle function *in vivo* or *ex vivo* were detected.

A number of *in vitro* investigations report potential mechanisms by which exogenous Vitamin D can regulate Ca^{2+}[i] in isolated primary myoblasts and immortalised cell lines (Vazquez et al., 1998). This has led to the notion that sub optimal circulating 25[OH]D concentrations may cause aberrant Ca^{2+} handling in skeletal muscle *in vivo* (Girgis et al., 2012). Indeed, in elderly populations there is correlative evidence to suggest that Vitamin D may correlate with muscle function supporting *in vitro* data (Bischoff-Ferrari et al., 2004a). These findings may however be flawed in that Vitamin D deficiency is only one of many factors that influence muscle health in the elderly. Severe comorbidity may cause muscle weakness and functional impairment and an indoor lifestyle may be attributable for the low serum 25[OH]D. Nevertheless, case reports have suggested that supplemental Vitamin D can improve muscle strength in those with chronic severe Vitamin D deficiency (Irani, 1976; Ziambaras & Dagogo-Jack, 1997). In non-aged/pathological muscle, findings are equivocal. The current data support the proposition that serum 25[OH]D concentrations typically observed in young individuals across Europe are unlikely to affect muscle function even at concentrations previously suggested to associate with muscle function impairments i.e. < 25 $nmol.L^{-1}$ (Stockton et al., 2011). All available evidence implies that in high-risk groups of frail, institutionalized individuals there is potential for Vitamin D to influence muscle function however in young persons with non-pathological muscle there is unlikely to be observable deficits in muscle function with serum concentrations ≥25 $nmol.L^{-1}$. However, it is still not understood whether serum 25[OH]D <12.5 $nmol.L^{-1}$ associated with

hypercalcemia, influences muscle function through disturbances in Ca^{+2}. Such concentrations are rare in adult normal populations and it will be a challenge to the researcher to obtain a sample large enough to study muscle function. As such, the direction of investigation will now be driven toward the potential role of Vitamin D in skeletal muscle regeneration as part of the second major aim of this thesis

Comparably to the link between Vitamin D and muscle function, data on muscle regeneration in the context of Vitamin D are largely observational with some in vitro reports defining potential mechanisms by which Vitamin D may mediate the regeneration process. It is important that well controlled, translational investigations are conducted to elucidate how Vitamin D may be important for muscle regeneration and to understand how to optimize Vitamin D status to support this function if a link exists.

Chapter 5

Vitamin D and Muscle Regeneration *In Vivo*

This chapter assesses the second major aim of this thesis; to investigate muscle regeneration in the context of Vitamin D. An in vivo model of muscle damage was employed to assess functional recovery in participants with Vitamin D deficiency prior to and following supplementation with Vitamin D. Findings suggest that supplemental Vitamin D may potentiate the recovery of force from damaging eccentric exercise when baseline Vitamin D status is insufficient.

5.1 Abstract

Purpose – Vitamin D concentration associates with the recovery of maximal force following eccentric exercise. Although potential mechanisms have been identified that may explain this, a definitive cause-effect relationship remains to be established by randomized controlled trials (RCTs). The main objective of the current study was to implement a randomized controlled trial to assess the relationship between Vitamin D concentration and maximal torque recovery following eccentric exercise.

Methods - A randomised, placebo-controlled trial was performed in twenty males with low serum 25[OH]D (45 ± 25 nmol.L^{-1}). Prior to and following 6-weeks of supplemental Vitamin D_3 (4,000 IU.day^{-1}) or placebo (50 mg cellulose), participants performed 20 × 10 damaging eccentric contractions of the knee extensors with peak torque measured over the following 7 days of recovery. Serum 25[OH]D was measured pre and post supplementation via LC-MS/MS.

Results - Supplemental Vitamin D_3 increased serum 25[OH]D (pre = 45 ± 25 vs. post = 115 ± 31 nmol.L^{-1}, $P < 0.005$) and improved recovery of peak torque (60 deg.sec^{-1}) at 48 hours (14 %) and 7 days (13.7 %) post-exercise. No changes were observed in perceived muscle soreness in either of the experimental groups.

Conclusion - These data are the first to characterise a role for Vitamin D in human skeletal muscle regeneration by implementing an appropriately controlled RCT and suggest that maintaining serum 25[OH]D may be beneficial for enhancing reparative/remodelling processes following eccentric exercise.

5.2 Introduction

Eccentric contraction, contusions and toxic insults cause skeletal muscle damage that leads to decrements in functional capacity. Yet skeletal muscle is uniquely equipped to regenerate via the activation, proliferation, migration and differentiation of resident muscle stem cells, known as satellite cells (Relaix & Zammit, 2012); indeed muscle regeneration is significantly compromised when satellite cells are ablated (Relaix & Zammit, 2012). Effective repair usually leads to full functional recovery of the damaged tissue whilst ineffective repair leads to fibrosis and sub optimal rescue of function (Mann et al., 2011).

Many intrinsic, local and systemic factors interact to orchestrate the repair process and thus identifying and targeting modifiable risk factors that compromise any step of this process can augment functional recovery. Recent insights from human trials have reported that Vitamin D_3, a member of a group of pleiotropic pro-steroid hormones is implicated in numerous biological processes. However, worldwide, low circulating Vitamin D concentrations (measured as total serum 25[OH]D) prevail in humans and are associated with numerous preventable disease states. Vitamin D may be implicated in skeletal muscle regeneration and remodelling (reviewed in (Owens et al., 2014)) and indeed, sufficient 25OHD positively correlates with muscle force recovery from damaging eccentric exercise (Barker et al., 2013a; Barker et al., 2013b). These studies, while promising, are compromised by the observational nature of their design and raise the question of the potential cause-effect mechanisms between Vitamin D and muscle repair and recovery.

Fragments of evidence from animal and cellular models have provided supporting preliminary human data for the observational insights in humans. *In-vivo* crush injury rat models, Vitamin D sufficiency culminates in significant increases in BrdU positive proliferating myoblasts, decreased apoptosis via staining tissue for DNA damage and subsequent improvements in maximal force recovery in comparison to deficient rats (Stratos

et al., 2013). In addition, Vitamin D supplementation, following $BaCl_2$ damage of murine tibilais anterior, culminates in improved regeneration and increased expression of the VDR (Haussler, Jurutka, Mizwicki, & Norman, 2011), highlighting the potential of the Vitamin D pathway in controlling aspects of the regeneration process (Srikuea et al., 2012). Complementing these *in vivo* rodent studies, *in vitro* studies using C_2C_{12} murine myoblasts also indicate that myoblast proliferation, differentiation, myotube hypertrophy and survival are mechanisms facilitated by Vitamin D, however, similar human muscle cell studies remain to be performed (Garcia et al., 2013; Salles et al., 2013; Srikuea et al., 2012). This is particularly relevant, given that circulating 25[OH]D and 1α,25$[OH]_2$D are undetectable in many rodent species. Taken together, a definitive cause-effect relationship between Vitamin D and skeletal muscle repair and remodelling is yet to be established in humans by well-controlled, translational investigations.

5.3 Aims and Hypotheses

The main aim of the investigation was to identify whether there is a causal relationship between Vitamin D concentration and muscle force recovery, indicative of muscle regeneration, following eccentric exercise induced damage by employing a randomized placebo controlled trial.

It was hypothesized that low serum 25[OH]D will associate with inferior recovery of peak torque over 7 days following eccentric contraction induced damage and that supplementation to correct elevate serum 25[OH]D to >75 $nmol.L^{-1}$ will correct this impairment as evidenced by quicker recovery of MVC force and a dampened perception of muscle soreness.

5.4 General Methodology

5.4.1 Participants

Twenty-five healthy males volunteered to partake in the current trial of which twenty participants (21 ± 1 years, 179 ± 4 cm, 84 ± 13 kg) presented with serum 25[OH]D < 75 nmol.L^{-1} and met the inclusion criteria described in the General Methods chapter of this thesis and completed all experimental protocol. Participants received detailed information regarding the study procedures and were required to complete a PAR-Q, a medical history questionnaire and provide written informed consent before inclusion in the trial.. Ethical approval was granted by the local ethics committee of Liverpool John Moores University and all data were collected in accordance with the Declaration of Helsinki.

5.4.2 Pilot data & Eccentric Exercise Protocol

In order to validly investigate the influence of Vitamin D on muscle force recovery from eccentric contraction induced damage, it was first necessary to validate our muscle damage protocol. The protocol developed aimed to induce damage significant enough to result in a significant loss of force by 24-48 hours post exercise. The protocol was based on previously published data that describe a significant loss of force immediately post exercise (47 ± 5%) that remained significantly lower than baseline at 23 hours post exercise (35 ± 6% force reduction) and significant elevation in muscle soreness at 47 hours post exercise (Paulsen et al., 2007). The exercise bout was performed on a Cybex isokinetic dynamometer and consisted of 200 unilateral eccentric contractions at $30^{o}.sec^{-1}$ (0.52 $rad.sec^{-1}$) executed as 20 sets of 10 contractions interspersed by 30 second rest intervals. Exercise was performed through the participants' full range of motion (ROM), thus was specific to each participant. To assess maximal voluntary contraction force (MVC), peak toque was generated by 4 maximal voluntary contractions at two fixed movement velocities of 60 and $180^{o}.sec^{-1}$ (1.047 and 3.14 $rad.sec^{-1}$, respectively).

Pilot data (N = 4) revealed that a significant interaction was present between peak torque and time at both 60 deg.sec^{-1} (P = 0.002) and 180 deg.sec^{-1} (P = 0.032). Post-hoc analysis of the data revealed significant losses in isokinetic torque at 60 deg.sec^{-1} immediately post damage (P = 0.015), which remained significantly lower than pre damage at 24 hours (P = 0.046). Torque remained low at 48 hours although statistically insignificant (P = 0.054) and recovered to 96% by day 7, post damage. Similarly, at 180 deg.sec^{-1} force was significantly impaired immediately post exercise (P = 0.05) and remained lower at 24 (45%) and 48 hours (28%) versus pre damage, although not statistically significant (Figure 5.1).

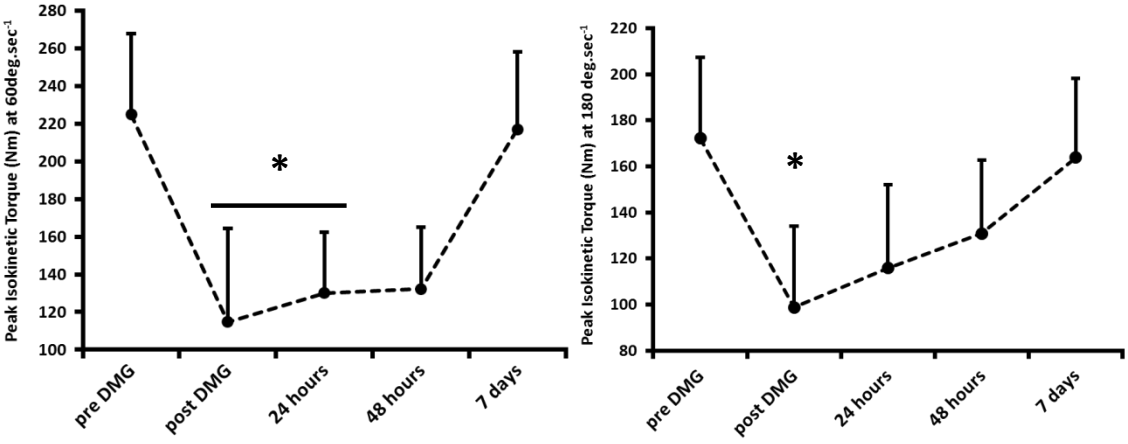


Figure 5.1 Pilot data demonstrating force recovery from 20 sets of 10 eccentric contractions at 60 and 180 deg.sec^{-1} immediately post damage (DMG), 24 hours, 48 hours and 7 days post damage. * denotes significance to pre DMG.

5.4.3 Familiarization

All participants were familiarized with the MVC protocol before undertaking any experimental procedures. Only when participants obtained a CV% <10 % were they permitted to undertake the experimental trials (Atkinson & Nevill, 1998). Mean CV% for peak torque (nm) at $60^{o}.sec^{-1}$ was 4% (± 3 %) and at $180^{o}.sec^{-1}$ was 4% (± 3 %). Mean torque values at both movement velocities can be found in Table 5.1.

5.4.4 Statistical Methods

Statistical analyses were performed as described in Chapter 3 with additional power calculations being performed in the same manner as described in Chapter 4 section 4.3.4.

5.5 Experimental Trial

5.5.1 Methodology

Once familiarized with the experimental procedures, participants were block randomized to either the Vitamin D treatment group (4,000 IU.day^{-1}) or placebo control based on peak torque attained at 60 deg.sec^{-1} and on baseline total serum 25[OH]D.

Table 5.1. Block randomization data including mean total serum 25[OH]D concentration presented as nmol.L^{-1} and mean peak isokinetic torque from a knee extension at 60 deg.sec^{-1}.

	PLB (n = 10)	**VITD (n = 10)**
Mean total 25[OH]D (nmol.L^{-1})	45	45
±SD	15	25
t-test	t = 0.017; *P* = 0.987	
Mean Isokinetic Peak Torque (Nm)	211	232
±SD	48	25
t-test	t = 1.113, *P* = 0.290	

Participants were instructed to abstain from alcohol, caffeine and other over the counter stimulants 48 hours prior to the experimental trials. On the day of the trial a measure of muscle soreness was taken via a pressure algometer at two points on the right quadriceps; 5cm from the patella and at the midpoint of the quadriceps as measured with a tape measure from the inguinal crease to the top of the patella when the leg was flexed at 90^{o}. The algometer was pressed with gradually increasing pressure at the two separate measurement points and removed when the participant indicated that pressure had become pain. The value given on the algometer in kg was recorded as a measure of muscle soreness. Participants were then seated in the isokinetic dynamometer as described in Chapter 4 IKD Methods (section 4.3.3) and allowed to perform four submaximal practice repetitions increasing in intensity at 60 ^{o}.sec^{-1} to activate the muscle to be tested.

Maximal voluntary torque was obtained by performing four isokinetic contractions at two fixed movement velocities of 60 and $180^{o}.sec^{-1}$, separated by a five-minute rest period. Muscle soreness was also measured prior to the eccentric exercise bout. Participants were then given a five-minute rest period following the MVC measures at which point they were asked to perform 20 sets of 10 maximal eccentric contractions as described in the pilot data section above. Immediately following the damaging procedure a second MVC measure was taken and a second muscle soreness measurement was obtained following which participants were allowed to leave the laboratory. MVC and muscle soreness measures were then taken at 24 hours, 48 hours and then at 7 days post damage to monitor recovery from the damaging event.

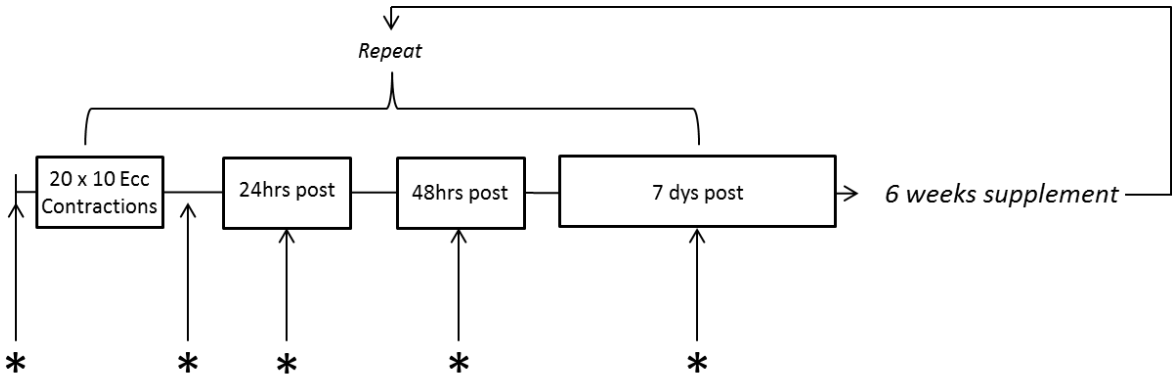


Figure 5.2. Schematic representation of experimental procedures. * denotes peak torque and muscle soreness measurement. Serum 25[OH]D measure before and after all experimental procedures. Blood was collected at baseline and then again following the six week supplementation period.

5.6 Results

5.6.1 Total Serum 25[OH]D

At baseline, no significant differences were detected between experimental group for total serum 25[OH]D ($P < 0.005$) with both groups presenting with mean serum concentrations of 45 ± 15 and 45 ± 25 nmol.L^{-1} for PLB and VITD, respectively. Following 6 weeks of supplementation with 4,000 IU.day^{-1} Vitamin D_3, a significant interaction effect was observed between treatment group and time ($P < 0.005$). Post-hoc analysis revealed that the VITD group showed a significant increase in total serum 25[OH]D at week 6 compared with baseline (pre = 45 ± 25 vs post = 115 ± 31 nmol.L^{-1}, $P < 0.005$) and a significant difference when compared with PLB ($P < 0.005$). Conversely, the PLB group demonstrated a significant decline in serum 25[OH]D at week 6 compared with baseline (pre = 45 ± 25 vs post = 33 ± 13 nmol.L^{-1}, $P = 0.013$).

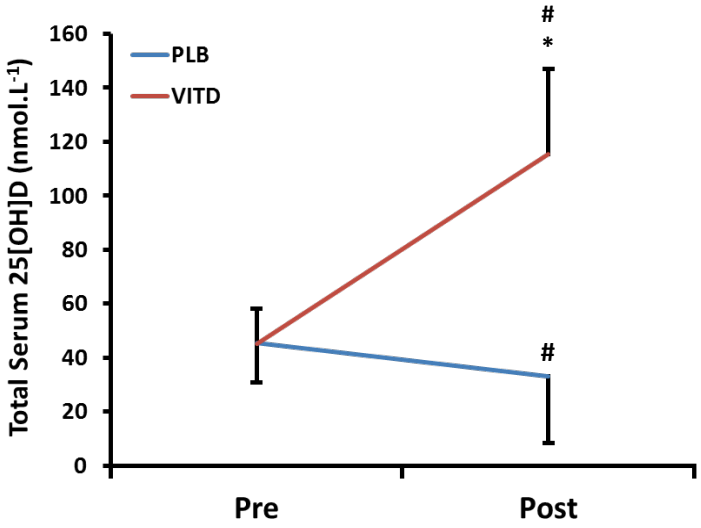


Figure 5.3. Response of total serum 25[OH]D to either 4,000 IU.day^{-1} Vitamin D_3 or placebo for 6 weeks from January to March. # denotes significance to baseline and * denotes significance to PLB.

5.6.2 Supplemental Vitamin D to Increase Serum 25[OH]D Improves Recovery From Eccentric Exercise Induced Muscle Damage

A significant interaction effect was observed between recovery time point, test week (pre-post supplementation) and treatment group at 60 deg.sec^{-1} (P = 0.049). Exploration of this interaction identified a significant improvement in force recovery in the supplemental VITD group following supplementation at 48 hours (10.7% improvement, P = 0.042) and 7 days post damage (12.4% improvement, P = 0.001) compared with pre supplementation (Figure 5.4). Although a slight improvement was observed for VITD post supplementation at 180 deg.sec^{-1} this result failed to meet statistical significance.

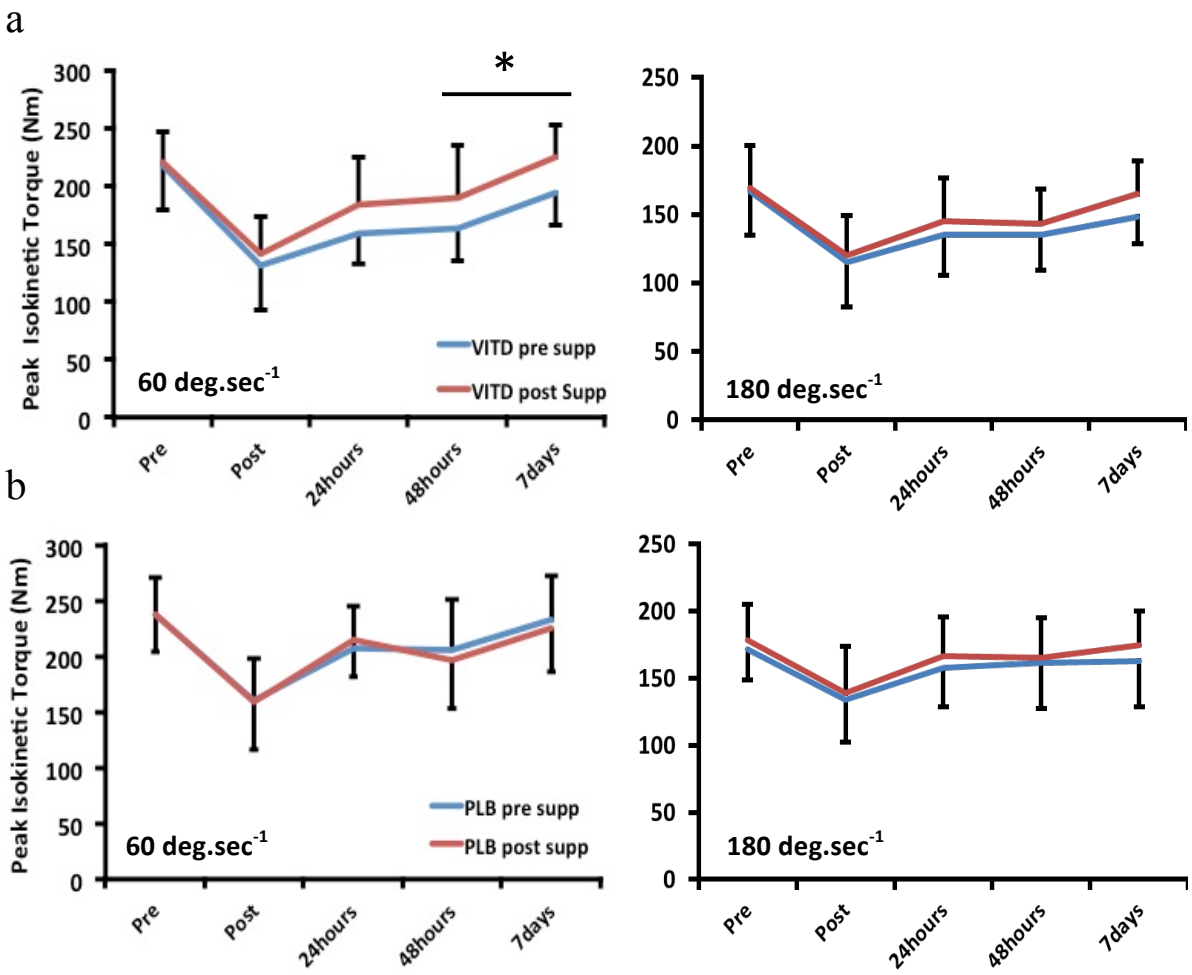


Figure 5.4. Recovery profile of peak isometric torque at 60 and 180 deg.sec^{-1} following six weeks supplementation with **a**) 4,000 IU.day^{-1} Vitamin D_3 or **b**) visually identical placebo. * denotes significance to post damage torque.

5.6.3 Supplemental Vitamin D Does Not Attenuate Muscle Soreness Following Eccentric Exercise Induced Damage

Both VITD and PLB demonstrated a significant increase in muscle soreness at both measurement sites ($P < 0.05$ mid quad and 5cm patella), however this was unchanged following supplementation at both 5cm patella ($P = 0.418$) and mid quadriceps ($P = 0.71$). Thus supplemental Vitamin D did not attenuate muscle soreness following damaging eccentric exercise (Figure 5.5).

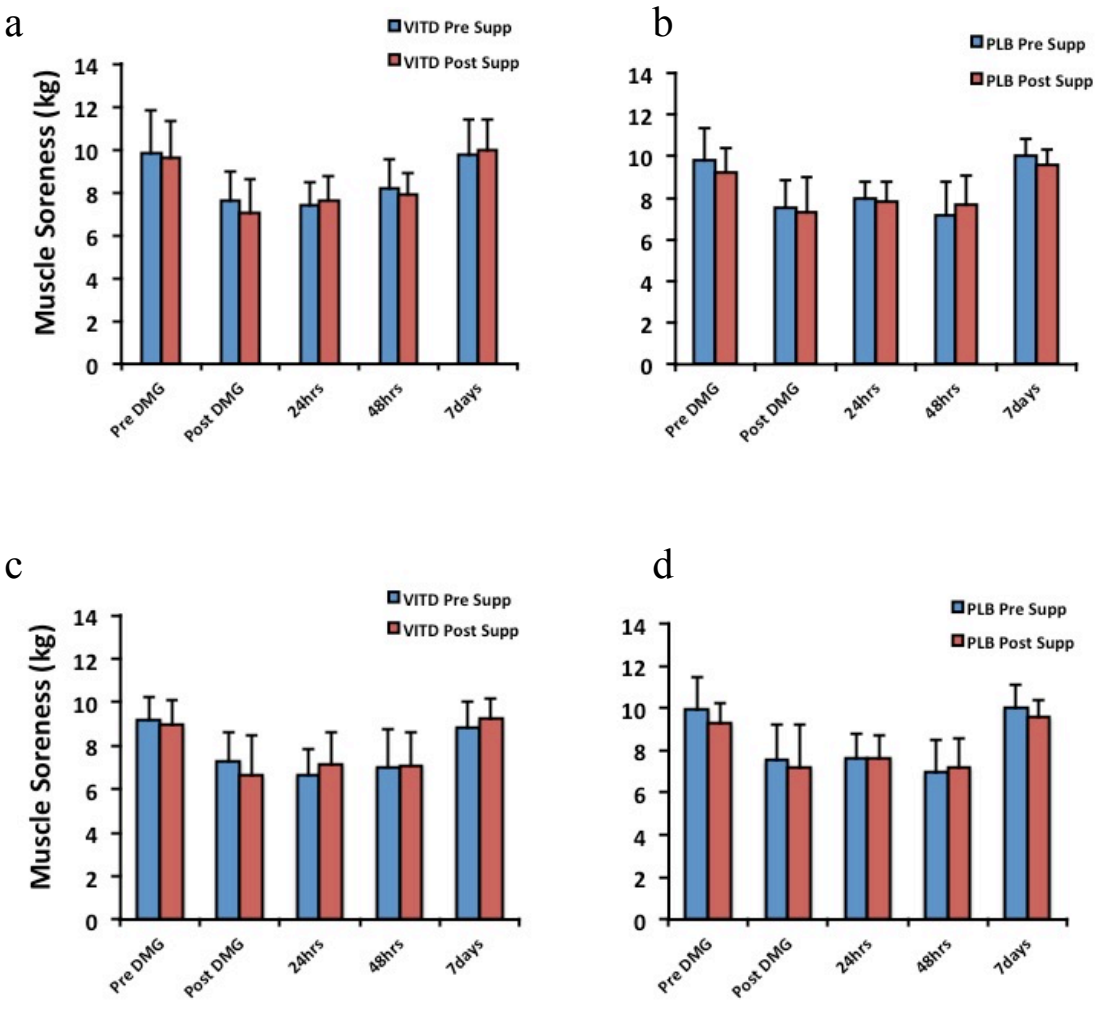


Figure 5.5. Upper bar charts represent muscle soreness scores 5 cm from the patella pre and post eccentric exercise and then at 24, 48 hours and 7 days post damage **a**) in the VITD supplemental group pre and post supplementation and **b**) the PLB group pre and post supplementation. Lower bar charts represent muscle soreness scores from the same time points as upper charts from the mid-quadriceps **c**) in the VITD supplemental group pre and post supplementation and **d**) the PLB group pre and post supplementation.

5.7 Discussion

The main aim of the investigation was to identify whether a causal relationship exists between Vitamin D concentration and muscle force recovery, indicative of muscle regeneration, following eccentric exercise induced damage. It was hypothesized that Vitamin D concentrations <75 nmol.L^{-1} would associate with inferior recovery of peak torque at two dynamic movement velocities over 7 days following eccentric contraction induced damage. Further it was proposed that supplementation with 4,000 IU.day^{-1} Vitamin D_3 to elevate serum 25[OH]D concentrations >75 nmol.L^{-1} would correct this impairment.

The main findings of the investigation partly support the proposed hypotheses. It was observed that supplementation with 4,000 IU.day^{-1} Vitamin D_3 for 6 weeks significantly elevated total serum 25[OH]D concentration >75 nmol.L^{-1} whereas supplementation with PLB led to a decrease in total serum 25[OH]D. This finding is similar to that observed in Chapter 4 of this thesis and many other published investigations supporting the efficacy of supplemental Vitamin D. The observed increase in serum 25[OH]D may also explain the improvement in force recovery observed in the VITD group at 48 (14 % improvement) hours and 7 days (13.7 % improvement) at 60 deg.sec^{-1}. Supporting this observation, the significant decrease in PLB total serum 25[OH]D corresponded to decreases in force recovery at 48 hours (-4 % decrement) and 7 days (-2.8 % decrement), although not statistically significant. Improving serum 25[OH]D did not however have any perceptible effect of muscle soreness as values were unchanged following the supplemental period in both VITD and PLB.

The current findings are in line with previous data from correlative investigations. It has been reported that supplemental Vitamin D resulted in ~70% increase in serum 25[OH]D and improved force recovery from eccentric exercise by 8% at 24 hours post damage without affecting muscle soreness (Barker et al., 2013b). Similarly, serum 25[OH]D has been shown to predict the severity of force loss following eccentric seated leg press exercise

in a small cohort of recreationally active adults (Barker et al., 2013a). Supplemental Vitamin D has also been shown to enhance the recovery of force following cruciate knee ligament injury (Barker et al., 2015).

There are a number of potential explanations for the current data and those observations made previously by others. The muscle regeneration process is a complex series of individual but overlapping and mechanistically linked events that involve intracellular, cellular and systemic processes to be successful. Theoretically, there are therefore a number of targets that Vitamin D may interact with to mediate the resolution of damaged muscle tissue. First, $1\alpha,25[OH]_2D_3$ may directly influence muscle progenitor cell (myoblast) behaviour. In order to regenerate skeletal muscle following different types of damaging exercise, the muscle SC is required (Hyldahl et al., 2014; Relaix & Zammit, 2012). The SC is first activated by one or a combination of many biochemical and biophysical cues and will undergo cellular division to become a daughter cell or a committed myoblast (Bentzinger, Wang, & Rudnicki, 2012). The myoblast must then undergo proliferative cycles to increase the population number, migrate to the site of damage and differentiate and fuse to that damaged site to regenerate the tissue. Data report that exogenous treatment of muscle progenitor cells with $1\alpha,25[OH]_2D_3$ modulates proliferation and differentiation as well as enhancing the expression of pro-myogenic growth factors (Buitrago et al., 2012; Garcia et al., 2011; Srikuea et al., 2012). Additionally, in vascular smooth muscle cells, Vitamin D treatment enhances migration capacity, although this has not been confirmed in skeletal myoblasts (Rebsamen, Sun, Norman, & Liao, 2002). Upon migrating and fusing to the damaged site, the newly added nuclei permit hypertrophy at the damaged site. Recent data also suggest that Vitamin D can regulate such growth pathways through MAPK/ERK (Buitrago et al., 2013; Ronda et al., 2007) and Akt/MTOR (Buitrago et al., 2012) whilst also enhancing the anabolic effect of insulin and leucine (Salles et al., 2013). The importance of these events in the short term recovery of skeletal muscle following eccentric exercise is evidenced by data that report increases in embryonic MHC at only 48 hours post eccentric

exercise in rodents, indicative of SC incorporation into the muscle (Peters et al., 2003). Notably, this increase in embryonic MHC was the only regenerative marker of those examined that was synchronous with recovery of force.

Vitamin D may also function indirectly to re-establish an appropriate vascular network to the damaged muscle. Vitamin D has been shown to improve angiogenic properties of endothelial progenitors (Grundmann et al., 2012) and enhances the migration of vascular smooth muscle cell progenitors (Rebsamen et al., 2002). Furthermore, in muscle progenitor cultures, exogenous treatment with $1\alpha,25[OH]_2D_3$ increases VEGFa and FGF-1: two pro-angiogenic growth factors that promote neo-vascularization and tissue regeneration, and decreases FGF-2 and TIMP-3: two myogenic and angiogenic inhibitors (Garcia et al., 2013). In a similar indirect manner, Vitamin D may improve recovery of muscle force through the optimised reinnervation of the damaged fibre. In a rat model of nerve regeneration it was observed that treatment with Vitamin D_3 at a high dose (500 IU/kg/day) induces a significant locomotor and electrophysiological recovery. Further, increases in the number of preserved or newly formed axons in the proximal nerve end, the mean axon diameter in the distal end, and neurite myelination in both distal and proximal nerve ends was observed with D_3 treatment (Chabas et al., 2013). In accordance, recent data provide evidence that Vitamin D enhances the expression of IGF-1, myelin basic protein, and VDR in rat primary Schwann cells following running exercise in rats. Signalling through the VDR was demonstrated to regulate neuromuscular maintenance and enhance locomotive ability following exercise (Sakai et al., 2015). Finally, it is possible that Vitamin D may also enhance reparative processes in skeletal muscle via the regulation of the immune response to muscle damage. Indeed, as discussed previously, the inflammatory response is necessary to regenerate muscle when fibre damage occurs (Arnold et al., 2007). Furthermore, in cell culture systems, inflammatory cells such as macrophages enhance the proliferation and differentiation of skeletal myoblasts (Saclier, Cuvellier, Magnan, Mounier, & Chazaud, 2013a; Saclier et al., 2013b).Vitamin D is known potent regulator of innate and acquired

immunity and functions in both stimulating and repressing immune processes (Hewison, 2010). Although direct evidence is not yet available, it is plausible that regulation of the inflammatory response by Vitamin D is a contributing factors to our observations.

To summarize, the investigation presented is the first to employ an appropriately controlled RCT to study Vitamin D status and muscle regeneration. The data suggest that supplemental Vitamin D may enhance maximal force recovery following eccentric exercise induced damage when baseline Vitamin D status is <75 $nmol.L^{-1}$ however it is unlikely that Vitamin D status has any influence on the perception of muscle soreness. Mounting evidence inclusive of the current data set strongly suggest that Vitamin D may function to facilitate aspects of the muscle regeneration process. However, limited insights are available that adequately describe cellular mechanisms by which this may occur. The muscle regeneration process requires a series of well-orchestrated cellular events that lead to the resolution of the damaged tissue. Efforts are now required to study these events in the presence or absence of exogenous Vitamin D to determine whether the sterols apparent role in tissue repair is direct on satellite cells and muscle progenitors or indirect through regulation of the inflammatory response, neuro-regeneration, angiogenesis or a combination of all of these processes required for tissue repair.

The final investigation of this thesis will aim to elucidate cellular mechanisms by employing an *in vitro* model of muscle wound repair and myogenesis in primary human muscle derived cells. The major aim is to increase the translational application of findings in this field and provide adequate evidence to suggest Vitamin D status causally interacts with muscle regeneration.

Chapter 6

Optimization of an *In Vitro* Model of Muscle Regeneration

This chapter describes pilot experiments undertaken to optimize an in vitro model of muscle regeneration to investigate cellular aspects of the regeneration process in the context of Vitamin D. A summary of optimal assay conditions is provided at the end of the chapter.

6.1 Abstract

Purpose – In order to identify potential cellular mechanisms that account for *in vivo* observations made in Chapter 5, an *in vitro* model of muscle repair was required. The main objective of the current chapter was to optimize the 2D scratch assay for the study of skeletal muscle regeneration in the presence and absence of Vitamin D.

Methods – The C_2C_{12} murine myoblast cell line and human derived muscle cells were used in a variety of experimental variations of the scratch assay. Pilot study 1 investigated the seeding density (4×10^4 vs 8×10^4 cells.ml^{-1}) and serum conditions (2 %, 20 % or serum free differentiation media) most appropriate to induce migration following damage. Pilot study 2 aimed to identify whether a lateral or horizontal wound induced differential migration characteristics across the three serum conditions used in pilot study 1. Pilot study 3 examined the influence of potentially interfering proliferative cycles on the migration process across 48 hours. Finally, pilot study 4 examined whether human muscle derived cells were capable of fusion following mechanical scrape wounding and treatment with an inhibitor of proliferation (mitomycin-C at 10 μg.ml^{-1}).

Results – The main outcomes from the studies indicated that a seeding density of 8×10^4 cells.ml^{-1} in 2% serum were optimal culture conditions for the study of migration following wounding. There were no differences between wound directions. Finally, significant proliferation occurred following scrape damage and this was inhibited by mitomycin-C pre treatment, which displayed no effect on the ability of cells to fuse following wound closure.

Conclusion – The 2D scratch assay first described by Dimchev et al. (2013), allows the study of myoblast migration on a protein substratum. Moreover, at 7 days post wound closure human derived myoblasts fuse normally to form mature myotubes. Using this system and implementing a cell seeding density of 8×10^4 and 2 % serum differentiation medium allows the study of cellular events specific to muscle following damage.

6.2 Introduction

In order to investigate the cellular mechanisms that account for *in vivo* observations in Chapter 5, an *in vitro* model of muscle regeneration was developed. Since myoblast migration to the site of damage and fusion at the site of damage are integral cellular processes in muscle regeneration, these aspects were necessary to incorporate into the model. To assess directional migration, the in vitro wound healing scratch assay was implemented. Other assays to study cell migration have previously been adopted including the Boyden chamber assay, in which a microporous membrane separates two chambers. Chemotactic agents are then added to one chamber and cells that migrate through the membrane toward the chemotactic signal are counted. Another commonly implemented assay is the Oris or stopper assay. As the name suggests, the assay utilises a plate with a circular 'stopper' at its centre to prevent cell growth. Upon removal of the stopper, cells will migrate into the central circular area where they are not contact inhibited by other cells. Both of the described methods have pitfalls that limit their usefulness when studying the migration of myoblasts. The Boyden chamber, although ideal for studying non-adherent cell types, is limited for investigation adherent mesenchymal cells such as myoblasts that will migrate through the porous membrane and adhere to the underside of the membrane. The Oris method, although accounting for the lack of application for adherent cell types typically displays slow migration due to the size of the plate. The 2D wound healing assays overcomes the drawbacks of the previously described assays. As demonstrated in the pilot trials described, an area of a cell monolayer is scratched (typically laterally) forming a wound area. This stimulates chemotactic agents to be released from the ruptured cells and triggers intrinsic repair mechanisms, potentially simulating more closely the migration mechanisms that would be activated following an injury *in vivo* (Goetsch, Myburgh, & Niesler, 2013). Following migration to the wound site, it was important to also assess myoblast fusion at the wound. A classic method of serum withdrawal was opted for as this technique provides the optimal conditions to stimulate replication arrest and allow induction of the myogenic

differentiation programme (Blau, Chiu, & Webster, 1983; Spizz, Roman, Strauss, & Olson, 1986).

For the pilot experiments described within this chapter the C_2C_{12} murine myoblast cell line was used. C_2C_{12} are derived from satellite cells, with behaviour corresponding to that of progenitor lineage. C_2C_{12} myoblasts will spontaneously differentiate in culture after serum removal (Blau et al., 1983). Sarcomeric actin and myosin are present also in undifferentiated myoblasts, but progressively acquire a structured pattern up to the appearance of sarcomeres and myofibrils at about 5 days after differentiation induction (Burattini et al., 2004). Therefore the use of C_2C_{12} myoblasts was an appropriate choice to use as a model for optimizing the model of regeneration to avoid depleting precious primary human muscle derived cells that were to be used for experimental procedures.

6.2 Pilot Study 1 - Determination of Optimal Seeding Density & Optimal Serum Concentration for Myoblast Migration

6.2.1 Aim & Hypothesis

Spatial proximity influences growth and differentiation of skeletal myoblasts *in vitro*. Furthermore, the presence or absence of growth factors in commercially available media and sera may have different effects on myoblast migration. Therefore pilot study 1 aims to determine the optimal seeding density and media composition to study myoblast migration *in vitro*. It is hypothesised based on published literature (Dimchev et al., 2013) that cells seeded at $8x10^4$ cells.ml^{-1} will provide an optimal cell density following 24 hours of culture to study myoblast migration following mechanical scrape injury and that a low serum media (2%) will allow migration into the wound site more effectively than high serum (20%) or serum free media.

6.2.2 Cell Culture and Treatments

Low passage C_2C_{12} myoblasts were seeded in pre-gelatinised six well plates at two densities of $8x10^4$ and $4x10^4$ cells.ml^{-1} in MGM. Following a 24 hour incubation, once ~80% cell confluence was reached, a lateral wound was inflicted using a sterilised pipette tip (~800-1000 μm diameter). MGM was then removed and cell monolayers washed twice with PBS. One of three media types was then added to the 6-well plates; MGM (20% serum), MDM (2%) or serum-free DMEM. Wound images were captured at 0, 24 and 48 hours following damage to assess wound closure and split into wound segments as described in Chapter 3, section 3.14. Experiments were conducted on three separate C_2C_{12} cell populations with technical duplicates performed for each condition. No significant differences were detected in wound size immediately following damage (DM = 931 ± 109 vs GM = 945 ± 121 vs SF = 933 ± 118 μm (P = 0.762)). See Figure 6.1 for schematic representation.

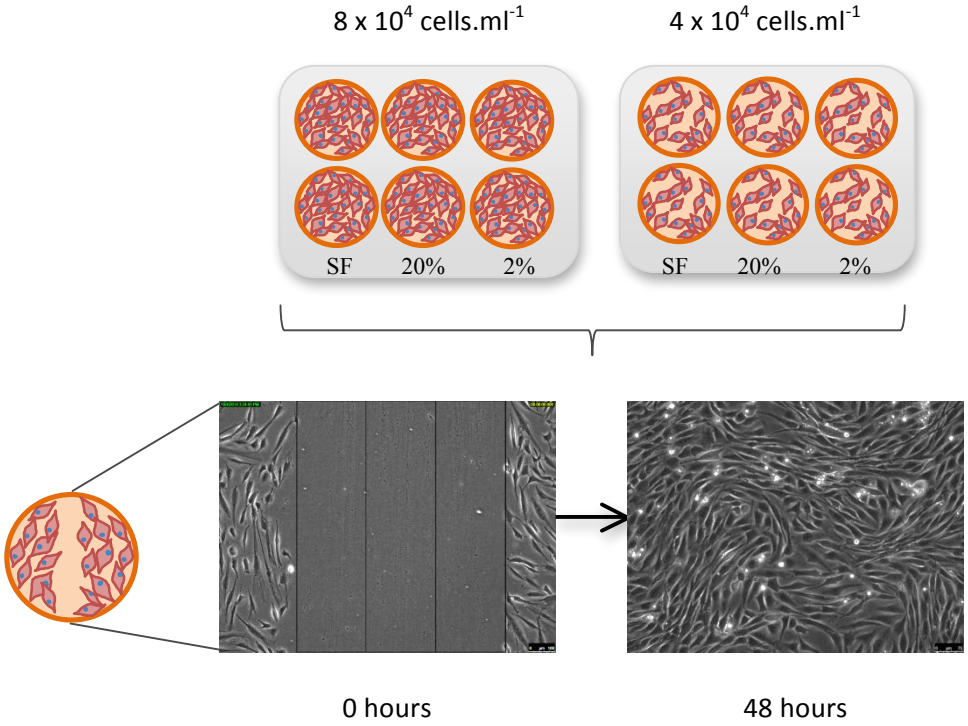
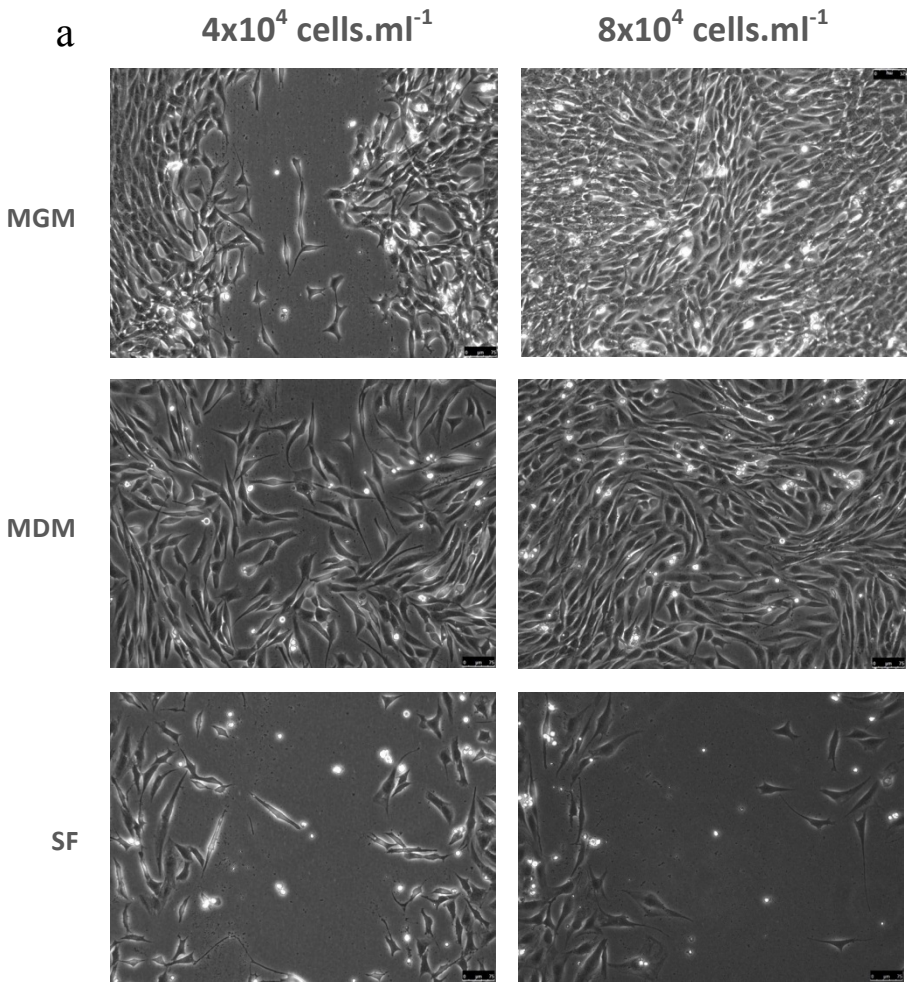


Figure 6.1. Experimental design employed to investigate the optimal conditions to study myoblast migration following a mechanical damaging scrape *in vitro*.

6.2.3 Results

A significant interaction between seeding density and serum concentration was detected ($P < 0.0005$) with both MGM and MDM being preferable when compared with SF ($P < 0.0005$) however no difference was detected between MGM and MDM ($P = 0.215$). Furthermore, a seeding density of $8x10^4$ cells.ml^{-1} resulted in significantly more migrating cells than $4x10^4$ cells.ml^{-1} ($P < 0.0005$). Although migration of cells into the inner wound space occurred with MGM/20% serum, qualitative morphological analysis suggested this to be as a result of excessive proliferative cycles 'pushing' cells into the wound rather than migrating i.e. more cells appeared to be undergoing mitosis/cellular division and not locomotion. This was substantiated by observing a lack of migration/movement towards the inner space after an initial round of mitosis. In conclusion, a cell seeding density of $8x10^4$ cells.ml^{-1} in MGM and dropped to 2% serum media following mechanical wounding is the most appropriate condition of those tested for 48 hour live cell imaging as wound closure as a result of predominantly migrating cells achieved within this time frame (Figure 6.2).



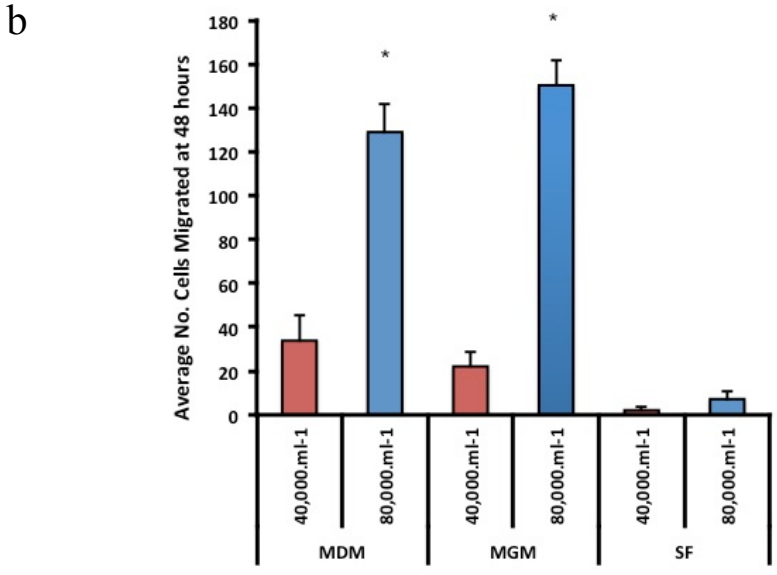


Figure 6.2 a) representative phase contrast images of C_2C_{12} myoblasts at 48 hours following mechanical scrape damage at $4x10^4$ or $8x10^4$ cells.ml^{-1} in MGM, MDM or SF media. Magnification is 15x, scale bar is 100µm. **b**) Graphical representation of average cell number in the wound site at 48 hours. * denotes significance within media condition.

6.3 Pilot Study 2 - Determination of Appropriate Wound Direction

6.3.1 Aims & Hypothesis

Pilot study 1 established that a seeding density of $8x10^4$ cells.ml^{-1} in 2% serum media (MDM) were the most effective conditions tested to study myoblast migration in *in vitro*. However, the direction of the wound is also a consideration to be investigated to optimize experimental conditions as it is unknown whether direction of the wound differentially effects cell-cell contact signalling. Therefore, the main aim of pilot study 2 was to establish whether a lateral or horizontal wound is most appropriate for studying muscle cell migration following mechanical scrap injury *in vitro*. It is hypothesised that C_2C_{12} myoblast cells damaged by a lateral or horizontal wound inflicted by a 1ml pipette tip and dropped to a low serum (2% MDM) media will migrate in a similar manner to the site of damage by 48 hours.

6.3.2 Cell Culture and Treatments

Low passage C_2C_{12} myoblasts were seeded in pre-gelatinised six well plates at $8x10^4$ cells.ml^{-1} in MGM as described in pilot trial 1. Following a 24 hour incubation, once cell confluence was reached, a wound was inflicted in one of two ways; vertical or horizontal scrape with a sterilised 1ml pipette tip (800-1000 μm). MGM was then removed, cell monolayers were washed twice with PBS and one of three media types (MGM, MDM or serum-free) was added to the 6-well plates as described in pilot trial 1. Wound images were then captured at 0, 24 and 48 hours post damage to analyse wound closure (Figure 6.3). No significant difference in wound size immediately post damage was detected between conditions (lateral = 989 ± 180 vs horizontal = 966 ± 125 μm (P = 0.512)).

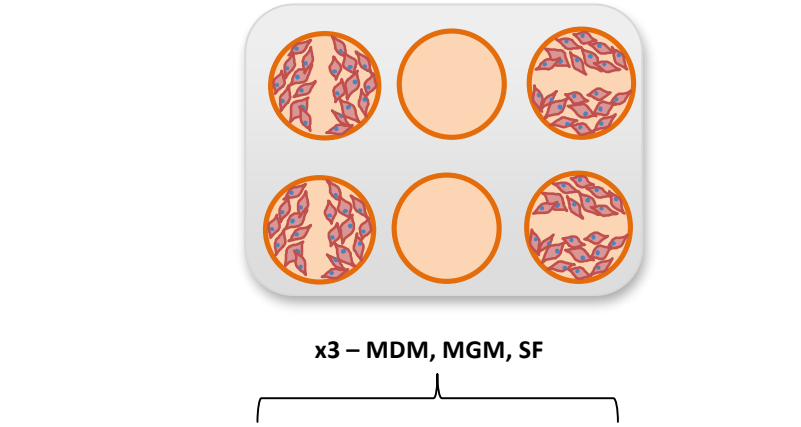


Figure 6.3. a) Schematic representation of the experimental set up of culture plates for the current pilot investigation **b**) Representative phase contrast images of C_2C_{12} myoblasts following mechanical scrape damage and seeded at $8x10^4$ cells.ml^{-1} in MGM, MDM or SF media and damaged with a lateral or horizontal wound. Magnification is 15x, scale bar is 100µm.

6.3.3 Results

A mixed design ANOVA was used to address difference in wound type (i.e. vertical versus horizontal) with serum type as three separate levels of analysis (MDM, MGM, SF). A significant main effect for serum type was detected ($P < 0.005$) as MGM resulted in significantly more cells in the wound space at 48 hours in comparison to both MDM and SF (Figure 6.4). MDM also resulted in significantly more cells in the wound space when compared with SF. However, there was no difference between wound types in any serum condition ($P = 0.943$) indicating that both wound types result in similar migration patterns.

Qualitative analysis of images suggested that the MGM condition resulted in more cells entering the wound space via mitosis rather than migration as described earlier, whilst MDM appeared to stimulated migration of cells rather than mitosis, likely due to a lack of growth factors as serum content was lower than the MGM condition. Since there was no effect of lateral of horizontal wound, the lateral wound with a 2% media was used for subsequent trials as has been reported previously (Dimchev et al., 2013).

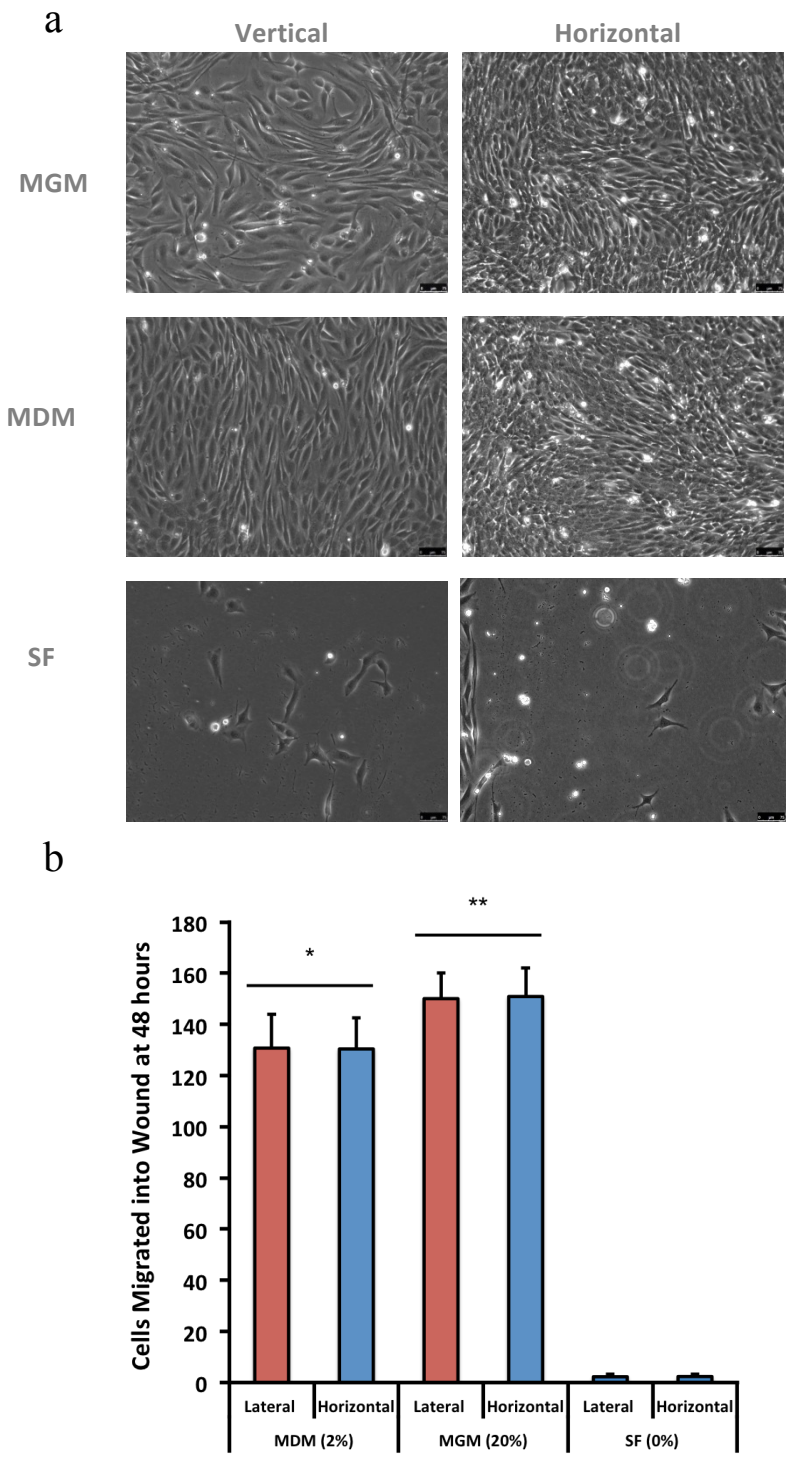


Figure 6.4. a) Representative phase contrast images of C2C12 myoblasts at 48 hours following mechanical scrape damage and seeded at $8x10^4$ cells.ml^{-1} in MGM, MDM or SF media and damaged with a lateral or horizontal wound. Magnification is 15x, scale bar is 100µm. **b**) graphical representation of average cell number in the wound site at 48 hours. ** denotes significance to MDM and SF, * denotes significant to SF.

6.4 Pilot Study 3 - Influence of Interfering Proliferation on Human Muscle Derived Cell Migration

6.4.1 Aims & Hypothesis

Mixed population human derived muscle cells (MDCs) (typically predominantly myogenic cells with a smaller proportion of non-myogenic cells such as fibroblasts) will be used for all experimental trials. To further optimise the experimental model, it is therefore important to investigate **1**) whether proliferation during wound healing impacts upon migration of human MDCs and **2**) whether relative myogenic cell proportion (myogenic:non-myogenic) influences MDC migration of one cell type.

It is hypothesised that inhibiting cell proliferation will result in fewer cells in the wound space at 48 hours post wounding by reducing the number of cells that enter the wound space through proliferative expansion and this will not incur any detrimental effects to the migration MDC populations. It is further hypothesized that the relative proportion of myogenic to non-myogenic cells will not favour the migration of one cell type over the other.

6.4.2 Cell Culture and Treatments

Human MDC populations retrieved from muscle biopsy specimens obtained as described in section 3.9.1 of the General Methods chapter of this thesis were resuscitated from liquid nitrogen, seeded at a density of $8x10^5$ cells.ml^{-1} and allowed to proliferate for ~48 hours to reach confluence. Although ~24 hours was needed for C_2C_{12} cultures to reach confluence, human MDCs grew at a slower rate vs. e.g. murine C_2C_{12} cells and therefore a longer incubation time in GM was required. Two populations were initially studied representing the extremes of both low and high myogenic (desmin positive - immunohistological analysis described in Chapter 3 section 3.9.3) populations (23% and 60% respectively). Once cell confluence was reached, GM was removed and cells were washed twice with PBS before being incubated for 20 hours in quiescent media (QM) in an attempt to allow cells that had

already began a proliferative cycle to complete and exit the cycle. Following 20 hours QM incubation, QM was removed and two wells were incubated for a further 3 hours in QM whilst two were incubated in QM in the presence of mitomycin-C (10 µg.ml^{-1} in QM). Mitomycin C is a potent DNA cross-linker thus acts to cause replication arrest. After incubation with QM or QM+mitomycin, cells were damaged with a vertical linear scrape inflicted by a 1ml pippette tip. Media was then aspirated, monolayers were washed three times with 1ml PBS per well to ensure removal of all pre-treatment media and DM was then added. Monolayers were then imaged and fixed periodically as described in the General Methods chapter of this thesis at 0, 6, 17, 26, 40 and 48 hours post wounding for subsequent morphological and immunocytochemical analysis. No significant difference was detected in wound size immediately post damage between treatments (Mitomycin-C = 949 ± 146 vs untreated control = 927 ± 103 µm).

6.4.3 Immunocytochemistry

Following fixation at each time point, monolayers were blocked and permeabilized following which they were probed for desmin (myoblast marker), TE7 (fibroblast marker) and counterstained with DAPI nuclear stain as described in the Chapter 3 of this thesis (section 3.9.3).

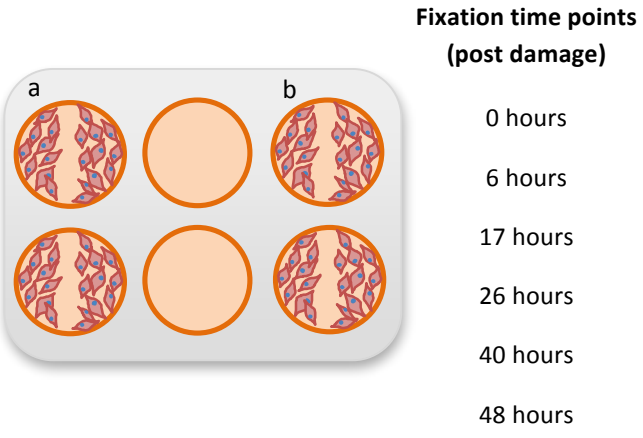


Figure 6.5. Migration time course pilot experiment schematic. Cells monolayers were grown for 48 hours in GM followed by a) 20 hours in QM or b) 17 hours in QM and 3 hours QM + mitomycin-C at 10 µg.ml^{-1}.

6.4.4 Results

A two way ANOVA was used to compare the effect of mitomycin-C treated versus untreated cells in segment 1 and 2 of the wound space as two separate levels. Comparison of mitomycin-C treated and untreated cells in segment 1 and 2 revealed a significant interaction between treatment group and segment (P = 0.030). Post hoc analysis identified a significant 'treatment group' main effect was present (P = 0.028) with on average, more untreated control cells migrating into the wound site when compared to mitomycin-C treated cells (Figure 6.6). Observation of images implied that the greater number of cells in segments 1 and 2 of the untreated group may be as a consequence of proliferative cycles following infliction of the wound.

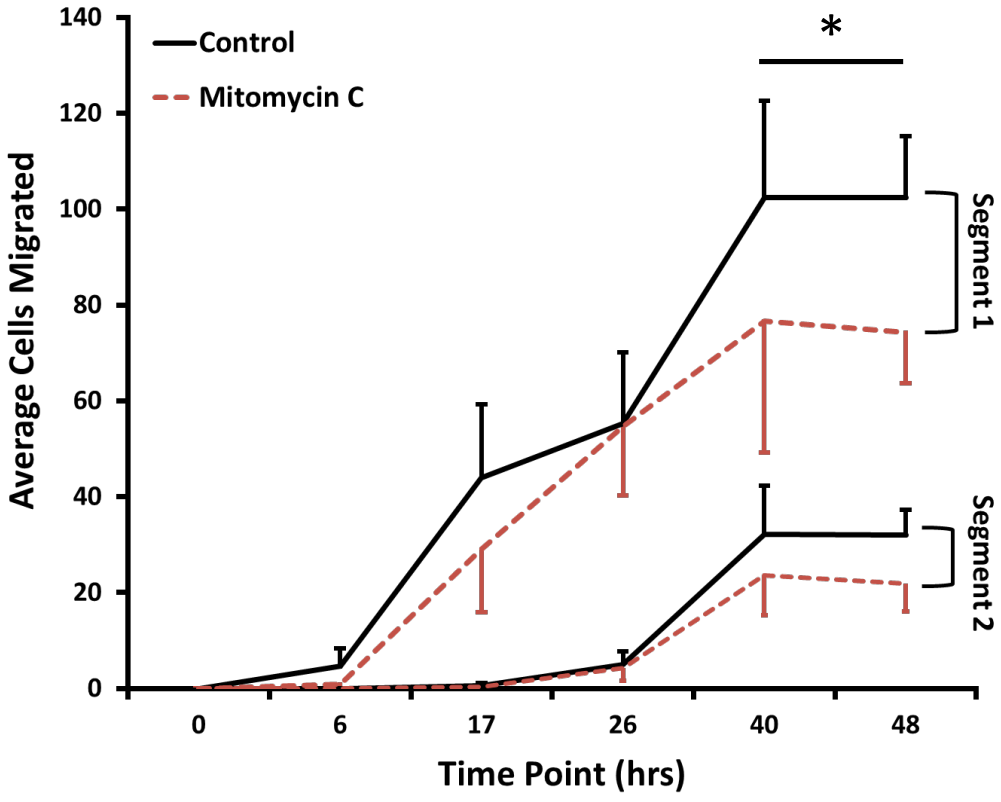


Figure 6.6. Effect of 3 hours mitomycin-C (10µg.ml^{-1}) pre-treatment on MDC migration across 48 hours following a damaging insult with a sterilised 1ml pipette tip. Segment 1 represents the areas adjacent to the wound edge whilst segment 2 represents the central wound area. * denotes significant difference between groups at time point for segment 1 and 2.

Condition 1: High Desmin^{+} without Mitomycin-C pre-treatment.

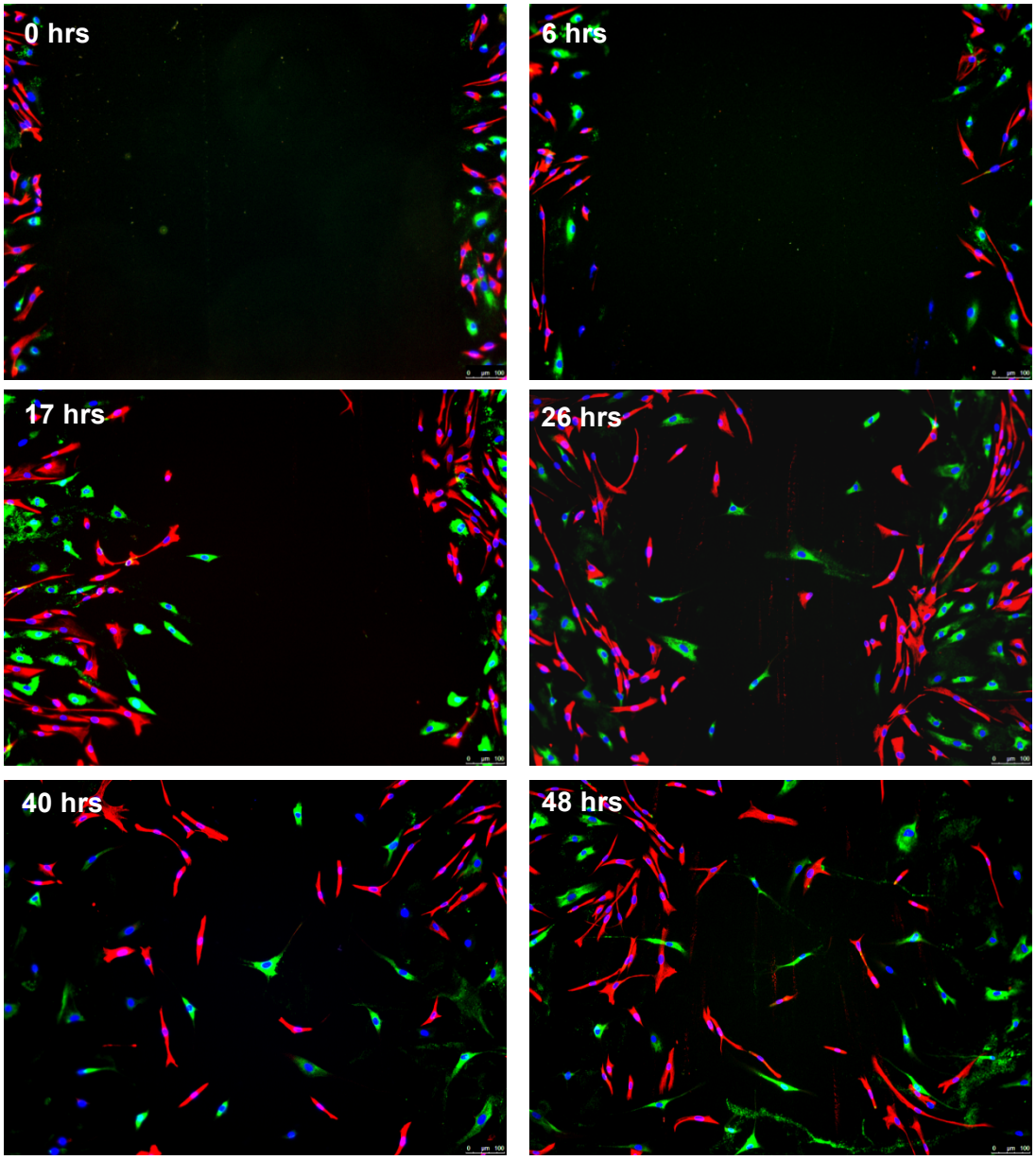


Figure 6.7. Representative images visualising the wound healing process over 48 hours in a high Desmin^{+} population of MDCs following 23 hours pre-incubation in QM. Myoblasts are stained for Desmin (Red/TRITC) and fibroblasts are stained for TE7 (Green/AlexaFluor488). Nuclei are blue (DAPI). Images were captured at 0, 6, 17, 26, 40 & 48 hrs post insult.

Condition 2: High Desmin^{+} with Mitomycin-C pre-treatment.

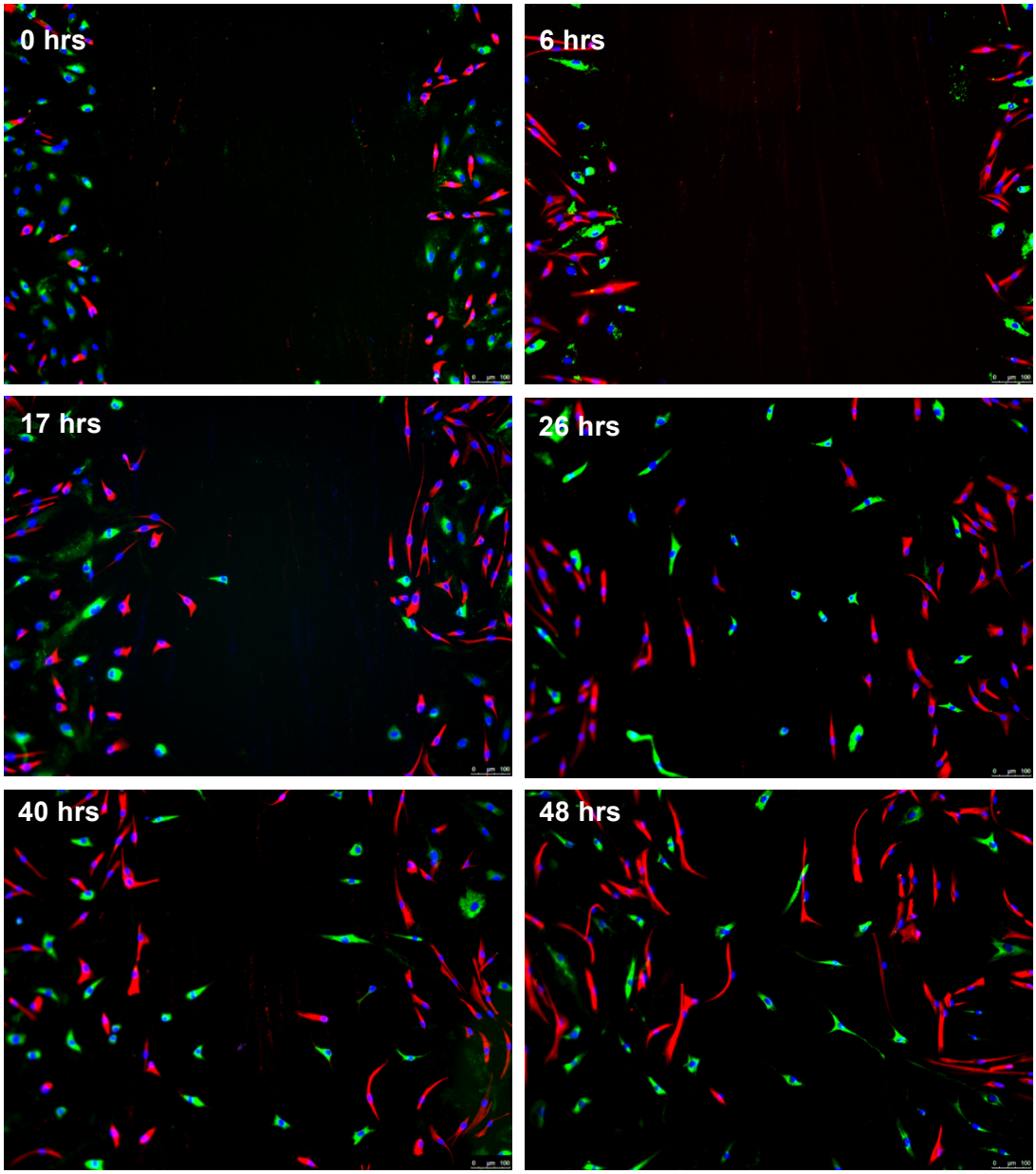


Figure 6.8. Representative images visualising the wound healing process over 48 hours in a high Desmin^{+} population of MDCs following 20 hours pre-incubation in QM and 3 hours of pre-treatment with mitomycin-C. Myoblasts are stained for Desmin (Red/TRITC) and fibroblasts are stained for TE7 (Green/AlexaFluor488). Nuclei are blue (DAPI). Images were taken at 0, 6, 17, 26, 40 & 48 hours post insult.

Condition 3: Low Desmin^{+} without Mitomycin-C pre-treatment.

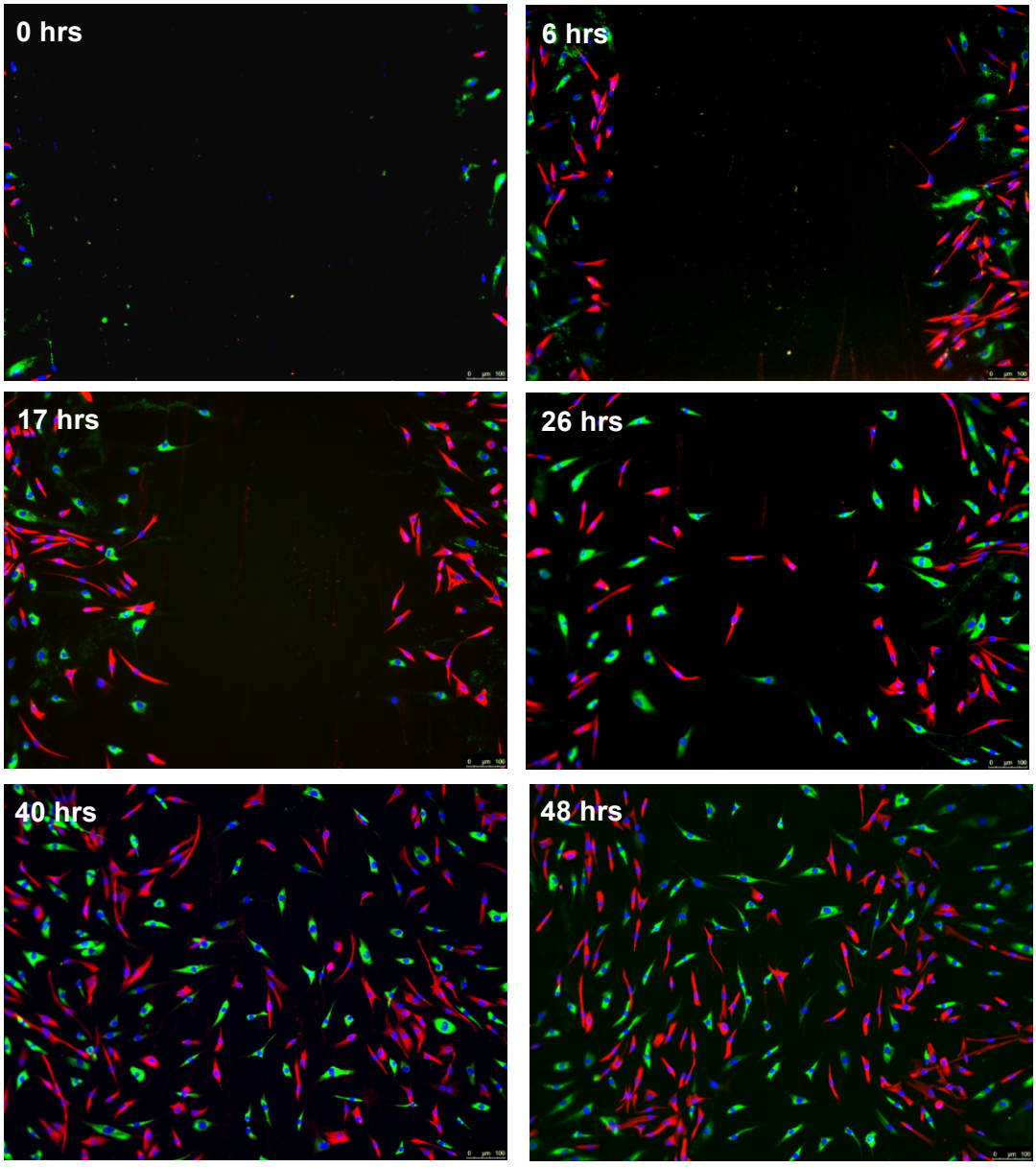


Figure 6.9. Representative images visualising the wound healing process over 48 hours in a low Desmin+ population of MDCs following 23 hours pre-incubation in QM. Myoblasts are stained for Desmin (Red/TRITC) and fibroblasts are stained for TE7 (Green/AlexaFluor488). Nuclei are blue (DAPI). Images were captured at 0, 6, 17, 26, 40 & 48 hours post insult.

Condition 4: Low Desmin^{+} with Mitomycin-C pre-treatment.

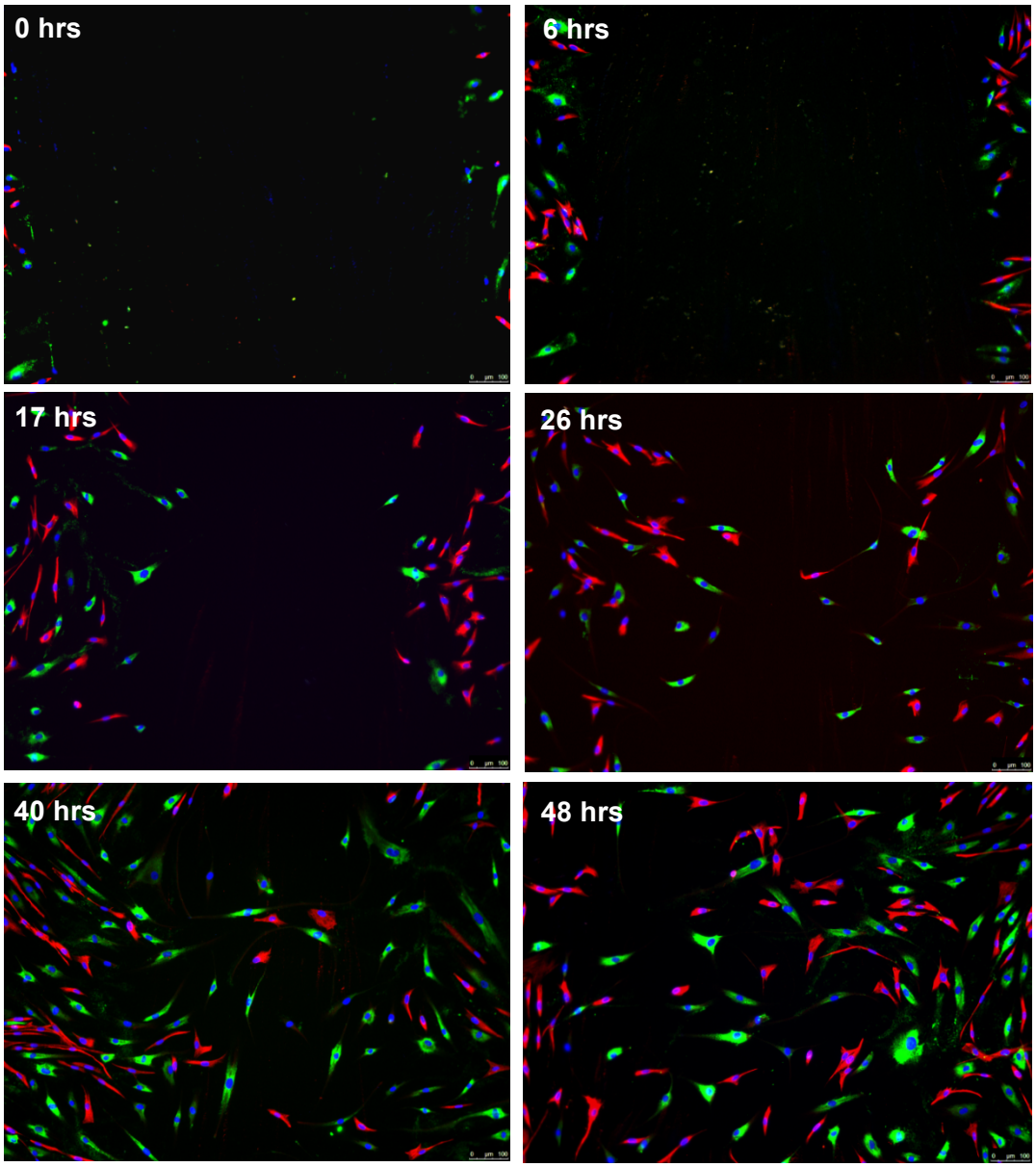


Figure 6.10. Representative images visualising the wound healing process over 48 hrs in a low Desmin^{+} population of MDCs following 20 hours pre-incubation in QM and 3 hrs of pre-treatment with mitomycin-C. Myoblasts are stained for Desmin (Red/TRITC) and fibroblasts are stained for TE7 (Green/AlexaFluor488). Nuclei are blue (DAPI). Images were captured at 0, 6, 17, 26, 40 & 48 hrs post insult. Scale bar is 100 µm.

A mixed design ANOVA was also implemented to identify whether the proportion of myogenic cells (i.e. High versus Lo desmin^{+}) affected the migration of one cell type or the other. Data analysis revealed that high Desmin positivity resulted in fewer cells of both cell types migrating into segments 1 and 2 at 48 hours following damage when compared with low Desmin positivity. However, no significant differences were observed in the relative proportion of Des^{+} or TE7^{+} migrating cells over 48 hours (Figure 6.11). This may suggest that it is unlikely that Desmin positivity was the factor that led to lower numbers of migrating cells in high versus low populations, rather the observed difference was due to individuals differences in the intrinsic capacity for migration of the MDCs obtained from two different participants.

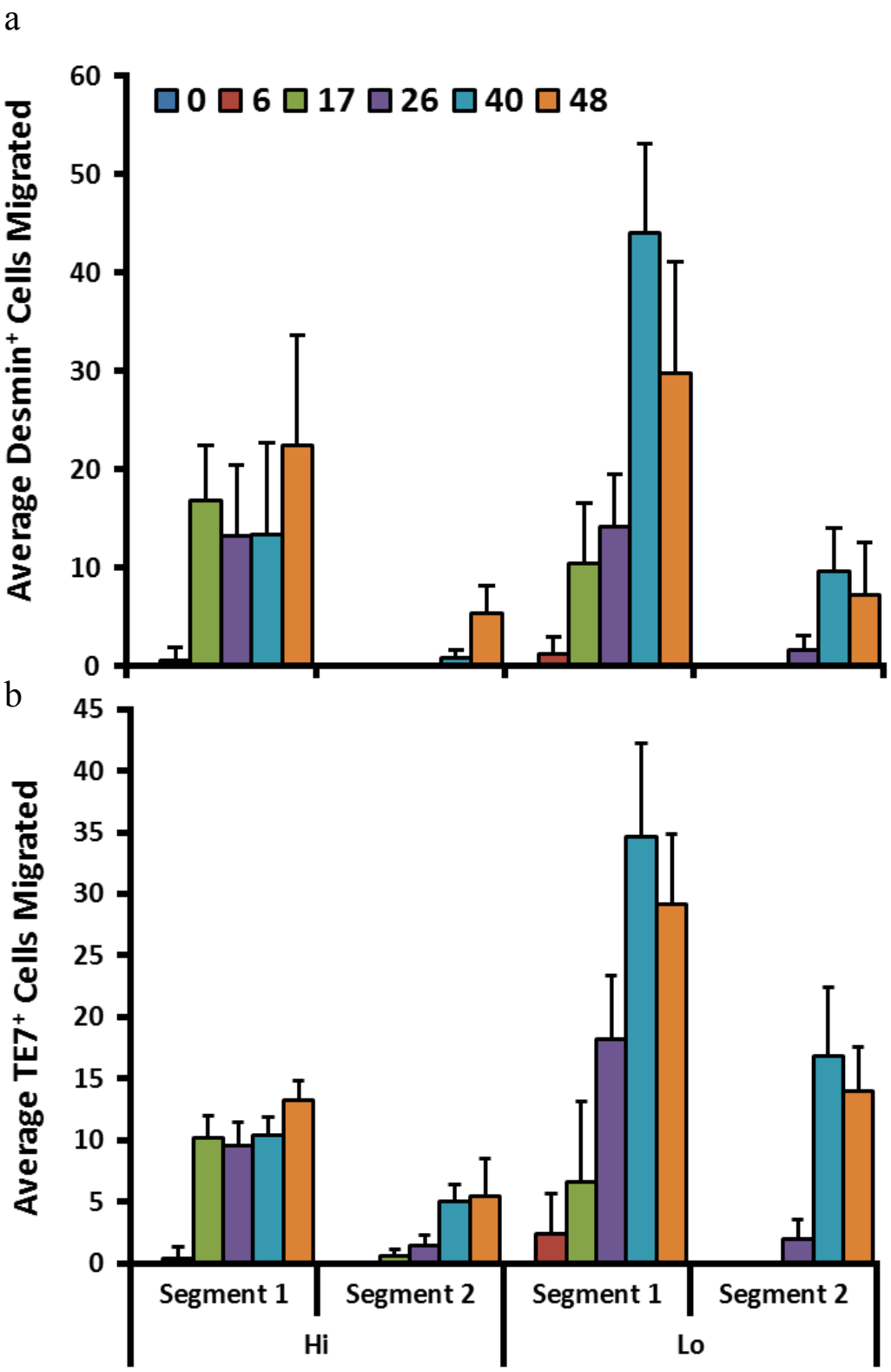


Figure 6.11. The effect of high (hi) and low (lo) myogenic cell populations on migration of **a**) myoblasts (Des^+) and **b**) fibroblasts ($TE7^+$) following mechanical scrape damage inflicted at 23 hours incubation in QM without Mitomycin-C.

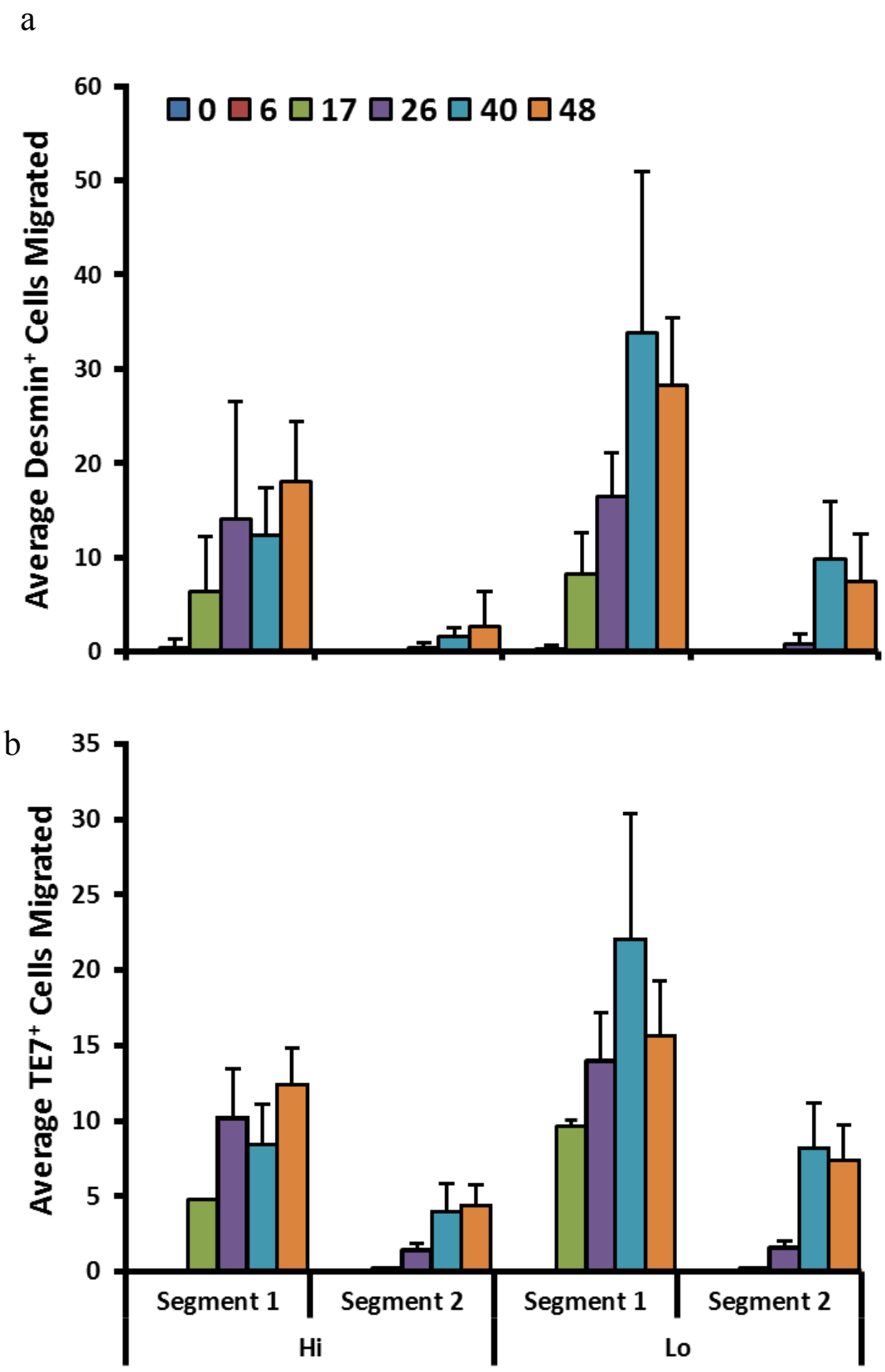


Figure 6.12. The effect of high (hi) and low (lo) myogenic cell populations on migration of myoblasts (Des^+) and fibroblasts ($TE7^+$) following mechanical scrape damage inflicted at 20 hours incubation in QM and 3 hours Mitomycin-C treatment (10 $\mu g.ml^{-1}$).

6.5 Pilot Trial 4 – Capability of Human MDCs to Form Mature Myotubes Following Mitomycin-C Pre-Treatment and Mechanical Scrape Damage

6.5.1 Aims and Hypothesis

Pilot studies 1-3 determined appropriate conditions for studying muscle derived cell migration in an *in vitro* wound-healing model. Although these data allow analysis of migration dynamics, it is also important to assess myoblast fusion at the wound space in order to create an appropriate model of muscle regeneration. This final pilot experiment therefore aims to simply establish whether MDCs are capable of fusing in the wound space following mitomycin-C pre-treatment and mechanical scrape damage. Furthermore, if fusion occurs in this model, the time course over which this occurs will be identified. It is hypothesized that human MDCs will be capable of fusion at the inner wound space but this will take longer than observed in typical models of myogenesis in which fusion occurs within 72 hours of serum depletion.

6.5.2 Cell Culture and Treatments

Two human MDC populations (n = 2) retrieved from muscle biopsy specimens were seeded at a density of $8x10^5$ cells.ml^{-1} and allowed to proliferate for ~48 hours to reach confluence. Once cell confluence was reached, GM was removed and cells were washed twice with PBS before being incubated for 20 hours in quiescent media (QM) in an attempt to allow cells that had already began a proliferative cycle to complete and exit the cycle. Following 20 hours QM incubation, QM was removed and two wells were incubated for a further 3 hours in QM whilst two were incubated in QM in the presence of mitomycin-C (10 μg.ml^{-1} in QM). After incubation with QM or QM+mitomycin cells were damaged with a vertical linear scrape inflicted by a 1ml pippette tip. Media was then aspirated, monolayers were washed twice with 1ml PBS per well and DM was added. Monolayers were then imaged and fixed

periodically as described in the General Methods chapter of this thesis at 0, 48, 7 and 10 days following wounding.

6.5.3 Immunocytochemistry

Following fixation at each time point, monolayers were blocked and permeabilized following which they were probed for desmin and counterstained with DAPI nuclear stain as described in the General Methods chapter of this thesis (section 3.9.3).

6.5.4 Myotube Morphology

Three images per well were captured at each time point from which total myotube number per field of view, nuclei per myotube, myotube are and myotube diameter were analysed via ImageJ. Myotubes were defined as having 2 or more nuclei and only myotubes for which the whole area of the cell was visible were considered in counting.

6.5.5 Results

Image analysis demonstrated that no myotube formation had occurred at 48 or 72 hours following wounding and therefore no statistical analysis was performed for these time points. A two-way ANOVA was used to analyse myotube number and morphology at 7 and 10 days following damage. There was a significant main effect of time for myotubes formed per field ($P < 0.005$) with 2 fold changes being observed in both groups between 7 and 10 days, however no treatment group differences were observed ($P > 0.05$). Myotube area (7 days; mitomycin C = 3438 ± 1318 vs untreated = 3582 ± 1261 μm^2 and 10 days; mitomycin C 4413 ± 1971 vs untreated = 3890 ± 1758 μm^2) and diameter (7 days; mitomycin C = 11.3 ± 3.3 vs 11 ± 2.9 μm and 10 days; mitomycin C = 12.7 ± 3.5 vs untreated = 11.9 ± 4.1 μm) increased similarly in both treatment groups but failed to meet statistical significance between 7 and 10 days. Myonuclear accretion did however show a significant main effect of time ($P = 0.001$) with more nuclei per myotube observed at 10 days versus 7 but no difference between treatment group (7 days; mitomycin C = 3 ± 1.7 vs untreated = 3 ± 0.7

nuclei per myotube and 10 days; mitomycin C = 3.8 ± 1.6 vs untreated = 3.6 ± 1.2 nuclei per myotube, $P > 0.05$). As no myotube parameters assessed showed statistical differences between groups this provides support that human MDCs are capable of fusion upon serum withdrawal following a mechanical scrape insult in co-culture with non-myogenic cells such as fibroblasts and furthermore that mitomycin-C pre-treatment at a concentration of $10\mu g.ml^{-1}$ in QM does not affect the ability of myoblasts to fuse (Figure 6.13).

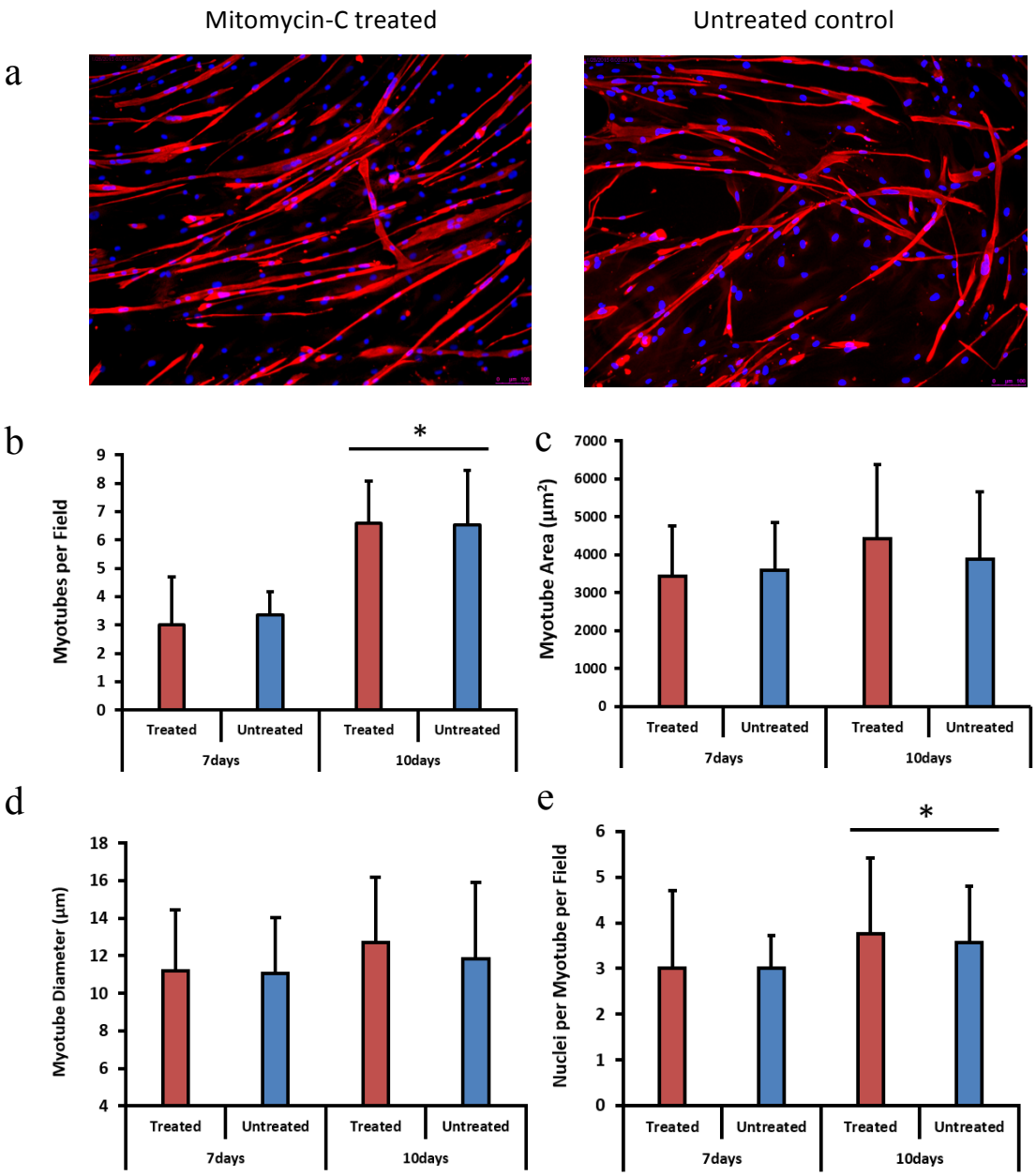


Figure 6.13. Fusion capability parameters of human muscle derived cells upon serum withdrawal at 7 and 10 days post mechanical scrape damage following a 3 hour incubation in QM with or without 10µg.ml^{-1} mitomycin C. **a**) representative fluorescent images captured at 10 days post wounding. Myotubes are staned for Desmin (Red/TRITC) and nuclei are stained with DAPI (blue). Scale bar is 100 µm; **b**) average myotubes per field of view; **c**) average myotube area (µm^2); **d**) average myotube diameter (µm); **e**) average nuclei per myotube per field and **f**) average myonuclear domain size (µm^2).

6.6 Discussion

The purpose of performing the described pilot experiments was to optimize the experimental conditions for studying the migration and fusion of human MDCs *in vitro* as a model of muscle damage and regeneration. Important considerations explored were seeding density, wound size, serum concentration of media, interfering proliferation, myogenic proportion of the cell population and fusion capability. The results gathered show that the optimal conditions for studying cell migration following a mechanical scrape insult is as follows:

Table 6.1. Optimal culture conditions for studying wound healing in an *in vitro* mechanical scrape model of muscle wound repair.

Variable	Optimum
Seeding density	$8x10^4$ cells.ml^{-1}
Wound direction	Vertical or horizontal linear
Wound size	1ml pipette tip (~800-1000μm)
Media	2% serum differentiation medium
Myotube formation in wound space	Min. 7 days

In addition to the experiments undertaken on C2C12 myoblasts, the data obtained from the final pilot trial show that primary human MDCs derived from human biopsies in an unsorted population of varying myogenicity migrate similarly between samples (within the range obtained in this study), regardless of myogenic cell proportion. Moreover, additional proliferative cycles are likely to give a false indication of migration capacity as treatment of cells with a inhibitor of cell proliferation (mitomycin-C; 10 μg.ml^{-1}) prior to wounding resulted in significantly fewer cells in the wound site at 48 hours post insult likely as a result of mitotic inhibition. Taken together, the conditions described in Table 6.1 are appropriate to study the migration and fusion of human MDCs in an *in vitro* model of muscle damage and repair.

Chapter 7

Vitamin D and Muscle Regeneration *In Vitro*

This chapter utilised the in vitro model that was optimised in chapter 6 to explore the role of Vitamin D in cellular aspects of muscle regeneration with the main aim of providing a mechanistic underpinning for the in vivo findings of chapter 5. The main outcomes suggest that treatment of human derived cells with 10 nmol 1α,25[OH]$_2$D$_3$ improves muscle derived cell migration over 48 hours and myoblast fusion at 7 and 10 days following a damaging mechanical scrape.

Publications resulted from this chapter: Owens D.J., Sharples, A.P., Polydorou, I., Alwan, N., Tang, J.C.Y., Donovan, T., Fraser, W.D., Morton, J.P., Stewart, C & Close, G.L. (2015). A Systems Based Investigation into Vitamin D and Skeletal Muscle Repair, Regeneration and Hypertrophy. Am J Physiol Endocrinol Metab. Doi: 10.11.1152/ajpendo.00375.2015.

7.1 Abstract

Purpose – Skeletal muscle is a direct target for Vitamin D. In Chapter 5 of this thesis it was demonstrated that raising serum 25[OH]D improved maximal torque recovery following eccentric exercise. However, mechanisms underlying this observation have not been established. In order to address this gap in knowledge in relation to damage repair, the main objective of the current study was to identify whether aspects of muscle regeneration are responsive to Vitamin D *in vitro*, utilising the model developed in Chapter 6.

Methods – Human skeletal muscle derived myoblast cells from biopsies of 14 males with low serum 25[OH]D (37 ± 11 nmol.L^{-1}) were subjected to mechanical wound injury, which enabled *in vitro* studies of muscle repair (migration, filmed over 48 hours), regeneration and hypertrophy (myotube morphology, biochemical and gene markers at 7 and 10 days post wound) in the presence and absence of 10 nmol or 100 nmol 1α,25$[OH]_2D_3$.

Results – Ten nmol 1α,25$[OH]_2D_3$ improved muscle cell migration dynamics (migration velocity and distance) and resulted in improved myotube fusion/differentiation at the biochemical (creatine kinase activity), morphological and molecular level together with increased myotube hypertrophy at 7 and 10 days post-damage.

Conclusion - Together with data from the study described in Chapter 5, these data provide a strong basis of support for the notion that Vitamin D interacts with skeletal muscle to mediate aspects of muscle regeneration and remodelling. Additionally, given the improved capability to recruit nuclei to each myotube, Vitamin D may also enhance post eccentric exercise remodelling and prime muscle for further bouts of mechanical stress.

7.2 Introduction

In Chapter 5 of this thesis, supplemental Vitamin D (4,000 IU.day^{-1} for 6 weeks) to increase serum 25[OH]D led to an improvement in the recovery of maximal isokinetic torque at 48 hours and 7 days post eccentric exercise. These data and available insights from other investigations may imply a role for Vitamin D in the regeneration of skeletal muscle *in vivo*. However, limited information is available to explain the mechanisms that underpin these observations at the cellular and molecular scale. As described in the Literature Review of this thesis, the cellular events of skeletal muscle cells that lead to muscle regeneration include activation of the resident muscle stem cell, expansion of the committed myoblast population, migration to the site of damage and fusion with damaged myofibres to repair the injury. Targeted ablation of the skeletal muscle stem cell results in an inability to regenerate the tissue (Relaix & Zammit, 2012), highlighting the importance of these cells in skeletal muscle homeostasis.

Available evidence suggests that 1α,25[OH]$_2$D$_3$ rapidly activates signalling networks that are linked to the control of cytoskeletal reorganization and cell migration, although a direct link between these signalling cascades and skeletal muscle cell migration is yet to be established. Such pathways activated in myoblasts in the presence of Vitamin D include Src/PI3K/Akt and MAPK/ERK (Buitrago et al., 2013). In other cell types such as vascular smooth muscle cells, 1α,25[OH]$_2$D$_3$ promotes migration through PI3K (Rebsamen et al., 2002) and indeed the requirement for PI3K in myoblast migration has previously been demonstrated (Dimchev et al., 2013). Moreover, Vitamin D has also been demonstrated to enhance other aspects of the repair process in immortalized cell models such as the C_2C_{12} murine myoblast cell line (Srikuea et al., 2012). Interestingly, these pro myogenic effect observed in C_2C_{12} cells is similar to that of other steroids such as testosterone that improve fusion capability and induce hypertrophy (Deane et al., 2013; Sinha-Hikim et al., 2002). Collectively, these data may imply that Vitamin D interacts with skeletal muscle to enhance muscle repair, however these data are yet to be produced.

Given that myoblast migration, fusion and hypertrophy are key processes in muscle regeneration and remodelling, the main objective of this study were to identify whether treatment of primary human MDCs isolated from biopsy specimens, with $1\alpha,25[OH]_2D_3$ following a mechanical scrape wound influences migration dynamics, including: migration velocity, distance and directionality. Furthermore, improved migration to the wound site is only impactful if the myoblasts subsequently fuse efficiently to effectively repair the site of damage. Thus, a further objective was to examine the cellular events post wound closure in the presence or absence of $1\alpha,25[OH]_2D_3$ to establish whether there is indeed a positive role in muscle regeneration.

7.1.1 Aims and Hypotheses

The main aims of this investigation were twofold; to identify whether exogenous treatment of MDCs following a mechanical injury would improve **1**) the migration characteristics and **2**) fusion capability of human MDCs. It was hypothesised that exogenous treatment with $1\alpha,25[OH]_2D_3$ (10 nmol (Lo) or 100 nmol (Hi)) following damage would improve the described aspects of cell migration and fusion in a dose dependent manner i.e. 100 nmol superior to 10 nmol and both treatment superior to a vehicle control.

7.2 General Methods

7.2.1 Participants

Following ethical approval and informed consent, fourteen male participants (age = 25 ± 3yrs; height = 181 ± 5cm; weight = 81 ± 10kg) volunteered to partake in the trial. After meeting the initial inclusion criteria (Chapter 3, section 3.2), participants provided a venous blood sample that was analysed for total serum 25[OH]D concentration by LC-MS/MS (Chapter 3, section 3.4). The average total serum 25[OH]D concentration for the group was 37±11 nmol.L^{-1} with the range represented in Fig 7.1. A biopsy specimen was also then harvested from the *Vastus Lateralis* from all participants (Chapter 3, section 3.5).

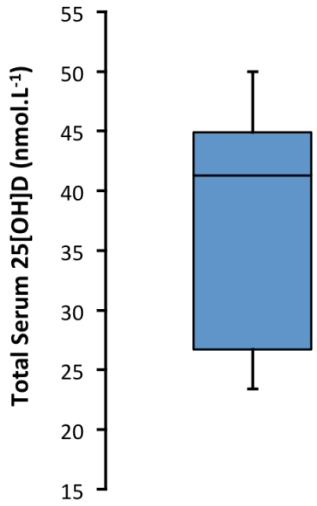


Figure 7.1. Box plot representing basal Vitamin D status of all participants from whom a biopsy was obtained. Data are presented as upper and lower quartiles, median and maximum/minimum values.

7.2.2 Cell Culture

Cell populations derived from individual biopsy samples were resuscitated from liquid nitrogen and seeded on pre gelatinised T25 cm^2 cell culture flasks for expansion. The seeding density was dependent on the cell number retrieved at passage 1 and ranged from 3x10^5 cells.ml^{-1} to 1 x 10^6 cells.ml^{-1}. Cells were grown in Hams F-10 GM and upon ~80% confluence were trypsinized, counted in the presence of Trypan blue and seeded at 8x10^4 cells.ml^{-1} on pre gelatinised 6 well culture plates for subsequent experimental treatments. All cell cultures were maintained in a controlled humidified environment of 37^{o}C, 5% CO_2. The composition and preparation of growth media is detailed in section Chapter 3, section 3.11.

7.2.3 Cell Treatments

Once cell monolayers reached a confluent state, GM was removed, monolayers were washed twice with PBS and GM was replaced with QM for 20 hours, following which QM was exchanged for QM + mitomycin-C (10 $\mu g.ml^{-1}$) for 3 hours to allow replication arrest as determined by the pilot experimentation described earlier and by previous work (Dimchev et al., 2013). Subsequent to 3 hours treatment with QM + mitomycin-C, cells were damaged by a vertical scrape with a 1 ml pipette tip. The mitomycin-C pre-treatment media was aspirated and damaged cell monolayers were washed three times with 1x PBS to remove cell debris and residual pre-treatment media. Each six well culture plate was subjected to a low dose of exogenous $1\alpha,25[OH]_2D_3$ (10 nmol total in 2ml DM, Lo), a high dose (100 nmol total in 2ml DM, Hi) or control vehicle (20 $\mu l.ml^{-1}$ of 100% EtOH, Veh) in DM; n=2 wells per dose/experiment. The doses of Vitamin D chosen were based on previously published research evidence. Garcia et al. (2011) reported a dose response study performed in the C_2C_{12} cell line, reporting 100 nmol as the optimal dose to elicit a cellular responses in their chosen model. This dose has been widely used in a number of other investigations to elicit cellular responses (see Table 7.1), therefore both 10 and 100 nmol doses were selected to determine whether potential responses in cell migration and fusion were dose dependent. It should be noted however that these widely used doses are supra-physiological as $1\alpha,25[OH]_2D_3$ circulates in pmol concentrations. Immediately following the addition of treatments, monolayers were placed in a controlled live imaging microscopy environment of 37^oC, 5% CO_2 (Chapter 3, section 3.13) and images were captured every 30 minutes for 48 hours. All media composition is detailed in Chapter 3, section 3.11.

Table 7.1. Summary of investigations implementing exogenous Vitamin D on skeletal muscle cells.

Authors	Model	Treatment concentration	Outcome variable
Girgis et al. (2014a)	C_2C_{12} myoblasts	1-100 nmol	Myoblast proliferation, differentiation and myotube formation.
Srikuea et al. (2012)	C_2C_{12} myoblasts	20 nmol	Myoblast proliferation.
Garcia et al. (2011).	C_2C_{12} myoblasts	100 nmol	myoblast cell proliferation, progression, and differentiation into myotubes.
Artaza, Sirad, Ferrini, and Norris (2010).	Mesenchymal multipotent cells	100 nmol	cell morphology, cell proliferation, cell cycle progression and apoptosis.
Cardus et al. (2006)	Vascular smooth muscle cells	5-100 nmol	VSMC proliferation.

7.2.4 Cell Migration Analysis

TIF files captured over the 48-hour filming period were exported from Leica Application Suite and loaded as TIF image stacks in ImageJ with a *Cell Counter* plug in. Cells in the outer (segment 1) and inner (segment 2) wound spaces were counted (refer to General Methods section 3.14). TIF files were also exported as TIF image stacks into ImageJ with a *Manual Tracking Tool* plug-in (ibidi GmbH: München, Germany). The individual trajectory of each cell was tracked in the *xy* axis and derived raw co-ordinate data exported in an ImageJ *Chemotaxis and Migration Tool* plug-in (ibidi GmbH: München, Germany) for analysis. The chemotaxis tool analyses raw data from the manual tracking tool and provides quantitative data on the migration of individual and grouped cell trajectories including; migration velocity, accumulated migration distance, Euclidean migration distance and directionality. These terms are defined below:

- **Migration Velocity (*V*)** is calculated by the rate of change in position of a cell per unit of time. In this case a change in the *xy* position in µm.sec^{-1}.
- **Accumulated Migration Distance (D_{Acc})** is the total distance covered of a cell trajectory (Figure 6.18). Individual and mean cell trajectory values are generated in µm.
- **Euclidean Distance (D_{Euc})** is the length of the line segment connecting the start and end point of a cell trajectory (Figure 6.18).
- **Directionality (D_i)** is determined by comparing the Euclidean distance to the accumulated distance. It gives a measure of the directness of cell migration and is calculated by the following equation:

$$D_i = \frac{d_{i,Euclidean}}{d_{i,accumulated}} \qquad D = \frac{1}{n}\sum_{i=1}^{n} D_i = \frac{1}{n}\sum_{i=1}^{n} \frac{d_{i,Euclidean}}{d_{i,accumulated}}$$

Single Cell Directionality **Averaged Directionality**

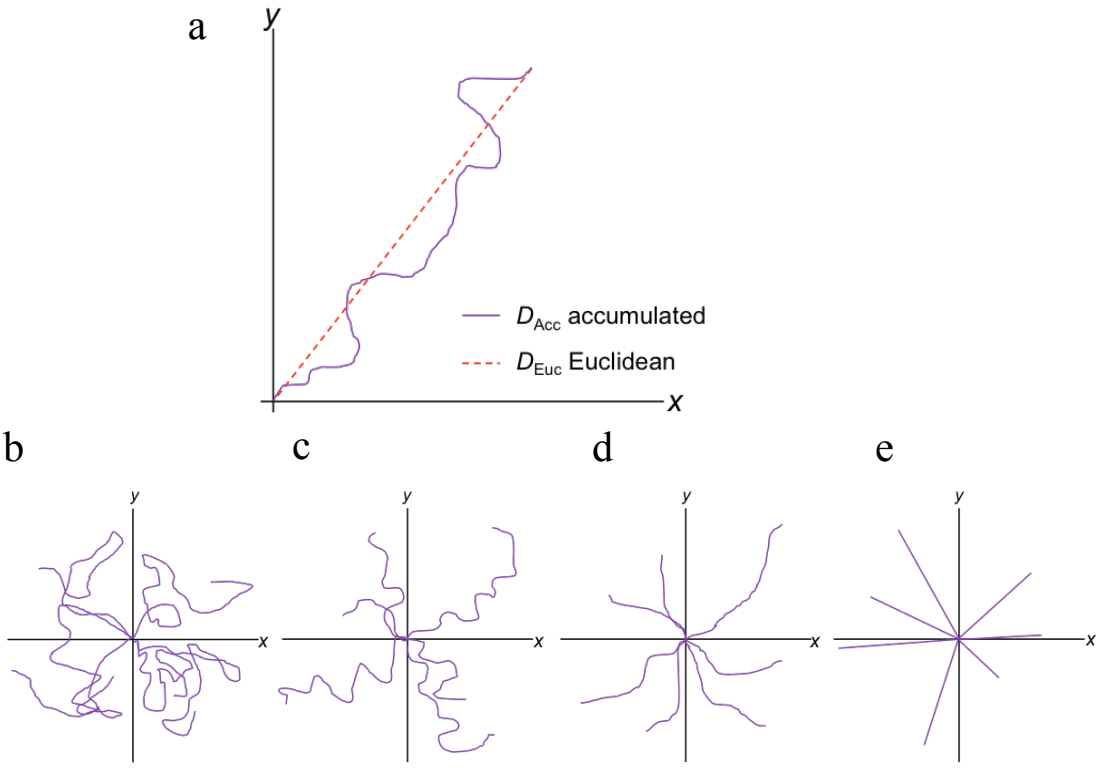


Figure 7.2. A) Example of a single cell trajectory as mapped by ImageJ. **B-E**) Examples of cell trajectories for a number of tracks with a range of directionality scores from 0 (B, far left) to 1 (E, far right).

Experiments were performed on six well plates of cell monolayers derived from 14 populations isolated from 14 individuals (n = 14). All experimental treatments were performed in duplicate. Two live imaging movies were captured per treatment well giving a total of four movies per treatment per sample (56 movies per treatment in total). From these movies, an average of 20 individual cell trajectories was tracked per movie. Cells were tracked from the leading wound edge only and tracking was terminated if a trajectory left the filmed space at any time.

7.2.5 Gene Expression

Upon cessation of filming, media was aspirated and monolayers washed three times with PBS following which total RNA was isolated and quantified as described in section 3.6 of the General Methods chapter of this thesis. Following RNA quality and quantity measurement, the expression of the VDR was assessed by RT-qPCR also described in section 3.6. Three reference genes were initially assessed for their stability and ribosomal protein L13a (*RPL13a*) was selected as a stable reference gene as the co-efficient of variation between PCR samples was 1.86%.

For the cell monolayers used for the analysis of myoblast fusion, total RNA was isolated and quantified as described above at 7 and 10 days. In addition to the VDR gene the late differentiation genes myogenic regulatory factor 4 (*MRF4*) and myogenin (*MYOG*) were also analysed at the 7 and 10 day time points. Ribosomal protein L13a (RPL13a) was again selected as a stable reference gene as the co-efficient of variation between PCR samples was 2%. Primer information and can be found in Table 3.2 (Chapter 3). Statistical analyses were performed as described in Chapter 3, section 3.15.

7.5.6 Creatine Kinase Activity

Immediately following injury (0 hours), 48 hours, 7 and 10 days following injury 1 well per treatment per sample was lysed in tris-mes-triton buffer (50 mM tris-mes, 1% Triton X100)

for determination of CK activity as a marker of differentiation. The bichinconinc acid (for protein content) and CK assays were performed as described in Chapter 3, section 3.8.

7.5.7 Morphology and Immunocytochemistry

In order to determine myotube formation at 7 and 10 days, damaged monolayers were imaged at 4 sites per well in the wound site immediately post damage (0 hours). These image co-ordinates were then saved to allow monitoring of a consistent wound site to avoid experimental bias. Images were obtained at 7 and 10 days, exported as TIFF image files and analysed in ImageJ. Morphology was assessed by myotubes per field of view, myotube diameter and myotube area. Myotubes were counted via ImageJ *cell counter* plug-in and only myotubes for which the entire length of the tube was visible in the field of view were considered. Myotubes were determined as cells containing 2 or more nuclei. Myotube area was determined by manually drawing a line around the sarcolemma of each myotube. By normalizing the pixel scale to the micron scale of each image a value expressed as μm^2 is obtained. To calculate myotube diameter, three equidistant diameters along the length of the myotube (left centre, centre and right centre) were measured and averaged. A total of 3 image per well were analysed per treatment, treatments were performed in duplicate and experiments performed on 14 cell populations from 14 different individuals. Therefore, 84 images per condition were assessed.

As intact monolayers were required determination of CK and extraction of RNA, experiments were repeated for immunocytochemical staining in order to visualise nuclei for the accurate determination of myonuclear fusion index myonuclear domain size. Monolayers were fixed and stained as described Chapter 3, section 3.9.3. Monolayers were stained for Desmin and DAPI nuclear stain. Fluorescent images were then captured at 10 days post damage and nuclei counted via ImageJ *cell counter* plug-in. A total of 3 image per well were analysed per treatment, treatments were performed in duplicate and experiments performed

on 7 cell populations from 7 different individuals. Although populations from 14 individuals were obtained, cell stocks were depleted from early passages from prior experiments leaving only 7 populations between passage 1-5 that could be used for the current experiments.

7.6 Results

Treatment of Muscle Derived Cells with 10 or 100 nmol 1α,25-dihydroxyvitamin$_2$D$_3$ Improves Migration Dynamics In Vitro

7.6.1. Live Cell Imaging Analysis

To establish the influence of Vitamin D on muscle derived cell migration, cell monolayers were treated with 10 and 100 nmol 1α,25[OH]$_2$D$_3$ or vehicle (EtOH) following a mechanical scrape insult. A one way ANOVA was implemented to compare means of the three conditions for the migration parameters V, D_{acc}, D_{Euc} and D_i. A significant treatment group effect was detected for V, D_{acc}, D_{Euc} and D_i ($P < 0.005$). Both Hi and Lo dose 1α,25[OH]$_2$D$_3$ significantly enhanced migration V compared to vehicle with 1.37 and 1.43 fold increases observed, respectively ($P < 0.0005$), versus control. Hi dose was also superior to Lo dose (0.331 ± 0.11 vs 0.318 ± 0.1 μm.min^{-1} respectively, $P = 0.033$). A similar observation was made in D_{Acc} as Hi treatment promoted greater migration (953 ± 305 μm) distances than Lo ((909 ± 281 μm) $P = 0.009$) whilst both Hi and Lo were superior to Veh ((666 ± 288 μm) $P < 0.0005$). However, although both Lo and Hi treatment improved D_{Euc} when compared with Veh, there were no differences between the two doses ($P = 0.193$), which would imply a loss of directionality in the Hi dose treatment. Indeed, analysis revealed that Hi dose treated MDCs demonstrated a loss of directionality (0.498 ± 0.21 AU) compared to Lo (0.546 ± 0.2 AU) and Veh (0.547 ± 0.23 AU) treated cells ($P < 0.005$) whereas Lo and Veh showed no differences. Data are presented graphically in Figure 7.3. No significant differences were evident in wound size between conditions (Lo = 949 ± 146 vs Hi = 927 ± 103 vs Veh = 925 ± 108 μm ($P = 0.810$)).

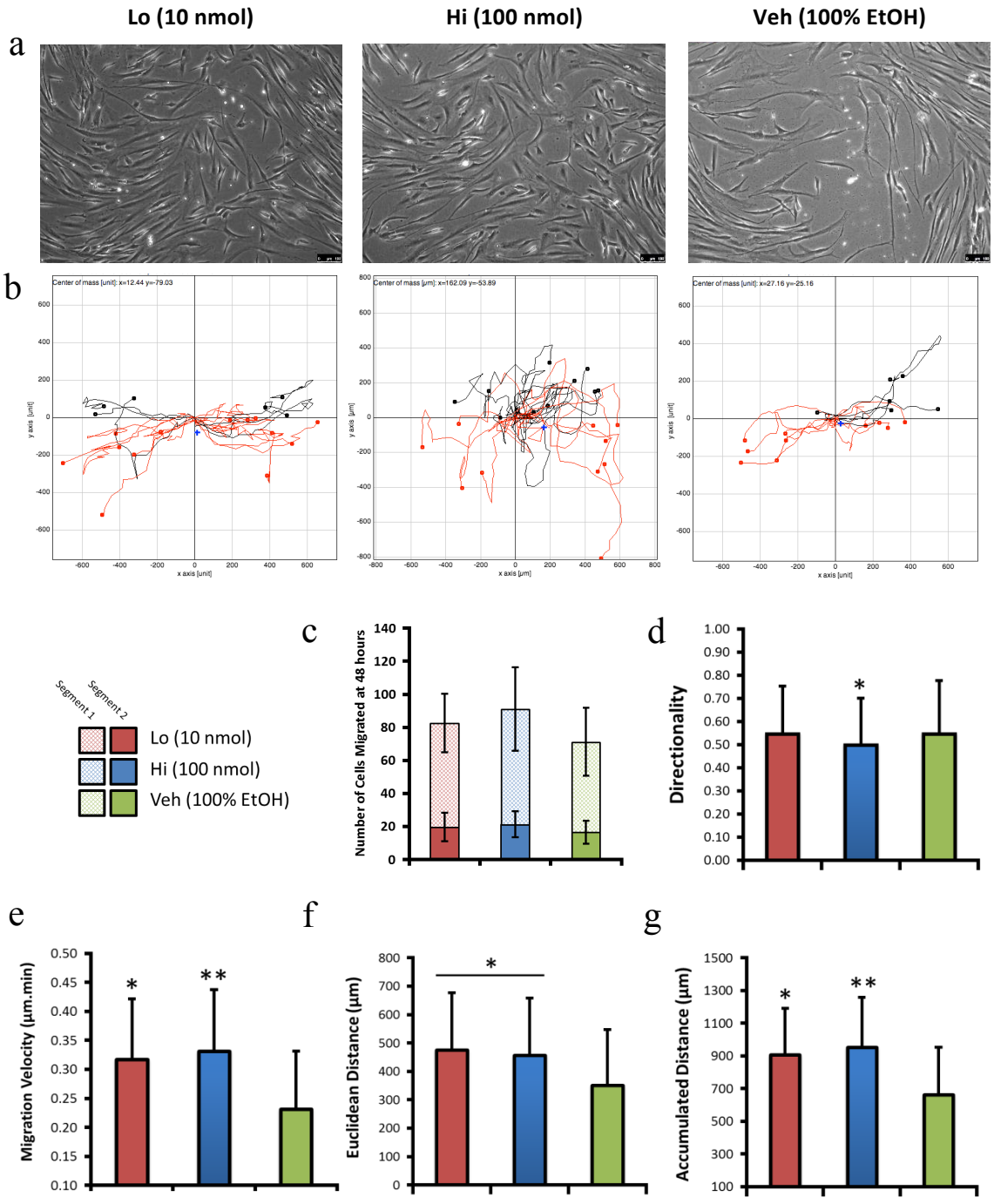


Figure 7.3. MDC migration dynamics following a mechanical scrape injury in the presence of Lo (10 nmol) or Hi (100 nmol) 1α,25[OH]$_2$D$_3$ or vehicle solution (100% EtOH); **a**) representative phase contrast microscope images captured at 48 hours post wounding. Scale bar is 100 µm. n = 14; **b**) representative migration plots produced via ImageJ chemotaxis tool; **c**) average number of cells migrated into segment 1 and 3 at 48 hours post damage; **d**) migration D$_i$ (0-1); **e**) migration V (µm.min^{-1}); **f**) migration D_{Euc} and **g**) migration D_{Acc} (µm); * denotes significance to Veh and ** denotes significance to all other treatments.

Treatment of Muscle Derived Cells with 10 nmol 1α,25-dihydroxyvitamin$_2$D$_3$ Enhances Myoblast Fusion Following Mechanical Scrape Injury In Vitro

7.6.2. Morphological Analysis

Treatment of MDC cultures with 10 or 100 nmol 1α,25[OH]$_2$D$_3$ resulted in greater migration dynamics, however the same dosing strategy led to differential effects on myoblast fusion at 7 and 10 days following the damaging event. Morphological data were analysed via a mixed design ANOVA and revealed that myoblast fusion was significantly inhibited as fewer myotubes were observed per field with 100 nmol 1α,25[OH]$_2$D$_3$ versus 10 nmol and vehicle at 7 days post damage (Hi = 2 ± 2 vs Lo = 6 ± 4 vs Veh = 3 ± 1 myotubes per field (P < 0.0005)). In contrast, Lo treatment led to significant improvements in myotube number compared with 100 nmol and Veh at both 7 (as above) and 10 days (Lo =10 ± 3 vs Hi = 6 ± 2 vs Veh = 6 ± 2 myotubes per field (P < 0.005)). This observation was also similar for myotube area with Lo treatment resulting in significantly greater myotube area versus Hi and Veh at 7 (Lo = 4984 ± 2776 vs Hi = 4603 ± 1697 vs 4227 ± 1768 μm^2 (P = 0.003)) and 10 days (Lo = 5488 ± 2853 vs Hi = 4671 ± 2932 vs Veh = 4388 ± 2312 μm^2 (P < 0.0005)). Myotube diameter was significantly greater at 7 days in both Lo and Hi dose treatments versus Veh (Lo = 14.13 ± 4 vs Hi = 13.7 ± 4.5 vs Veh = 12.12 ± 3.8 μm (P = 0.005)), however this effect was lost at 10 days (Lo = 13.6 ± 4 vs Hi = 12.6 ± 4.1 vs Veh = 12.1 ± 3.7 μm (P = 0.256)). All morphological data are presented graphically in Figure 7.4.

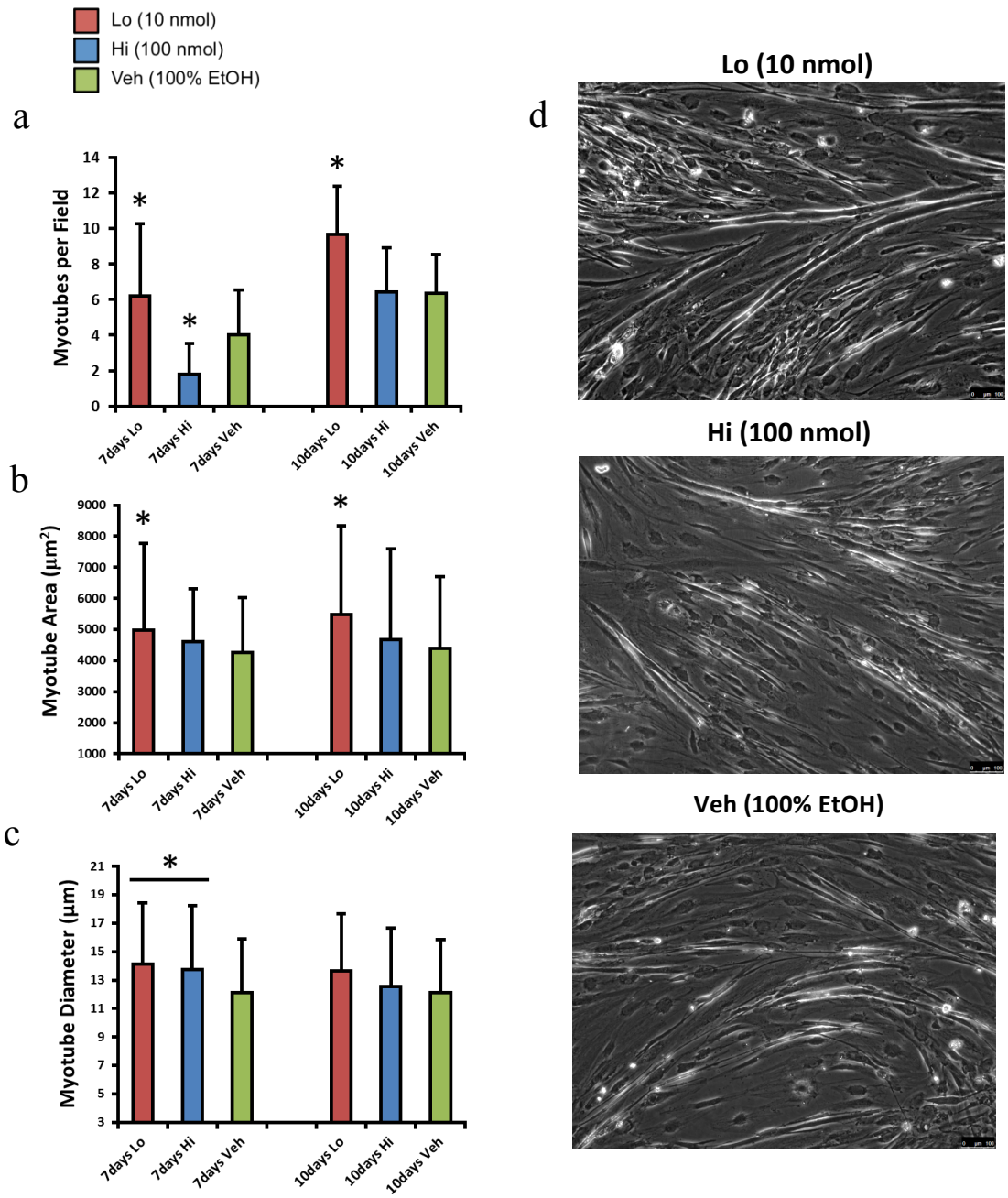


Figure 7.4. Effect of Lo or Hi dose 1α,25[OH]$_2$D$_3$ versus Veh on myotube morphology following 7 and 10 days in DM following mechanical scrape wounding**; a**) Average myotubes per field of view; **b**) average myotube area (µm^2); **c**) average myotube diameter (µm) and **d**) representative phase contrast microscope images captured at 10 days in the wound space of each condition. Scale bar is 100µm.

7.6.3. Biochemistry and immunocytochemistry

Findings from biochemical and fluorescent imaging were in agreement with morphological findings. Creatine kinase activity data were analysed via a mixed design ANOVA. Results show CK activity was elevated above Veh with Lo treatment and repressed with Hi compared with both Lo and Veh at 7 days (Lo = 302.5 ± 173 vs Hi = 186.7 ± 132 vs Veh = 290 ± 160.5 mU.mg.ml^{-1}), although this effect did not reach statistical significance. At 10 days, Lo treatment cells demonstrated significantly higher CK activity compared with both Hi and Veh (Lo = 340.4 ± 183 vs Hi = 258.4 ± 188 vs Veh = 229.4 ± 139.5 mU.mg.ml^{-1} (P = 0.017)). Myonuclei and myonuclear domain size were analysed by one-way ANOVA. The CK observations correlated with a significantly greater accretion of myonuclei in Lo treated cells versus Hi and Veh at 10 days post damage (Lo = 5.9 ± 2.3 vs Hi = 3.5 ± 1.4 vs Veh = 3.4 ± 1.2 nuclei per myotube per field (P < 0.0005), however myonuclear domain size was smaller at 10 days with Lo treatment (Lo = 986.5 ± 439.4 vs Hi = 1460 ± 726.3 vs Veh 1257.3 ± 584 μm^2 (P < 0.0005)).

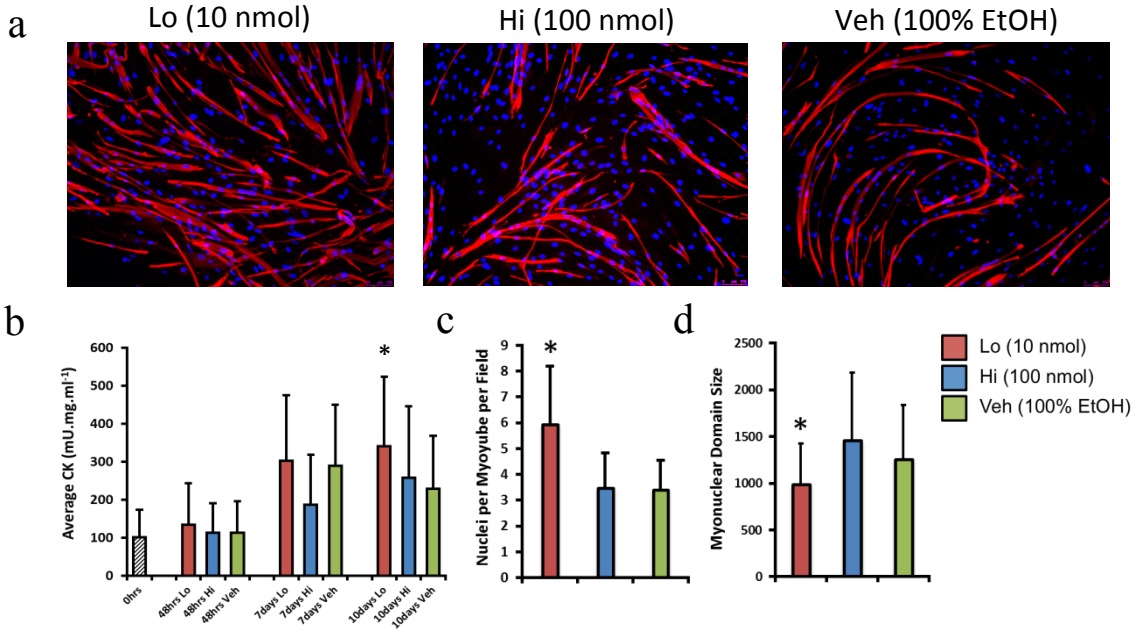


Figure 7.5. Effect of Lo or Hi dose 1α,25[OH]$_2$D$_3$ versus Veh on biochemical aspects of myotube formation and immuncytochemical analysis of nuclear accretion; **a**) Representative fluorescent microscopy images taken at 10 days following damage. Scale bar is 100μm; **b**) Average CK activity (mU.mg.ml^{-1}) at 48 hours, 7 and 10 days following damage. * denotes significance to other conditions at a time point; **c**) average nuclei per myotube per field of view at 10 days following damage.

7.6.4. Gene expression

To determine the impact of treatment on gene expression, ΔΔCt analysis was performed by comparing the fold change in target gene expression against both a non-treated 0 hours control and a stable reference gene (RPL13a). Data were then analysed by a mixed design ANOVA. Results demonstrated that on average Lo treated cells up regulated MRF4 expression to a higher extent at both 7 and 10 days (3.2 and 3.7 fold) than Hi (-0.5 fold and no change) and Veh treated cells (2.9 and 2.9 fold). Similarly, Lo treated cells showed an increased myogenin expression at 10 days (84 fold) compared with Hi (64 fold) and Veh (62 fold), however these data failed to meet statistical significance, likely due to the large variation in basal expression of the MRFs (Figure 7.6). VDR expression showed no discernible difference between groups at any time point, however all significantly increased expression in a similar trend with myogenin between 48 hours and seven days ($P = 0.022$) suggestive of a role for VDR in myoblast fusion. Interestingly, Hi treated cells also showed an impairment (although no statistically significant) in the ability induce both MRFs at 7 days post insult versus control and Lo treated cells which correlates with biochemical observations demonstrating lower CK activity in Hi treated cells at 7 days.

Having obtained large variation in basal expression, raw cycle threshold (Ct) values were also analysed and are also presented with ΔΔCt in Figure 7.6. Analysis of raw Ct values showed similar results, with all treatment groups demonstrating a main effect for time in myogenin and VDR expression, with significantly increased expression from 48 hours to 7 days. A main effect of time was not detected for MRF4, however observation of Ct values (Figure 7.6) suggests a trend of increasing expression with time in keeping with ΔΔCt scores.

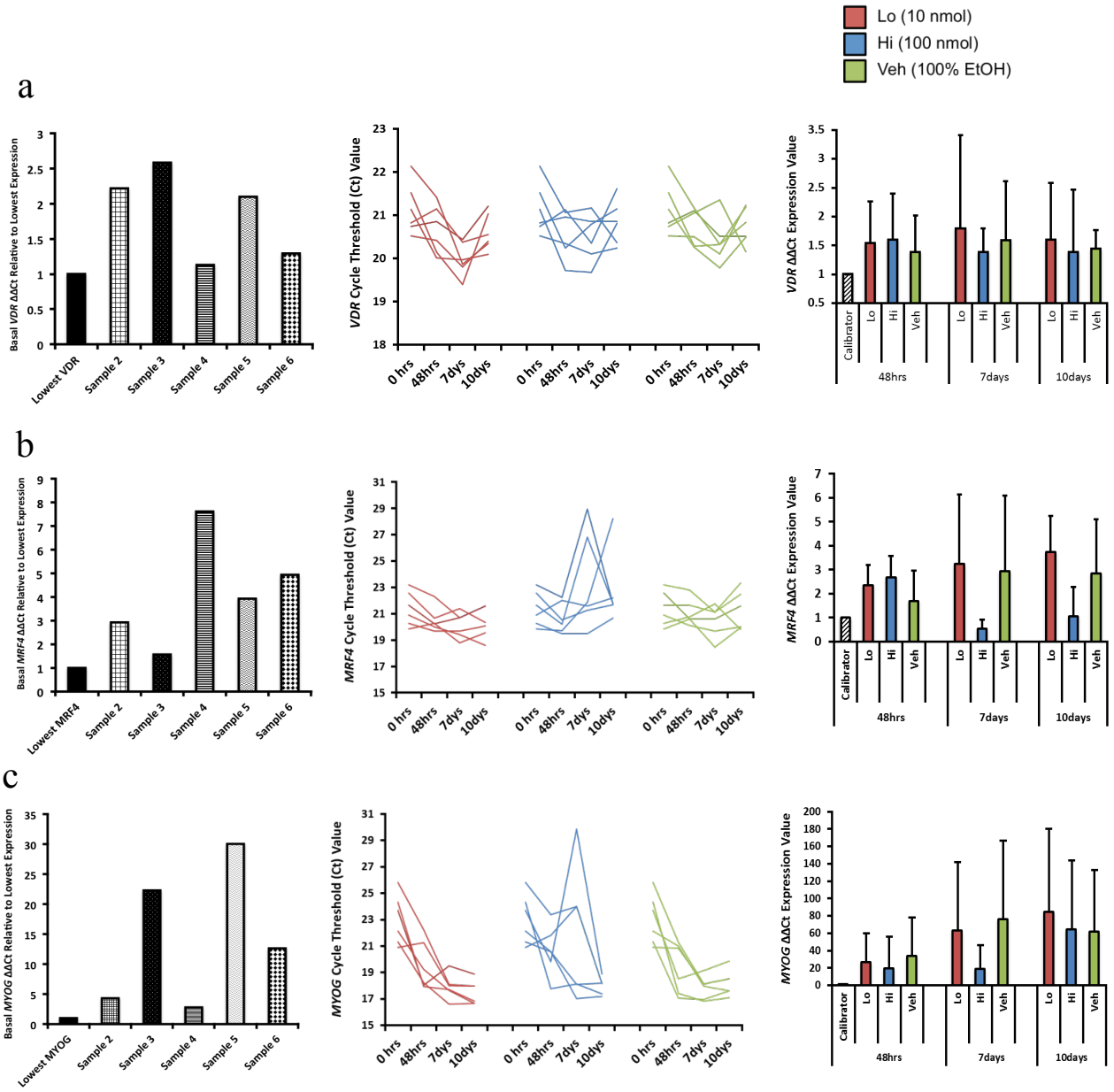


Figure 7.6. Time course effects of 1α,25[OH]$_2$D$_3$ on mRNA expression of **a**) Vitamin D Receptor; **b**) Myogenic regulatory factor 4 and **c**) myogenin. From left panels, figures represent basal variation of genes presented as fold changes versus lowest expressing sample, raw Ct values of individual samples and ΔΔCt fold changes against a stable reference gene (RPL13a) and an internal calibrator (0 hours sample).

7.7 Discussion

The investigations in this chapter aimed to establish whether *in vivo* findings of Chapter 5 could be explained by improved migration and fusion aspects of the regeneration process in the presence of Vitamin D. It was hypothesized that exogenous $1\alpha,25[OH]_2D_3$ would improve aspects of migration and fusion, regardless of whether the dose was 10 or 100 nmol, providing potential cellular mechanisms by which Vitamin D appears to mediate muscle regeneration *in vivo*.

The main findings from the investigations suggest that $1\alpha,25[OH]_2D_3$ has the capacity to improve MDC migration dynamics including speed, accumulated and Euclidean distance when administered at both low (10 nmol) and high (100 nmol) doses. The Lo dose promoted faster migration with sustained directionality whilst the Hi dose further improved migration speed but at the expense of directionality. As both treatments resulted in significantly more cells in the wound space at 48 hours versus vehicle treatment, both treatments were equally beneficial in closing the wound space over 48 hours. A superior effect of the lower 10 nmol dose was revealed by morphological analysis of myoblast fusion in the wound site at 7 and 10 days following the scrape wounding. Ten nmol $1\alpha,25[OH]_2D_3$ promoted both hypertrophy and hyperplasia above that of Hi treatment and vehicle control in the wound site at 7 and 10 days post damage. This finding was reinforced by biochemical analysis of CK activity, which showed small increases above Hi and Veh at 7 days and significant increases at 10 days following injury. Myonuclear domain size was however significantly smaller with Lo treatment at 10 days, indicating an improved ability to recruit myoblasts to a single myotube (myonuclear accretion) and perhaps a hypertrophic response that had not peaked by the final sampling stage at 10 days. Analysis of the late differentiation myogenic regulatory factors, MRF4 and myogenin showed large variability between samples and although no significant treatment group effects were detected, $1\alpha,25[OH]_2D_3$ administered at 10 nmol up-regulated both MRF4 and myogenin to a greater extent than 100 nmol $1\alpha,25[OH]_2D_3$ and slightly greater than vehicle solution, peaking with marked increases at 7 days and a

continued trend of increasing expression at 10 days. Interestingly, peak expression of VDR coincided with the induction of myogenin at 7 days post damage, suggestive of a role for VDR myoblast fusion and consistent with reports of increased VDR expression in C_2C_{12}myoblasts at 7 days following $BaCl_2$ treatment (Srikuea et al., 2012). However, this was consistent between conditions raising new questions as to what the role of the VDR is in myogenisis.

A second important finding of the current work was that Hi dose 1α,25$[OH]_2D_3$ treatment of MDC monolayers resulted in delayed fusion of myoblasts at the wound site when compared with Lo treatment. Hi treated monolayers formed fewer myotubes at 7 days post damage, only reaching control (Veh) levels by day 10 which was supported by significantly lower CK activity and lower expression of the MRFs compared with Lo treatment. An interesting observation on this point was that regardless of basal MRF expression, there appeared to be a critical 'threshold', particularly for myogenin, that must be reached to allow fusion of myoblasts (approximately PCR cycle 18 in the current study, Figure 7.6). Lo treated cells appeared to reach this threshold more effectively across all samples, whilst Hi treated did not meet this threshold until 10 days with some samples not reaching the threshold at all. These interesting changes in gene expression may account for the morphological observations made.

The positive influence of Lo 1α,25$[OH]_2D_3$ administration on cell migration is analogous to effects observed in other cell types. For example, exogenous treatment of vascular smooth muscle cells with 1α,25$[OH]_2D_3$ induced migration following activation of PI3 kinase, with observed effects being abolished by the addition of the PI3 kinase inhibitor, LY294002 (Rebsamen et al., 2002). Indeed the importance of PI3K in myoblast migration has previously been characterized (Dimchev et al., 2013), pointing towards stimulation of PI3K activity as a main mediator of improved MDC migration dynamics in the current trial. It is likely that this response occurs through direct interaction of ligand bound VDR with Src kinase (Buitrago et al., 2000) and does not require transcriptional activity, as addition of

RNA polymerase inhibitor, 5,6-dichlorobenzimidazole riboside is reported to have no effect on 1α,25$[OH]_2D_3$ mediated PI3K activation (Rebsamen et al., 2002). Activation of PI3-kinases and their lipid product PI(3,4,5)P3 leads to increases in GTP bound Rac, which is an important small GTPase along with Rho in the control of downstream signalling that generates filamentous actin branching and lamellipodia formation (Raftopoulou & Hall, 2004). Lamellipodia are transient membrane protrusions that along with effective rear edge detachment allow migration of many cell types including myoblasts and fibroblasts. Tight regulation of the networks that lead to lamellipodia formation by positive and negative feedback loops are responsible for directional persistence and migration speed and 'steering' of the cell toward specific stimuli (Krause & Gautreau, 2014). It could therefore be postulated based on available evidence that Vitamin D may function to stimulate myoblast migration through the activation of PI3 kinases and increased downstream activity of small Rho GTPases resultantly altering actin cytoskeletal dynamics. Future research should aim to investigate this pathway in the context of Vitamin D and examine lamellipdodia formation with high-resolution microscopy techniques implementing kymography and fluorescent probes.

The finding that 1α,25$[OH]_2D_3$ improved myoblast fusion is perhaps not surprising as Vitamin D metabolites are a closely related hormone system with homologous receptors, with some of the characteristics of true steroids as receptor ligands, such as testosterone. Indeed testosterone has repeatedly been demonstrated to enhance myoblast differentiation *in vitro* (Deane et al., 2013; Sculthorpe et al., 2012). In a similar observation to the current study, these investigations also observed significant increases in hyperplasia and hypertrophy. However, hypertrophy in these reported trials was evidenced by increased myotube diameter, whereas the current study reported increases in myotube area but not diameter. Furthermore, the current trial saw decreases in myonuclear domain size as a consequence of both nuclear accretion and myotube area but no increases in diameter. This may be due to the different models employed as the current trial implemented human

derived myoblasts in co-culture with fibroblasts. Therefore, the immediate environment and extracellular regulatory signals are incomparable between models and may account for these differences. Alternatively, the observation may be as a consequence of a hypertrophic response that had not reached its peak in the Lo treated population as a result of a more sustained phase of nuclear accretion delaying subsequent hypertrophy. Nevertheless, exogenous treatment of MDCs with 10nmol 1α,25$[OH]_2D_3$ showed comparable effects on myogenesis with that of other true steroid hormones *in vitro*. These findings show similarities and contrasts with other models of myogenesis in the context of Vitamin D. As a contrasting example, increases in myotube diameter and MHC type II were detected following 100 nmol treatment of C_2C_{12} myoblasts versus vehicle, indicative of a positive myogenic effect (Garcia et al., 2011). In another trial, following serum depletion 100 nmol 1α,25$[OH]_2D_3$ was shown to suppress myotube formation in a similar manner to the current study with decreased mRNA expression for myogenin and myf5. However, the treatment led to a 1.8-fold increase in cross-sectional size of individual myotubes associated with slightly decreased myostatin expression (Girgis et al., 2014a). The disparity between the current work and previous findings may lie in fundamental differences in the metabolism of Vitamin D in humans and rodents. Although both species express the same components of the Vitamin D endocrine system, how they respond to various doses is unknown. Furthermore, modelling Vitamin D deficiency in rodents is a fundamentally dietary challenge whereas in humans it is environmental. Differences between species in the capacity to metabolism Vitamin D should be considered further in future work modelling Vitamin D deficiency *in vivo* and *in vitro*.

A surprising finding from the current study was the variable response observed in MDC migration and fusion in response to 100nmol 1α,25$[OH]_2D_3$. These observations could be explained by negative regulation of the Vitamin D metabolic pathway. Large quantities of 1α,25$[OH]_2D_3$ have been demonstrated to cause a dose dependent induction of the CYP24A1 gene encoding for the enzyme 24-hydroxylase (Girgis et al., 2014b). This enzyme

is responsible for 24-hydroxylation and subsequent inactivation of 1α,25[OH]$_2$D$_3$ (to 24,25[OH]D), thus acts to 'block' 1α,25[OH]$_2$D$_3$ mediated VDR signalling activity. Moreover, unpublished observations from our laboratory have demonstrated that high dose Vitamin D$_3$ supplementation *in vivo* causes rapid increases in total serum 25[OH]D and subsequently 1,25[OH]$_2$D$_3$ that cause a concomitant rise in 24,25[OH]D concentration, supporting these *in vitro* data and the current hypothesis.

Taken together, these data provide novel evidence for a functional role of Vitamin D in skeletal muscle regeneration when administered at 10 nmol *in vitro*. Coupled with *in vivo* evidence there is now stronger data to further investigate the link between Vitamin D metabolites and the regeneration processes in a variety of populations.

Chapter 8

Thesis Synthesis

This chapter provides a synthesis of the main aims and outcomes of the investigations described in this thesis. Limitations and future direction of research are also discussed.

8.1 Realisation of Aims

8.1.1. Aim 1 – To investigate the role of Vitamin D concentration in human skeletal muscle function in vivo.

This aim was addressed in Chapter 4. Skeletal muscle function was assessed using an RCT in which individuals with total serum 25[OH]D <100 nmol.L^{-1} (41 ± 18.2 nmol.L^{-1}) were supplemented with 10,000 IU.day^{-1} Vitamin D_3 for 12 weeks. Voluntary and involuntary skeletal muscle function measures determined that despite increasing serum concentrations >100 nmol.L^{-1} this had no effect on skeletal muscle contractile properties. Furthermore, in a small sub group of individuals with more pronounced Vitamin D deficiency at baseline (<25 nmol.L^{-1}), supplementation was also ineffective. Therefore, the hypothesis that improving serum 25[OH[D >100 nmol.L^{-1} would improve contractile properties could not be accepted.

8.1.2. Aim 2 – To investigate the role of Vitamin D concentration in human skeletal muscle function ex vivo.

Aim 2 was also addressed in Chapter 4. It was decided upon to isolate single skeletal muscle fibres from biopsy specimens obtained from individuals with Vitamin D concentrations < 25 nmol.L^{-1} and analyse contractile properties *ex vivo*. Although a small n = 2 was analysed, no detectable differences in contractile properties were detected following a supplementation period of 8 weeks with 4,000 IU.day^{-1} Vitamin D_3 despite significant increases in serum 25[OH]D from <25 nmol.L^{-1} to >75 nmol.L^{-1}. Thus the hypothesis that improving serum 25[OH]D from a concentration of <25 nmol.L^{-1} to >75 nmol.L^{-1} would improve single fibre contractile properties was rejected. Taken together with findings from study 1, there is strong evidence to support the notion that skeletal muscle contractile properties of young otherwise healthy males is not affected by serum 25[OH]D ranging from 18 – 75 nmol.L^{-1}. However, it remains to be established whether serum concentrations associated with

hypercalcemia (<12.5 nmol.L^{-1}, severely deficient) also show no association with skeletal muscle contractile properties.

8.1.3. Aim 3 – To investigate the role of Vitamin D concentration in human skeletal muscle regeneration in vivo.

This aim was addressed in Chapter 5. Twenty males with serum 25[OH]D <75 nmol.L^{-1} (45 ± 20 nmol.L^{-1}) underwent a bout of eccentric exercise (20 x 10 eccentric contractions) prior to and following supplementation with either 4,000 IU.day^{-1} Vitamin D_3 or placebo. Maximal voluntary contraction force was assessed by isokinetic dynamometry at both 60 and 180 deg.sec^{-1} immediately prior to-, following and at 24, 48 hours and then at 7 days following the exercise bout. Supplemental Vitamin D_3 significantly elevated serum 25[OH]D and resulted in a 10.7% and 12.4% improvement in MVC force at 60 deg.sec^{-1} at 48 hours and 7 days following eccentric exercise, respectively. The placebo group showed no change prior to or following supplementation despite a significant reduction in serum 25[OH]D during the 6 weeks supplementation period. These findings allow acceptance of the hypothesis that increasing total serum 25[OH]D >75nmol.L^{-1} would lead to an improved recovery of MVC force following eccentric exercise. Therefore, it was necessary to explore potential cellular mechanisms that may account for these observations.

8.1.4. Aim 4 – To investigate the role of exogenous Vitamin D on the regeneration of human derived muscle cells in vitro.

Aim 4 was addressed in Chapters 6 & 7. Chapter 6 optimised the methodological aspects of an *in vitro* model of muscle regeneration. Muscle regeneration was then investigated in the context of Vitamin D in Chapter 7. As the muscle regeneration process involves numerous complex and overlapping steps, it was important to isolate aspects that could be replicated appropriately *in vitro*. Therefore, muscle derived cell migration to the site of damage and myoblast fusion were investigated in the presence or absence of 10 and 100 nmol $1\alpha,25[OH]_2D_3$. Findings from these investigations allowed partial acceptance of the

hypothesis that both 10 and 100 nmol doses would improve migration characteristics and fusion in a dose dependent manner. Both 10 and 100 nmol stimulated faster MDC migration speeds and distances, however only the 10 nmol dose led to improved biochemical and morphological aspects of myoblast fusion at 7 and 10 days post damage, whereas 100 nmol was detrimental to this process. These findings imply that Vitamin D_3 is involved in the processes that mediate cellular responses to muscle damage providing novel cellular aspects by which Vitamin D concentration associates with muscle regeneration *in vivo*.

8.2 Thesis Limitations

8.2.1. Chapter 4 – There were a number of limitations in Chapter 4. Markers of Vitamin D toxicity were not analysed thus compromising the efficacy of the supplementation protocol. An early indication of adverse effects to Vitamin D supplementation is hypercalciuria (Vieth, 2007); however this was not monitored in the current study. Symptoms of Vitamin D toxicity are wholly attributable to hypercalcemia/hypercalciuria, including nausea, dehydration and lethargy. No participants reported any of the associated symptoms. The dose of 10,000 $IU.day^{-1}$ was selected as previous trials including our own (Close et al., 2013a) have failed to achieve the desired serum 25[OH]D concentrations >100 $nmol.L^{-1}$ with lower doses. Additionally, data have demonstrated that oral Vitamin D intakes of 9,600 $IU \cdot day^{-1}$ are needed to obtain serum 25[OH]D concentrations above 100 $nmo.L^{-1}$ in 97% of a large cohort of 3667 participants (Garland, French, Baggerly, & Heaney, 2011). Clinical trial evidence and dermal photosynthesis of Vitamin D following UVB exposure supports our assumption that a prolonged daily dose of 10,000 $IU.day^{-1}$ is unlikely to cause adverse effects in otherwise healthy persons (Vieth, 2007). Toxicity monitoring and analysis of 24,25[OH]D (the product of Vitamin D catabolism) in future trials that employ high daily doses of Vitamin D will aid in establishing a safe upper limit for Vitamin D_3. A further limitation was that the sample size calculation for the current study determined that to enable

a 10% (21 Nm) increase in peak torque between pre-supplementation and post-supplementation with 80% power, n = 24 was required. Although 29 participants completed supplementation and blood sampling, only 22 completed all muscle function trials. This is a potential confounding factor, however it may be argued given that no trends were detected and observed *P*-values were strongly non-significant that it is unlikely a 2 participant deficit significantly affected these findings.

Single skeletal muscle fibres were isolated in order to study the effect of increasing serum 25[OH]D on single fibre contractile properties. Although a number of biopsies were obtained, only n = 2 were analyzed pre and post supplementation. This was due to constraints on the accessibility of the equipment needed to perform the analyses. Such a small sample does not allow definitive conclusions to be made and therefore larger samples should be analyzed to confirm the findings of section 4.8. Furthermore, the fibres analysed were not 'fibre-typed'. As described in the literature review of this thesis (2.1.2), skeletal muscle fibres expressing different myosin heavy chain isoforms exhibit different contractile properties. In order to make definitive conclusions about the fibres analyzed pre and post supplementation, future work should aim to increase the sample size analyzed and determine myosin heavy chain isoform expression via electrophoretic separation of isolated protein from the fibres and characterization by silver staining or immunoblotting.

8.2.2. Chapter 5 – The experimental design and interpretation of data in Chapter 5 are limited in that muscle damage was assumed to have occurred by measures of impaired voluntary contractile function without any histological or biochemical evidence. Therefore, it may be argued as to whether damage was indeed present since there are no direct markers provided. However, in argument against this notion, histological damage and uptake of extracellular dyes has been evidenced in animals and humans following eccentric exercise (Friden, Sjostrom, & Ekblom, 1981; Hamer, McGeachie, Davies, & Grounds, 2002; McNeil & Khakee, 1992; Newham et al., 1983). Furthermore, activation of satellite cells has been observed after a single bout of eccentric exercise (Hyldahl et al., 2014) suggesting the need

for adaptive remodeling in response to the damaging event. Future studies could expand on the work described in Chapter 5 by retrieving multiple biopsy specimens during the recovery from the exercise bout to assess the *in vivo* cellular response to eccentric exercise in Vitamin D deficient and sufficient individuals.

The sample population recruited in the current study were healthy, physically active males engaging in regular exercise training outside of day-to-day activity. However, comparatively to that of the elite performer, the current cohort is still considered novice. Untrained muscle is known to be more susceptible to exercise induced damage than that of trained individuals (Newton, Morgan, Sacco, Chapman, & Nosaka, 2008) and therefore the likelihood of seeing an augmented response to a nutritional aid or therapy is increased in an untrained population. Nevertheless, trained muscle still experiences muscle damage and therefore low Vitamin D concentration may indeed hamper the regenerative process. In keeping with discussion of damage susceptibility, the 'repeated bout effect' is the phenomenon that describes the reduced susceptibility of muscle to damage from eccentric exercise with repeated bouts. It may be argued that the individuals in the VITD treatment group were less trained than those of the PLB group hence the potentiated recovery following supplementation. Although efforts were made to control for this by block randomizing participants based on MVC torque and employing a strict inclusion criteria, it is unknown whether the individuals of the PLB group regularly participated in exercise programmes that incorporate eccentric loading. Neural mechanisms proposed for this effect are an increased recruitment of type I motor units thus spreading the workload of high fibre stress and/or recruitment of a larger motor unit pool. Mechanical mechanisms may be increased dynamic muscle stiffness and increased passive stiffness and cellular adaptations may be the longitudinal addition of sacromeres following eccentric exercise, adaptations in the inflammatory response and adaptations to maintain excitation contraction coupling (McHugh, 2003) Whether 6 weeks is sufficient to lose any of these proposed adaptations gained from a single bout of eccentric exercise is debatable (Nosaka, Clarkson, McGuiggin, & Byrne, 1991; Paddon-Jones, Muthalib, &

Jenkins, 2000; Smith et al., 1994). Finally, it is reasonable to question why an improvement in peak torque recovery was only observed at 60 deg.sec^{-1} and not at 180 deg.sec^{-1}. Observation of the data presented in Figure 5.3a shows that indeed an improvement did also occur in torque recovery of at 180 deg.sec^{-1} in the VITD group (3.6% improvement at 48 hours and 8.4% improvement at 7 days). Although this does not reach statistical significance there is still an observed physiological benefit. It is likely that a significant change was undetected as maximal force production is known to be elicited at slower dynamic movement velocities (Baltzopoulos & Brodie, 1989) and therefore the ability to detect subtle changes in peak torque may be lost at faster velocities.

8.2.3. Chapter 6 - For the optimisation of the *in vitro* model of muscle regeneration, the C_2C_{12} was used. These cells line are derived from murine satellite cells and are a purely myogenic cell line. Therefore, their capacity for myogenisis is arguably greater than the mixed populations of human derived cells used in the experimental trials. However, the C_2C_{12} cell line represents the best model to study myogenisis aside of primary cell lines isolated from whole tissue. Given that primary cells reach replicative senescence unlike immortalised cell lines, it was opted for to not deplete primary cell populations and utilise these stocks for experimental trials.

8.2.4. Chapter 7 - For the experimental trials described in Chapter 7, human MDCs were used. It was decided that cells retrieved from biopsy specimens would not be sorted (for example by magnetic activated cell sorting or MACS) to isolate a purely myogenic cell population. This approach was adopted due to the importance of non-myogenic cell types, such as fibroblasts in the wound healing process. Indeed, the migration and proliferation of fibroblasts is an important step preceding secretion of temporary ECM components and is fundamental to allow efficient migration and alignment of myoblasts and also serves to stabilize the tissue for the formation of new myofibres *in vivo* (Goetsch, Hawke, Gallardo, Richardson, & Garry, 2003). Moreover, reciprocal interactions between fibroblasts and muscle progenitors have been found to contribute more effective muscle regeneration *in*

vitro (Murphy, Lawson, Mathew, Hutcheson, & Kardon, 2011) a finding that has been replicated in other organized co-culture trials (Rao et al., 2013). Thus although it may be argued that varied populations of skeletal myoblasts and fibroblasts were used, implementing a co-culture model permits better modelling of *in vivo* wound healing in an *in vitro* assay.

A 2D model was utilised to study muscle regeneration *in vitro*. Although this method has strengths (see Chapter 6, section 6.1) and is appropriate for studying the migration of adherent cell types as well as the maturation of skeletal muscle myoblasts, there are aspects of the *in vivo* skeletal muscle niche that are not present in this model. Indeed mechanical stimuli, local and systemic factors implicated in cell behaviour during muscle regeneration are missing from 2D models of myogenesis. Bioengineering of 3D muscle constructs, a relatively new technique for the *in vitro* modelling of myogenisis partly overcomes the constraints of 2D systems by allowing better alignment and manipulation of mechanical stress (Sharples et al., 2012). Replication of the studies described in Chapter 7 of this thesis utilising a 3D cell culture system will provide more physiological insights as to how Vitamin D metabolites influence skeletal muscle regeneration.

Additionally, 10 and 100 nmol doses of $1\alpha,25[OH]_2D_3$ were utilised for the experimental trials described in Chapter 7. As discussed in section 7.3.3. these doses have widely been used to study the role of Vitamin D in numerous cell types including skeletal muscle. However, given that 100 nmol resulted in a detrminetal outcome for myoblast directional migration and fusion, it remains to be established what the most apporiate dose of exogenous $1\alpha,25[OH]_2D_3$ would be in a cell culture system to elicit beneficial responses. Although 10 nmol improved both migration and fusion of human skeletal myoblasts, this dose may also be higher than the optimal concentration to maximise these processes. Thus if this work were to be repeated, a broad dose response study should be implemented.

8.3 Future Directions

Although Vitamin D has been strongly implicated in skeletal muscle function, the data generated from this thesis suggest that non-pathological muscle of Vitamin D insufficient males is unlikely to show perturbations in contractile properties. It may be speculated that although data point towards a role of Ca^{2+} regulation as the factor by which Vitamin D which in theory may regulate muscle function, this may not be the mechanism by which Vitamin D treatment has previously resulted in positive outcome trials. As discussed, positive outcome data are limited to elderly cohorts in whom a loss of muscle mass and function occurs with age regardless of Vitamin D status. It may be the case that Vitamin D contributes to the preservation of muscle mass with age thus preventing further functional deficits. In vitro, Vitamin D regulates pathways related to control of muscle mass such as Notch signalling (Domingues-Faria et al., 2014), PI3K/Akt/mTOR (Buitrago et al., 2013; Garcia et al., 2011; Salles et al., 2013) and the ubiquitin proteasome degradation pathway (Bhat et al., 2013). Further, Vitamin D can enhance the stimulatory effect of insulin and leucine on muscle growth in cultured myoblasts (Salles et al., 2013). In Vivo, recent data support this idea as a high whey protein-, leucine, and Vitamin D-enriched supplement preserves appendicular muscle mass in older adults during a hypocaloric diet and resistance exercise program compared with compared with isocaloric control (Verreijen et al., 2015).

What is currently still unknown is whether cases of severe Vitamin D deficiency < 12 $nmol.L^{-1}$ may impact muscle function. Such concentrations are known to alter systemic Ca^{2+} homeostasis and may indeed also influence $Ca^{2+}[i]$ in skeletal muscle (Ceglia & Harris, 2013). However, the prevalence of such concentrations in young healthy individuals is uncommon according to literature reports and thus obtaining a large enough sample to study the relationship between such 25[OH]D concentrations and muscle function will be a challenge to the researcher. The data presented here do not disprove previous findings *per se* but pose new questions as to what Vitamin D concentration is important for normal skeletal muscle function:

1. Since Vitamin D concentrations above ~20 nmol.L^{-1} do not associate with changes in skeletal muscle function, do individuals with severe deficiency (<12.5 nmol.L^{-1}) show detectable changes in muscle function?
2. As Vitamin D deficient elderly populations have demonstrated improvements in muscle function with supplemental Vitamin D, does this suggest Vitamin D concentration is only important in aged muscle when comorbidities exaggerate the loss of muscle function experienced with age?

It was also demonstrated that Vitamin D might play a more important role in aspects of the muscle regeneration. The data presented demonstrate that increasing serum 25[OH]D concentrations improves the recovery from eccentric exercise and also provide novel insights implicating cellular aspects of the muscle regeneration process are responsive to Vitamin D. However, the molecular networks that orchestrate these cellular effects are yet to be established thus warranting further investigation. First steps were taken to characterise these pathways as it was shown that VDR is up regulated during myoblast fusion regardless of treatment although MRF expression was up regulated differentially based on treatment, suggesting VDR expression and activity may not be coupled. Additionally, although myoblast migration and fusion are important steps in muscle regeneration and regulated by Vitamin D, the activation and expansion of satellite cells are also steps preceding those investigated in the current work that should be explored in the context of Vitamin D. Taken together, the data suggest a causal relationship between Vitamin D and muscle regeneration, posing new research questions:

1. At what serum concentration of serum 25[OH]D is muscle regeneration impaired? Although a 75 nmol.L^{-1} inclusion criteria was implemented in the study described in Chapter 5, basal serum 25[OH]D ranged from 16-75 nmol.L^{-1}.
2. At what serum 25[OH]D concentration are no further improvements in maximal torque recovery observed?
3. What signalling pathways mediate the cellular remodelling response to Vitamin D?

4. Why does excessive Vitamin D treatment *in vitro* lead to impaired myoblast fusion?
5. Is VDR expression regulated regardless of ligand availability in myogenesis and does ligand availability influence VDR activity?
6. Is Vitamin D implicated in the activation and expansion of satellite cells?

A final consideration for this field of research is whether the Vitamin D classification system is useful for the study of individual systems. A vibrant debate at present is what serum concentrations should constitute deficiency and sufficiency. As described in Chapter 2, Section 2.4.10., governing bodies such as the US IoM suggest the classification system highlighted in Table 2.3. However, the current data would suggest that for skeletal muscle function these classifications are not meaningful since individuals with low serum 25[OH]D concentrations were tested and showed no changes at the whole tissue and single fibre scale of analysis. Defining the individual response of different tissues to Vitamin D and the interaction of genetic mutations in the Vitamin D endocrine system will help to define more appropriate recommendations on dietary Vitamin D requirements. However, at present the complexities and tissue specificity of Vitamin D signalling makes gross classifications impossible.

8.4 Conclusions and Implications

As described in the opening chapter of this thesis, low serum 25[OH]D concentrations are prevalent worldwide. Most tissues of the human body express the necessary components of the Vitamin D endocrine system to respond functionally to Vitamin D, including skeletal muscle. This thesis has shown that in non-pathological muscle, there are no disturbances to contractility in males with low serum 25[OH]D and raises new questions as to whether Vitamin D is implicated in contractile function. In contrast to muscle function, the data in this thesis have provided a systems based insight into Vitamin D and skeletal muscle damage demonstrating that Vitamin D is an important factor in skeletal muscle regeneration. Given the importance of skeletal muscle regeneration in training, performance and in ageing where an impaired ability to regenerate skeletal muscle is evident (Shi & Garry, 2006), it may be important to maintain optimal serum 25[OH]D concentration to optimise skeletal muscle remodelling in response to muscle damage. Screening of 25[OH]D is advisable to determine whether supplemental Vitamin D is required to maintain serum 25[OH]D concentrations of approximately 75 nmol or more. The data presented in this thesis show that this is easily achievable with 4,000 $IU.day^{-1}$ Vitamin D_3 and falls within the European Food Safety Authority (EFSA) safe upper limits for supplemental Vitamin D and may therefore be suggested as good practice to prevent impairments in the regenerative capacity of human skeletal muscle.

It is now a challenge to the field to determine what precise concentrations (both low and high) of Vitamin D are detrimental to muscle regeneration and to identify factors of the Vitamin D endocrine system that introduce variability in the response to supplementation. The widespread prevalence of low serum 25[OH]D should not be ignored as there is now ample evidence providing strong support that this is detrimental to many parameters of human health.

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ORIGINAL ARTICLE

Vitamin D supplementation does not improve human skeletal muscle contractile properties in insufficient young males

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Abstract
Purpose Vitamin D may be a regulator of skeletal muscle function, although human trials investigating this hypothesis are limited to predominantly elderly populations. We aimed to assess the effect of oral vitamin D_3 in healthy young males upon skeletal muscle function.
Methods Participants ($n = 29$) received an oral dose of 10,000 IU day^{-1} vitamin D_3 (VITD) or a visually identical placebo (PLB) for 3 months. Serum 25[OH]D and intact parathyroid hormone (iPTH) were measured at baseline and at week 4, 8 and 12. Muscle function was assessed in $n = 22$ participants by isokinetic dynamometry and percutaneous isometric electromyostimulation at baseline and at week 6 and 12.
Results Baseline mean total serum 25[OH]D was 40 ± 17 and 41 ± 20 nmol L^{-1} for PLB and VITD, respectively. VITD showed a significant improvement in total 25[OH]D at week 4 (150 ± 31 nmol L^{-1}) that remained elevated throughout the trial ($P < 0.005$). Contrastingly, PLB showed a significant decrease in 25[OH]D at week 12 (25 ± 15 nmol L^{-1}) compared with baseline. Despite marked increases in total serum 25[OH]D in VITD and a decrease in PLB, there were no significant changes in any of the muscle function outcome measures at week 6 or 12 for either group ($P > 0.05$).
Conclusions Elevating total serum 25[OH]D to concentrations > 120 nmol L^{-1} has no effect on skeletal muscle function. We postulate that skeletal muscle function is only perturbed in conditions of severe deficiency (<12.5 nmol L^{-1}).

Keywords Cholecalciferol · 25-Hydroxyvitamin D · Supplementation · Isokinetic dynamometry · Electromyostimulation · Fatigue

Abbreviations

25[OH]D	25-Hydroxyvitamin D
1,25[OH]D	1,25-Dihydroxyvitamin
Ca^{2+}	Calcium, Ionized
ECLIA	Enzyme-linked chemiluminescent immunosorbent assay
EMS	Electromyostimulation
FDA	Food and Drug Agency
iPTH	Intact parathyroid hormone
IU	International units
IKD	Isokinetic dynamometry
LC–MS/MS	Liquid chromatography tandem mass spectrometry
LLOQ	Lower limit of quantification
N	Newtons
Nm	Newton metres
nmol L^{-1}	Nanomoles per litre
PAR-Q	Physical activity readiness Questionnaire
Pmol L^{-1}	Picomoles per litre
rad s^{-1}	Radians per second

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RDI Recommended daily intake
US IoM United States Institute of Medicine
UVB Ultraviolet B

Introduction

Vitamin D is classically regarded as a vital endocrine regulator of bone health via its role in calcium and phosphate homeostasis (Anderson et al. 2012). However, a myriad of non-skeletal effects of the steroid hormone are now known and are predominantly attributable to the identification of the vitamin D receptor in virtually all tissues (Rosen et al. 2012) including skeletal muscle (Srikuea et al. 2012). Genome-wide analyses suggest that any improvement in vitamin D status significantly affects the expression of a large number of genes that have many biological functions (Hossein-nezhad et al. 2013). Specifically regarding skeletal muscle, it has long been understood that patients with osteomalacia resultant from inadequate dietary vitamin D intake often display an accompanying skeletal muscle myopathy presenting as a proximal muscle weakness that is responsive to vitamin D supplementation (Ziambaras and Dagogo-Jack 1997; Irani 1976; Smith and Stern 1967; Al-Said et al. 2009). Emerging data now suggest at a cellular level that skeletal muscle is indeed a tissue that responds functionally to vitamin D. Recent data have demonstrated in vivo improvements in mitochondrial oxidative function when severe vitamin D deficiency is corrected in humans (Sinha et al. 2013). Moreover, studies in chick embryo myoblast cultures have provided robust evidence that vitamin D is a regulator of intracellular Ca^{2+} concentration (Capiati et al. 2000; Morelli et al. 1993), which has also been observed in vivo in chicks (Vazquez et al. 1995) and in vitro in fully differentiated soleus muscle (de Boland et al. 1988). Vitamin D may therefore be implicated in skeletal muscle contractility, which early reports from vitamin D depleted rats have alluded to (Rodman and Baker 1978). Aside from these observations, reports suggest a role for the bioactive vitamin D compound 1,25-dihydroxyvitamin D (or 1,25[OH]D) in signalling pathways which involved myoblast proliferation and differentiation indicating a key role for vitamin D in muscle cell development and survival (Garcia et al. 2013; Srikuea et al. 2012; Stratos et al. 2013). Despite such evidence, vitamin D deficiency is still widespread around the globe (van Schoor and Lips 2011). We have previously shown that this observation is consistent in sub-populations of professional athletes and healthy, young athletic cohorts (Morton et al. 2012; Close et al. 2013a, b).

A number of trials have sought to assess the effect of vitamin D status on muscle function at a macroscopic scale. Insights from these investigations have established a link between vitamin D status and falls and various other physical performance measures such as handgrip, lower limb strength, balance and gait speed (Bischoff-Ferrari et al. 2004; Gerdhem et al. 2005; Houston et al. 2007; Marantes et al. 2011). However, such trials are limited to predominantly geriatric or clinical populations, and it is difficult to generalise such findings to young healthy populations, due to age-related skeletal muscle disease (i.e. sarcopenia). The few trials that have been conducted in young otherwise healthy cohorts have resulted in equivocal findings, with some investigations reporting beneficial effects of vitamin D supplementation in muscle (Sinha et al. 2013; Close et al. 2013b) and others observing no perceptible changes following supplementation (Close et al. 2013a). Hampering such findings further is large heterogeneity in outcome measures implemented to assess muscle function, low participant numbers, heterogeneity in supplementation protocols and varying baseline vitamin D status of the sample population making the cross comparison between findings difficult.

Adding further complexity to this issue is that the daily requirement for vitamin D needed to elevate total serum vitamin D concentrations to a level necessary for optimal physiological function is a point of debate. With regard to dose, the United States Institute of Medicine (US IoM) has set the RDI for vitamin D at 600 IU day^{-1} for young adults and tolerable upper intake at 4,000 IU day^{-1} (IoM 2011), although the US IoM set the 'no observed adverse effect limit' (NOAEL) at 10,000 IU day^{-1} (IoM 2011). In relation to total serum 25-hydroxyvitamin D (or 25[OH]D) concentration, the US IoM suggest >50 nmol L^{-1} as adequate whilst suggesting that concentrations exceeding 125 nmol L^{-1} may be detrimental to health. Others have suggested that these guidelines are too conservative and claim values exhibited in those living outdoors in sun-rich climates (~100 nmol L^{-1}) to be adequate, since they reflect the values present when the human genome evolved in such rich environments around equatorial Africa (Vieth 2011; Heaney 2011). An '*optimal*' 25[OH]D concentration for muscle function is currently still elusive and no study till date has tested the hypothesis that elevating serum 25[OH]D concentrations to >120 nmol L^{-1} is optimal for muscle function in healthy recreationally active individuals, as previously suggested (Heaney 2011). Our group has previously implemented doses of 20,000 and 40,000 IU $week^{-1}$ oral vitamin D_3, but failed to elevate serum concentrations >100 nmol L^{-1} (Close et al. 2013a). Thus higher doses may be necessary to achieve a total serum 25[OH]D concentration >100 nmol L^{-1}.

Given the available evidence and a clear lack of data in the area, we sought to investigate the impact of vitamin D status on lower limb muscle function using valid macroscopic techniques including percutaneous isometric

myostimulation (IMS) and isokinetic dynamometry (IKD) in healthy, recreationally active young men. We hypothesized that supplementing with high dose oral vitamin D_3 (cholecalciferol) to elevate total serum 25[OH]D concentrations > 100 nmol L^{-1} (suggested optimal Zittermann 2003; Heaney 2011; Vieth 2011) would have a beneficial effect on our chosen outcome measures of muscle function.

Methodology

Participants

Twenty-nine male participants (age = 22.7 ± 3 years; height = 179.3 ± 5.9 cm; weight = 76.1 ± 16.2 kg) volunteered to partake in the current trial. Participants received detailed information regarding the study procedures and were required to complete a PAR-Q, a medical history questionnaire and provide written informed consent before inclusion in the trial. Strict exclusion criteria were implemented for participant safety and validity of the investigation. Participants were excluded if they had underlying health problems as identified by completion of the medical history questionnaire, were sunbed users or taking vitamin D supplementation, standard oral multivitamins or, currently unfit to perform physical exercise, currently taking prescribed medication and those who expected a drastic change in their physical activity/training routine during the trial. After meeting these initial inclusion criteria, participants provided a venous blood sample that was analysed for total 25-hydroxyvitamin D (25[OH]D) concentration. Those presenting with total serum 25[OH]D concentrations ≥100 nmol L^{-1} were excluded from the trial. Ethical approval was granted by the local ethics committee of Liverpool John Moores University, and all data were collected in accordance with the Declaration of Helsinki.

Participants were block randomized based on their baseline 25[OH]D concentration and electrically evoked 20 Hz isometric knee extensor force into two experimental groups. Of the 29 initial participants, 22 completed all test procedures whilst the remaining 6 only took part in supplementation and blood sampling and one dropped out. This was due to an inability on the participants' behalf to commit sufficient time for each testing point. Fourteen participants were assigned to an oral vitamin D treatment group (VITD) and 15 participants were initially assigned to a visually identical placebo control group (PLB) (see Table 1).

Table 1 Block randomization data including mean total serum 25[OH]D concentration presented as nmol L^{-1} and mean isometric force from a single stimulation at a frequency of 20 Hz and stimulation intensity of 130 mA

	PLB	VITD
Mean total 25[OH]D (nmol L^{-1})	40	41
±SD	17	21
t test	$t = 0.867$, $P = 0.876$	
Mean isometric force (N)	267	307
±SD	69	67
t test	$t = -1.086$, $P = 0.303$	

Vitamin D supplementation

Participants allocated to VITD received 10,000 IU day^{-1} of vitamin D_3 (Bio-Tech Pharmacal. Arkansas, USA) taken as two 5,000 IU gelatin capsules. Those allocated to PLB received two visually identical placebo capsules each containing 90 mg cellulose (Bio-Tech Pharmacal. Arkansas, USA). The supplementation period lasted 12 weeks between the months of January and April to achieve a plateau in serum 25[OH]D response, as previously shown in a dose response trial with 10,000 IU day^{-1} vitamin D_3 cholecalciferol (Heaney et al. 2003). Both researcher and participant were blinded until completion of the study procedures.

Blood sampling

For the analysis of vitamin D metabolites and iPTH, participants provided a fasted venous blood sample collected from the antecubital vein into two K_2EDTA plasma (10 ml) vacutainers and two serum separator [(SST) 8.5 ml] vacutainers (Beckton Dickinson. Oxford, UK) prior to supplementation and then at 4, 8 and 12 weeks post-supplementation. SST samples were allowed to clot for 1 h at room temperature whilst K_2EDTA samples were immediately stored on ice, following which all samples were centrifuged for 15 min at 1,500 rcf at 4 °C. Serum and plasma were extracted and stored at −80 °C until required for analysis.

Analysis of vitamin D metabolites and intact parathyroid hormone (iPTH) concentration

For the analysis of vitamin D concentration 25(OH)D_2, 25(OH)D_3 were extracted from serum samples, following zinc sulphate protein precipitation, using Isolute C18 solid phase extraction cartridges. Potential interfering compounds were removed by initial elution with 50 % methanol followed by elution of the vitamins using 10 % tetrahydrofuran in acetonitrile. Dried extracts were reconstituted prior to injection into a high performance liquid chromatography tandem mass spectrometer (LC–MS/MS) in the multiple reaction mode. The multiple reaction mode transitions (*m*/*z*) used were 413.2 > 395.3, 401.1 > 383.3 and, 407.5 > 107.2 for 25(OH)D_2, 25(OH)D_3, and hexa-deuterated [OH]D_3, respectively. The assay

Table 2 (A) Imprecision of the LC–MS/MS assay and (B) Recovery efficiency of the assay

A

nmol L^{-1}	Chromsystem 1		Chromsystem 2		Calf serum	
	Vitamin D3	Vitamin D2	Vitamin D3	Vitamin D2	Vitamin D3	Vitamin D2
Intra-assay precision						
Mean	42.9	43.4	80.2	81.1	6.9	7.8
SD	4.1	3.4	7.8	8.6	0.7	0.8
%CV	9.5	7.8	9.7	10.6	10.1	10.2
Inter-assay precision						
Mean	40.4	32.4	76.8	95.3	7.7	7.8
SD	2.4	3.3	7.2	8.3	0.7	0.9
%CV	6	10	9.3	8.7	9.1	10.8

B

	Endogenous 25[OH]D_3 (nmol L^{-1})	Spiked (nmol L^{-1})	Measured value (nmol L^{-1})	%Recovery
Sample 1	5.5	50	53.7	96.4
Sample 2	10.2	100	103.4	93.8
Blank	0	500	491	98.2
	Endogenous 25[OH]D_2 (nmol L^{-1})	Spiked (nmol L^{-1})	Measured value (nmol L^{-1})	%Recovery
Sample 1	37.0	50	84	96.5
Sample 2	80.2	100	171.3	95
Blank	0	500	479	95.8

Intra precision was assessed by running quality control materials (Chromsystems) and commercially bought material (calf serum) ten times ($n = 10$) within a single run, and separately over 15 runs ($n = 15$) for inter-assay assessment. Variation is expressed as standard deviation (SD) and percentage of coefficient of variation (%CV)

was validated against published acceptance criteria (FDA 2001). Assay sensitivity was determined by the lower limit of quantification (LLOQ): 25[OH]D3 = 2.5 nmol L^{-1} and 25[OH]D2 = 2.5 nmol L^{-1}. Coefficients of variation (CVs) for the assay were 10 % across a working range of 2.5–625 nmol L^{-1} for both 25(OH)D_2 and 25(OH)D_3. Data regarding assay precision, detection and recovery are provided in Table 2A, B. Table 2A demonstrates imprecision of the assay and 2B shows recovery efficiency. Intra-assay precision was assessed by running quality control materials (Chromsystems Instruments and chemicals. Gräfelfing, Germany) and commercially bought material (calf serum) ten times ($n = 10$) within a single run, and separately over 15 runs ($n = 15$) for inter-assay assessment. Values used to determine assay variation are expressed as SD and %CV. Acceptance criteria define that %CV should not exceed 10 % in both intra- and inter-assay (Table 2A). Extraction recovery was assessed by determining the amount of vitamin D_3/D_2 recovered from the amount spiked prior to extraction. The percentage recovery was calculated by the measured value against the sum of endogenous value and spiking concentration. Spiked recovery is determined by adding known quantity of 25[OH]D3/D2 to serum samples with different levels of endogenous 25[OH]D (Table 2B). The data demonstrate good assay recovery, indicating that the procedure was able to efficiently extract vitamin D from matrix and the method was able to determine the amount recovered accurately. MassCheck calibration materials and controls are traceable against NIST 972 reference material. Furthermore, the LC–MS/MS method of analysis has previously been validated against other commercially available assays and is regarded as the most valid and reliable technique for the assessment of vitamin D metabolites (Snellman et al. 2010).

Classification of vitamin D status was based upon the US IoM (2011) guidelines (see Table 3).

For the analysis of iPTH, an Elecsys electrochemiluminescent immunoassay (ECLIA) by Cobas (Roche Diagnostics. Indianapolis, USA) was implemented. Fifty µL of plasma, a biotinylated monoclonal PTH-specific antibody and monoclonal PTH-specific antibody labelled with a ruthenium complex were formed into a sandwich complex. After addition of streptavidin-coated microparticles, the complex became bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture was aspirated into the measuring cell where the microparticles

Table 3 The US IoM (2011) guidelines for vitamin D status and suggested optimal concentrations (Zittermann 2003)

Serum 25[OH]D (nmol L^{-1})	Status
<12	Severely deficient
12 to <30	Deficient
30 to 50	Inadequate
>50	Adequate
>100 to 250	Suggested optimal (Zittermann 2003)
>120 to 225	Suggested optimal (Heaney 2011)

were magnetically captured onto the surface of the electrode. Unbound substances were then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results were determined via a calibration curve which is instrument-specifically generated by two-point calibration and a five-point master curve provided via the reagent barcode. The measuring range of the assay was 0.127–530 pmol L^{-1} (defined by the lower detection limit and the maximum of the master curve). The Elecsys ECLIA has previously been validated by multicentre evaluation as a valid and reliable measure of iPTH concentration (Hermsen et al. 2002).

Assessment of lower limb muscle function

A number of outcome measures were employed to validly assess muscle function of the lower limb. Participants completed both an IKD protocol and EMS protocol. All tests were completed at baseline, week 6 and week 12 of supplementation apart from IKD, which was only completed at baseline and week 12.

Isokinetic dynamometry

Isokinetic torque was assessed on a Biodex isokinetic dynamometer (Biodex Medical Systems Inc. Shirley, NY, USA), previously validated for its use in reliable assessment of muscle function variables related to force production (Drouin et al. 2004). Participants were seated as per the manufacturer's guidelines with a 90° flexion of the hip and non-extendable straps crossing the chest and abdomen and across the quadriceps to maximise isolation of the target muscle groups. The test protocol consisted of four consecutive maximal extension and flexion movements (Baltzopoulos and Brodie 1989) of the right quadriceps and hamstrings at two different fixed angular velocities, 1.05 rad s^{-1} (60 deg s^{-1}) and 3.14 rad s^{-1} (180 deg s^{-1}) from which peak torque (Nm) was generated, separated by a 5-min rest to allow full recovery of the high energy phosphate pool (Soderlund and Hultman 1991). Testing at two angular velocities produces valuable information as to how peak torque is produced by during different activation patterns (Baltzopoulos and Brodie 1989) as it is understood that neurological activation patterns of motor units changes as the angular velocity increases e.g. from 60 to 180 deg s^{-1} (Lesmes et al. 1978) thus replicating different locomotive activities. All participants were familiarized with the protocol until the coefficient of variation for each participant was <10 % (Atkinson and Nevill 1998) (Table 4).

Table 4 Co-efficient of variation for each isokinetic dynamometry variable

Measurement variable	Flexion 1.05 rad s^{-1}	Extension 1.05 rad s^{-1}	Flexion 3.14 rad s^{-1}	Extension 3.14 rad s^{-1}
CV%	3.9	3.7	4.9	4.1

Data are presented as group means from familiarization trials. 1.05 rad s^{-1} = 60 deg s^{-1}; 3.14 rad s^{-1} = 180 deg s^{-1}

Percutaneous isometric electromyostimulation

Stimulation was applied through surface electrodes, delivered via a BIOPAC systems MP100 stimulator (BIOPAC systems inc, Santa Barbara, CA, USA) and knee extension forces were recorded with a tension compression load cell (Tedea-Huntleigh, Vishay Precision Group Inc. Malvern, PA, USA) connected to a non-extendable strap attached to the participants' lower leg approximately 2–3 inches from the malleolus. Information collected from the force transducer was visualized and analysed using AcqKnowledge v.3.7.2 software (BIOPAC systems inc. Santa Barbara, CA, USA). Muscles were stimulated at an intensity of 130 mA for each test procedure as described previously (Gerrits et al. 2002). For the determination of muscle contractile properties, contraction was evoked by a 1-Hz twitch. For the determination of fatigue resistance, contractions were evoked by 20 Hz, 200-ms square wave pulses, previously defined to be representative of normal voluntary human motor neurone firing rates (Bigland-Ritchie et al. 1979), and thus physiologically valid when used to assess the fatigue resistance of human skeletal muscle.

For every session, the position of the participant in the isometric chair (Lido Active, Loredan, Davis, CA, USA) was standardized in accordance with guidelines previously published (Morton et al. 2005). Two 3 × 5 inch (8 × 13 cm) oval self-adhesive stimulating electrodes (Chattanooga, DJO Global, CA, USA) were placed approximately 2 inches above the knee joint across the vastus medialis (VM) and proximal to the acetabulofemoral joint across the vastus lateralis (VL). Participants were required to fully extend and contract the quadriceps to make the muscle body more apparent prior to electrode placement

to minimise antagonist coactivation, although this was not monitored with electromyography during stimulation.

To determine time to peak twitch and half relaxation time, a 1-Hz, 200-ms square wave pulse was administered. Time to peak twitch was determined as the time taken to reach peak isometric force from the onset of stimulation. Half relaxation time was determined as the time from peak stimulated force to reach half peak stimulated force. Test–retest reliability data revealed that the CV % for time to peak twitch and for ½ relaxation time as determined by a 1-Hz twitch at 130 mA was 4.5 and 5.7 %, respectively. Fatigue resistance was determined from 2 min repeated stimulation with 20 Hz, 200-ms square wave pulses every 750 ms (total of 160 evoked contractions equating to 32 s of total stimulation). Fatigue index was calculated as absolute force loss from the first stimulated contraction to the final stimulated contraction.

Statistical methods

A power calculation was performed by a priori using Minitab software (v.16) to allow for any dropouts based on detecting a significant change in serum 25[OH]D and increased peak torque in response to vitamin D supplementation. Pilot work from our laboratories during the winter months suggested that the standard deviation for test–retest serum 25[OH]D concentrations (taken 6 weeks apart) in young athletes is ~12 nmol L^{-1}. Furthermore, previous work from our laboratory demonstrated that standard deviation in isokinetic force at 1.04 rad s^{-1} (60 deg s^{-1}) is ~35 Nm. To enable the detection of a 50 nmol L^{-1} increase in total serum 25[OH]D concentration between pre-supplementation and post-supplementation with 80 % power, $n = 3$ participants per group was required. To enable a 10 % (21 Nm) increase in peak torque between pre-supplementation and post-supplementation with 80 % power, $n = 12$ per experimental group was required. Therefore, we initially recruited 30 participants to allow for dropouts and maintain statistical power.

For determination of test–retest reliability in familiarization trials, a paired sample t test was used. For the analyses of total serum 25[OH]D, PTH and all muscle function measurement variables, a two-way mixed design ANOVA was implemented. Where an interaction between independent variables was detected, simple main effects were explored with a one-way ANOVA. Significant main effects were explored using the Bonferroni post-hoc procedure and α was set at 0.05. In addition, a linear regression was plotted using Pearson's correlation to assess whether baseline 25[OH]D concentration was a predictor of Δ 25[OH]D in response to supplementation. All analyses were performed using SPSS (Predictive Analytics Software v.20, IBM Corporation, NY, USA). Data are presented as mean ± SD.

Results

Total serum 25[OH]D and iPTH

There was a significant interaction between group and time for total serum 25[OH]D ($P < 0.005$). Serum 25[OH]D concentration showed a significant main effect for time ($P < 0.005$) and treatment group ($P < 0.005$). Participants in VITD demonstrated significantly elevated total serum 25[OH]D concentrations at week 4 compared with baseline that remained elevated throughout the trial. Contrastingly, participants in PLB displayed total serum 25[OH]D concentrations significantly lower at week 12 compared with baseline (see Fig. 1a). In addition, regression analysis revealed that pre-treatment vitamin D concentration was a strong predictor of 25[OH]D response to supplementation in VITD, where 83 % of the variation in response to supplementation was attributable to baseline concentration (see Fig. 1b). VITD showed no significant change in iPTH concentration throughout the trial ($P = 0.322$), whereas PLB showed a significant increase in iPTH concentration at week 4 ($P < 0.005$), which remained elevated throughout the trial (see Fig. 1c) showing an inverse relationship with serum 25[OH]D.

Maximum voluntary contraction

There were no differences between experimental groups in peak torque production at either of the fixed movement velocities or for flexion/extension ($P = 0.610$). Furthermore there was no effect of experimental treatment as no changes were observed in peak torque production for either of the movement velocities or for flexion/extension following supplementation with 10,000 IU day^{-1} vitamin D_3 ($P = 0.345$, see Fig. 2).

Fatigue resistance

The effects of vitamin D supplementation on fatigue resistance of the VL and VM muscles can be seen in Fig. 3. Data are presented as absolute maximal force (Fig. 3a) and percentage of maximal force (Fig. 3b), although statistical analysis was performed on the fatigue ratio of absolute data only. No significant interaction was detected between treatment group and time i.e. baseline, week 6 and week 12 ($P = 0.134$). However, approaching significant main effect was also not observed for experimental group ($P = 0.063$).

Time to peak twitch and half relaxation time

No significant differences were detected between treatment groups for time taken to reach peak isometric twitch force ($P = 0.968$) or half relaxation time ($P = 0.924$).

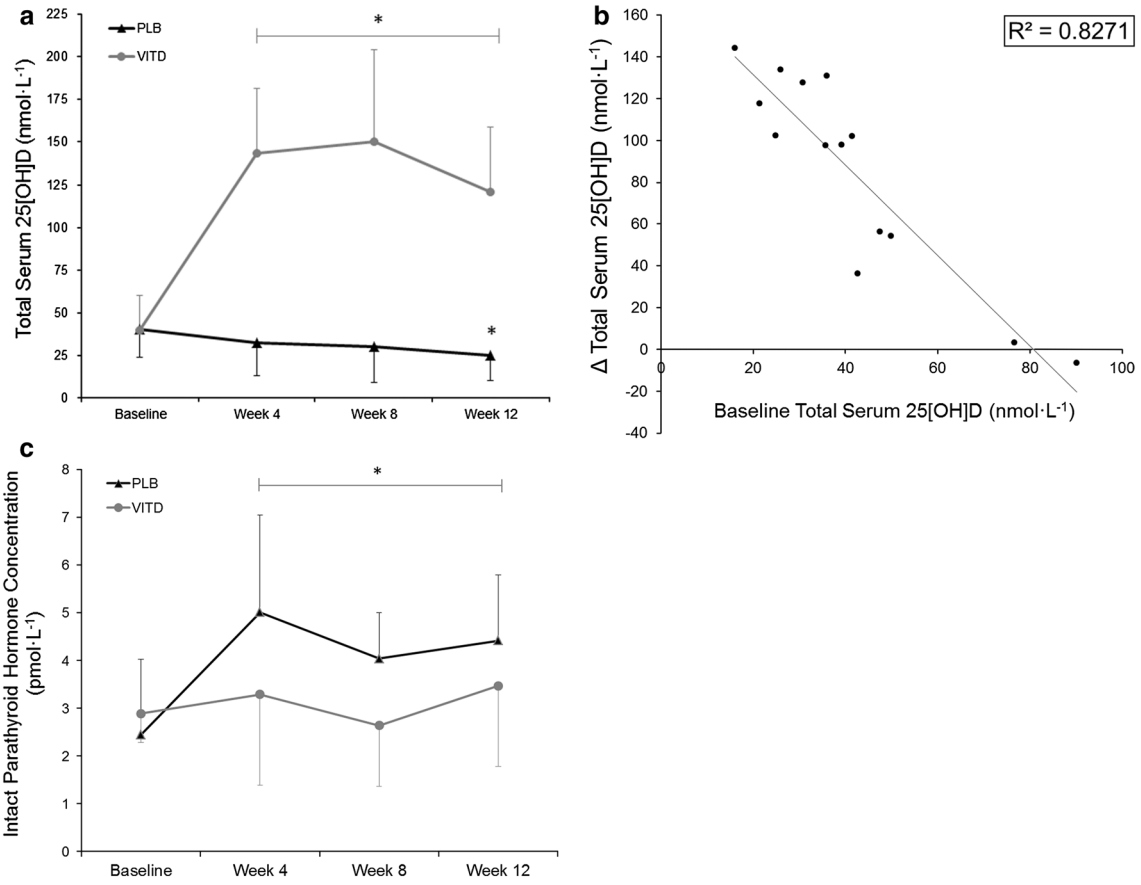

Fig. 1 **a** Response of total serum 25[OH]D to 12-week supplementation with 10,000 IU day^{-1} vitamin D_3 or placebo. **b** Linear regression describing the relationship between baseline vitamin D status and response to supplementation. Baseline total 25[OH]D concentration is the predictor variable whilst change in total 25[OH]D at 12 weeks is the response variable. **c** Response of intact parathyroid hormone to 12-week supplementation with 10,000 IU day^{-1} vitamin D_3 or placebo. (*Asterisk*) Denotes significance to baseline ($P < 0.05$)

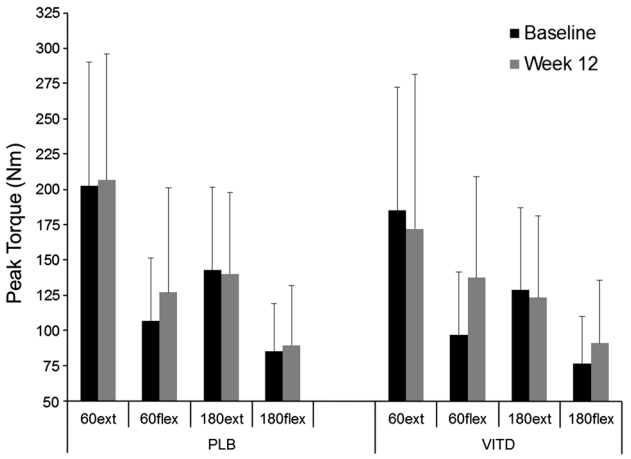

Fig. 2 Peak torque data from maximal extension (ext) and flexion (flex) of the right lower limb at two fixed velocities of 1.05 rad s^{-1} (60° s^{-1}) and 3.14 rad s^{-1} (180° s^{-1}) prior to and following 12-week supplementation with 10,000 IU day^{-1} or placebo

Furthermore, there was no significant effect of time for either time to peak twitch ($P = 0.861$) or half relaxation time ($P = 0.293$) indicating that a treatment effect was not observed (see Fig. 4).

Discussion

The aim of the current study was to investigate the impact of vitamin D status on muscle function in healthy, recreationally active young men. We hypothesized that supplementing with oral vitamin D_3 (cholecalciferol) to elevate total serum 25[OH]D concentrations > 100 nmol L^{-1} (suggested optimal Zittermann 2003; Heaney 2011; Vieth 2011) would have a beneficial effect on muscle function. However, we demonstrate that in a population of predominantly vitamin D inadequate young men, supplementation with 10,000 IU day^{-1} effectively elevated total serum 25[OH]D

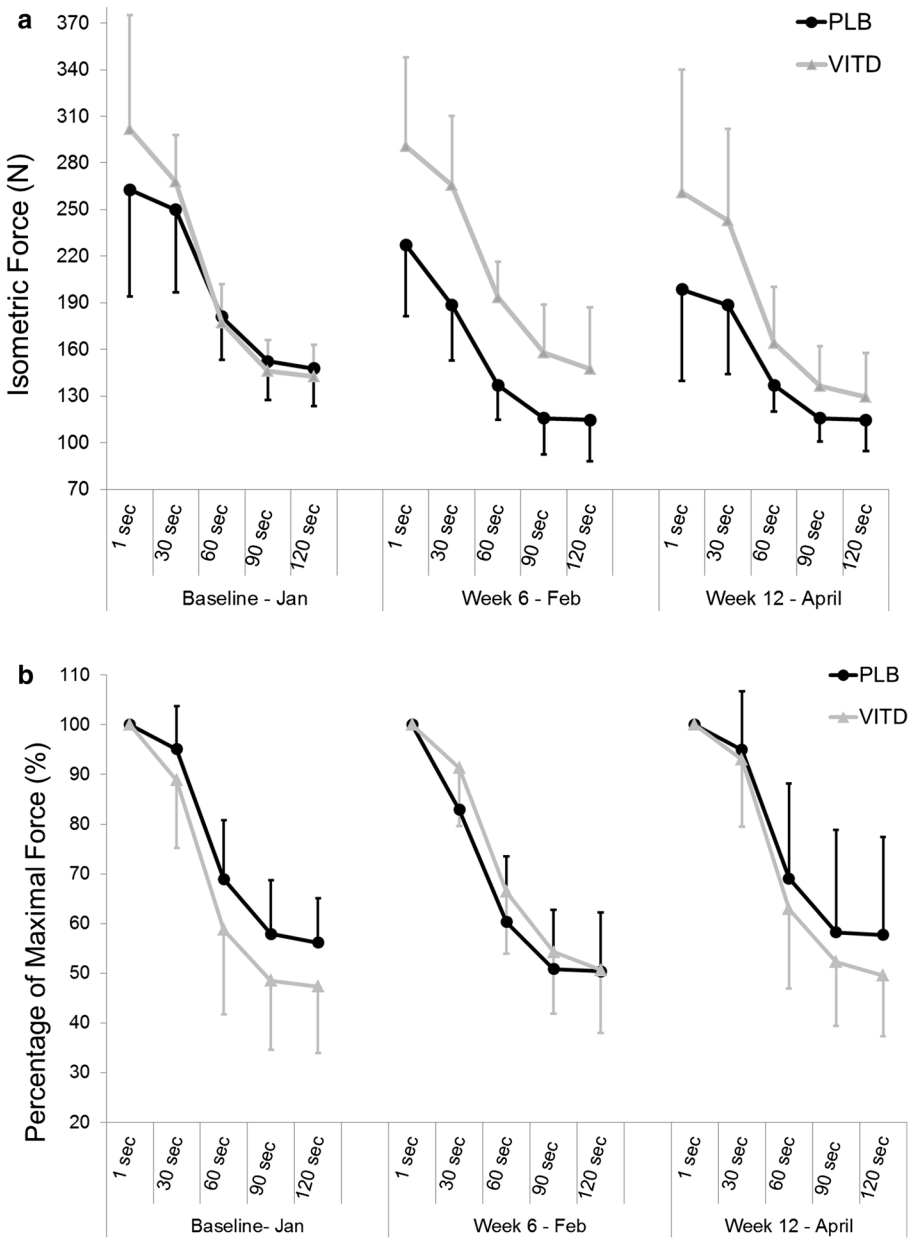

Fig. 3 **a** Absolute isometric force and **b** percentage force loss during 120 s of repeated unilateral lower limb stimulation from an electrically evoked square wave pulse of 200 ms, 20 Hz and a stimulation intensity of 130 mA, every 750 ms. Data presented prior to supplementation (baseline) and then at week 6 and 12 of supplementation

concentration to values >100 nmol L^{-1}; this had no effect (positive or negative) on muscle force, muscle fatigue or muscle contractile properties.

Our primary findings are in agreement with other randomized interventional trials assessing the impact of vitamin D supplementation on skeletal muscle function. Previous data from our group has demonstrated in a sample of 30 club level athletes with a mean 25[OH]D concentration of 51 nmol L^{-1} (SD ± 24 nmol L^{-1}) that neither 20,000 or 40,000 IU $week^{-1}$ of vitamin D_3 for 12 weeks were effective in improving 1-RM bench press, leg press or vertical jump height, although both doses achieved a significant elevation of total serum 25[OH]D (20,000 and 40,000 IU day^{-1} vitamin D_3 = 85 ± 10 nmol L^{-1} and 91 ± 24 nmol L^{-1}, respectively) after 12 weeks (Close et al. 2013a). Similarly, other studies predominantly undertaken in elderly cohorts have failed to detect improvements in strength parameters following supplementation (Kukuljan et al. 2011; Kenny et al. 2003). As highlighted in a systematic review and meta-analysis, trials in which the sample population display serum concentrations >25 nmol L^{-1} have failed to show improvements in muscle strength parameters following supplementation (Stockton et al. 2011).

We also report that elevating serum 25[OH]D > 100 nmol L^{-1} does not negate the force producing capacity, fatigue resistance or contractile properties of skeletal muscle in humans, an idea that has previously

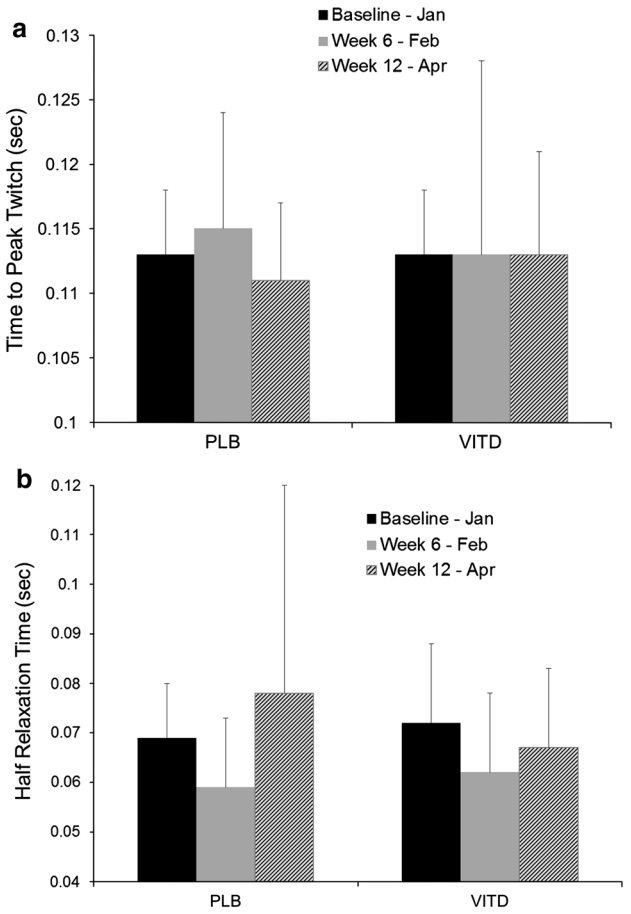

Fig. 4 Muscle contractile properties from an electrically evoked 1-Hz twitch and a stimulation intensity of 130 mA. Data are displayed from baseline, 6 and 12 weeks of supplementation with 10,000 IU day^{-1} vitamin D_3 or placebo

been suggested; One randomized controlled trial has previously reported that annual high dose vitamin D_3 (500,000 IU) effectively elevated serum concentrations of 25[OH]D to approximately 120 nmol L^{-1} in community dwelling older women (Sanders et al. 2010). However, this resulted in an *increased* risk of falls and fractures compared with a placebo control group. This observation could be attributable to the dosing strategy implemented, as smaller but more frequent doses in other RCT's do not show comparable findings. Furthermore, although Sanders et al. state that baseline prevalence of risk factors for falls and fractures was similar in both the supplemented and placebo groups, the physical activity profile of the two experimental groups is not reported. This is a confounding factor given that physical activity is a valuable counter measure for sarcopenia (Forbes et al. 2012), thus disparity in the physical activity profile of the two experimental groups is of importance when interpreting this data.

We also report that measurement of iPTH displayed an inverse relationship with serum 25[OH]D, in line with previous data (Sai et al. 2011). PLB showed elevated iPTH concentration whereas the opposite was observed for VITD, which may be explained by the understanding that 1,25-dihydroxyvitamin D represses the transcription of the PTH gene and also indirectly regulates PTH secretion by regulating the expression of calcium sensing receptors (Kumar and Thompson 2011). Thus, in addition to providing evidence that our findings produce no negative effect of high serum 25[OH]D concentrations (>100 nmol L^{-1}) on skeletal muscle function, we further demonstrate that our supplementation protocol offers a positive effect on the maintenance of iPTH concentrations. It is reported that high serum 25[OH]D concentrations (>100 nmol L^{-1}) may be necessary for disease prevention (Heaney 2011; Vieth 2011), thus we demonstrate that such concentrations needed to optimise other biological functions are unlikely to be detrimental to the normal function of skeletal muscle.

It is important to also consider the available evidence that suggests vitamin D supplementation has a *beneficial* impact on muscle function. Case studies have previously described a proximal muscle weakness associated with nutritional osteomalacia that is responsive to supplementation with vitamin D (Ziambaras and Dagogo-Jack 1997; Irani 1976; Smith and Stern 1967; Al-Said et al. 2009). Recently, in contrast to our current findings, we have observed an improvement in 10-m sprint times and vertical jump height following supplementation in a group of vitamin D deficient young athletes (Close et al. 2013b). A recent trial has also demonstrated in a sample of severely deficient individuals (all <15 nmol L^{-1}) that supplementation with 20,000 IU of vitamin D_3 on alternate days for 10–12 weeks significantly elevated serum 25[OH]D (>100 nmol L^{-1}) and resulted in significant improvements in phosphocreatine recovery half-time (τ½ PCr) of the soleus muscle, indicative of improved mitochondrial oxidative function as measured by ^{31}P-magnetic resonance spectroscopy (Sinha et al. 2013). Furthermore, in a systematic review previously mentioned (Stockton et al. 2011), the authors also discuss that though participants with baseline 25[OH]D >25 nmol L^{-1} show no change in muscle strength with vitamin D supplementation, a limited number of trials demonstrate an increase in proximal muscle strength in adults with vitamin D *deficiency*. A common underlying feature of these positive outcome trials is that a large portion of the sample population exhibited *severe* vitamin D deficiency (<12 nmol L^{-1}) at baseline that was corrected with vitamin D_3 supplementation. It is reasonable to postulate based on such findings that skeletal muscle function only displays perceptible perturbations in function when vitamin D status of the sample population reaches concentrations <12 nmol L^{-1}.

Taking the available evidence into account, we propose a possible reason that an improvement in muscle function was not detected in the current study, because muscle function was not impaired to begin with as no participants had serum 25[OH]D concentrations in the range <12 nmol L^{-1}. If one assumes a sigmoidal physiological response of skeletal muscle to vitamin D, it may be difficult to detect a response when nutrient status is already on the ascending limb or plateau of the response curve (Lappe and Heaney 2012). Thus skeletal muscle may only display sharp decrements in function at serum 25[OH]D concentrations <12 nmol L^{-1}, but thereafter shows no perceptible added benefit of the increasing concentrations. This could be due to a disturbance of systemic and intracellular calcium concentrations that occur in severe deficiency, affecting both contractile activity and intracellular signalling, and a notion previously put forward (Girgis et al. 2012). Although speculative, this would explain much of the human data available on vitamin D status and muscle function.

We acknowledge that there were limitations in the current study design regarding supplemental dose and response. We did not measure markers of vitamin D toxicity thus compromising the efficacy of our supplementation protocol. An early indication of adverse effects to vitamin D supplementation is hypercalciuria (Vieth 2007); however, this was not monitored in the current trial. Symptoms of vitamin D toxicity are wholly attributable to hypercalcemia/hypercalciuria, including nausea, dehydration and lethargy. No participants reported any of the associated symptoms. The dose of 10,000 IU day^{-1} was selected as previous trials including our own (Close et al. 2013a) have failed to achieve the desired serum 25[OH]D concentrations >100 nmol L^{-1} with lower doses. In addition, data have demonstrated that oral vitamin D intakes of 9,600 IU day^{-1} are needed to obtain serum 25[OH]D concentrations above 100 nmol L^{-1} in 97 % of a large cohort of 3,667 participants (Garland et al. 2011). Clinical trial evidence and dermal photosynthesis of vitamin D following UVB exposure supports our assumption that a prolonged daily dose of 10,000 IU•day^{-1} is unlikely to cause adverse effects in otherwise healthy persons (Vieth 2007). The authors are, however, in support of toxicity monitoring in future trials that employ high daily doses of vitamin D. Further, our sample size calculation determined that to enable a 10 % (21 Nm) increase in peak torque between pre-supplementation and post-supplementation with 80 % power, $n = 24$ was required. Although 29 participants completed supplementation and blood sampling, only 22 completed all muscle function trials. This is a potential confounding factor, however, it may be argued given that no trends were observed, and *P*-values were strongly non-significant that it is unlikely a two-participant deficit that significantly affected our findings.

Conclusions

In summary, the current investigation has added to a body of evidence that suggests young healthy adults present with inadequate and deficient vitamin D concentrations in the winter months. We have demonstrated that elevating serum 25[OH]D concentrations in a predominantly insufficient group to concentrations >100 nmol L^{-1} may be effectively achieved by daily supplementation with 10,000 IU vitamin D_3 (cholecalciferol); however, this does not improve or negate skeletal muscle function. We propose that muscle function of healthy young adults is likely only aberrant in a state of severe vitamin D deficiency (<12 nmol L^{-1}) and thus future work should assess this. Due to the inter-individual variation in baseline vitamin D status and response to supplementation, it is crucial that the baseline vitamin D concentration of the sample population being tested is considered in any interventional trial as this may have a significant impact upon the outcome of the trial.

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Conflict of interest The authors declare no conflicts of interest.

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Vitamin D and the athlete: Emerging insights

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REVIEW ARTICLE

Vitamin D and the athlete: Emerging insights

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Abstract
Interest in Vitamin D has risen considerably recently with many athletes now advised to take daily vitamin D supplements. The reason for this interest is partly not only attributed to the resurgence of the Vitamin D-deficient disease rickets but also due to the discovery of a Vitamin D receptor in many tissues suggesting a more global role for Vitamin D than previously considered. Unlike the other vitamins that are obtained through the diet, Vitamin D is unique since endogenous synthesis following ultraviolet B (UVB) exposure is the predominant route of entry into systemic circulation. Moreover, Vitamin D could be better classed as a seco-steroid, given that its structure is similar to that of a steroid, and its production is derived from a cholesterol precursor (7-dehydrocholesteol) in the skin. The classification of Vitamin D status is currently subject to considerable debate with many authors opposing governing body recommendations. Regardless of the suggested optimal concentration, there is now growing evidence to suggest that many athletes are in fact Vitamin D deficient, especially in the winter months largely as a consequence of inadequate sun exposure, combined with poor dietary practices, although the consequences of such deficiencies are still unclear in athletic populations. Impaired muscle function and reduced regenerative capacity, impaired immune function, poor bone health and even impaired cardiovascular function have all been associated with low Vitamin D in athletes, however, to date, the majority of studies on Vitamin D have described associations and much more research is now needed examining causation.

Keywords: *25-hydroxyvitamin D, 1α,25-dihydroxyvitamin D, muscle, bone, supplementation, ultraviolet B radiation*

1. Introduction

There are numerous examples that provide evidence that many of the great civilisations heralded the sun for its apparent benefit to human health. In ancient Greece, Herodotus was said to have recommended 'solaria' as a cure for weak and flabby muscles, and thus the ancient Greek Olympians were told to train and lie under the sun's rays (Mercola, 2008). Such observations were made long before the realisation that sunlight was essential for Vitamin D synthesis. In the past decade, we have witnessed a considerable increase in research attention towards the 'sunlight vitamin' (Vitamin D), which is in part due to the re-emergence of the preventable bone disorder rickets. In recent years, there have been a number of key findings which have advanced the field considerably, particularly the identification of the Vitamin D receptor (VDR) in many tissues through which Vitamin D exerts many of its effects (reviewed by Demay, 2006). Moreover, the generation of the VDR knockout mouse (Li et al., 1997) has provided great insight into the multiplicity of roles for Vitamin D. It is now understood that aspects of innate and acquired immunity (Chun, Liu, Modlin, Adams, & Hewison, 2014), bone health (Ebeling, 2014), cardiovascular health (Lavie, Dinicolantonio, Milani, & O'Keefe, 2013) and biological processes within skeletal muscle are just some of the physiological features thought to be regulated by Vitamin D. This review will explore Vitamin D synthesis, considerations when assessing and interpreting measurement of Vitamin D status, the biological actions of Vitamin D relevant to the athlete and protocols for supplementation with Vitamin D.

2. Vitamin D synthesis

The human genome has developed the mechanism for Vitamin D synthesis in the skins' dermis that is

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activated by sunlight, or more specifically, ultraviolet B (UVB) radiation (Holick, 1995). During this photosynthetic reaction, the B-ring of 7-dehydrocholesterol (precursor to cholesterol and previtamin D_3) is cleaved and resultantly, the thermodynamically unstable previtamin D_3 (pre-cholecalciferol) is formed. Thermal isomerisation following this cleavage yields Vitamin D_3, a seco-steroid hormone. In human skin, the thermal isomerisation process converts ~80% of previtamin D_3 to Vitamin D_3 within 8 hours of exposure to UVB (Tian, Chen, Matsuoka, Wortsman, & Holick, 1993).

There are many factors that can affect the dermal synthesis of Vitamin D. For example, the amount of radiation that actually reaches the biosphere (thus available for cutaneous Vitamin D photosynthesis) is a product of wavelength and the amount of ozone that solar radiation must pass, which itself is a function of the solar zenith angle dependent on latitude, season and time of day (Chen, Chimeh et al., 2007). If UVB reaches the skin, another influential factor impacting the subsequent photosynthetic reaction is skin pigmentation as melanin competes with 7-dehydrocholeterol for UVB radiation. Resultantly, those with darker skin require exposure to a stronger source of UVB or more prolonged exposure time to elicit comparable changes in circulating Vitamin D concentration seen in lighter skinned persons (Chen, Chimeh et al., 2007; Clemens, Henderson, Adams, & Holick, 1982).

An obvious alternative route for obtaining vital nutrients not naturally synthesised (such as the other vitamins) is through dietary intake. Indeed, there are some, but very few, dietary sources of Vitamin D, although as will become evident, it is unlikely that humans obtain enough Vitamin D from such sources to compensate for a lack of sun exposure. Unlike dermal synthesis that solely produces Vitamin D_3, dietary intake provides both Vitamin D_2 and D_3. Dietary sources of Vitamin D include oily fish, eggs, fortified breakfast cereals, shitake mushrooms and powdered milk. However, intake of these foods appears to be poor in developed countries (Department of Health National Diet and Nutrition Survey, 2011; Hill, O'Brien, Cashman, Flynn, & Kiely, 2004; Moore, Murphy, Keast, & Holick, 2004; Tylavsky, Cheng, Lyytikainen, Viljakainen, & Lamberg-Allardt, 2006). Large-scale investigations have identified that <2% of individuals studied meet the recommended daily allowance (RDA) for Vitamin D intake from foods (Hill, Jonnalagadda, Albertson, Joshi, & Weaver, 2012). Worryingly, Chen, Chimeh et al. (2007) provide evidence that many foods stating fortification with Vitamin D may in fact contain <80% of the Vitamin D claimed on the label, whilst 14% of samples tested contained no detectable Vitamin D.

3. Vitamin D metabolism

Regardless of how Vitamin D is obtained (through dermal synthesis or obtained from dietary sources), both forms will travel in the circulation bound to the Vitamin D-binding protein (DBP) (Cooke & Haddad, 1989). It is of importance to note that Vitamin D_2 (ergocalciferol) which enters systemic circulation from dietary food sources undergoes the same hydroxylation steps as Vitamin D_3; however, D_3 has a relative potency to Vitamin D_2 of 9.5:1 (Armas, Hollis, & Heaney, 2004).

Once circulating bound to DBP, Vitamin D will undergo two important hydroxylation steps occurring in the liver (via 25-hydroxylase) and in the kidney (via 1α-hydroxylase) or tissues directly expressing 1α, 25-hydroxylase. The resultant seco-steroid, 1α,25-dihydroxyvitamin D (1,25$[OH]_2$D), is metabolically active, and it is this form of Vitamin D that exerts its biological effects. The efficacy of tissues expressing 1α,25-hydroxylase to convert DBP-bound 25[OH]D will be dependent upon the ability of the cell to internalise the 25[OH]D-DBP complex. In skeletal muscle, this is likely to be achieved by the expression of membrane-bound megalin that has recently been identified (Abboud et al., 2013).

Specifically relating to skeletal muscle, following this final activating hydroxylation step, 1α,25$[OH]_2$D will bind to the VDR and exert genomic and non-genomic effects. Non-genomic effects appear to be rapid and research focus has pointed to a prominent role for 1α,25$[OH]_2$D in stimulating signalling pathways including the PI3/Akt and mitogen-activated protein kinase/extracellular signal related kinase (MAPK/ERK) pathways (Buitrago, Pardo, & Boland, 2013). Genomic effects rely on heterodimerisation of the 1α,25$[OH]_2$D-VDR complex with retinoic x receptor (RXR) acting on Vitamin D response elements (VDREs). The VDR-RXR complex interacts with transcription machinery and recruits specific coactivators with chromatin modifying enzymatic activity that consequently recruit transcription factors to gene promoter regions. The complex may also recruit corepressors allowing regulation of target gene expression related to the function of the cell. In this way, cellular demand may be translated to cellular action (Ingraham, Bragdon, & Nohe, 2008). The pathway from vitamin D synthesis to cellular effect is summarised in Figure 1.

4. Assessing Vitamin D status

4.1. What, why and how to measure it

Over 40 metabolites of Vitamin D have been identified (Bouillon, Okamura, & Norman, 1995), but most of these have a very short half-life in the circulation and so are currently of minimal interest.

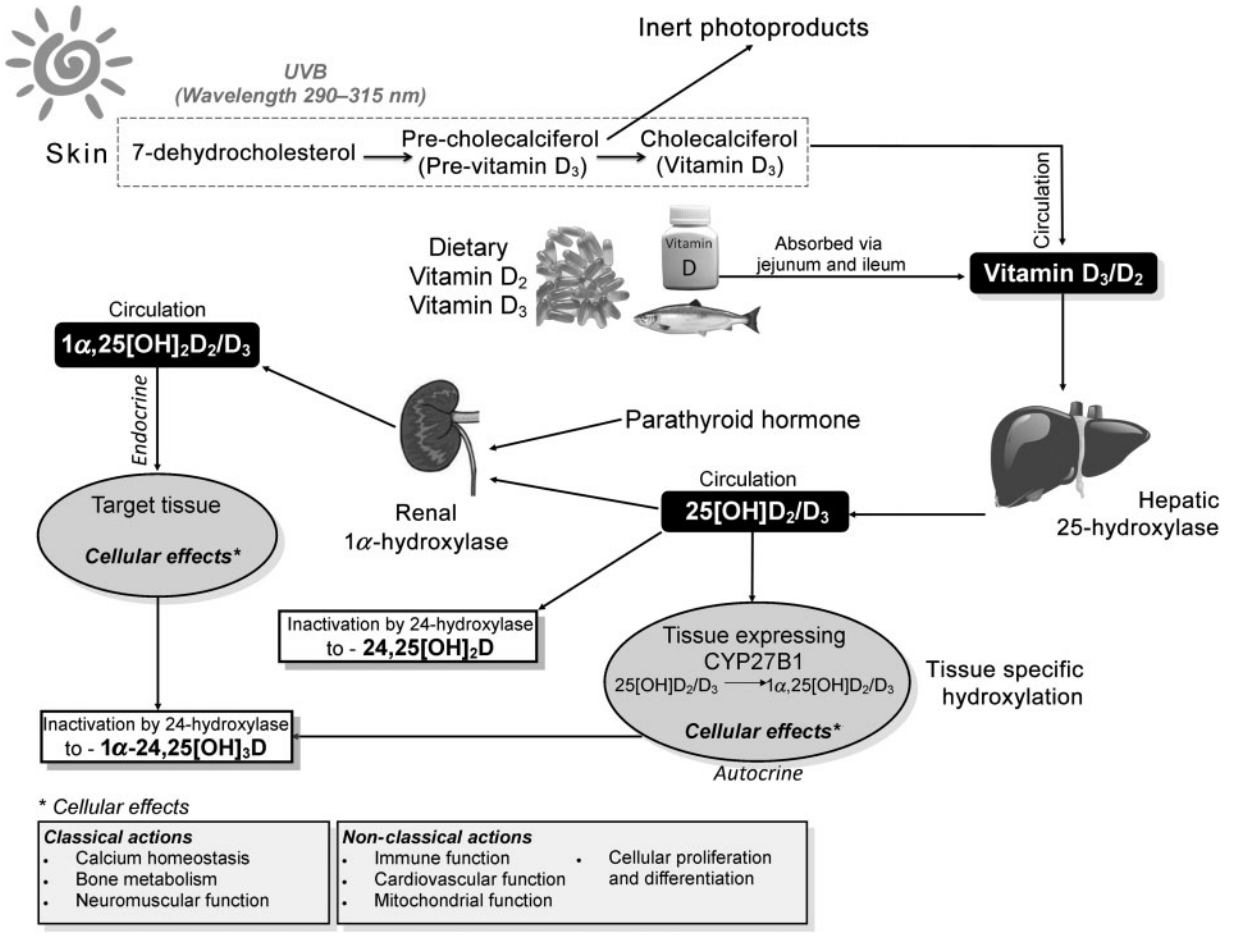

Figure 1. Schematic representation of the Vitamin D synthetic and metabolic pathways. Vitamin D is obtained via dermal synthesis, a self-regulated process that produces vitamin D_3 or inert photoproducts when sufficient production is achieved. Vitamin D is also obtained in both D_2 and D_3 forms from the diet. Both endogenous production and exogenous intake elevate serum concentrations of vitamin D, which undergoes two hydroxylation steps (via hepatic 25-hydroxylase and renal 1α-hydroxylase or target tissues expressing CYP27B1) to activation. The active 1α-25[OH]$_2$D exerts its effects via the VDR and stimulates rapid non-genomic and genomic effects. Inactivation of the active 1α-25[OH]$_2$D occurs via hydroxylation at C-24 catalysed by the 24-hydroxylase enzyme.

Although the parent sterol Vitamin D has a half-life close to 24 h (Clemens, Actams, Nolan, & Holick, 1982), this is relatively short compared to 25[OH]D with a half-life of 21–30 days (Zehnder et al., 2001). The measurement of circulating 25[OH]D is a better indicator of Vitamin D stores whether obtained from UVB exposure (contributing 80–90% of 25[OH]D) or dietary sources (contributing 10–20%). The most potent physiologically active, circulating metabolite produced by humans is 1α,25 dihydroxyvitamin D (1α,25[OH]$_2$D), which has a half-life of 4–15 h, while 25[OH]D circulates in nmol.L^{-1} concentrations, 1α,25[OH]$_2$D is present in much lower concentrations (pmol.L^{-1}).

Supplementation of foods and health products or physician treatment with either Vitamin D_2 (ergocalciferol) or D_3 (cholecalciferol) can increase the percentage derived from exogenous sources, and so assay technology needs to be able to measure both D_2 and D_3 metabolites. The main rate-limiting step in the pathway of Vitamin D metabolism is the 25 hydroxylation that takes place in the liver. This step is primarily dependent on the substrate concentration (Vitamin D) and is the reason why seasonal variability exists related to UVB exposure. 1-hydroxylation mainly takes place in the kidney but can also happen in bone, skin, placenta and granuloma tissue (sarcoid and tuberculosis) (Zehnder et al., 2001) and requires 25OHD$_2$/D$_3$ as the substrate. The rate of 1α,25[OH]$_2$D production by the kidney can be influenced by the prevailing calcium (Ca) and parathyroid hormone (PTH) concentration. For these reasons, as well as the short half-life, measurement of total 1,25[OH]$_2$D is a poor indicator of overall Vitamin D status as total 25[OH]D needs to decrease to around 10 nmol.L^{-1} for total 1,25[OH]$_2$D to decrease significantly (Need et al., 2008).

Isotope dilution liquid chromatography–mass spectrometry (LC-MS/MS) is considered the gold standard method for measuring serum total 25[OH]D and can simultaneously quantitate 25[OH]D$_2$ and 25 [OH]D$_3$ with summation of the two values, resulting

in an accurate and precise measure of total 25[OH]D. All immunoassays evaluated appropriately have a problem with the recognition of 25[OH]D_2 and under-recover this molecule whether added in the natural/synthetic form or estimated in the circulation following ingestion. Detailed comparison of the performance of commercially available assays inclusive of assay data can be found in Snellman et al. (2010).

4.2. What do the numbers mean?

The two most commonly reported units of measurement for 25[OH]D are ng.ml^{-1} and nmol.L^{-1}, where 1 ng.ml^{-1} = 2.496 nmol.L^{-1}. The SI unit for Vitamin D is nmol.L^{-1} and will be used herein. Following quantification by the assay of choice, data are produced giving an indication of circulating Vitamin D in the sample tested. 25[OH]D_2 and 25[OH]D_3 may both be analysed and referred together as total serum 25[OH]D. At present, there is disparity as to what serum 25[OH]D concentrations constitute deficiency, adequacy and an 'optimal' status. The US Institute of Medicine (US IoM) at present provide guidelines that should be accepted until revision (<12 nmol.L^{-1} severely deficient; 12–<30 nmol.L^{-1} deficient; 30–50 nmol.L^{-1} inadequate; >50 nmol.L^{-1} adequate). However, it is noteworthy that numerous scientific researchers disagree with such guidelines and propose that they are too conservative and outdated.

4.3. Supplementation efficacy and dose

As with the measurement units used for circulating metabolites of Vitamin D, there is more than one unit of measurement for supplemental Vitamin D. It is extremely important to distinguish between international units (IU) and μg (100 IU = 2.5 μg Vitamin D_2/D_3). Clearly, confusing these two units could result in extremely large or very low and ineffective doses of Vitamin D. A further consideration before supplementation is whether Vitamin D_2 or D_3 is more effective. Quite simply, Vitamin D_3 is approximately 87% more potent in raising and maintaining serum 25[OH]D concentrations and produces two- to three-fold greater storage of Vitamin D than does equimolar D_2 (Heaney, Recker, Grote, Horst, & Armas, 2011). This is intuitive, given that the primary route of obtaining Vitamin D is via dermal synthesis, a process that produces Vitamin D_3.

A further consideration to the efficacy of supplementation is that there exists great inter-individual differences in baseline 25[OH]D concentration, and this can have a profound impact upon the response to supplementation. For example, baseline Vitamin D concentration is a strong predictor of the response to supplementation, i.e. those with low basal serum 25[OH]D show the greatest response to supplementation (see Figure 2, redrawn from Close, Leckey, et al., 2013; Owens et al., 2014). Moreover, individuals with similar baseline 25[OH]D may not respond to supplementation in a comparable way, given that genetic variation in the Vitamin DBP gene also appears to influence responsiveness to supplementation (Nimitphong, Saetung, Chanprasertyotin, Chailurkit, & Ongphiphadhanakul, 2013).

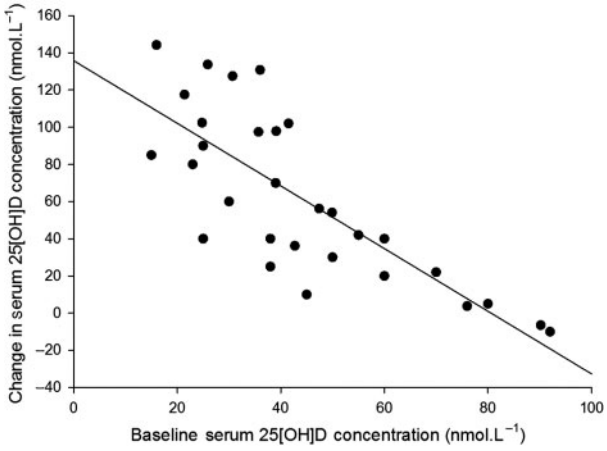

Figure 2. Relationship between baseline serum 25[OH]D concentrations and the magnitude of change in serum 25[OH]D in response to Vitamin D_3 supplementation. Data presented are an amalgamation of Close, Russell, et al. (2013) and unpublished data from our laboratory. Oral Vitamin D_3 administered was between 2000 and 10,000 IU per day.

Serum 25[OH]D responds to supplementation in a dose-dependent manner (Heaney, Davies, Chen, Holick, & Barger-Lux, 2003). However, along with basal 25[OH]D, the desired 25[OH]D concentration will influence the supplementation dose. This remains a topic of hot debate, and a universal serum concentration for optimisation of all physiological functions affected by Vitamin D is far from being established. However, it is becoming more commonly accepted that the health of many biological systems and processes including the immune system, cardiovascular system, the nervous system and the cell cycle is better at serum 25[OH]D concentrations >75 nmol.L^{-1} (Heaney, 2013). To achieve serum concentrations >75 nmol.L^{-1}, we have previously demonstrated that supplementation with oral Vitamin D_3 at a dose of 5000 IU.day^{-1} for 8 weeks can effectively achieve this (Close, Russell, et al., 2013). Other authors suggest that estimated serum concentrations needed for other health benefits are above 100 nmol.L^{-1} and that a daily requirement of 9600 IU.day^{-1} is needed to maintain such serum concentrations (Garland, French, Baggerly, & Heaney, 2011). In support, Holick and colleagues have demonstrated that adults in a bathing suit exposed

to 1 minimal erythemal dose tanning bed (UVB) radiation raises blood levels of Vitamin D to levels equivalent to those achieved by ingesting between 10,000 and 25,000 of Vitamin D (Holick, 2002). Notably, these supplemental doses would be considered ~8× and ~16× of the recommended daily intake (RDI), respectively, as the US Institute of Medicine (US IoM) has set the RDI for Vitamin D at 600 IU.day^{-1} (for young adults) and tolerable upper intake (TUI) at 4000 IU.day^{-1} (IoM, 2011) which is in accordance with recent guidance by the European Food Safety Authority (EFSA, 2012). Thus scientific opinion of numerous research groups is in contrast to that of governing bodies.

When interpreting such opinions, it is vitally important to consider that dermal synthesis of Vitamin D via UVB exposure is self-regulated with previtamin D being converted to inactive photoproducts when synthesis is no longer required (Holick, MacLaughlin, & Doppelt, 1981). Such a biological mechanism is not available for oral ingestion, thus it is prudent to adhere to the current TUI of 4000 IU. day^{-1} suggested by both the IoM and the EFSA until further evidence is generated.

5. Vitamin D status of athletes

From the available literature and our own findings, it is evident that there is great inter-individual variation in basal Vitamin D concentration in participants tested at the same point of the year. As an example, Figure 3 demonstrates cumulative findings from a trial conducted in our laboratory during the winter months across a range of athletic disciplines. A notable observation from the data is that many tested athletes present with Vitamin D concentrations which are considered deficient by the US IoM.

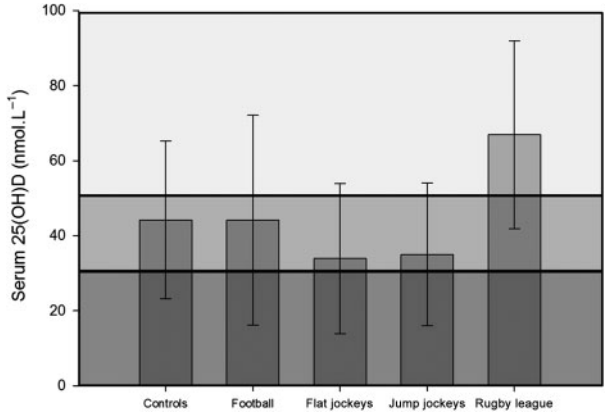

Figure 3. Serum 25[OH]D concentration of a variety of athletes (and age-matched controls) in relation to current classification guidelines; dark grey area represents deficiency (<30 nmol.L^{-1}), lighter grey area insufficiency (<50 nmol.L^{-1}) and lightest grey area sufficiency (>50 nmol.L^{-1}). (Data redrawn from Close et al., 2012).

Furthermore, other reports from around the world have shown similar findings to our own, supporting the notion that Vitamin D deficiency and insufficiency is widespread among athletic communities (Allison et al., 2014; Bescos Garcia & Rodriguez Guisado, 2011; Magee et al., 2013; Wolman et al., 2013). This may have numerous adverse effects that may ultimately attenuate normal physiological function, as will be described. It must be stressed that due to the 'mega-dose' supplementation culture in professional and amateur sport, it is also important to consider that although supplementation guidelines exist, athletes do indeed take doses of vitamin D well above the TUI level. It is, therefore, vital to be aware of the adverse effects of extreme vitamin D supplementation, which will be discussed later in Section 7.

6. Physiological roles of Vitamin D relevant to the athlete

6.1. Vitamin D and muscle

It has long been understood that a proximal muscle weakness (myopathy) associates with nutritional osteomalacia (caused by severe Vitamin D deficiency) and is responsive to supplementation with Vitamin D (Al-Said, Al-Rached, Al-Qahtani, & Jan, 2009; Irani, 1976; Smith & Stern, 1967; Ziambaras & Dagogo-Jack, 1997). Recently, our group has observed an improvement in 10-m sprint times and vertical jump height following supplementation with 5000 IU day^{-1} in a group of Vitamin D-deficient young athletes (Close, Russell, et al., 2013). Others have also demonstrated in a sample of severely deficient individuals (<15 nmol·L^{-1}) that supplementation with 20,000 IU of Vitamin D_3 on alternate days for 10–12 weeks significantly elevated serum 25[OH]D (> 100 nmol·L^{-1}) and resulted in significant improvements in phosphocreatine recovery half-time (τ½ PCr) of the soleus muscle, indicative of improved mitochondrial oxidative function as measured by ^{31}P-magnetic resonance spectroscopy (Sinha, Hollingsworth, Ball, & Cheetham, 2013). A feature underlying all of these positive outcome trials is a large portion of the sample population exhibit *severe* Vitamin D deficiency (<12.5 nmol.L^{-1}) at baseline that is corrected with Vitamin D_3 supplementation.

Some investigations have failed to detect improvements in muscle function following supplementation with Vitamin D. Systematic review and meta-analysis of the current literature show that participants with baseline 25[OH]D > 25 nmol.L^{-1} show no change in muscle strength following Vitamin D supplementation including our own recently published observations (Owens et al., 2014) in which we

have sensitively assessed skeletal muscle contractile properties and peak torque production.

At the cellular and molecular level, positive findings have been consistently reported for Vitamin D-related investigations. Specifically, control of intracellular Ca^{2+} (Vazquez, de Boland, & Boland, 1997, 1998), enhanced stimulatory effect of Leucine (Salles et al., 2013), stimulation of mitogen-activated protein kinases (MAPKs; (Buitrago et al., 2013; Buitrago, Ronda, de Boland, & Boland, 2006) and promotion of promyogenic and angiogenic growth factors (Garcia, Ferrini, Norris, & Artaza, 2013; Garcia, King, Ferrini, Norris, & Artaza, 2011) have all been reported. If these findings are translated *in vivo*, improvements in muscle contraction, growth and regeneration following damage may be observed when vitamin D status is optimal; however, such work is lacking considerably at present.

6.2. Vitamin D and muscle regeneration

A fascinating feature of human skeletal muscle is its ability to respond to positive and negative stimuli, a characteristic that is vital in adaptation to a training bout or in recovery from damaging exercise. This regenerative process relies on the resident muscle stem cells termed 'satellite cells', wedged between the sarcolemma and basal lamina (Mauro, 1961). Briefly, in response to appropriate cues satellite cells will exit quiescence, proliferate and differentiate to regenerate areas of damaged muscle. An exciting novel role emerging for Vitamin D is related to this process of skeletal muscle regeneration as the seco-steroid appears to display an ability to mediate myoblast proliferation and differentiation (Buitrago et al., 2013; Garcia et al., 2011, 2013; Srikuea, Zhang, Park-Sarge, & Esser, 2012). Findings have demonstrated that in the absence of 25[OH]D, 1α,25$[OH]_2$D or the VDR, signalling of pathways necessary for growth and survival such as MAPKs, Src and Akt (PKB) are reduced and muscle cell development is impaired (Buitrago et al., 2013; Garcia et al., 2011).

Aside from the ability to proliferate and differentiate in response to appropriate stimuli, migration of myoblasts to and within the site of damage is also a key step in the regenerative process of adult skeletal muscle (Saini, Faulkner, Al-Shanti, & Stewart, 2009). Following muscle damage, myoblasts have the capability to migrate towards the site of repair (Watt, Karasinski, Moss, & England, 1994). Furthermore, the ability to migrate is necessary for proper alignment and fusion of myoblasts to form multinucleated myotubes (Leloup, Mazeres, Daury, Cottin, & Brustis, 2006; Vaz, Martins, Thorsteinsdottir, & Rodrigues, 2012). Recently, insights into the signals involved in myoblast migration have alluded to an important role for PI3/Akt and MAPK/ERK pathways in myoblast migration (Dimchev, Al-Shanti, & Stewart, 2013). As described, these pathways are indeed mediated by Vitamin D, at least in C_2C_{12} myoblast models. Figure 4 provides a graphical interpretation highlighting the potential points at which Vitamin D may influence skeletal muscle regeneration.

Recently, one group has demonstrated that a high-dose Vitamin D treatment in rats improves the cellular turnover and recovery of soleus muscle crushed *in vivo* (Stratos et al., 2013). Muscle sampling in this study revealed Vitamin D enhanced cell proliferation and attenuated apoptosis when compared with rats treated with a vehicle solution. Additionally, the expression of prolyl-4-hydroxylase-β was increased in Vitamin D-treated rats, evidence of increased extracellular matrix proteins. In another study employing a rat model, investigators observed a decline in rotator cuff healing in diet-induced Vitamin D-deficient rats (Angeline et al., 2014). Angeline and colleagues demonstrated significant decreases in load to failure and in collagen matrix proteins in deficient rats. Indeed, the skeletal muscle extracellular matrix is comprised of such matrix proteins and is crucial in signalling events that occur in skeletal muscle regeneration (Lund & Cornelison, 2013).

In humans, data on the role of Vitamin D in muscle recovery are still lacking. However, promising preliminary observations suggest that basal Vitamin D concentration predicts immediate and persistent muscle weakness after damaging exercise. In particular, correlative data suggest that serum concentrations >75nmol.L^{-1} may be necessary for optimal force recovery following damage (Barker et al., 2013). Given the available evidence, this area of research warrants further attention as it may have direct implications for monitoring the Vitamin D status of athletes to promote optimal muscle recovery.

6.3. Vitamin D and bone health

Vitamin D is essential for skeletal health as it promotes calcium absorption from the bowel and enables mineralisation of newly formed osteoid tissue in bone (Francis, Anderson, Patel, Sahota, & van Staa, 2006). The major manifestation of Vitamin D deficiency is osteomalacia in adults and rickets in children, which the Department of Health (DoH) suggests are generally associated with a serum total 25[OH]D concentration of <20 nmol.L^{-1} (DoH, 1991). Vitamin D insufficiency may lead to secondary hyperparathyroidism, bone loss and fragility fractures in older people (Bischoff-Ferrari, Giovannucci, Willett, Dietrich, & Dawson-Hughes, 2006; Lips, 2001; Sahota, Gaynor, Harwood, & Hosking, 2001; Sahota et al., 2004), although data in young otherwise healthy individuals is less documented.

The Institute of Medicine (IoM) Report on Dietary Reference Intakes for Calcium and Vitamin D investigated the relationship between Vitamin D status and bone health (IoM, 2011) using evidence from two systematic reviews commissioned by the Agency for Healthcare Research and Quality (AHRQ), from the University of Ottawa (Chung et al., 2009) and the Tufts Evidence-Based Practice Centre (Cranney et al., 2007). These examined the relationship between serum total 25[OH]D as a marker of Vitamin D exposure and PTH concentration, calcium absorption, calcium balance, bone mineral density (BMD), fracture risk and rickets/osteomalacia as potential indicators of bone health. From these analyses, the IoM highlighted the inconsistency that exists with different threshold serum total 25[OH]D concentrations above which PTH reaches a plateau, ranging from <30 nmol.L^{-1} to 100–125 nmol.L^{-1}. The IoM also suggested that most people with a serum total 25[OH]D between 30 and 50 nmol.L^{-1} have adequate calcium absorption.

Meta-analyses of randomised controlled trials (RCTs) investigating the effect of Vitamin D supplementation on fractures indicate that combined calcium and Vitamin D supplementation modestly decrease the risk of hip and other non-vertebral fractures, whilst Vitamin D alone is ineffective (DipartGroup, 2010; Hathcock, Shao, Vieth, & Heaney, 2007). AHRQ-Tufts concluded that combined Vitamin D and calcium supplementation decreased fracture risk in institutionalised older people, but the effect in community-dwelling older people was inconsistent. They suggested that a serum 25[OH]D of 40 nmol.L^{-1} is sufficient to meet the Vitamin D requirement for bone health in 50% of the population, whilst 50 nmol.L^{-1} would be sufficient for 97.5% of the population. They, therefore, concluded that people are at risk of deficiency when serum 25[OH]D is <30 nmol.L^{-1} but suggest that some people are potentially at risk of inadequacy when serum 25[OH]D is 30–50 nmol.L^{-1}. Although some authors recommend much higher values for serum total 25[OH]D (>75 nmol.L^{-1}) for optimal bone health, there is significant debate regarding the data and the analysis that leads to these conclusions. No randomised placebo controlled trial has confirmed the optimal target for circulating serum total 25[OH]D to be above 75 nmol.L^{-1} when the outlook is bone health. It should, however, be noted that many athletes do present with serum 25[OH]D <50 nmol.L^{-1} with some <10 nmol.L^{-1} and may, therefore, benefit from targeted interventions to improve bone health. Notably, we have recently demonstrated that professional flat and national hunt jockeys often present with serum 25[OH]D concentrations of 37.6 ± 28 and 35.1 ± 14 nmol.L^{-1}, respectively, along with poor markers of bone health such as elevated C-terminal telopeptide (β-CTX) concentrations and negative Dual Energy X-ray Absorptiometry (DXA) T scores (Wilson et al., 2013).

6.4. *Vitamin D and immune function*

The VDR and Vitamin D metabolising enzymes are present in many cells of the immune system. Indeed, one of the most established roles for Vitamin D is its

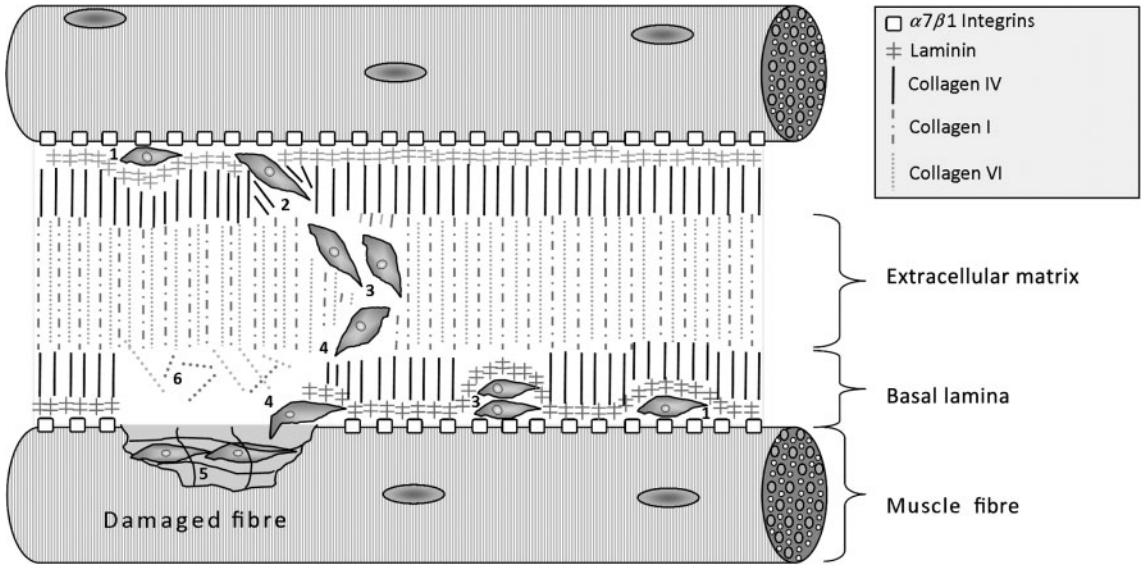

Figure 4. Schematic representation of the proposed roles of Vitamin D in skeletal muscle regeneration. (**1**) Quiescent satellite cell (**2**) activated satellite cell (**3**) proliferating myoblasts, known to be mediated by Vitamin D (Garcia et al., 2011; Srikuea et al., 2012) (**4**) migrating myoblast that requires PI3/Akt and MAPK/ERK pathways, both mediated by Vitamin D (Buitrago et al., 2013) (**5**) differentiating myoblasts, known to be mediated by Vitamin D (Garcia et al., 2011) (**6**) formation of ECM proteins, which Vitamin D has been implicated in (Angeline et al., 2014; Stratos et al., 2013).

ability to regulate aspects of innate and adaptive immunity. Therefore, Vitamin D deficiency is likely to directly compromise optimal functioning of aspects of the immune system causing increased infection frequency.

Immune cells from the adaptive arm of the immune system (T and B cells) express the VDR as well as the Vitamin D metabolising enzymes. In particular upon activation, the VDR is up-regulated in these cells, leading to profound modulation of Vitamin D responsive genes implicated in proliferation and in differentiation (Chen, Sims et al., 2007). In accordance, data have demonstrated that the immunomodulatory effects of Vitamin D on the adaptive immune system appear to be directed through the control of proliferation and differentiation of B cells and various T cell subtypes (Baeke, Takiishi, Korf, Gysemans, & Mathieu, 2010). This has important implications for normal antibody production and prevention of an exaggerated immune response (Prietl, Treiber, Pieber, & Amrein, 2013).

In relation to innate immunity, Vitamin D appears to enhance chemotaxis and the phagocytic capability of macrophages and monocytes (Baeke et al., 2010). Additionally, 1,25$[OH]_2$D up-regulates the transcription of antimicrobial peptides (AMPs), acting as a key link between toll-like receptor (TLR) activation and the antimicrobial response. AMPs are produced by epithelial cells and macrophages and essentially act as broad spectrum antibiotics, thus are an important first line of defence against invading microbes. Accordingly, data have demonstrated that Vitamin D-deficient persons exhibit lower concentrations of AMPs such as cathelicidin (Jeng et al., 2009) and low Vitamin D status associates with an increased frequency of upper respiratory tract infections (URTI) (Ginde, Mansbach, & Camargo, 2009; Laaksi et al., 2007). Aside from directly influencing antimicrobial activity, Vitamin D targets antigen-presenting cells such as monocytes and dendritic cells; 1,25$[OH]_2$D may alter the morphology of such cells to a more tolerogenic state (Baeke et al., 2010). These effects are likely to induce T regulatory cells (Treg) which are pivotal in controlling normal immune responses (Lemire, Adams, Sakai, & Jordan, 1984).

In relation to the athlete, it is well known that regular moderate exercise is associated with reduced infection frequency when compared with sedentary individuals. In contrast, prolonged bouts of strenuous exercise can lead to transient depression of aspects of immune function, lasting from 3 to 24 hours after exercise. Further, periods of overreaching may compound this immunosuppressive response (see Gleeson, 2007 for comprehensive review). This, therefore, potentially places Vitamin D-deficient athletes in a heightened immunocompromised state at greater infection risk. Recently, Mike Gleeson's laboratory has provided the first evidence that confirms such a hypothesis. The group found that in a cohort of 267 athletes (He et al., 2013), significantly more participants who presented with Vitamin D deficiency (<30 nmol.L^{-1}) experienced URTI symptoms compared with participants with concentrations >120 nmol.L^{-1}, and immune function markers also showed improvement in individuals with higher Vitamin D concentrations.

In summary, Vitamin D deficiency is likely to directly compromise optimal functioning of aspects of the immune system causing increased infection frequency. Given the available data, this appears to hold true for athletic performers, putting periodised training programmes at risk of disturbance and increases the probability of suboptimal adaptation to training stimuli. The discussed findings provide robust evidence to support the maintenance of adequate Vitamin D concentrations, particularly during the winter months when sunlight exposure is low and infection frequency is high.

7. Vitamin D toxicity: When is too much a problem?

Most investigators believe that Vitamin D toxicity manifests through hypercalcaemia. This is rarely seen unless the Vitamin D dose is very high as a result of inappropriate high-dose treatment or accidental overdosing (Hathcock et al., 2007). The high serum calcium leads eventually to soft tissue calcification and resultant renal and cardiovascular damage. An IoM report summarised the evidence from a number of supplementation studies of Vitamin D (IoM, 2011), which covered a range of doses (800–300,000 IU.day^{-1}) and duration (months–years) and concluded that Vitamin D below 10,000 IU.day^{-1} is not usually associated with toxicity, whereas doses equal to or above 50,000 IU.day^{-1} for several weeks or months are frequently associated with toxicity with documented hypercalcaemia. Notably, however, both the IoM and the EFSA declare 4000 IU.day^{-1} as the TUI as a preventative measure to avoid rare cases of toxicity occurring with vitamin D intakes <10,000 IU.day^{-1}.

Studies that report a reverse J-shape curve for the relationship between mortality and 25[OH]D, show a beneficial effect as serum total 25[OH]D concentrations increase to 30 nmol.L^{-1}, with lowest mortality at 50 nmol.L^{-1} and then increased risk above 75 nmol.L^{-1} (Durup et al., 2012). However, like many Vitamin D studies, the paper by Durup et al. was not able to assign cause and effect, and therefore, caution must be taken when interpreting these data. Studies in post-menopausal women have also raised concerns regarding high-dose replacement therapy. The use of a 500,000 IU Vitamin D dose resulted in an increase in falls and fractures in the

group receiving Vitamin D and in a further study of 300,000 IU single dose of Vitamin D an increase in falls was observed. Therefore, whilst toxicity does appear to be rare, there is a genuine risk, and athletes should certainly be discouraged from unmonitored *mega dose* supplements and encouraged to follow evidence-based guidelines provided by the US IoM and the EFSA.

8. Summary and future directions

Despite the resurgence in interest into the effects of Vitamin D on athletic performance, due to a lack of high-quality research studies, particularly those trying to establish cause and effect, it is still difficult to give definitive advice to the athlete. What is clear is that many athletes tested in the winter months would appear to be Vitamin D deficient, and this does not exclude athletes that live in sun-rich environments such as Western Asia (Hamilton, Grantham, Racinais, & Chalabi, 2010). It would, therefore, appear sensible to supplement these athletes with Vitamin D_3 to correct such deficiencies, which in turn has the *potential* to improve muscle function, bone health, immune health and preliminary data suggest even cardiac structure (Allison et al., 2014). Whilst at present it is not possible to give an *ideal* serum concentration of 25[OH]D that athletes should strive to achieve, there are data to suggest that values >75 nmol.L^{-1} have the potential to improve immune health and skeletal muscle regeneration following muscle injury (Barker et al., 2013; He et al., 2013). What is clear, however, is that extreme deficiencies (i.e. <25 nmol.L^{-1}) could impair skeletal muscle function, and it is therefore sensible to test all athletes and recommend supplementation as appropriate. As far as a dose is concerned, we have demonstrated that doses of 2000–5000 IU per day for 8 weeks increase serum 25 [OH]D > 75 nmol.L^{-1}. Considering current guidance provided by the US IoM and the EFSA and given the fact that our doses are well within the safe upper limit suggested by the IoM (i.e. <10,000 IU), daily dosing with 4000 IU may be a sensible dose for supplementation. Future research must now focus on establishing if Vitamin D deficiencies are having detrimental physiological effects or are simply correlated with such events. High-quality mechanistic studies are now clearly warranted adopting a systems biology approach to answer such questions. Only then will it be possible to understand the role of Vitamin D in athletic performance and begin to identify if there is such a thing as an 'optimal Vitamin D concentration' for athletes.

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A systems-based investigation into vitamin D and skeletal muscle repair, regeneration, and hypertrophy

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[1]*Research Institute for Sport and Exercise Science, Liverpool John Moores University, Liverpool, United Kingdom;* [2]*Department of Sport and Exercise Sciences, Glyndwr University, Plas Coch Campus, Wrexham, United Kingdom;* [3]*Faculty of Medicine and Health Science, Norwich Medical School, University of East Anglia, Norwich, United Kingdom; and* [4]*Medical Research Council-Arthritis Research UK Centre for Integrated Research into Musculoskeletal Ageing, University of Liverpool, Liverpool, United Kingdom*

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Owens DJ, Sharples AP, Polydorou I, Alwan N, Donovan T, Tang J, Fraser WD, Cooper RG, Morton JP, Stewart C, Close GL. A systems-based investigation into vitamin D and skeletal muscle repair, regeneration, and hypertrophy. *Am J Physiol Endocrinol Metab* 309: E1019–E1031, 2015. First published October 27, 2015; doi:10.1152/ajpendo.00375.2015.—Skeletal muscle is a direct target for vitamin D. Observational studies suggest that low 25[OH]D correlates with functional recovery of skeletal muscle following eccentric contractions in humans and crush injury in rats. However, a definitive association is yet to be established. To address this gap in knowledge in relation to damage repair, a randomised, placebo-controlled trial was performed in 20 males with insufficient concentrations of serum 25(OH)D (45 ± 25 nmol/l). Prior to and following 6 wk of supplemental vitamin D_3 (4,000 IU/day) or placebo (50 mg of cellulose), participants performed 20 × 10 damaging eccentric contractions of the knee extensors, with peak torque measured over the following 7 days of recovery. Parallel experimentation using isolated human skeletal muscle-derived myoblast cells from biopsies of 14 males with low serum 25(OH)D (37 ± 11 nmol/l) were subjected to mechanical wound injury, which enabled corresponding in vitro studies of muscle repair, regeneration, and hypertrophy in the presence and absence of 10 or 100 nmol 1α,25$(OH)_2D_3$. Supplemental vitamin D_3 increased serum 25(OH)D and improved recovery of peak torque at 48 h and 7 days postexercise. In vitro, 10 nmol 1α,25$(OH)_2D_3$ improved muscle cell migration dynamics and resulted in improved myotube fusion/differentiation at the biochemical, morphological, and molecular level together with increased myotube hypertrophy at 7 and 10 days postdamage. Together, these preliminary data are the first to characterize a role for vitamin D in human skeletal muscle regeneration and suggest that maintaining serum 25(OH)D may be beneficial for enhancing reparative processes and potentially for facilitating subsequent hypertrophy.

muscle damage; regeneration; vitamin D

ECCENTRIC CONTRACTION, CONTUSIONS, AND TOXIC INSULTS cause skeletal muscle damage that leads to decrements in functional capacity. Yet skeletal muscle is uniquely equipped to regenerate via the activation, proliferation, migration, and differentiation of resident muscle stem cells known as satellite cells (43); indeed, muscle regeneration is significantly compromised when satellite cells are ablated. Effective repair usually leads to full functional recovery of the damaged tissue, whereas ineffective repair leads to fibrosis and suboptimal rescue of function, such as in severe muscular dystrophies (32).

Many intrinsic, local, and systemic factors interact to orchestrate the muscle repair process, and thus identifying and targeting modifiable risk factors that compromise any step of this process can augment functional recovery. Recent insights from human trials have reported that vitamin D_3, a member of a group of pleiotropic prosteroid hormones, is implicated in numerous biological processes. Vitamin D is synthesized primarily in the skins dermis following exposure to ultraviolet B radiation (sunlight exposure), which results in the conversion of 7-dehydrocholesterol to pre-vitamin D_3 and subsequently to vitamin D_3. Nutritionally, both vitamin D_2 and D_3 can be obtained from dietary sources, albeit in far less quantity than can be synthesized following sunlight exposure. It is important to consider that presently, little biological significance can be attributed to vitamin D_2, and therefore Vitamin D_3 is thought to be the main contributor to vitamin D status (reviewed in Ref. 34). Whether vitamin D_3 is obtained from dietary sources or via ultraviolet B exposure, the metabolite will undergo hydroxylation at the liver to form 25-hydroxyvitamin D [25(OH)D], the main marker of vitamin D status, and a further hydroxylation step in the kidney to form the biologically active 1α-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$]. The bioactive metabolite can interact with the vitamin D receptor (VDR) to modulate genomic and nongenomic processes that function to control biological events in the cell (25). Classically, vitamin D is understood to be a major regulator of calcium and phosphate homeostasis, being required for normal bone mineralization. Indeed, the bone disorder rickets is caused by insufficient vitamin D_3 exposure and is reversible with supplemental vitamin D (27). Research evidence from the past decade, however, is now indicative that many tissues of the human body are responsive to vitamin D and function suboptimally when exposure to vitamin D is limited (26). Despite the understanding of the importance of vitamin D in human health, low circulating vitamin D concentrations [measured as total serum 25(OH)D] prevail in humans and are indeed associated with numerous preventable disease states. One such tissue that may be affected by low vitamin D concentrations is skeletal muscle. Recently, it has been suggested that the sterol is implicated in skeletal muscle regeneration and remodeling (reviewed in Ref. 34), and indeed, sufficient 25(OH)D positively correlates with muscle force recovery from damaging eccentric exercise (6, 7).

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These studies, while promising, are compromised by the observational nature of their design and raise the question of the potential cause-effect mechanisms between vitamin D and muscle repair and remodeling.

Fragments of evidence from animal and cellular models have provided supporting preliminary data for the observational insights in humans. In in vivo crush injury rat models, vitamin D sufficiency culminates in significant increases in bromodeoxyuridine-positive proliferating myoblasts, decreased apoptosis via staining tissue for DNA damage, and subsequent improvements in maximal force recovery compared with deficient rats (51). In addition, vitamin D supplementation, following $BaCl_2$ damage of murine tibilais anterior, culminates in improved regeneration and increased expression of the VDR (25), highlighting the potential of the vitamin D pathway in controlling aspects of the regeneration process (50). Complementing these in vivo rodent studies, in vitro studies using C_2C_{12} murine myoblasts also indicate that myoblast proliferation, differentiation, myotube hypertrophy, and survival are mechanisms facilitated by vitamin D; however, similar human muscle cell studies remain to be performed (21, 45, 50). This is particularly relevant given that circulating 25(OH)D and $1\alpha,25(OH)_2D$ are undetectable in many rodent species.

Taken together, a definitive cause-effect relationship between vitamin D and skeletal muscle repair and remodeling is yet to be established in humans by well-controlled, translational investigations. Therefore, the aim of the current work was to implement a systems-based trial to delineate the role of vitamin D in humans using an in vivo and in vitro design, and our aims were with twofold objectives: *1*) to investigate the effect of low serum 25(OH)D on functional recovery from eccentric exercise, implementing a randomized controlled trial (RCT), and *2*) to identify aspects of muscle cell regeneration that are responsive to supplemental vitamin D, using human primary muscle-derived cells, from deficient male participants in an in vitro model of muscle damage, repair, and regeneration in the presence or absence of vitamin D_3. This in vivo/in vitro model allowed us to identify the impact of vitamin D on whole tissue muscle recovery of function and the cellular adaptations that vitamin D may modulate during functional repair of skeletal muscle following a damaging event.

It was hypothesized that *1*) raising serum 25(OH)D from a low level with supplemental vitamin D_3 could improve the functional recovery from eccentric exercise and *2*) treatment of isolated muscle cells from humans with the active vitamin D metabolite $1\alpha,25(OH)_2D_3$ would improve migration, the capacity for differentiation/fusion, and myotube hypertrophy following damage in vitro.

MATERIALS AND METHODS

Inclusion Criteria and Ethical Approval

Strict inclusion criteria were implemented for both trials. Inclusion was limited to males aged 18–30 with no underlying medical ailments as identified by a medical history questionnaire, physical activity readiness questionnaire, and screening by a trained physician. Those taking fish oils, multivitamins, or vitamin D supplements or using sun beds were excluded from the trials. Finally, only individuals undertaking ≥3 h/wk of physical activity above daily tasks were included in the trial. Following informed consent and the meeting of the initial inclusion criteria, participants provided a venous blood sample that was analyzed for total serum 25(OH)D (nmol/l) and were excluded if serum concentration was ≥75 nmol/l, suggestive of adequate vitamin D concentration (26, 31).

Ethical approval for *study 1* (in vivo) was granted by the Liverpool John Moores University Research Ethics Committee and for *study 2* (in vitro) by the NHS West Midlands National Research Ethics Committee (NREC approval no. WM/09/13). All data were collected and stored in line with the Declaration of Helsinki and the Human Tissues Act.

Blood Sampling and Analysis of Vitamin D Metabolites

For the analysis of vitamin D metabolites (D_2 and D_3) in both *study 1* and *study 2*, following informed consent, serum was harvested from fasted venous blood samples collected from the antecubital vein. Serum was stored at −80°C until it was required for analysis.

For the analysis of total serum 25(OH)D concentration (sum of D_2 and D_3 metabolites), high-pressure liquid chromatography tandem mass spectrometry (LC-MS/MS) was implemented. The LC-MS/MS method of analysis has been validated against other commercially available assays and is regarded as the gold standard for the assessment of vitamin D metabolites (48). Analyses were performed in a Vitamin D External Quality Assurance Scheme-accredited laboratory. Assay procedures were conducted as previously described (35).

Randomized Controlled Trial Methods

Twenty volunteers (21 ± 1 yr, 179 ± 4 cm, 84 ± 13 kg) met the inclusion criteria for *study 1* and were allocated to the RCT. Participants were first block randomized based on their basal serum 25(OH)D and maximal isokinetic torque of the right knee extensors at 60°/s (1.05 rad/s). On the day of muscle damage, participants were instructed to produce a maximal voluntary contraction (MVC) prior to and following the bout of eccentric exercise. MVC torque was subsequently measured at 24 h, 48 h, and 7 days following the exercise bout to monitor functional recovery. Participants then received either an oral vitamin D_3 supplement [VITD; 4,000 IU/day European Food Safety Authority standard safe upper limit (18)] or a visually identical placebo capsule (PLB; 50 mg of cellulose) for 6 wk, following which a second blood sample was drawn for analysis of vitamin D metabolites and the eccentric exercise bout and MVC protocol were repeated (Figure 1).

MVC torque was assessed on a Biodex isokinetic dynamometer (Biodex Medical Systems, Shirley, NY) that was previously validated for its use in reliable assessment of muscle function variables related to torque production (17). Participants were seated as per the manufacturers' guidelines with a 90° flexion of the hip and nonextendable straps crossing the chest and abdomen and across the quadriceps to maximize isolation of the target muscle group. The test protocol consisted of four consecutive maximal extension movements of the right quadriceps at two different fixed movement velocities, 60 (1.05 rad/s) and 180°/s (3.14 rad/s), separated by a 5-min rest period to allow full recovery of the high-energy phosphate pool, from which peak torque (Nm) was calculated (49). All participants were familiarized with the protocol until the coefficient of variation for each participant was <10% (5).

The eccentric exercise bout was a modified version of that described previously and known to cause muscle damage (36). Furthermore, similar eccentric exercise protocols with a smaller volume of work have been shown to result in the activation and proliferation of satellite cells (30), an important consideration to allow for the transfer of findings between our in vitro and in vivo models. Exercise was performed on a Cybex isokinetic dynamometer and consisted of 200 unilateral eccentric contractions at 30°/s (0.52 rad/s) executed as 20 sets of 10 contractions interspersed by 30-s rest intervals. Exercise was performed through the participants' full range of motion and thus was specific to each participant. Muscle soreness was measured at the same time points as MVC measurements and was determined via

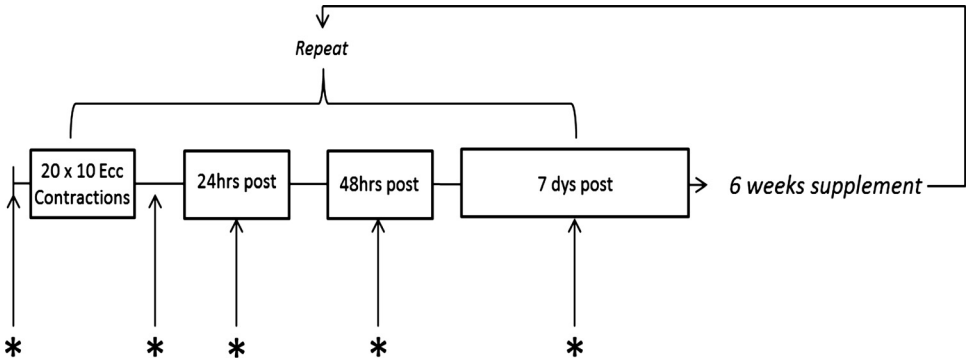

Fig. 1. Schematic representation of experimental procedures. *Peak torque and muscle soreness measurement, which were performed immediately prior to, immediately after (post), and then at 24 h, 48 h, and 7 days following an eccentric exercise protocol consisting of 20 sets of 10 eccentric contractions performed at 30°/s and separated by 30-s rest intervals between sets. Blood was collected presupplementation and then again following the 6-wk supplementation period for measurement of total serum 25-hydroxyvitamin D [25(OH)D].

pressure algometry. The distance from the inguinal crease to the tip of the patella was measured, and muscle soreness readings were then obtained 5 cm from the patella and at the midpoint of the quadriceps. Participants indicated the point at which pressure from the algometer became painful, and the reading indicated by the algometer was noted (in kg).

Methods for the In Vitro Model of Muscle Regeneration

Reagents, chemicals, and solvents. Growth media (GM) used for the expansion of human muscle-derived cell populations consisted of Hams F-10 nutrient mix (Lonza, Basel, Switzerland) with added L-glutamine (2.5 mM), 10% heat-inactivated fetal bovine serum (hiFBS; Gibco, Thermo Fisher Scientific, Altincham, UK), 10% newborn calf serum (NBCS; Gibco), 1% penicillin-streptomycin (PS; 50 units of penicillin-50 μg of streptomycin; Life Technologies, Warrington, UK), and 1% amphotericin B (2.5 μg/ml; Gibco). Differentiation media (DM) consisted of α-MEM (Lonza), 1% hiFBS, 1% NBCS, 1% PS, and 1% amphotericin B (2.5 μg/ml). Quiescent media (QM) used in human skeletal muscle cell pretreatments consisted of the same components as DM; however, hiFBS and NBCS were added at a concentration of 0.1% each (0.2% serum total). Phosphate-buffered saline (PBS; Sigma-Aldrich) was used to wash cell monolayers. The active vitamin D metabolite 1α,25-dihydroxyvitamin D_3 was purchased from Sigma-Aldrich and reconstituted as per the manufacturers guidelines in 100% ethanol (40 μl/well in DM, final volume for highest $1\alpha,25(OH)_2D_3$ treatment). Desmin polyclonal rabbit anti-human antibody (Ab 15200) was purchased from Abcam (Abcam, Cambridge, UK), and TE7 monoclonal mouse anti-human antibody (Ab CBL271) was purchased from Merck Millipore. Secondary fluorophore (TRITC goat anti-rabbit) and nuclear counterstain (DAPI) were purchased from Life Technologies.

Muscle biopsy procedure. Fourteen volunteers (age = 25 ± 3 yr, height = 181 ± 5 cm, weight = 81 ± 10 kg) were included in *study 2* and provided a skeletal muscle biopsy. The average total serum 25(OH)D concentration for the group was 37 ± 11 nmol/l. Participants were instructed to avoid exercise training 48 h preceding the biopsy procedure. On arrival at the laboratory, participants were asked to relax in a supine position on a hospital bed while the biopsy site was prepared. Briefly, the incision site (vastus lateralis) was shaved to maximize sterility and washed with an alcohol swab and Hydrex surgical scrub (ECOLAB Leeds, UK), following which a sterile sheet was used to maintain sterility. To anesthetize the biopsy site, 1.5 ml of bupivacaine hydrochloride (Astra Zenica, Luton, UK) was administered at a concentration of 5 mg/ml. A sterile single-use scalpel was used to penetrate the skin and deep muscle fascia and a Bard disposable core biopsy instrument (12 g × 10 cm; CR Bard, Crawley, UK) to retrieve a biopsy of muscle (~20–30 mg tissue).

Isolation and Characterization of Human Muscle-Derived Cells

Mixed populations of myoblasts and fibroblasts were harvested from all 14 biopsy specimens by implementing a modified method of that described previously (9) and characterized by immunofluorescent staining (described in detail below) for the detection of specific proteins expressed by myoblasts (desmin) and fibroblasts (TE7). Myogenic proportion ranged from 25 to 65% (median = 45%), and all populations were included for analysis. Pilot data revealed that the proportion of myoblasts to fibroblasts does not affect the ability of either cell type to migrate or the ability of myoblasts to fuse (data not shown). Thus, cell populations were not sorted into pure populations due to the importance of the presence of fibroblasts in myogenesis (33, 41). Biopsy samples were transferred in precooled transfer media (TM; containing Hams F-10, 2% hiFBS, 1% PS, and 1% amphotericin-B) to the laboratory (maximum 30 min). To dissociate the tissue, biopsy samples were carefully dissected with a sterile scalpel in petri dishes to remove visible connective and adipose tissue while still in Hams F-10 TM. Following three washes with ice-cold PBS (0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl, pH 7.4, in dH_2O) and antibiotics (1% PS and 1% amphotericin-B), 5 ml of trypsin-EDTA was added, and the samples were scissor minced to fragments <1 mm^3. The dissected sample was triturated on a magnetic stirring platform at 37°C. The trypsinization process was repeated two times in succession, and the supernatant derived following each treatment was collected and pooled with horse serum at a concentration of 10% of the total volume to inhibit further protease activity. Once trypsin treatments were complete, pooled cell supernatant was centrifuged at 1,300 rpm for 5 min to produce a cell pellet. The supernatant was discarded and the cell pellet resuspended in GM and plated on a T25 cm^2 culture flask for cell population expansion. Following ~10 days in culture, T25 cm^2 culture flasks reached ~80% confluence and were passaged via trypsinization. Cells were counted using Trypan Blue exclusion and frozen in GM with 10% dimethyl sulfoxide (DMSO) as a cryopreservant or replated to expand the population. All experiments were performed on cells between passages 3 and 5 to avoid issues of senescence (3).

Cell Culture

All cell culture experiments were performed under a Kojair Biowizard Silverline class II hood (Kojair, Vippula, Finland). Cells were incubated in a HERAcell 150i CO_2 Incubator (Thermo Scientific, Cheshire, UK). Cell populations were cultured on T75- (Nunc, Roskilde, Denmark) and T25-cm^2 (Corning, Life Sciences) culture flasks, and experiments were performed in sterile six-well plates (Nunc). Culture flasks and six-well plates were coated with a 2 mg/l porcine gelatin solution (~90–110 g, Bloom; Sigma-Aldrich, Dorset, UK) to allow cell adhesion.

Cell Treatments

For the expansion of cell populations, cells were grown in GM, which was changed every 48 h following two brief washes with 1× PBS. Once cell monolayers reached a confluent state, GM was removed, monolayers were washed twice with PBS, and GM was replaced with QM for 20 h, following which QM was exchanged for

QM + mitomycin C (10 μg/ml) for 3 h to allow replication arrest, as determined by pilot experimentation and by previous work (15). This method was implemented to study migration in the absence of interfering proliferation. Subsequent to 3 h of treatment with QM + mitomycin C, cells were damaged by a vertical scrape with a 1-ml pipette tip. The mitomycin C pretreatment media was aspirated, and damaged cell monolayers were washed three times with 1× PBS to remove cell debris and residual pretreatment media. Each six-well culture plate was subjected to a low dose of exogenous $1\alpha,25(OH)_2D_3$ (10 nmol in DM; Lo), a high dose (100 nmol in DM; Hi), or control vehicle (20 μl/ml 100% EtOH; Veh) in DM; $n = 2$ wells per dose per experiment (experiments performed on $n = 14$). The doses of vitamin D selected were based on previously published research (11, 22, 23, 50) and were used to determine whether potential responses in cell migration, fusion, and hypertrophy were dose dependent. Immediately following the addition of treatments, monolayers were placed in a controlled live imaging microscopy environment (Leica DMB 6000 equipped with PeCon incubation system and gas control system) of 37°C with 5% CO_2 and images, were captured every 30 min for 48 h for the analysis of cell migration dynamics (migration distance, velocity, and directionality; see below).

Wound-Healing Assay and Migration Analysis

TIF files captured over the 48-h filming period were exported from Leica Application Suite and loaded as TIF image stacks in ImageJ with a cell counter plug-in. Cells in the outer (*segment 1*) and inner (*segment 2*) wound spaces were counted (see Fig. 2). TIF files were also exported as TIF image stacks into ImageJ with a manual tracking tool plug-in (IBIDI, Munich, Germany). The individual trajectory of each cell was tracked in the *x- and y*-axes and derived raw coordinate data exported in an ImageJ chemotaxis and migration tool plug-in (IBIDI) for analysis. The chemotaxis tool analyzed raw data from the manual tracking tool and provided quantitative data on the migration of individual and grouped cell trajectories, including migration velocity (V; in μm/min), accumulated migration distance (D_{Acc}; in μm), Euclidean migration distance (D_{Euc}; in μm) and directionality (Dir; in arbitrary units, with 0 being random migration and 1 being a straight line).

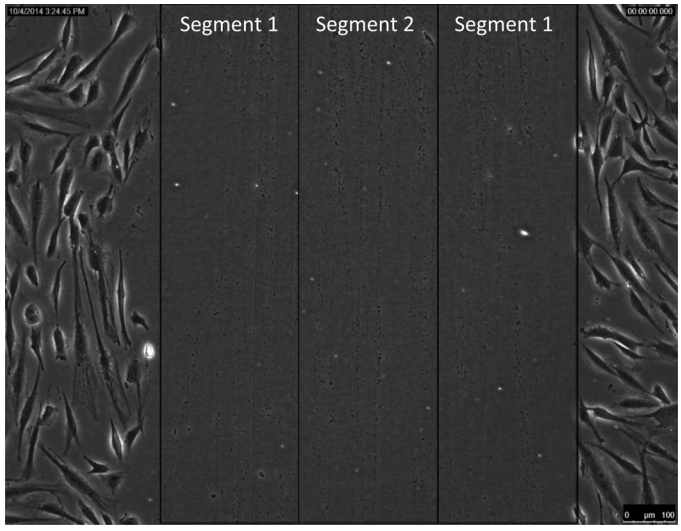

Fig. 2. Representative image of a scrape wound inflicted with a 1-ml pipette tip. The wound area is 900 μm in width and split into 3 × 300-μm segments. Magnification is ×10.5, and scale bar is 100 μm. The outmost segments are referred to as *segment 1* and the single inner segment is referred to as *segment 2*.

Creatine Kinase Activity

At 0 and 48 h and 7 and 10 days following the mechanical scrape insult, creatine kinase (CK) activity was analyzed as a marker of muscle cell differentiation/fusion into myotubes, as described previously (2, 20, 44, 47). Cell monolayers were first lysed with 300 μl/well of 50 μM Tris-mes and 1% Triton-X 100, pH 7.8 (TMT). Ten microliters of TMT cell lysate was loaded in duplicate wells on a 96-well UV plate and used for quantification of CK activity. The CK reaction reagent and diluent (Catachem) were prepared as per the manufacturer's instructions and heated for 2 min at 37°C. When reconstituted, the reagent contained the following active ingredients: 30 mmol/l PCr, 2 mmol/l ADP, 5 mmol/l AMP, 2 mmol/l NAD, 20 mmol/l *N*-acetyl-L-cystine, 3,000 U/l hexokinase, 2,000 U/l G-6-PDH, 10 mmol/l^{-1} Mg^{2+}, 20 mmol/l D-glucose, 10 μmol/l di(adenosine 5′) pentaphosphate, and 2 mmol/l EDTA. The reagent mixture was then added to the samples and the change in absorbance monitored continuously over 10 min in a spectrophotometer (Thermo Multiskan Spectrum plate reader) at a wavelength of 340 nm. Final concentrations were relativized to total protein are reported as $mU \cdot mg^{-1} \cdot ml^{-1}$.

For the quantification of total protein, a bicinchoninc acid (BCA) assay was also performed on lysed samples. BSA protein standards were prepared at 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, and 0 mg/ml. The BCA reaction materials were purchased as part of a Pierce BCA protein assay kit (Rockford, IL) and prepared as per the manufacturer's guidelines. Two hundred microliters of the working reagent was added to all wells containing 10 μl of sample using a multichannel pipette (excluding the blank, which was TMT alone), and the plate was incubated at 37°C for 60 min. Following background subtraction, the absorbance was recorded at 30 and 60 min at 595 nM using a Thermo Multiskan Spectrum plate reader. The standard curve was generated by plotting the average blank-corrected 595-nM measurement of each BSA standard against its preprogrammed known concentration in milligrams per milliliter. Sample concentrations were calculated from the standard curve.

Morphology and Immunocytochemistry

To determine myotube formation at 7 and 10 days, damaged monolayers were imaged at four sites per well in the wound site immediately postdamage (0 h). These image coordinates were then saved to allow monitoring of a consistent wound site to avoid experimental bias. Images were captured at 7 and 10 days, exported as TIFF image files, and analyzed in ImageJ. Morphology for assessment of muscle cell fusion/differentiation was assessed by myotubes per field of view and myotube hypertrophy via the assessment of myotube diameter and myotube area. Myotubes were counted via ImageJ cell counter plug-in, and only myotubes for which the entire length of the tube was visible in the field of view were considered. Myotubes were determined as cells containing three or more nuclei. Myotube area was determined by manually drawing a line around the sarcolemma of each myotube. By normalizing the pixel scale to the micron scale of each image, a value expressed as μm^2 is obtained. To calculate myotube diameter, three equidistant diameters along the length of the myotube (left center, center, and right center) were measured and averaged. A total of three images per well per treatment were analyzed, treatments were performed in duplicate, and experiments were performed on 14 cell populations from 14 different individuals. Therefore, 84 images per condition were assessed.

Experiments above were repeated for immunocytochemical staining to visualize nuclei for the accurate determination of myonuclear fusion index and myonuclear domain size. Monolayers were fixed at 10 days postinsult via 5-min graded methanol incubations [25, 50, and 100% vol/vol methanol in 1× Tris-buffered saline (TBS)] and stored wet in 1× TBS until they were required for staining. Monolayers were permabilized and blocked for 2 h prior to staining with 5% goat serum and 0.2% Triton X-100 in 1× TBS. Cells were incubated overnight at 5°C with 1° Desmin antibody (1:200). After overnight incubation, 1°

antibody was removed, and the cells were washed three times with 1× TBS. Secondary antibody TRITC antibody (1:200) was then applied and left for 2 h at 5°C. Finally, following removal of 2° antibody and three TBS washes, a nuclear counterstain (Sytox-Green; 1:5,000) was applied, and monolayers were incubated for 1 h before a final three TBS washes. Fluorescent images were then captured at 10 days postdamage and nuclei counted via ImageJ cell counter plug-in. A total of three images per well were analyzed per treatment, and treatments were performed in duplicate (n = 2/condition/sample). This immunostaining procedure was also used in the characterization of the cell populations described above in which cells were stained with desmin (1:200) and TE7 (1:200) to determine the relative proportion of myoblasts to fibroblasts.

Gene Expression: RNA Isolation, Primer Design, and RT-Quantitative PCR

Total RNA was isolated at 0 h post mechanical damage via wound infliction and then at 48 h and 7 and 10 days postdamage. Monolayers were washed with 1× PBS (1 ml/well), and RNA was extracted using 300 μl/well TRI Reagent (Sigma-Aldrich, Dorset, UK). RNA was isolated via guanidium thyocyanate phenol cholorform extraction. Concentration and purity were assessed via UV spectroscopy using a Nanodrop spectrophotometer 3000 (Fisher Scientfic, Rosklide, Denmark). Only samples with a purity ratio between 1.9 and 2.2 were used for the downstream application of RT-quantitative PCR (qPCR).

Purified RNA was diluted to 7.3 ng/ml in 9.5 μl of DNase RNase-free H_2O (Sigma-Aldrich) to create a final reaction concentration of 70 ng. RNA was reverse transcribed and amplified with specific primer sequences in a rotor-gene Q (Qiagen, Manchester, UK) PCR machine using a one-step SYBR Green I RT-qPCR kit (Qiagen). Briefly, double-stranded cDNA was first synthesized at 50°C for 10 min with the use of dTP oligonucleotides and reverse transcriptase. cDNA was denatured to single-stranded DNA at 95°C for 10 s, and combined primer annealing and extension were initiated at 60°C for 30 s. Primers sequences were designed using National Centre of Biotechnology Primer-BLAST software and RTprimerDB (http://www.rtprimerdb.org/), and highly purified salt free primer for each sequence was purchased from Sigma-Aldrich. Detailed primer information can be found in Table 1. A relative method of mRNA expression was used as described previously with a stable reference gene (*RPL13a*; %coefficient of variation = 1.8%) and a 0-h untreated control sample ($\Delta\Delta C_T$ method; see Ref. 38). Melt curve analysis was performed to confirm all PCR products demonstrated a clear single peak (same) melt temperature showing that only one gene target had been amplified and that primer-dimer issues were not present.

Additionally, amplified PCR products were electrophoretically separated to ensure correct end product length of amplified genes. Eight microliters of Norgen FullRanger 100-bp DNA ladder and 8 μl of sample were loaded after preparation with a DNA loading dye (Geneflow, Staffordshire, UK) into an agarose gel [2% agarose (Bioline Reagents, London, UK) in 1× Tris-acetate-EDTA buffer (Invitrogen, Life Technologies Paisley, UK)] prepared with Midori Green nucleic acid stain (Nippon Genetics Europe, Düren, Germany) at a dilution of 1:200. Samples were electrophoretically separated at 50 V for 20 min, followed by 70 V for an additional 20 min. Following electrophoretic separation, gels were placed on a UV transilluminator for visualization and analysis.

Statistical Analysis

All statistical analyses were performed using SPSS Predictive Analytics Software (version 20; IBM). For the comparison of two group means a t-test was used, and where comparison of multiple groups of means was required, an analysis of variance (ANOVA) was used. Data sets were first checked for normal distribution, and where data violated the assumption of normality, an appropriate correction factor was used. If data violated the assumption of sphericity, Greenhouse-Geiser or Huyn-Feldt correction factors were used. Where significant main effect and interactions were present, the Bonferroni post hoc pairwise comparisons test was used to detect where significances lay between paired comparisons, an analysis that includes correction for an ANOVA's multiple comparisons. Significance was assumed when α reached ≤0.05. All data are presented as mean ± SD.

RESULTS

Randomized Controlled Trial

At baseline, no significant differences were detected between experimental groups for total serum 25(OH)D ($P >$ 0.05), with mean serum concentrations of 45 ± 15 and 45 ± 25 nmol/l for PLB and VITD, respectively. Following 6 wk of supplementation with 4,000 IU/day vitamin D_3, a significant interaction effect was observed between treatment group and time ($P < 0.005$). Post hoc analysis revealed that the VITD group showed a significant increase in total serum 25(OH)D at *week 6* compared with presupplementation (pre = 45 ± 25 vs. post = 115 ± 31 nmol/l, $P < 0.005$) and a significant difference when compared with PLB ($P < 0.005$). Conversely, the PLB group demonstrated a significant decline in serum 25(OH)D at *week 6* compared with presupplementation (pre = 45 ± 25 vs. post = 33 ± 13 nmol/l, $P = 0.013$; see Fig. 3*E*).

At both pre- and postsupplementation test points, VITD and PLB demonstrated comparable, significant losses of peak torque immediately postexercise at both 60 and 180°/s (all at $P < 0.005$), indicating that the eccentric exercise protocol effectively caused skeletal muscle damage. At the presupplementation time point, no interaction effect was detected between treatment group and maximal torque recovery over 7 days for either 60 ($P = 0.281$) or 180°/s ($P = 0.310$). However, following supplementation, a significant interaction effect between recovery time point, test week (pre/postsupplementa-

Table 1. *Gene primer sequences for human MDC samples and amplicon lengths*

Gene	Accession No.	Primer Sequence	Amplicon Length, bp	Exon Junction
VDR	NM_001017536	GACCTGTGGCAACCAAGACT (forward) GGACGATCTGGGGAGACGA (reverse)	174	Reverse: 1,575/1,576
MYOG	NM_002479.5	TCCCAGATGAAACCATGCCC (forward) AGGCCCCTGCTACAGAAGTA (reverse)	103	None
MRF4	NM_002469.2	ACCCTTCCTGGCCTAATCCT (forward) ACCCTTCCTGGCCTAATCCT (reverse)	198	None
RPL13A	NM_012423	GGCTAAACAGGTACTGCTGGG (forward) AGGAAAGCCAGGTACTTCAACTT (reverse)	105	Reverse: 230/231

MDC, muscle-derived cell; *VDR*, vitamin D receptor; *MYOG*, myogenin; *MRF4*, myogenic regulatory factor 4; *RPL13A*, ribosomal protein L13a. All primers were used in the same PCR cycling conditions.

tion), and treatment group at 60°/s ($P = 0.049$) was detected. Exploration of this interaction identified a significant improvement in torque recovery in the supplemental VITD group following supplementation at 48 h (14% improvement, $P = 0.042$) and 7 days post-eccentric exercise (13.7% improvement, $P = 0.001$) compared with presupplementation. Although a slight improvement was observed for VITD postsupplementation at 180°/s (48 h = 5.6% improvement, 7 days = 9.9% improvement), this result failed to meet statistical significance (Fig. 3). We then also plotted the change in peak torque recovery at 7 days against the change in total serum 25(OH)D as a linear regression to determine whether a relationship existed (Fig. 3*F*). Interestingly, 88% of the variation in peak torque recovery could be explained by the change in serum 25(OH)D ($r^2 = 0.88$). Despite improvements in functional capacity, there was no interaction between treatment group and time for measurements of muscle soreness at either midquadriceps ($P = 0.71$) or 5-cm patella ($P = 0.418$), which was due possibly to the highly subjective nature of the sensation of pain and a disconnect between the mechanisms regulated by vitamin D in the repair process and those that regulate the sensation of pain.

In Vitro Model of Muscle Damage to Assess Repair and Regeneration

Having ascertained a beneficial impact on strength recovery after eccentric exercise induced damage in the presence of vitamin D, we next wished to establish the influence of vitamin D on muscle-derived cell migration, a key initial event in muscle repair. Cell monolayers were treated with 10 and 100 nmol $1\alpha,25(OH)_2D_3$ or vehicle (EtOH) following a damaging mechanical scrape insult and live-imaged for 48 h. A significant treatment group effect was detected for *V*, D_{acc}, D_{Euc} and Dir ($P < 0.005$). Both Hi- and Lo-dose $1\alpha,25(OH)_2D_3$ significantly enhanced cell migration *V* compared with vehicle, with 1.37- and 1.43-fold increases observed, respectively ($P < 0.0005$), vs. control. Hi dose was also superior to Lo dose

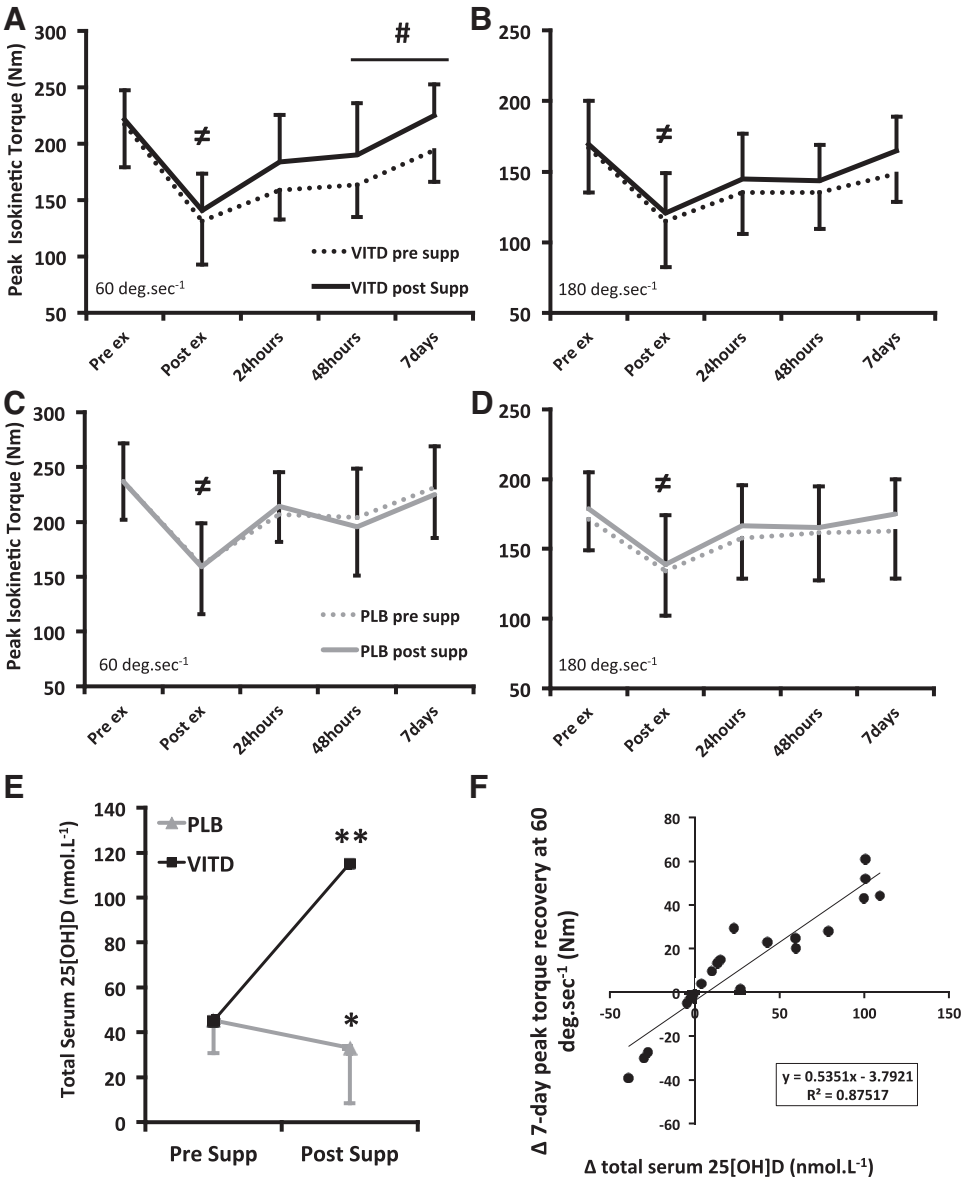

Fig. 3. *A–D*: recovery profile of peak isokinetic torque at 60 and 180°/s following 6-wk supplementation with 4,000 IU/day vitamin D_3 (*A* and *B*) or visually identical placebo (*C* and *D*). #Significance to presupplementation torque at the highlighted time point; ≠significance for the loss of both pre- and postsupplementation torque immediately following eccentric exercise (post ex) when compared with preexercise (pre ex) values. *E*: total serum 25(OH)D response to either 4,000 IU/day vitamin D_3 or placebo. **Significance to presupplementation and placebo; *significance to baseline. *F*: linear regression describing the relationship between Δtotal serum 25(OH)D (nmol/l) between pre- and postsupplementation and the Δpeak torque (Nm) recovery at 7 days postexercise between pre- and postsupplementation.

(0.331 ± 0.11 vs. 0.318 ± 0.1 μm/min, respectively, $P = 0.033$). A similar observation was made in D_{Acc}, as Hi treatment promoted greater migration (953 ± 305 μm) distances than Lo (909 ± 281 μm, $P = 0.009$) whereas both Hi and Lo were superior to Veh (666 ± 288 μm, $P < 0.0005$). However, although both Lo and Hi treatment improved D_{Euc} when compared with Veh, there were no differences between the two doses ($P = 0.193$), which would imply a loss of directionality in the Hi-dose treatment. Indeed, analysis revealed that Hi-dose-treated skeletal muscle cells demonstrated a loss of directionality [0.498 ± 0.21 arbitrary units (AU)] compared with Lo-dose- (0.546 ± 0.2 AU) and Veh-treated (0.547 ± 0.23 AU) cells ($P < 0.005$), whereas Lo and Veh showed no differences. Taken together, both Lo and Hi resulted in more cells at the inner wound space (Lo = 20 ± 9, Hi = 21 ± 8 cells) vs. Veh at 48 h (16 ± 7 cells) as a consequence of improved migration speed and distance. No significant differences were evident in wound size between conditions, indicating that this is unlikely to have affected results (Lo = 949 ± 146 vs. Hi = 927 ± 103 vs. Veh = 925 ± 108 μm, $P = 0.810$). Data are presented in Fig. 4.

Treatment of cultures with 10 or 100 nmol 1α,25(OH)$_2$D$_3$ resulted in superior migration dynamics compared with vehicle alone; however, the same dosing strategy led to differential effects on myoblast fusion at 7 and 10 days following the damaging event in the current investigation. Morphological data analysis revealed that myoblast fusion was significantly inhibited, as fewer myotubes were observed per field with 100 nmol 1α,25(OH)$_2$D$_3$ vs. 10 nmol and vehicle at 7 days postdamage (Hi = 2 ± 2 vs. Lo = 6 ± 4 vs. Veh = 3 ± 1 myotubes/field, $P < 0.0005$). In contrast, Lo treatment led to significant improvements in myotube number compared with 100 nmol and Veh at both 7 (as above) and 10 days (Lo = 10 ± 3 vs. Hi = 6 ± 2 vs. Veh = 6 ± 2 myotubes/field, $P < 0.005$). This observation was also similar for myotube area with Lo treatment, resulting in significantly greater myotube area vs. Hi and Veh at 7 (Lo = 4,984 ± 2,776 vs. Hi = 4,603 ± 1,697 vs. 4,227 ± 1,768 μm^2, $P = 0.003$) and 10 days (Lo = 5,488 ± 2,853 vs. Hi = 4,671 ± 2,932 vs. Veh = 4,388 ± 2,312 μm^2, $P < 0.0005$). Myotube diameter was significantly greater at 7 days in both Lo- and Hi-dose treatments vs. Veh (Lo = 14.13 ± 4 vs. Hi = 13.7 ± 4.5 vs. Veh = 12.12 ± 3.8 μm, $P = 0.005$); however, this effect was lost at 10 days (Lo = 13.6 ± 4 vs. Hi = 12.6 ± 4.1 vs. Veh = 12.1 ± 3.7 μm, $P = 0.256$).

Findings from biochemical analyses and fluorescent imaging were in agreement with morphological findings. Creatine kinase (CK) activity data were analyzed via a mixed-design ANOVA. Results show that CK activity was elevated above Veh with Lo treatment and repressed with Hi compared with both Lo and Veh at 7 days (Lo = 302.5 ± 173 vs. Hi = 186.7 ± 132 vs. Veh = 290 ± 160.5 mU·mg^{-1}·ml^{-1}), although this effect did not reach statistical significance. At 10 days, Lo treatment cells demonstrated significantly higher CK activity compared with both Hi and Veh (Lo = 340.4 ± 183 vs. Hi = 258.4 ± 188 vs. Veh = 229.4 ± 139.5 mU·mg^{-1}·ml^{-1}, $P = 0.017$). Myonuclei and myonuclear domain size were analyzed by one-way ANOVA. The CK observations correlated with a significantly greater accretion of myonuclei in Lo-treated cells vs. Hi and Veh at 10 days postdamage (Lo = 5.9 ± 2.3 vs. Hi = 3.5 ± 1.4 vs. Veh = 3.4 ± 1.2 nuclei per myotube per field, $P < 0.0005$); however, myonuclear domain size was smaller at 10 days with Lo treatment (Lo = 986.5 ± 439.4 vs. Hi = 1,460 ± 726.3 vs. Veh 1,257.3 ± 584 μm^2, $P < 0.0005$; see Figs. 5 and 6).

To determine the impact of treatment on myogenic gene expression, $\Delta\Delta C_T$ analyses were performed by comparing the fold change in target gene expression against both a nontreated 0-h control and a stable reference gene (RPL13a). Results demonstrated that on average Lo-treated cells upregulated myogenic regulatory factor 4 (MRF4) expression to a greater extent at both 7 and 10 days (3.2 ± 2.7- and 3.7 ± 1.5-fold) than Hi- (−0.5 ± 0.3- and 0 ± 0.2- fold) and Veh-treated cells (2.4 ± 2.8- and 2.8 ± 2.4-fold). Similarly, Lo-treated cells showed an increased myogenin expression at 10 days (84 ± 95-fold) compared with Hi (64 ± 79-fold) and Veh (62- ± 70-fold); however, these data failed to meet statistical significance ($P > 0.05$), which was likely due to very large variation in basal expression of the MRFs. VDR expression showed no discernible difference between groups at any time point; however, all significantly increased expression in a similar trend with myogenin between 48 h and 7 days as a main effect for time was detected ($P = 0.022$). Interestingly, Hi-treated cells also showed an impairment (albeit not a statistically significant one) in the ability induce both MRFs at 7 days postinsult vs. control and Lo-treated cells, which correlates with biochemical observations demonstrating lower CK activity in Hi-treated cells at 7 days (Fig. 7).

DISCUSSION

The aims of the current investigation were twofold. First, we looked to identify the effect of increasing serum 25(OH)D from a level of insufficiency on the functional recovery of skeletal muscle following eccentric work. Second, we aimed to establish cellular aspects of muscle regeneration and remodeling that may be responsive to vitamin D, providing a novel mechanistic underpinning for in vivo observations. It was hypothesized that increasing total serum 25(OH)D with supplemental vitamin D$_3$ would lead to an improvement in peak torque recovery following eccentric exercise. Furthermore, we postulated that myogenic progenitor migration, fusion, and myotube hypertrophy would be improved in the presence of exogenous 1α,25(OH)$_2$D$_3$.

The data presented provide novel insights that point toward an important role for vitamin D in muscle recovery in vivo and repair, regeneration, and hypertrophy in vitro. The main findings from this work demonstrate that elevating serum 25(OH)D from ~40 to >75 nmol/l with supplemental vitamin D$_3$ (4,000 IU/day) results in improved functional recovery from eccentric exercise at 48 h and 7 days postexercise vs. a placebo control group, which showed no changes in recovery rate. In an attempt to provide initial insights into the mechanisms responsible, we also uncovered novel roles for vitamin D$_3$ in muscle progenitor migration, fusion, and myotube hypertrophy following an artificial wound injury in vitro. Both the migration velocity and distance traveled into the wound site were significantly enhanced with both 10 and 100 nmol 1α,25(OH)$_2$D$_3$ treatments, implying that vitamin D$_3$ may function to stimulate cell migration in a positive manner when considered in the context of muscle repair. Furthermore, the 10-nmol treatment

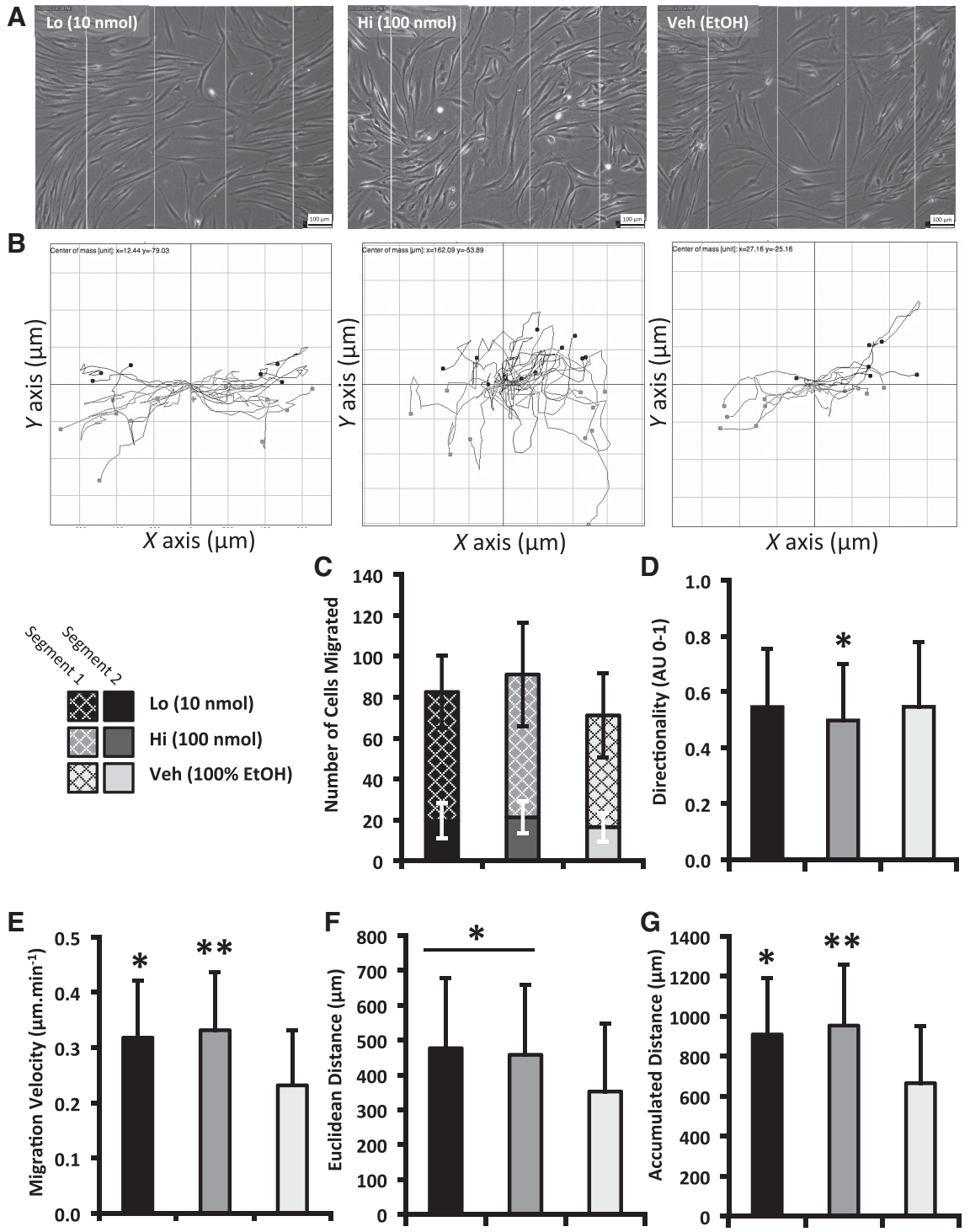

Fig. 4. Muscle-derived cell (MDC) migration dynamics following a mechanical scrape injury in the presence of low-dose (Lo; 10 nmol) or high-dose (Hi; 100 nmol) 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] or vehicle solution (Veh; 100% EtOH). *A*: representative phase contrast microscope images captured at 48 h postwounding. Scale bar, 100 μm; $n = 14$. *B*: representative migration tracking plots produced via ImageJ chemotaxis tool. Trajectories are mapped in μm. *C*: average no. of cells migrated into *segments 1* and *2* at 48 h postdamage. *D*: directionality (Dir; 0–1). *E*: migration velocity (V; μm/min). *F*: Euclidean migration distance (D_{Euc}). *G*: accumulated migration distance (D_{Acc}; μm); *Significance to Veh; **significance to all other treatments.

also led to elevated CK activity above that of 100 nmol and vehicle at all time points, reaching significance at 10 days postinsult. In accord with these data, myotube number and size were both significantly elevated in the 10-nmol treatment at 10 days vs. 100 nmol and vehicle, which was likely attributable to an increased ability to accrete myonuclei, which was further supported by a reduction in myonucelar domain that suggests there were more nuclei in longer/larger myotubes with less domain to serve per myotube. Interestingly, the 100-nmol dose of vitamin D_3 suppressed this effect, and fusion capability only

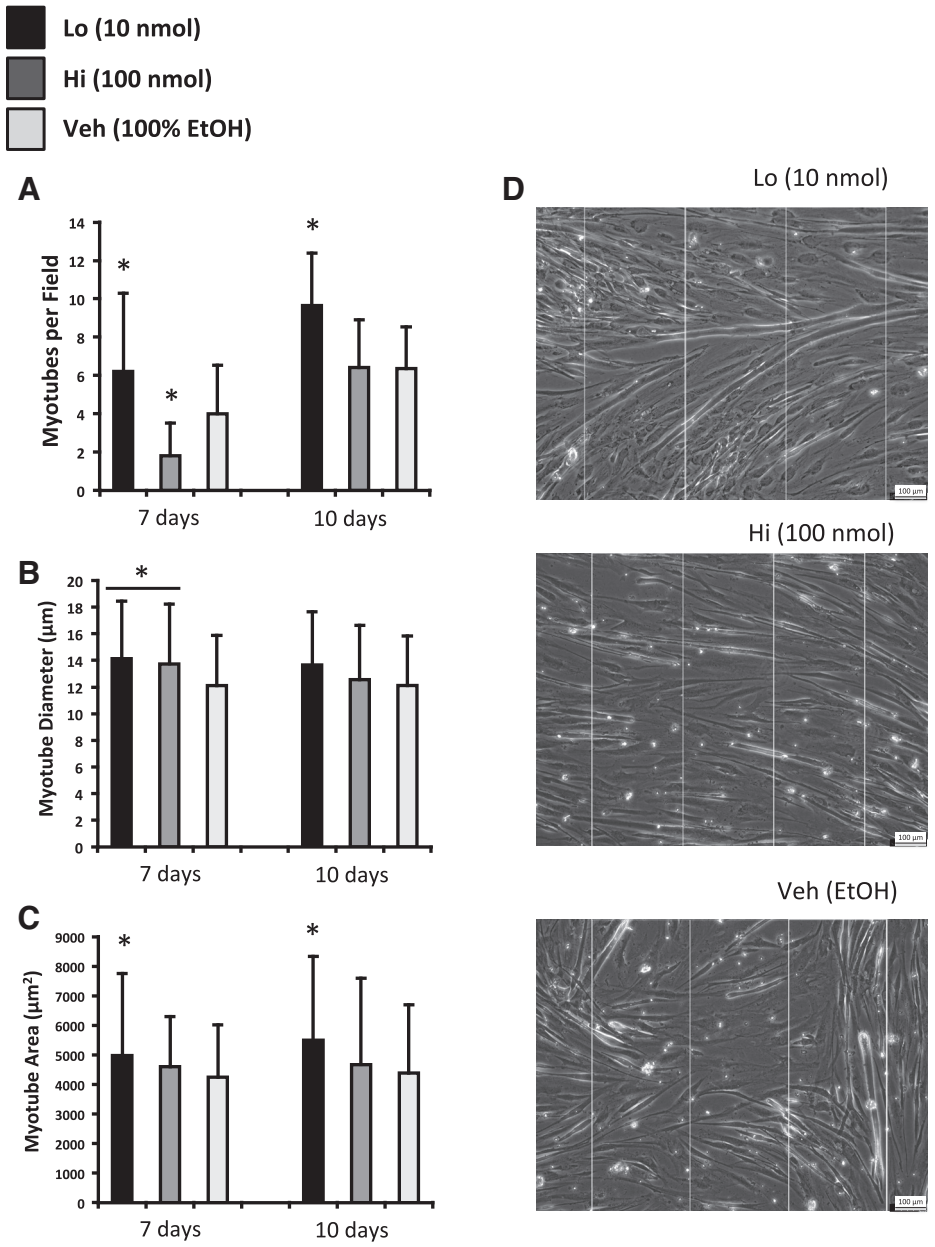

Fig. 5. Effect of Lo or Hi 1α,25(OH)$_2$D$_3$ vs. Veh on myotube morphology following 7 and 10 days of mechanical scrape wounding in DM. *A*: average myotubes per field of view. *B*: average myotube diameter (μm). *C* and *D*: average myotube area (μm^2; *C*) and representative phase contrast microscope images (*D*) captured at 10 days in the wound space of each condition. Scale bar, 100 μm. *Significance to all other treatments at that time point.

reached the level of the vehicle control by *day 10*. Together, these data imply that enhanced cellular characteristics of the muscle regeneration process may partly explain improved functional recovery of skeletal muscle with higher serum 25(OH)D in vivo and may also point toward a positive role for vitamin D in muscle remodeling given the increases in myotube size and nuclear accretion.

The positive influence of 10 nmol of 1α,25(OH)$_2$D$_3$ administration on cell migration is analogous to effects observed in other cell types. For example, exogenous treatment of vascular smooth muscle cells with 1α,25(OH)$_2$D$_3$ induced migration following activation of phosphatidylinositol 3-kinase (PI3K), with the observed effects being abolished by the addition of the PI3K inhibitor LY-294002 (42). Indeed, the importance of PI3K in myoblast migration has been characterized previously (15), and stimulation of this pathway by 1α,25(OH)$_2$D$_3$ has been reported previously in skeletal myoblasts (11), pointing toward stimulation of PI3K activity as a potential mediator of improved muscle-derived cell (MDC) migration dynamics observed in the current trial. Activation of PI3Ks and their lipid product PI(3,4,5)P$_3$ leads to increases in GTP-bound Rac, which is an important small GTPase along with Rho involved in the control of downstream signaling that generates filamentous actin branching and lamellipodia formation (40). Therefore, it could be postulated based on available evidence that vitamin D may function to stimulate MDC migration through the activation of PI3K and increased downstream activity of small Rho GTPases resultantly altering actin cytoskeletal dynamics. Taken together, vitamin D possesses the capability to improve the velocity of which skeletal muscle progenitors can reach a site of damage to permit repair and remodeling of the area. Future research should aim to investigate this pathway in the context of vitamin D

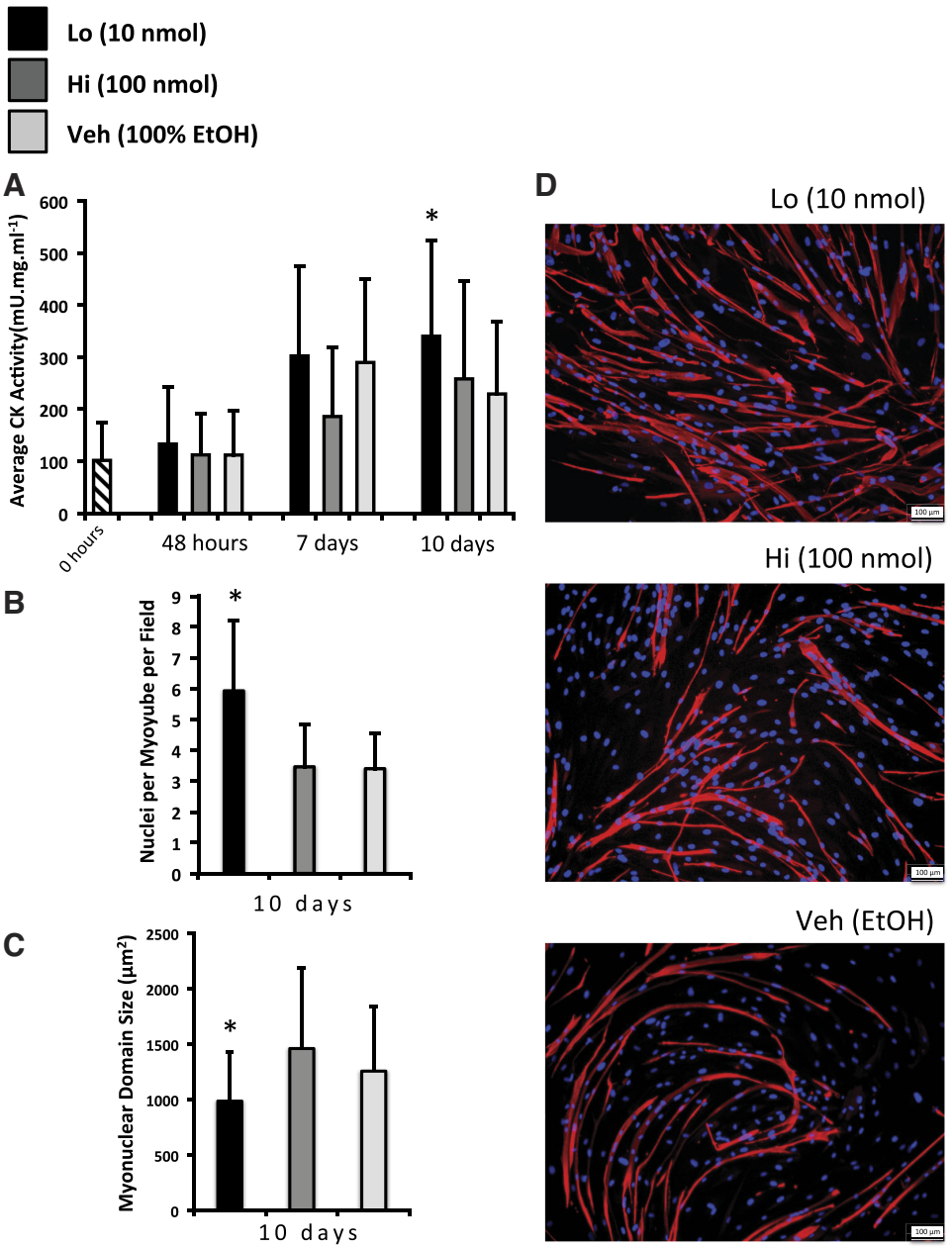

Fig. 6. Effect of Lo or Hi 1α,25(OH)$_2$D$_3$ vs. Veh on biochemical aspects of myotube formation and immuncytochemical analysis of nuclear accretion. *A*: average creatine kinase (CK) activity (mU·mg^{-1}·ml^{-1}) at 48 h and 7 and 10 days following damage. *Significance to other conditions at a time point. *B* and *C*: average nuclei per myotube per field of view (*B*) and myonuclear domain size (*C*) at 10 days following damage. *Significance to all other conditions. *D*: representative fluorescent microscopy images taken at 10 days following damage. Scale bar, 100 μm.

and examine cytoskeletal dynamics. Furthermore, the relevance of enhanced muscle cell migration in vivo should also be investigated.

The finding that 1α,25(OH)$_2$D$_3$ improved myoblast fusion is perhaps not surprising since vitamin D is a closely related hormone system with some of the characteristics of true steroids as receptor ligands, such as testosterone. Indeed, testosterone has repeatedly been demonstrated to enhance myoblast differentiation in vitro (14, 46). In a similar observation to the current study, these investigations also detected significant increases in hyperplasia and hypertrophy in the presence of the steroid. Interestingly, reports suggest that the significant myonuclear accretion observed during overload-induced hypertrophy is not lost during a 3-mo period of severe disuse atrophy (10). Furthermore, treatment of mice with testosterone propionate was demonstrated to induce nuclear accretion and hypertrophy that, following a period of testosterone withdrawal, led to atrophy but a sustained elevated myonuclear number that enhanced retraining-induced fiber hypertrophy (19).

Together, these data imply that steroid exposure stimulates adaptive remodeling that primes the muscle for adaptation in subsequent bouts of mechanical stimuli. Therefore, the finding that vitamin D increased nuclear accretion, accounting for a hypertrophic effect in vitro, is particularly interesting and raises new questions as to whether the sterol may also function similarly to testosterone treatment. Mechanisms accounting for the hypertrophic response elicited by vitamin D are yet to be established, although recent trials have provided evidence that vitamin D treatment improves breast meat yield in male broiler chickens through the mTOR pathway (52), a known major regulator of mechanical overload-induced muscle growth (24).

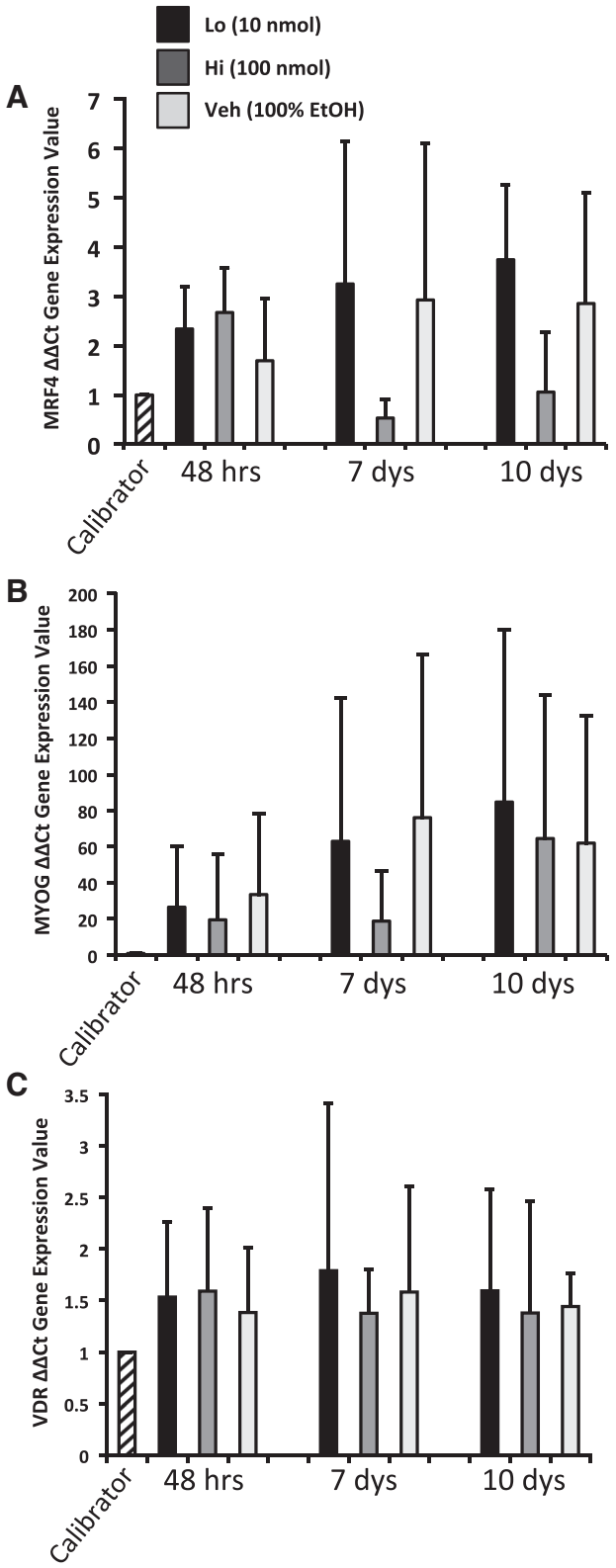

Fig. 7. Time course effects of $1\alpha,25(OH)_2D_3$ on $\Delta\Delta C_T$ mRNA expression levels of myogenic regulatory factor 4 (MRF4; *A*), myogenin (MYOG; *B*), and vitamin D receptor (VDR; *C*). Fold changes were calculated against a stable reference gene (RPL13a) and an internal calibrator (0-h sample).

Furthermore, proteasomal enzyme activities, expression of the E_2 ubiquitin-conjugating enzyme, and ubiquitin conjugates are increased in vitamin D-deficient vs. replete rats (8). Recently, an in vivo report also demonstrated that type IIa skeletal muscle fibers are increased in number and that myostatin mRNA is downregulated with vitamin D supplementation in human males during a resistance training program (1), providing preliminary support for the adaptive remodeling observations made in vitro in the current work.

Our in vitro findings also show similarities and disparities with other models of myogenesis in the context of vitamin D. As a contrasting example, increases in myotube diameter and MHC type II have been detected following 100-nmol treatment of C_2C_{12} myoblasts, which is indicative of a positive myogenic effect (22). In another trial, following serum depletion of 100 nmol of $1\alpha,25(OH)_2D_3$ was shown to suppress myotube formation, which was analogous to the current study. However, the treatment led to a 1.8-fold increase in cross-sectional size of individual myotubes associated with slightly decreased myostatin expression (23). The inconsistency between the current work and previous findings may lie in fundamental differences in the metabolism of vitamin D in humans and rodents. Although both species express the same components of the vitamin D endocrine system, rodents typically obtain all vitamin D from dietary sources, which are minimal amounts (29). As a result, many rodent species show negligible quantities of the major circulating vitamin D metabolites despite showing no typical symptoms of vitamin D deficiency, such as hypercalcemia, hypercalciuria, and elevated PTH concentration. Moreover, these species show discrimination of the D_2 metabolite over the D_3 form, which is the reverse in humans (28). An additional difference is that the current work implemented a coculture model in which fibroblasts were also present with myoblasts. Indeed, fibroblasts are known to positively regulate alignment and fusion of skeletal myoblasts (41), and therefore, it may be argued that our data present a more physiologically relevant insight into the regulation of myogenesis by vitamin D.

It is important to postulate whether our in vitro observations could be attributable for the improved resolution of MVC torque seen in vivo. Indeed, the activation and increased activity of satellite cells is evident within 24 h following eccentric work in humans (12, 16, 30). Moreover, increases in embryonic MHC content have been observed from 2 days, peaking at 7 days following eccentric work indicative of the incorporation of committed myoblasts into myofibers, which also shows a strong trend with functional recovery of peak torque that starts to recover at 24 h and peaks at 7 days (37). To confirm such a link, analysis of biopsy specimens in parallel to torque recovery is necessary.

It must be considered that myoblast migration, fusion, and hypertrophy are only some of the cellular mechanisms that underlie the resolution of damaged skeletal muscle as a result of eccentric work. Satellite cell activation and expansion, an immune response, reinnervation, recapillarization, and extracellular matrix remodeling are also important events that result in full function recovery of damage muscle tissue. It is well characterized that both innate and acquired immunity are potently regulated by vitamin D (39), nerve recovery and remyelination are improved in the presence of vitamin D (13), proangiogenic growth factors are upregulated in regenerating

vitamin D-treated myoblasts in vitro (21), and increased secretion of extracellular matrix components have been reported in vitamin D-deficient rats using a rotator cuff repair model (4). Taken together, it is possible that vitamin D had the profound effect observed in vivo as a result of interaction with a number of events that orchestrate muscle repair and remodeling. Thus work is now warranted to investigate these processes collectively in the presence and absence of vitamin D to fully characterize how regenerating human skeletal muscle is affected by the availability of the sterol.

Although the present study provides a promising concept of a relationship between vitamin D and muscle repair and remodeling, there are some limitations to be considered. First, the sample size of the RCT is indeed small, and larger scale studies will be needed to firmly establish the findings made in the current trial. This will also allow for subgroup analysis and perhaps the detection of subtle functional changes that could not be detected in the supplemental vitamin D group for peak torque recovery at 180°/s. Moreover, the chosen model of exercise is highly specific, with movements performed at two fixed velocities. Although this is ideal for experimental repeatability, such regimens are not common in everyday life and training. Thus, future experimental designs should expand on the current work and employ "real world" protocols resulting in muscle damage, such as downhill running or resistance training, employing negative repetitions of common strength training exercises. Serial biopsies alongside functional measurements of muscle recovery following eccentric work will also help to solidify the link between the in vivo and in vitro findings described in the current work. Finally, relating to our in vitro model, we utilized commonly implemented supraphysiological doses of $1\alpha,25(OH)_2D_3$; however, we now beleive that estimation of the concentration of $1\alpha,25(OH)_2D_3$ that skeletal muscle is exposed to is the next step toward further optimizing studies of vitamin D and skeletal muscle in vitro.

To conclude, this study is the first to identify a novel role for vitamin D in human skeletal muscle regeneration. In vivo observations made here demonstrate that low serum 25(OH)D is easily elevated with supplemental vitamin D_3 and may benefit skeletal muscle recovery, regeneration, and hypertrophy. A challenge to the field is to now expand these preliminary findings by employing larger sample sizes and aiming to characterize all aspects of muscle regeneration that are responsive to vitamin D. Furthermore, new questions are raised as to whether "at risk" populations susceptible to muscle damage and/or vitamin D inadequacy, such as the elderly, who are known to exhibit low serum 25(OH)D, experience aggravated declines in regenerative capacity and remodeling when serum 25(OH)D is low.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

D.J.O., A.P.S., T.F.D., R.G.C., W.D.F., J.P.M., C.S., and G.L.C. conception and design of research; D.J.O., A.P.S., I.P., N.A., J.T., R.G.C., and C.S. performed experiments; D.J.O., I.P., N.A., T.F.D., J.T., W.D.F., J.P.M., and G.L.C. analyzed data; D.J.O., A.P.S., I.P., N.A., T.F.D., J.T., W.D.F., J.P.M., C.S., and G.L.C. interpreted results of experiments; D.J.O. and J.P.M. prepared figures; D.J.O. drafted manuscript; D.J.O., A.P.S., I.P., T.F.D., W.D.F., J.P.M., C.S., and G.L.C. edited and revised manuscript; D.J.O., A.P.S., I.P., N.A., T.F.D., J.T., R.G.C., W.D.F., J.P.M., C.S., and G.L.C. approved final version of manuscript.

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