The Effect of Vitamin D on the Development and Function of Skeletal Muscle

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Forward

This thesis is comprised of original research in a style accepted for or appropriate for publication. Therefore, all results chapters are presented in the format of a manuscript containing their own introduction, methods, results, and discussion sections. A separate materials and methods chapter has been included to provide a comprehensive overview of all experiments carried out. Where relevant, the appendix contains manuscripts in their published format.

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Statement of Contribution and Authorship

I declare that all of the work within this thesis is my own original work and was produced with the support and assistance of the people stated below. All contributions are fully credited and referenced. This thesis has not been submitted in whole, or in part, for any other academic degree or professional qualification. I agree to have this text assessed for originality.

PhD supervision: Dr. John Brameld (JB), Dr. Tim Parr (TP), Dr. Preeti Jethwa (PJ).

Manuscript 1: Effects of Biologically Active Vitamin D on Myogenesis: A Systematic Review.

Katie Alliband (KA) and JB contributed to the conception. KA researched the literature, wrote the original draft manuscript, acquired and analysed the data, created graphs and interpreted the data. Sofia Kozhevnikova contributed to abstract screening. JB, TP, and PJ provided supervision and contributed to reviewing and editing. JB had final approval of the version published.

I confirm that KA contributed 80% towards manuscript 1.

Manuscript 2: Active vitamin D increases myogenic differentiation in C2C12 cells via a vitamin D response element on the myogenin promoter.

KA and JB designed experiments. All laboratory work was carried out my KA. Data analysis, statistics and interpretation of data was carried out initially by KA, then discussed with JB and TP. Writing was carried out by KA and review and editing by JB, TP and PJ. Supervision throughout laboratory work and writing was carried out by JB, TP and PJ. JB had final approval of the version published.

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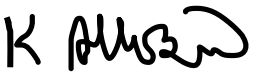
Manuscript 3: Effects of maternal and infant vitamin D deficiency on offspring growth, metabolic health, strength, and physical activity.

Project licence was written by PJ. Animal experiments were mostly carried out by KA, SK and PJ. JB and Matthew Elms (ME) contributed to some cull days. Generation of graphs and statistical analysis in manuscript 3 was carried out by KA. Interpretation of the data was carried out by KA, JB, PJ, TP and ME. Writing of manuscript 3 was carried out by KA. Review and editing were carried out by JB and PJ. Supervision throughout the animal trail and writing was carried out by JB, TP and PJ.

I confirm that KA contributed 75% towards manuscript 3.

Signature



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Abstract

Vitamin D deficiency is a major public health concern affecting around 15% of adults in the UK. Pregnant women have been identified as a sub-group with increased risk of deficiency, with around 20-40% of pregnant women being vitamin D deficient. Currently in the UK, vitamin D supplementation advice for pregnant women is the same as for the general adult population (10µg/day), with no pregnancy specific recommendations, despite pregnant women being an at-risk group. This is a concern for the fetus considering that cord blood contains around 80% of maternal vitamin D levels, therefore infants born to vitamin D deficient mothers are also likely to be vitamin D deficient. Vitamin D is essential for many developmental processes within the fetus including myogenesis, the development of skeletal muscle.

The research within this thesis aimed to investigate the effects of vitamin D deficiency on myogenesis and offspring health and establish a mechanism behind these effects. To do this, the current literature was systematically reviewed, *in vitro* experiments to investigate the effects of vitamin D on myogenesis were carried out and an *in vivo* study was carried out to investigate the impact of maternal vitamin D deficiency on offspring health.

The first research article systematically reviewed twelve studies looking at effects of vitamin D on myogenesis *in vitro*. It was found that vitamin D inhibited proliferation and increased myotube size, but effects on myogenic regulatory factor and myosin heavy chain expression were unclear. This systematic review revealed problems with variability between studies, particularly in terms of day of measurement, and highlighted the need for time course data within the literature. The second research paper carried out an eight-day differentiation time course in C2C12 cells (a muscle cell line), measuring at six time points, with four concentrations of active vitamin D,

spanning physiological range. Similarly to the systematic review, it was found that active vitamin D inhibited proliferation and increased myotube size, but also that active vitamin D resulted in an earlier increase in expression of myogenin and the myosin heavy chain isoforms, showing that vitamin D causes cells to move through differentiation more quickly. Additionally, a vitamin D response element was identified on the myogenin promoter, and the vitamin D receptor was shown to bind to this, via ChIP assay, showing that one of the mechanisms behind the effects of vitamin D on myogenesis is the ability of the vitamin D receptor to bind directly to the myogenin promoter and increase transcription. The third research paper investigated the impact of maternal and infant vitamin D deficiency on offspring health using mice as an *in vivo* model. Vitamin D deficiency during pregnancy, lactation and infancy was found to affect male offspring to a greater extent than females causing stunted growth of whole-body weight and tissue weights and reduced muscle strength. However, in both male and female offspring, vitamin D deficiency caused insulin resistance and a reduction in physical activity.

Overall, the work in this thesis shows clear effects of vitamin D on myogenesis and a negative impact of maternal and infant vitamin D deficiency on offspring health. This highlights the importance of future work needed in this area to give pregnancy specific vitamin D supplementation guidelines and to try to tackle the global issue of vitamin D deficiency during pregnancy.

Chapter 1 Introduction

1.1 Vitamin D

1.1.1 Definition and Discovery

Rickets, a disease characterised by bone softening and deformity (Korkmaz et al., 2023), was described extensively during the mid-17th century, however the cause of this disease remained unknown until scientists in the 1800s observed that both cod liver oil and sunlight had the ability to cure rickets, as reviewed by Chang and Lee (2019). In 1922, it was discovered that a single factor within cod liver oil had the ability to cure rickets, which was termed 'vitamin D' (McCollum et al., 1922). Following this, in 1936 the chemical structure of vitamin D_2 (calciferol) was revealed (Windaus et al., 1936). Between 1936 and 1955, several vitamin D metabolites were discovered and the photochemical and thermal reaction steps from 7-dehydrocholesterol (pre-vitamin D found within the skin) to active vitamin D_3 were discovered, as summarised by Wolf (2004). Vitamin D is now known to be a fat-soluble vitamin which is classed as having hormone functions, regulating several processes within the body (Marazziti et al., 2021).

1.1.2 Sources and Synthesis

Vitamin D is a fat soluble vitamin which can be synthesised in the skin or consumed within foods such as oily fish, egg yolks, dairy products, liver and fortified foods such as cereals (Chang and Lee, 2019). In supplemented foods, vitamin D can be in the form of D_2 or D_3 (Borel et al., 2015). UV-B radiation (290-315 nm) is required for the synthesis of vitamin D in the skin and accounts for 80-90% of vitamin D within the body (Halfon et al., 2015). 7-dehydrocholesterol, which is found within the skin, is converted to pre-vitamin D upon exposure to UV-B radiation (Halfon et al., 2015).

Both melanin content of the skin and the use of sunscreen significantly reduce this conversion (Christakos et al., 2016). Pre-vitamin D is then converted into cholecalciferol (known as D_3) which is able to bind to vitamin D binding protein in the blood (Wiciński et al., 2019), which transports it to the liver (Halfon et al., 2015). Within the liver, an enzyme called 25-hydroxylase converts cholecalciferol to $25(OH)_2D_3$ or calcidiol (Kohlmeier, 2003). Calcidiol is the major circulating form of vitamin D (Chang and Lee, 2019).

There is evidence to suggest that there is more than one 25-hydroxylase enzyme because multiple genes encoding for this enzyme have been found, for example: CYP2R1, CYP27A1, CYP2C11 and CYP34A1 (Christakos et al., 2016). However, some of these have been ruled out: CYP2C11 has been found to be only expressed in rats and not humans, CYP27A1 knockout models appear to have normal $25(OH)_2D_3$ levels, and CYP34A1 appears to lack the ability to hydroxylate cholecalciferol (Dusso et al., 2005). On the other hand, crystal structure analysis of the liganded CYP2R1 hydroxylase shows that within the active site, the 17β-aliphatic side chain of cholecalciferol is present in the correct configuration for 25-hydroxylation (Christakos et al., 2016). In addition, CYP2R1 is expressed at a high level within the liver and mutations in this gene lead to low serum $25(OH)_2D_3$ levels (Dusso et al., 2005) suggesting that CYP2R1 plays a major role in cholecalciferol hydroxylation. Finally, $25(OH)_2D_3$ is converted to $1,25(OH)_2D_3$, or calcitriol, the biologically active form of vitamin D, in the kidney via the 1α-hydroxylase enzyme (Halfon et al., 2015). A schematic outlining the synthesis of vitamin D, taken from Kitson and Roberts (2012) is shown in Figure 1.1.

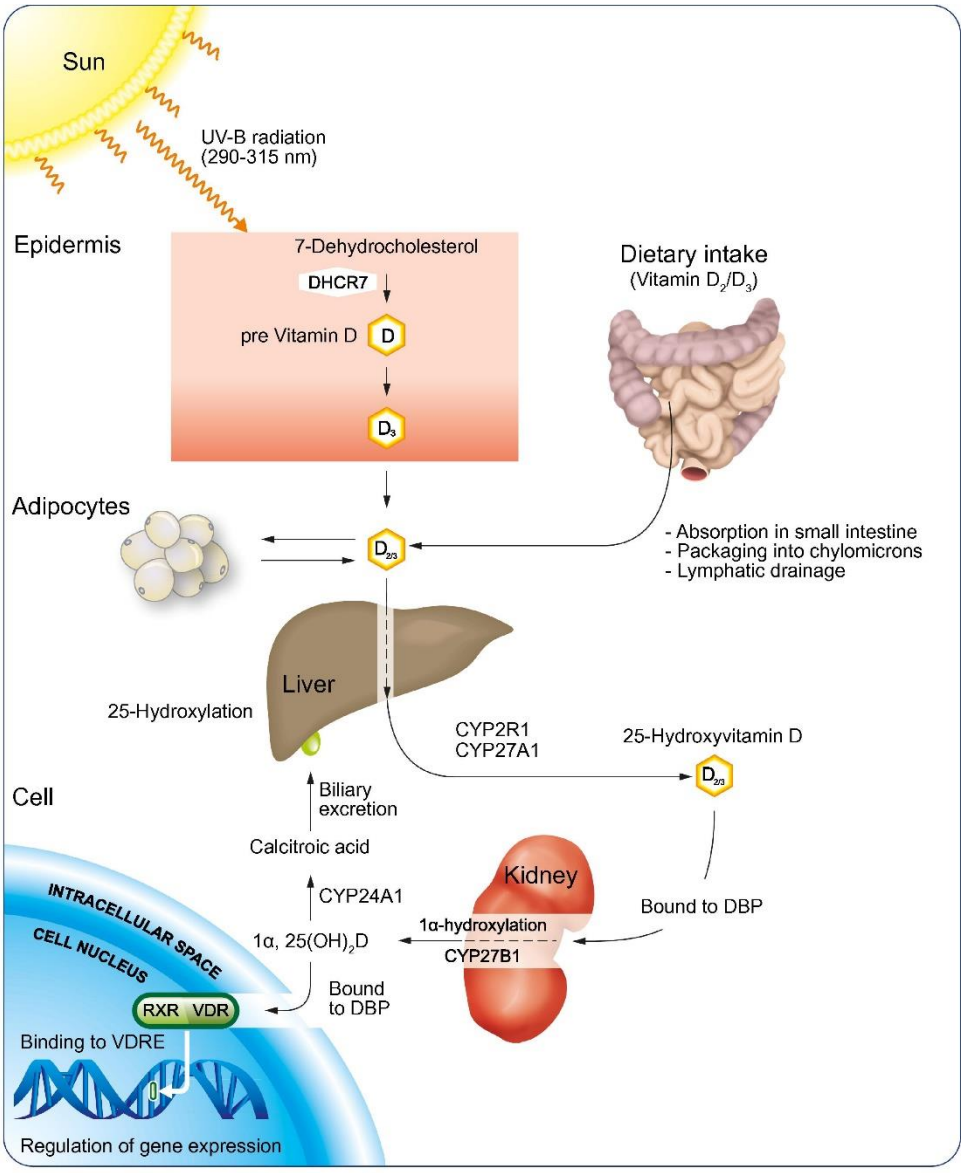

Figure 1.1 A schematic taken from Kitson and Roberts (2012) detailing the synthesis of vitamin D from 7-dehydrocholesterol through to $1,25(OH)_2D_3$, as well as the storage pathway for vitamin D within the adipose tissue, and also showing the genomic effects of vitamin D via the vitamin D receptor which is discussed further in section 1.1.7.1.

1.1.3 Bioavailability

It was assumed that vitamin D is absorbed in a similar manner to that of major lipids where several steps including emulsification, solubilisation into mixed micelles and permeation across the enterocyte membrane occurs (Tso and Fujimoto, 1991). However, the evidence suggests that different forms of vitamin D are absorbed via different mechanisms. Calcidiol ($25(OH)_2D_3$) and calcitriol ($1,25(OH)_2D_3$) are less

incorporated into micelles than cholecalciferol (D_3), which is the form often added to supplemented foods (Borel et al., 2015). Whilst D_3 can be solubilised into mixed micelles and absorbed via the lymphatic route, $25(OH)_2D_3$ and $1,25(OH)_2D_3$ are more polar and therefore possess the ability to become solubilised in water, so are mainly absorbed via the portal route (Maislos et al., 1981). This was confirmed by Maislos and Shany, (1987), who studied the effects of the absence of bile salts on the absorption into the portal vein system for hydroxylated and non-hydroxylated forms of vitamin D in rats. It was shown that $1,25(OH)_2D_3$ absorption was unaffected by a lack of bile salts, $25(OH)_2D_3$ was decreased, and absorption of D_3 was completely abolished, revealing that more polar vitamin D metabolites can be absorbed directly into the portal vein system without the involvement of bile salts and micelle formation. The main site of absorption for vitamin D is poorly understood in humans, but in rat studies, uptake was observed in both the jejunum and the ileum, with the ileum being the main site of absorption (Hollander et al., 1978, Reboul and Borel, 2011). The absorption rate of D_3, the main form of vitamin D in supplements and supplemented foods, has been shown to be between 55% and 99% in humans, with mean absorption being 78% (Thompson et al., 1966).

1.1.4 Transport and Activation

The major circulating form of vitamin D, $25(OH)_2D_3$, is lipophilic and therefore has low aqueous solubility so has to be transported by vitamin D binding protein (DBP) within the serum (Dusso et al., 2005). Only a very small number of vitamin D metabolites (<1%) circulate freely within the serum, the rest are bound to DBP (Gattineni and Friedman, 2015). The $25(OH)_2D_3$-DBP complex travels to the kidneys where $25(OH)_2D_3$ is converted into $1,25(OH)_2D_3$ (Halfon et al., 2015). $1,25(OH)_2D_3$ is the biologically active form of vitamin D and has a half-life of around 4-6 hours

(Chang and Lee, 2019). The enzyme responsible for the conversion of inactive to active vitamin D is called 1α-hydroxylase (Kohlmeier, 2003) and the process involves a single hydroxylation on carbon 1 of the A ring to form $1,25(OH)_2D_3$ (Christakos et al., 2016). This hydroxylation occurs within renal proximal tubules and is known to involve facilitated uptake and intracellular delivery of the precursor to 1α-hydroxylase (Dusso et al., 2005). $1,25(OH)_2D_3$ travels in the serum bound to DBP (Gattineni and Friedman, 2015) which binds to $1,25(OH)_2D_3$ with a much higher affinity than $25(OH)_2D_3$. However, levels of DBP are around 20 times higher than total vitamin D metabolites to ensure that almost all circulating vitamin D metabolites are bound (Dusso et al., 2005). Degradation of $1,25(OH)_2D_3$ is achieved via the enzyme 24-hydroxylase which catalyses reactions at carbon 23 and 24 resulting in side chain cleavage (Dusso et al., 2005). The 24-hydroxylase enzyme is tightly regulated by levels of phosphate, parathyroid hormone, fibroblast growth factor 23 and negative feedback of $1,25(OH)_2D_3$ itself (Gattineni and Friedman, 2015). Serum levels of $1,25(OH)_2D_3$ must be very tightly regulated to avoid toxicity (Dusso et al., 2005). Mice which lack the 24-hydroxylase enzyme have abnormally high serum levels of $1,25(OH)_2D_3$ due to their inability to degrade it (Husseini and St-Arnaud, 2018). Renal excretion of vitamin D is very low (<5%) (Gil et al., 2018).

As mentioned previously, $25(OH)_2D_3$ is the major circulating form of vitamin D (Chang and Lee, 2019). Levels of this vitamin D metabolite are not tightly regulated and fluctuate depending on dietary intake, hence it is considered a very good biomarker to assess vitamin D status (Dusso et al., 2005). In addition to being much more tightly controlled, serum levels of $1,25(OH)_2D_3$ only provide a measure of renally derived active vitamin D because locally produced $1,25(OH)_2D_3$ is used rapidly and is unlikely to leak into the circulation (Ameri et al., 2013). Therefore, serum

$25(OH)_2D_3$ level is a good indication of globally available vitamin D within the body (Ameri et al., 2013).

1.1.5 Physiological range and deficiency

Vitamin D deficiency is considered to be a world-wide issue affecting around 1 billion individuals globally (Bollen et al., 2022). The National Institute of Health advise that vitamin D insufficiency occurs when serum $25(OH)_2D_3$ levels are between 25-50 nmol/L and that deficiency occurs when levels fall below 25 nmol/L (Holick, 2017). In the UK, it is estimated that between 13 and 16% of adults (around 1 in 6) have a deficient or insufficient vitamin D status (GOV.UK, 2022). Groups at greater risk of deficiency include people with dark skin, people who spend most of their time indoors, pregnant women and the elderly, with around 20-40% of pregnant women worldwide being considered as vitamin D deficient, resulting in many children being born vitamin D deficient (Urrutia-Pereira and Solé, 2015). In the UK, it is currently advised that everybody considers taking a 10µg (400IU) vitamin D_3 supplement from October to March, because UV-B radiation levels during autumn and winter are not sufficient for adequate synthesis via the skin, and that at risk groups consider taking a supplement year-round (GOV.UK, 2022). However, it should be noted that some authors suggest that this 10µg/day recommendation is too low due to findings that 28µg/day was required to maintain serum 25(OH)D levels above 50nmol/L in 97.5% in the study sample (Cashman et al., 2008). Additionally, suncreams of SPF 30 or above reduce vitamin D synthesis by around 95% so people who wear sun cream during the summer months are also advised to consider supplementation year-round (Urrutia-Pereira and Solé, 2015). Although vitamin D supplements are available in both the D_2 and the D_3 form, D_3 supplementation raises serum $25(OH)_2D_3$

levels significantly more than D_2 and therefore D_3 is considered more efficacious at raising vitamin D status than D_2 (Tripkovic et al., 2012).

As mentioned, serum $25(OH)_2D_3$ levels are considered to be a good marker of globally available vitamin D and therefore, this metabolite is measured to assess an individual's vitamin D status (Ameri et al., 2013). However, only around 50% of $25(OH)_2D_3$ within the body is within the circulation whilst the rest is stored within the adipose and muscle tissues (Urrutia-Pereira and Solé, 2015). Within the circulation, 85-90% of $25(OH)_2D_3$ is bound to vitamin D binding protein, around 10-15% is bound to albumin and around 1% is free, with the latter two forms being bioavailable (Dzik and Kaczor, 2019). Some authors argue that in order to identify true vitamin D deficiency, bioavailability should be taken into consideration. Interestingly, one study showed that although black people had a lower serum $25(OH)_2D_3$ level compared with white people, the levels of bioavailable vitamin D were similar between the two groups (Powe et al., 2013). It is also suggested that free, or bioavailable, vitamin D status is a better predictor of bone health, especially in groups with darker skin, than total vitamin D status (Montenegro et al., 2019). Therefore, assessing vitamin D deficiency may be more complex than simply measuring serum $25(OH)_2D_3$ status.

The association between vitamin D and bone health has long been recognised with deficiency leading to diseases such as osteoporosis and rickets, as first recognised by McCollum et al, (1922). However, more research is now being undertaken into the relationship between vitamin D and other diseases and processes (Reid and Bolland, 2014). Conditions with evidence to suggest that they are associated with vitamin D include, but are not limited to: cardiovascular disease, muscle weakness and sarcopenia, inflammatory bowel disease, autism, diabetes, kidney disease, some

cancers (breast, colorectal, ovarian, lung and prostate) and skin conditions such as psoriasis and eczema, as reviewed in Sahota, (2014). During pregnancy, a low vitamin D status is associated with negative health outcomes including pre-eclampsia, insulin resistance, gestational diabetes, and increased frequency of caesarean delivery (Urrutia-Pereira and Solé, 2015). However, it should be noted that these negative health outcomes are also associated with maternal obesity, and that obesity is associated with lower serum vitamin D levels due to increased storage within adipose tissue (Alhomaid et al., 2021), so it is difficult to draw conclusions on whether these outcomes are as a result of vitamin D deficiency or increased risk of obesity. Studies have shown that maternal vitamin D ($25(OH)_2D_3$) levels in the lowest quartile (<9.6 ng/ml) significantly increases risk of pre-term birth and low birth weight compared to maternal vitamin D levels within the highest quartile (>29.5 ng/ml) (Mansur et al., 2022). Maternal vitamin D deficiency is also the main risk factor for deficiency in childhood. For the first 6-8 weeks of life, newborns rely heavily upon the vitamin D transferred across the placenta before birth with newborn vitamin D levels corresponding to 60-89% of maternal levels (Hillman and Haddad, 1974). To ensure the fetus receives vitamin D during pregnancy, maternal renal 1α-hydroxylase is upregulated between two to five fold (Fenton and Britton, 1980) and the placenta also expresses 1α-hydroxylase thereby converting $25(OH)D_3$ from the maternal serum to active $1,25(OH)_2D_3$ (Ashley et al., 2022). Few studies have measured vitamin D status or determined optimal maternal vitamin D levels during pregnancy. One meta-analysis found that oral supplementation with 2000 IU (50µg) vitamin D_3 during pregnancy significantly reduced the risk of pre-eclampsia (Irwinda et al., 2022) whilst another meta-analysis found that vitamin D_3 supplementation (dose ranging from 200 IU (5µg)/day to 600,000 IU (15,000µg) one off dose) during pregnancy reduced the risk of low birth weight and was associated

with an increased length and head circumference of newborns (Maugeri et al., 2019).

Considering that globally around 1 billion people are thought to be vitamin D deficient (Bollen et al., 2022), it is of great importance that the role vitamin D plays in processes within the body and in disease is understood, so that recommendations and interventions can aim to reduce this.

1.1.6 Vitamin D receptor

Biologically active vitamin D ($1,25(OH)_2D_3$) exerts its effects via the vitamin D receptor (VDR) which is a receptor belonging to the steroid receptor family including receptors for retinoic acid, thyroid hormone, sex hormones and adrenal steroids such as cortisol (Christakos et al., 2016). The VDR has nuclear hormone receptor properties, with the ability to translocate from the cytoplasm into the nucleus upon ligand binding, thereby affecting transcription of target genes (Wang et al., 2005).

It is thought that the VDR evolved some 550 million years ago in boneless fish and the oldest identified evolutionary function of the VDR was its involvement in energy metabolism and its role in immune function (Kimura, 1983). In humans, the VDR gene is located on chromosome 12 and includes eight coding exons and at least six non-coding exons (Christakos et al., 2016). The VDR gene is most highly expressed in metabolic tissues such as the kidneys, bone and intestine, but low to moderate expression is found in nearly all of the 250 human tissue and cell types (Dzik and Kaczor, 2019). The receptor is comprised of an N terminal domain, which has two zinc fingers giving the receptor its ability to bind to DNA, a C terminal ligand binding domain, which binds to the active form of vitamin D ($1,25(OH)_2D_3$), and an unstructured region linking the two (Pike and Meyer, 2012). The ligand binding C terminal domain has been solved by X ray crystallography and is comprised of 12

alpha helices (Rochel et al., 2000). Upon binding of $1,25(OH)_2D_3$ to the ligand binding pocket, a conformational change occurs which allows two protein-protein interactions to occur on the surface of the VDR (Pike and Meyer, 2012). One conformational change facilitates interaction with a protein partner, where the VDR can form a homodimer with another VDR, or a heterodimer with the retinoid acid receptor (RAR) or the retinoid X receptor (RXR), with the latter being the predominant heterodimer formed (Carlberg and Molnár, 2015). The other conformational change allows recruitment of co-regulatory complexes required for gene transcription such as p160 coactivators and steroid receptor activators 1, 2 and 3 (Pike and Meyer, 2012). These co-regulatory complexes typically contain a VDR interacting unit as well as other units which are usually involved in ATPase-containing nucleosome remodelling ability, methyl- and dimethyl-transferases and complexes which can recruit RNA polymerase II (Pike and Meyer, 2012). The VDR/RXR heterodimer binds to vitamin D response elements (VDREs) on target genes (Rochel, 2022). Active vitamin D directly increases transcription of the VDR and therefore a lower vitamin D status usually results in a lower level of the VDR (Dzik and Kaczor, 2019).

Studies in VDR knockout mice show both VDR dependent and VDR independent actions of $1,25(OH)_2D_3$ (Dusso et al., 2005). Likewise, studies in mice lacking the 1α-hydroxylase enzyme indicate that there is a $1,25(OH)_2D_3$ independent action of the VDR (Dusso et al., 2005) suggesting that both receptor and ligand may have some biological functions independent of each other, or that more than one ligand and receptor may be involved.

1.1.7 Vitamin D signalling

It is known that active vitamin D ($1,25(OH)_2D_3$) exerts genomic effects via the VDR, which acts as a transcription factor, however it also exerts effects which are too rapid to be accounted for by genomic effects and so these effects are termed as non-genomic actions (Bikle, 2014).

1.1.7.1 Genomic Effects

As mentioned previously, in order to exert genomic effects, $1,25(OH)_2D_3$ binds to the VDR and dimerisation occurs with either the retinoid X receptor (RXR), retinoid acid receptor (RAR) or with another VDR (Carlberg and Molnár, 2015). This heterodimer interacts with VDREs, which can range in position from the proximal promoter to thousands of base pairs from the transcriptional start site of the genes they regulate, to modulate transcription of target genes (Jeong et al., 2018). Studies show that the active transcription unit which binds to VDREs is predominantly, but not exclusively

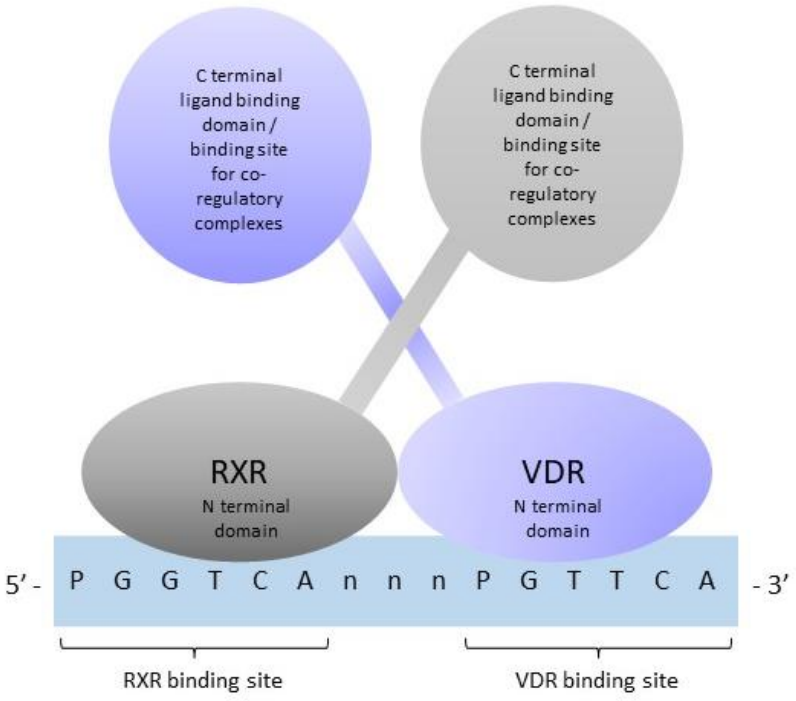

Figure 1.2. Illustration based on Haussler et al, (2011) to show the 'optimal' VDRE sequence with the highest binding affinity for the VDR/RXR heterodimer (PGGTCAnnnPGTTCA) where P is a purine base (A or G) and n can be any nucleotide. The RXR binding site is on the 5' side whilst the VDR binding site is on the 3' side. The ligand binding N terminal domain of the VDR and RXR bind to the VDRE via zinc fingers.

the VDR/RXR heterodimer (Bikle, 2014). There is some variability amongst VDRE sequences but those with the highest affinity for the VDR/RXR are direct hexameric repeats separated by a 3 nucleotide spacer (Tsoumpra et al., 2020). The 'optimal' VDRE with the highest binding affinity contains the RXR binding site on the 5' side with the sequence 5'-PGGTCA-3' and the VDR binding site on the 3' side with a sequence of 5'-PGTTCA-3' where P is a purine base (A or G) (Haussler et al., 2011), Figure 1.2.

Once bound to the VDRE, the VDR/RXR can recruit co-regulatory complexes which can be both gene specific and also cell specific, thereby enabling selectivity of vitamin D action between different cell types and even between different genes within the same cell (Bikle, 2014). The VDR/RXR complex has been shown to recruit co-activators such as the p160 co-activators and steroid receptor activators 1, 2 and 3 which have histone acetylase activity resulting in the unwinding of DNA, allowing access for transcriptional machinery (Christakos et al., 2016). Once bound, the p160 coactivators can recruit secondary coactivators such as CREB-binding protein and p300 which also have histone acetylase activity, resulting in the formation of stable, multi-subunit complexes that inhibit the interaction between the DNA and histones (Haussler et al., 2013). The liganded VDR also has the ability to interact with other transcription factors such as TFIIB which facilitates formation of a pre-initiation complex prior to transcription (Christakos et al., 2016). In their review of the genomic actions of vitamin D, Pike and Meyer (2010) outlined six key principles for VDR/RXR action on target genes: 1. The number of VDR binding sites on target genes is specific to the cell type; 2. The active transcription factor is predominantly, but not exclusively, the VDR/RXR heterodimer; 3. VDREs consist of predominantly, but not exclusively two hexameric repeats separated by a 3 nucleotide spacer; 4. Enhancer regions can be located both in the proximal promoter and in the distal promoter

regions, or a combination of both; 5. Enhancers are modular in nature, containing several subunits which can interact with several transcription factors; 6. Enhancers are usually cell type specific.

Over 900 genes have been found to be affected by vitamin D (Wang et al., 2005). This includes genes involved in several cellular processes such as: bone metabolism, mineral homeostasis, renal phosphate reabsorption (Haussler et al., 2011), bile acid metabolism, degradation of xenobiotic compounds in many tissues, differentiation of keratinocytes in the skin, development and cycling of hair follicles, functioning of the immune system (Bikle, 2014), and cell proliferation and differentiation (Wiciński et al., 2019).

1.1.7.2 Non-genomic effects

Although it is established that the VDR is present within the cytoplasm, with the ability to translocate to the nucleus and exert genomic actions, it has also been identified that the VDR can be anchored to the cell membrane within caveolae/lipid rafts (Dzik and Kaczor, 2019). When anchored to the membrane, the VDR is positioned to be able to activate kinases, phosphatases and ion channels in a fast manner, which is too fast to be accounted for by genomic effects (Bikle, 2014). Binding of vitamin D to a surface anchored VDR can activate c-Src (a non-receptor tyrosine kinase) and phoshoinositide-3 kinase, which then leads to activation of phospholipase C and thus IP3, which leads to the rapid release of calcium from the sarcoplasmic reticulum into the cytosol (Montenegro et al., 2019). The well-established effects of vitamin D on bone are predominantly indirect and are mediated by calcium and phosphate homeostasis (Bouillon et al., 2019). Studies have also shown a dose dependent increase of intracellular calcium levels in cultured muscle cells following treatment with $1,25(OH)_2D_3$ which may have an impact on

muscle contractility (Capiati et al., 2000, Vazquez et al., 1995). Src also has the ability to activate MAPK and ERK1/2 (Li et al., 2006). MAPK signalling is required for the maintenance of skeletal muscle mass whereby inhibition of the MAPK pathway leads to muscle atrophy both *in vitro* and *in vivo,* and ERK1/2 is thought to be involved in muscle hypertrophy effects induced by IGF1 (Dzik and Kaczor, 2019).

1.1.7.3 Vitamin D signalling in muscle

The vitamin D receptor has been identified to be present within muscle cells indicating that vitamin D has the ability to exert effects within muscle (Olsson et al., 2016). Studies in human skeletal muscles have identified the presence of the VDR, and that expression of the VDR is essential for uptake of $1,25(OH)_2D_3$ into muscle cells (Dzik and Kaczor, 2019). It is well established that low serum vitamin D levels are associated with a reduction in muscle strength (Mendoza-Garcés et al., 2021, Kalliokoski et al., 2013, Toffanello et al., 2012) as well as impaired muscle function, particularly within the elderly population (Aspell et al., 2019). Vitamin D supplementation, in deficient individuals, has been shown to improve muscle function and physical performance (Książek et al., 2019) and serum vitamin D status in athletes and physically active individuals is strongly correlated with exercise performance (Koundourakis et al., 2016). Finally, animal models show that vitamin D deficiency during pregnancy leads to reduced muscle size in the offspring (Max et al., 2014). Although there are clear effects of vitamin D signalling within muscle, the mechanisms behind these effects are largely unknown (Braga et al., 2017). Therefore, more research is needed to establish the nature of these effects, and the mechanisms involved.

1.2 Myogenesis

1.2.1 Skeletal Muscle

Skeletal muscle represents the largest tissue mass in the body, accounting for approximately 40% of total body weight. It contains 50-75% of total body proteins and accounts for 30-50% of protein turnover within the body (Frontera and Ochala, 2015). Skeletal muscle is highly organised containing several bundles of multinucleated muscle fibers (myofibers), each one representing a muscle cell, with its basic contractile unit called the sarcomere (Mukund and Subramaniam, 2020). Skeletal muscle contributes to many functions within the body. Firstly, it provides a mechanical role by converting chemical energy into mechanical energy to generate force and power in the form of movement. It also contributes to metabolism with a positive correlation between muscle mass and basal metabolic rate, it serves as a store for amino acids and carbohydrates, and plays a role in the maintenance of core body temperature due to the production of heat as a by-product when oxygen and fuel are used during movement or exercise (Frontera and Ochala, 2015). A decrease in skeletal muscle mass leads to negative health outcomes such as weakness, increased fatigue, decreased exercise capability, an impaired ability to cope with stress and chronic illness, and decreased quality of life (Yin et al., 2021).

1.2.2 Myogenesis – Formation of Skeletal Muscles

Within the growing embryo, skeletal muscles arise from somites which are embryonic structures originating from the paraxial mesoderm (Chal and Pourquié, 2017). Cells migrate from the somite towards the limb buds and it is only at this point, when they reach the limb site, that they begin to express myogenic determination genes, such as Myf5, and commit to a myogenic lineage (Tajbakhsh and Buckingham, 1994). Prior to the formation of skeletal muscle, myogenic

precursor cells undergo extensive proliferation. Transcription factors such as Pax3 are involved in maintaining this proliferative phase (Tajbakhsh et al., 1997). The pre-muscular masses for the limbs, which later go on to split up and form individual, distinct muscles, contain two parts: an outer layer of Pax3 and Myf5 expressing proliferating muscle precursor cells, and a deeper layer of differentiating myoblasts which begin to express differentiation factors such as MyoD and skeletal muscle proteins such as type I and embryonic myosin heavy chains, α-actin and desmin (Babai et al., 1990, Ott et al., 1991, Christ and Brand-Saberi, 2002). In order to enter this differentiative stage, cells must cease to proliferate by exiting the cell cycle. To do this, myoblasts begin to express p21, which becomes transcriptionally activated by MyoD (Christ and Brand-Saberi, 2002). p21 is a potent cyclin dependent kinase (CDK) inhibitor, effectively inhibiting CDK2, CDK3, CDK4 and CDK6, blocking the ability of cyclin/CDK complexes to form and thus preventing cells from being able to progress through the cell cycle (Harper et al., 1995). This results in irreversible exit from the cell cycle, initiating the differentiation stage. Once myoblasts have entered the differentiation stage, re-addition of mitogens (proteins which stimulate cell division) does not supress p21 expression, nor does it re-activate DNA synthesis, suggesting that irreversible cell cycle withdrawal correlates with irreversible activation of p21 (Walsh and Perlman, 1997).

Once myoblasts have exited from the cell cycle, and committed to differentiation, myoblast fusion occurs, which in mammals is a 2-stage process (described as the embryonic stage and the fetal stage). In the first phase, myoblast-myoblast fusion occurs to form multinucleated cells which form nascent myotubes. In the second phase, additional myoblasts fuse with the nascent myotube leading to an increased number of nuclei in the growing myotube. Due to repulsion forces between lipid head groups on opposing membranes, fusion of plasma membranes has to be

catalysed by muscle specific cell-surface proteins called fusogens (Hernández and Podbilewicz, 2017). This includes Myomaker (MymK) and Myomerger/Myomixer/Minion (Mymx) as well as a host of other additional protein and lipid species to ensure a favourable biochemical event, as reviewed by Millay (2022). This also increases the cytoplasmic volume and protein synthesis becomes elevated, leading to an increase in myotube size (Lehka and Rędowicz, 2020).

Embryonic skeletal muscle growth occurs due to a fine balance between proliferation and differentiation. It is important that some cells continue to proliferate to allow muscle growth and enlargement of muscle size, whilst differentiation must also occur in order to develop established and functioning muscles (Christ and Brand-Saberi, 2002), see figure 1.3.

The first muscle fibers to appear are the primary fibers, followed by the secondary fibers which form around the primary ones. In mice, these primary fibers appear around embryonic day 11-14 with the secondary fibers appearing around day 14-16 (Ontell and Kozeka, 1984). In humans, the primary fibres form between 6 and 8 weeks and the secondary fibres form between 8 and 18 weeks during gestation (Barbet et al., 1991). Primary fibers become slow twitch (type 1) muscle fibers with secondary fibers becoming the fast twitch (type 2) fibers (Evans et al., 1994). Slow and fast fibers can be identified by distinct morphological and biochemical properties as well as expressing different types of myosin heavy chain proteins (Page et al., 1992). Slow twitch fibers are oxidative in nature, have a well-established blood supply, are fatigue resistant and have a slow contractile speed whereas fast twitch fibers are glycolytic in nature and therefore are less vascular, have a fast contractile speed and fatigue more quickly, as reviewed by Wang and Pessin (2013). It is thought that commitment to either a slow or fast fiber type lineage occurs within the myogenic precursor cells whilst they are still within the somite (Nikovits et al.,

2001). In some species (pig, sheep, bovine and human) a third wave of tertiary fibers form following primary and secondary fiber formation (Brameld et al., 2003a). In the final stages of fiber formation, a last wave of myogenic cells are formed, which lie adjacent to the existing muscle fibers, called satellite cells. These satellite cells form during the final period of embryogenesis and enter a quiescent state within the tissue, but remain primed for activation, and are responsible for skeletal muscle regeneration and repair during the extent of an individual's lifetime (Hernández-Hernández et al., 2017).

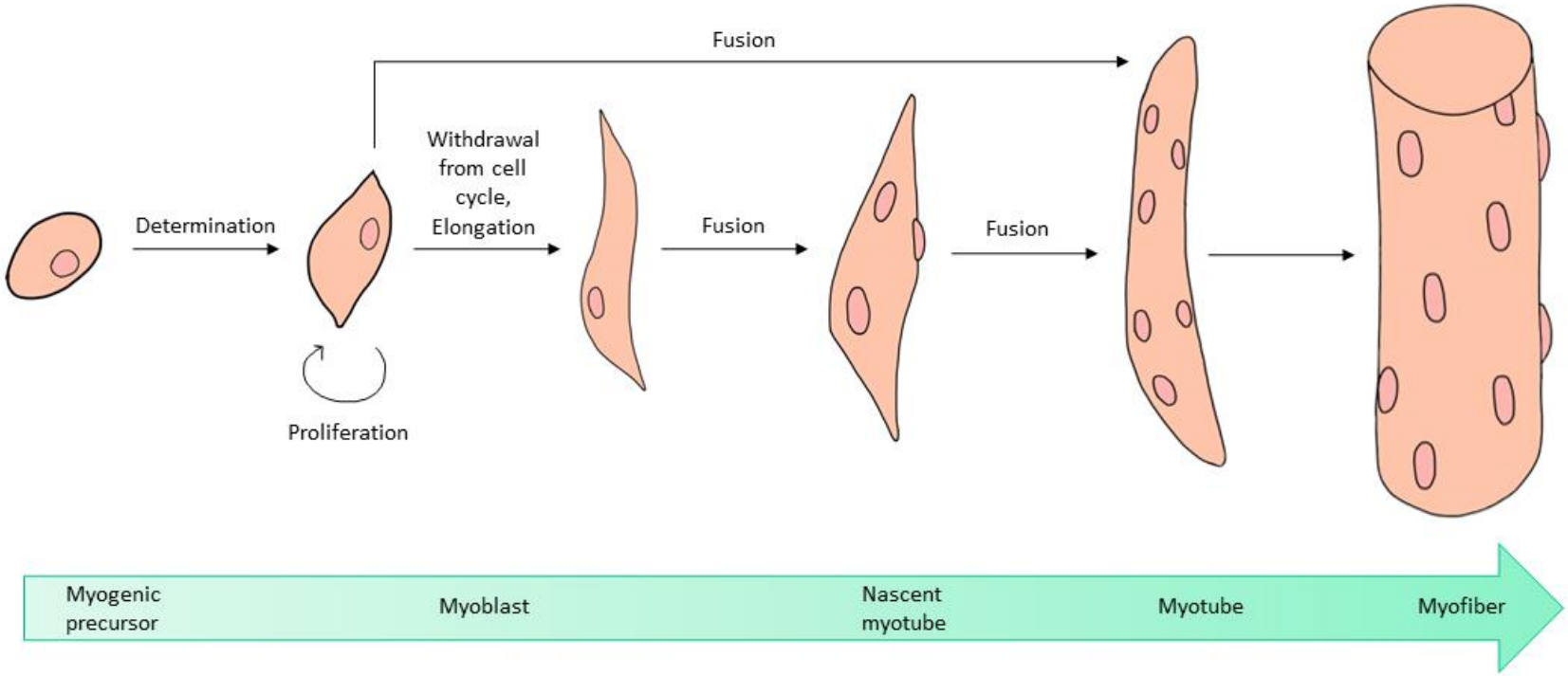

Figure 1.3. The stages of myogenesis. Myogenic precursor cells begin to express myogenic specific genes which causes determination to the myogenic lineage and cells become myoblasts which proliferate extensively to increase cell number. Upon initiation of differentiation, myoblasts exit the cell cycle and begin to elongate before fusing together to form nascent myotubes. Further myoblasts fuse onto the nascent myotube to form large, multinucleated myotubes. Protein expression of muscle specific proteins increases (such as myosin heavy chain, myosin light chain and actin) and cytoplasmic volume increases causing secondary myotubes to mature into myofibers. Figure drawn by K. Alliband based on information from Lehka and Rędowicz (2020).

1.2.3 Myogenic regulatory factors (MRFs)

The myogenic regulatory factors (MRFs) are a group of four muscle-specific transcription factors which include MyoD, Myf5, myogenin and myogenic regulatory factor 4 (MRF4). They are basic helix-loop-helix transcription factors which regulate transcription of target genes (Lehka and Rędowicz, 2020). Upon heterodimerisation with a member of the ubiquitously expressed E-protein family, the MRF-E protein dimers are able to bind directly to DNA via recognition of E box sequences (CANNTG) (Hernández-Hernández et al., 2017). These factors have multiple roles within muscle development and function and are involved in establishing skeletal muscle phenotype through regulation of proliferation, irreversible cell cycle arrest of myogenic precursor cells and differentiation. The location, timing and level of expression for each of these MRFs during myogenesis is finely modulated to allow cells to move through the process accurately in order to form functional skeletal muscle (Hernández-Hernández et al., 2017).

MyoD was the first MRF to be discovered in 1987 (Davis et al., 1987), whilst the other three MRFs were discovered shortly after by screening of cDNAs with similar homology to MyoD (Weintraub et al., 1991). Structurally, MyoD and Myf5 are more similar to each other (sharing 53% homology) than either of them are to myogenin (40% and 38% respectively) or to MRF4 (40% and 43%). Similarly, myogenin and MRF4 are more similar to each other (53%) than to MyoD or Myf5 (Megeney and Rudnicki, 1995). It is thought that there is an evolutionary relationship between the MRFs where MRF4 arose from Myf5, myogenin then arose from MRF4 and MyoD arose from Myf5. This theory originates from phylogenetic analysis which suggests that all four MRFs are likely to have evolved from one single myogenic factor (Atchley et al., 1994).

Following their discovery, the function and pattern of expression of the MRFs was investigated. In mice, the first MRF (Myf5) is detected within the myotome around embryonic day 8. Sonic hedgehog (Shh) signalling induces Myf5 expression via the mab-related transcription factor 2 (Dmrt2) (Borycki et al., 1999, Sato et al., 2010). At embryonic day 10.5, MyoD is expressed. Both Shh and Wnt signalling (an ancient and evolutionarily conserved pathway which regulates cell fate, cell migration, polarity and formation of tissues during embryogenesis) activates expression of MyoD (Hernández-Hernández et al., 2017). When Shh is knocked out within cultured mouse embryonic limb bud cells, they fail to express both Myf5 and MyoD, which results in failure to form differentiated limb muscle myotubes (Krüger et al., 2001). Gli2 is a transcription factor which induces expression of Shh (Pan et al., 2006). Shh then directly induces the expression of Myf5 through binding to responsive elements within the Myf5 enhancer region, leading to the determination of myogenic progenitor cells to a myogenic lineage (Anderson et al., 2012). It has also been shown that Gli2 is able to bind to MyoD gene elements to activate the transcription of MyoD, and that it also has the ability to bind to the MyoD protein itself to regulate MyoD activity at the protein level (Voronova et al., 2013).

Over-expression of MyoD in non-muscle cells results in conversion to myoblast-like cells, giving the first suggestion that MyoD is important in the commitment towards a myogenic lineage (Lassar et al., 1991). In double MyoD/Myf5 knockout mouse models, there is a complete lack of skeletal muscle formation due to an absence of any population of myoblast precursor cells (Rudnicki et al., 1993). These mice are born alive but die within a few minutes of birth. In the absence of MyoD and Myf5, cells within the somite, which would normally become myoblasts, do not migrate correctly to the sites of myogenesis and instead adopt other cell fates (Buckingham et al., 2003), therefore demonstrating the role of MyoD and Myf5 as myogenic

determination factors. Mice lacking only the MyoD gene are viable and do not show any apparent skeletal muscle abnormalities. However, the level of Myf5 mRNA is drastically increased in these mice suggesting that an increase in Myf5 may be able to substitute for the absence of MyoD during embryogenesis (Rudnicki et al., 1992). When Myf5 is knocked out, MyoD expression occurs normally, followed by normal myogenin expression, suggesting that MyoD activation is independent of Myf5 and that MyoD can also substitute for the absence of Myf5 (Rudnicki et al., 1993).

Both myogenin and MRF4 are expressed later in myoblasts, after the onset of differentiation (Nikovits et al., 2001). Myogenin acts downstream of MyoD and Myf5 and is essential for the differentiation of myoblasts into myotubes (Montenegro et al., 2019). When either MyoD or Myf5 are knocked out, myogenin is still expressed, but when both MyoD and Myf5 are absent, myogenin expression is severely affected (Hernández-Hernández et al., 2017). When myogenin itself is mutated, mice are born immobile and die immediately after birth (Hasty et al., 1993). In these mice, myoblasts are formed as normal but there is a complete lack of differentiated skeletal muscle. This was the first evidence to suggest that myogenin is responsible for the later stages of differentiation, following the process of myogenic determination regulated by MyoD and Myf5. The myoblasts present in these myogenin$^{-/-}$ mice were mononucleated and expressed normal levels of MyoD but lacked expression of myosin heavy chain isoforms and actin. As a result of this, myogenin is described as a differentiation specific factor which is important for the fusion of myoblasts to form multinucleated myotubes and activation of muscle specific genes (Hernández-Hernández et al., 2017).

MRF4 shows a bi-phasic pattern of expression during mouse embryogenesis whereby it is first detected around embryonic day 9, becomes down-regulated by day 11.5 but then re-appears around day 16 within differentiated muscle fibers,

suggesting a dual role in both determination and differentiation (Sassoon et al., 1989). MRF4 knockout mouse models show inconsistent results despite being similar in design. One study found that knocking out MRF4 results in inhibited formation of muscles within the deep back and rib which resulted in death at birth (Braun and Arnold, 1995), whilst another found that a lack of MRF4 resulted in severe rib phenotype abnormalities but mice were viable (Patapoutian et al., 1995). Finally, a third MRF4 knockout study found that mice had a few misshapen, bifurcated rib muscles, but were fully viable and appeared healthy (Zhang et al., 1995). Mice in this latter study also showed an upregulation of myogenin suggesting a possible compensatory role of myogenin for MRF4. Double MyoD/MRF4 knockout mice die at birth and show a phenotype extremely similar to myogenin knockout mice. In these MyoD/MRF4$^{-/-}$ mice myogenin is still expressed, suggesting that neither of these factors play a crucial role in activation of myogenin expression, but that myogenin expression in the absence of MyoD and MRF4 is insufficient to support normal myogenesis (Rawls et al., 1998). Under normal circumstances, in differentiated, mature muscle fibers, expression of MyoD, Myf5 and myogenin is down-regulated, whereas MRF4 continues to be expressed at high levels and acts as the predominant MRF in mature muscle (Hinterberger et al., 1991).

1.2.4 Myosin heavy chains

A myosin molecule consists of two myosin heavy chains (MHC), two essential myosin light chains and two regulatory myosin light chains (Schiaffino et al., 2015). MHC is a large (200kDa) protein molecule which comprises an N-terminal globular 'head' domain, a neck region where the light chains bind and a C-terminal long alpha-helical tail domain (Schiaffino and Reggiani, 1996). Hundreds of MHCs come together to form a 'thick filament' via the tail domains aligning into a distinct

pattern. This thick filament contains hundreds of myosin 'heads' each containing binding sites for both ATP and actin. The myosin heads are able to bind to the actin filaments and slide over each other, using energy from ATP, resulting in shortening of the sarcomere and thus muscle contraction (Guhathakurta et al., 2018), Figure 1.4.

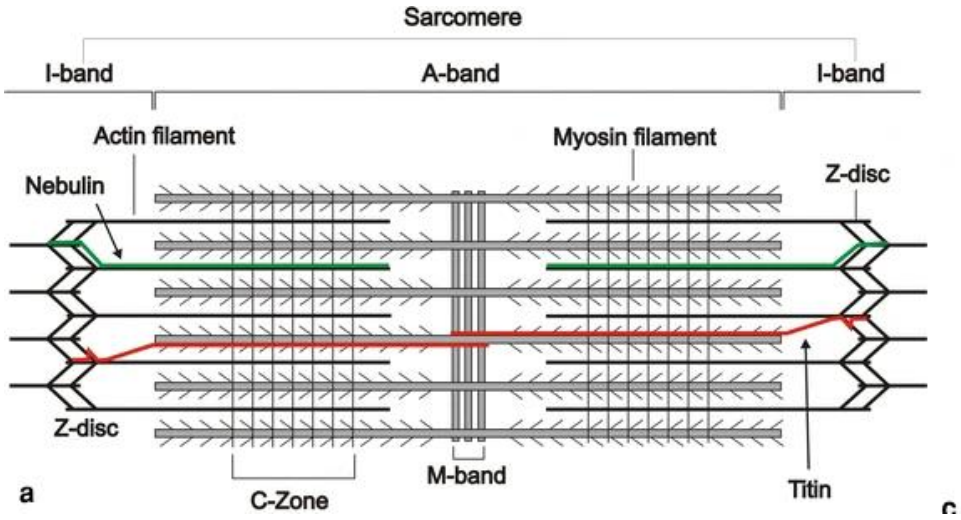

Figure 1.4. Structure of the sarcomere taken from Luther (2009). Key components of the sarcomere, the basic contractile unit within a muscle cell, are labelled.

Skeletal muscle is comprised of four major MHC isoforms, type I, IIa, IIx and IIb, with two further isoforms, embryonic (emb) and neonatal (neo), which are generally only expressed during fetal development or during muscle regeneration following injury or disease where they are transiently re-expressed (Sartore et al., 1982). The speed of contraction of a muscle fiber depends on which MHC isoform is predominant. Different MHC isoforms have different ATPase activity which gives rise to different speeds of contraction. The predominant MHC isoform varies from muscle to muscle, depending mainly on the function of the muscle, and muscles can be very broadly classified 'fast' or 'slow' twitch (Bárány, 1967). Therefore, slow (or type I), and fast (or type II) muscle fibers can be defined by their expression of different MHC isoforms. Type II fibers can be sub-divided into three major subtypes (IIa, IIx and IIb)

(Talbot and Maves, 2016). The expression of these different MHC isoforms depends on the muscle itself, but can also be influenced by exercise, age and disease (Resnicow et al., 2010). Type I fibers are oxidative in nature, possess a slower contractile speed and are resistant to fatigue. Type IIa fibers are classed as oxidative glycolytic and have a faster contractile speed than type I and are only somewhat resistant to fatigue. Type IIx fibers are described as fast glycolytic and have a fast contractile speed, but fatigue quickly. Finally, type IIb fibers are also glycolytic and have the fastest contractile speed, but fatigue very quickly (Plotkin et al., 2021). Whilst MHC IIb is highly expressed in fast skeletal muscles of small mammals, it is not detectable in humans where IIx is the predominant fast isoform present (Schiaffino and Reggiani, 1994). The developmental MHCs (emb and neo) have been shown to have low ATPase activity and so are considered slow, likely due to the low impact/force that the muscles are subjected to during fetal development (Drachman and Johnston, 1973).

Brown et al, (2012), showed that the pattern of mRNA expression for the MHC isoforms during myogenic differentiation *in vitro* occurs in two distinct cohorts whereby the slow isoforms (emb, neo and type I) increase during the early to mid-stages of differentiation, then decrease in expression, then the fast isoforms increase towards the later stages of differentiation, after the type I isoforms begin to decrease, in the order IIa, IIx, IIb. *In vivo* MHC emb is first detected in the mouse embryo around day 9.5 and MHC neo at day 10.5 (Lyons et al., 1990). MHC emb and neo then disappear in most skeletal muscles following birth and the adult fast MHC isoforms begin to be expressed. In rats, the transcripts for IIa, IIx and IIb can be detected a few days post birth and become more predominant in the following weeks (DeNardi et al., 1993). In mice this occurs earlier, with small amounts of type II isoform transcripts detectable just before birth, however at the protein level, new

born mouse muscles contain around 70% MHC neo and around 30% MHC emb with traces of MHC I, and MHC fast isoforms are undetectable (Agbulut et al., 2003). Following this, there is a switch from the developmental isoforms to the adult isoforms. In mice, MHC neo becomes totally absent in most muscles 21 days post-birth, and by 49 days, in fast contracting hind limb muscles, type II isoforms make up 90% of the total MHC content (Resnicow et al., 2010).

Although several factors involved in myogenesis have been well studied, and the functions of the MHC isoforms in adult skeletal muscle are well understood, little is known about the role of MHCs during differentiation. Despite not being expressed during fetal development, mice lacking either MHC IIx or IIb are significantly smaller at birth, weighing less than controls, have reduced grip strength and increased muscle weakness (Acakpo-Satchivi et al., 1997). Mutations in MHC emb can lead to Freeman-Sheldon syndrome (FSS), an exceptionally rare disorder affecting muscles of the face and skull, as well as other congenital contracture syndromes (Toydemir et al., 2006). It has also been shown that MHC emb is involved in regulation of muscle fiber size, fiber number and fiber type as well as being involved in regulation of progression through differentiation. In both extensor digitorum longus (EDL) and soleus muscles, loss of MHC emb results in a greater number of myofibers and in the soleus there is a reduction in fiber size, showing that MHC emb could be involved in regulation of fiber number in both fast and slow muscles and fiber size in slow muscles (Agarwal et al., 2020). It is clear that MHCs, particularly the developmental isoforms, have a vital, but poorly understood, role during fetal development.

1.2.5 Muscle Cell Lines

C2C12 is a commonly used muscle cell line derived from satellite cells from the thigh muscle of 8-week-old female C3H mice. C2C12 cells readily proliferate in high serum

media (media supplemented with 10-20% fetal bovine serum) and differentiation can be induced by switching to a low serum media (typically 2% horse serum), resulting in mature myotube formation within approximately 7 days (Diokmetzidou et al., 2016). By 8-10 days of differentiation, some C2C12 myotubes have been shown to start spontaneously contracting (McMahon et al., 1994). Alongside C2C12 cells, L6 cells (derived from newborn rat thigh muscle) are also commonly used. C2C12 cells have been shown to resemble human muscle tissue more closely in terms of myosin content and are often the chosen cell line for studying effects on contraction and muscle atrophy and hypertrophy (Abdelmoez et al., 2020). L6 cells are commonly used for the study of metabolism due to their higher level of insulin and glucose responsiveness compared to C2C12s (Kase et al., 2013).

Cell lines such as C2C12 and L6 can be more suitable for cell culture experiments compared to primary muscle cells because they tend to proliferate and differentiate much more quickly with the average doubling time for C2C12 cells being 14 hours compared to 76 hours for primary human skeletal muscle myoblasts (Abdelmoez et al., 2020). Therefore, proliferation and differentiation studies can be carried out in a relatively short period of time.

One limitation of muscle cell lines is that every cell, when stimulated, will differentiate from a myoblast to a myotube. This does not reflect myogenesis *in vivo* where some cells enter a quiescent state, instead of differentiating, to become satellite cells. These cells become activated in response to stimuli such as muscle damage, differentiate, and fuse onto existing myotubes to enable muscle repair (Hernández-Hernández et al., 2017).

1.3 Aims and Objectives

Whilst the understanding of skeletal muscle myogenesis has greatly improved since the 1990s, when the MRFs were first discovered, the effects of nutritional factors such as vitamin D on muscle development are still poorly understood with a large amount of conflicting evidence published to date. With 20-40% of pregnant women worldwide having vitamin D deficiency, it is vital that a better understanding of the effects of vitamin D deficiency on myogenesis is gained. The aim of this thesis was to carry out the following:

1. To summarise the current body of *in vitro* evidence on the effects of vitamin D on myogenesis and to establish any issues within the current literature to enable better quality experiments to be carried out.
2. To use information gained from 1. to carry out *in vitro* experiments to analyse the effects of vitamin D on proliferation and differentiation of C2C12 cells.
3. To investigate molecular mechanisms involved in the effects of vitamin D on myogenesis by looking at regulation of genes such as the myogenic regulatory factors and the myosin heavy chain isoforms as well as bioinformatic work to identify genes which the vitamin D receptor may be able to bind to.
4. To carry out an *in vivo* mouse trial where mice are fed a diet deficient in vitamin D and to study offspring of these mice to look at the effects on serum vitamin D status, body weight, tissue weights, insulin responsiveness, muscle strength, physical activity, and behaviour.

Chapter 2 – Manuscript 1

Alliband K. H., Kozhevnikova S. V., Parr T., Jethwa P. H. & Brameld J. M. 2021. Effects of Biologically Active Vitamin D on Myogenesis: A Systematic Review. *Front Physiol*, 12, 736708.

2.1 Abstract

Vitamin D (VD) deficiency is associated with muscle weakness. A reduction in the incidence of falls in the elderly following VD supplementation and identification of the VD receptor within muscle cells suggests a direct effect of VD on muscle, but little is known about the underlying mechanisms. Here we systematically searched the literature to identify effects of active VD ($1,25(OH)_2D_3$) on skeletal muscle myogenesis *in vitro*, with no restriction on year of publication. Eligibility was assessed by strict inclusion/exclusion criteria and agreed by two independent investigators. Twelve relevant papers were identified using four different cell types (C2C12, primary mouse satellite cells, primary chick myoblasts and primary human myoblasts) and a range of myogenic markers (myoD, myogenin, creatine kinase, myosin heavy chain and myotube size). A clear inhibitory effect of $1,25(OH)_2D_3$ on proliferation was reported, while the effects on the different stages of differentiation were less consistent, probably due to variation in cell type, time points and doses of $1,25(OH)_2D_3$ used. However, myotube size was consistently increased by $1,25(OH)_2D_3$. Overall, the evidence suggests that $1,25(OH)_2D_3$ inhibits proliferation and promotes differentiation of myoblasts, but future studies should use time courses to gain a clearer understanding.

2.2 Introduction

The link between vitamin D (VD) and bone health has been studied extensively, but recent evidence points towards a relationship between VD and skeletal muscle

function (Wiciński et al., 2019). Muscle biopsies from VD deficient individuals show muscle wasting (mostly type II fibre atrophy), large interfibrillar spaces and fat infiltration within the muscle (Ceglia, 2008). In general, deficiency occurs when levels of $25(OH)D_3$ (inactive vitamin D) fall below 25nmol/L, however this cut-off point can vary within the literature (Halfon et al., 2015). In the elderly population, VD deficiency has been linked to an increased risk of falls which is thought to be partly due to muscle weakness and wasting (Garcia et al., 2011).

Around 80-90% of VD is obtained via UV-B induced synthesis in the skin in humans, whilst 10-20% comes from dietary intake (Halfon et al., 2015). In the skin, 7-dehydrocholesterol is converted to pre-vitamin D upon UV-B radiation. As age increases, the concentration of epidermal 7-dehydrocholesterol decreases, therefore lowering the capacity of the skin to produce pre-vitamin D in older individuals (MacLaughlin and Holick, 1985). This pre-vitamin D is then converted to cholecalciferol which becomes bound to VD binding globulin and this complex is transported to the liver where it undergoes hydroxylation by 25-hydroxylase to form $25(OH)D_3$ or calcidiol (Hamilton, 2010). $25(OH)D_3$ is the major circulating form of VD and is measured as a marker of VD status (Pojednic and Ceglia, 2014). A final step, to produce the biologically active form of VD, involves hydroxylation by 1α-hydroxylase to produce $1,25(OH)_2D_3$ otherwise known as calcitriol (Hamilton, 2010), Figure 2.1. 1α-hydroxylase is expressed largely in the kidney, which contributes to active VD in the circulation, however the enzyme is also expressed within other tissues such as muscle and immune cells which allows local conversion of inactive to active VD (Ceglia, 2008).

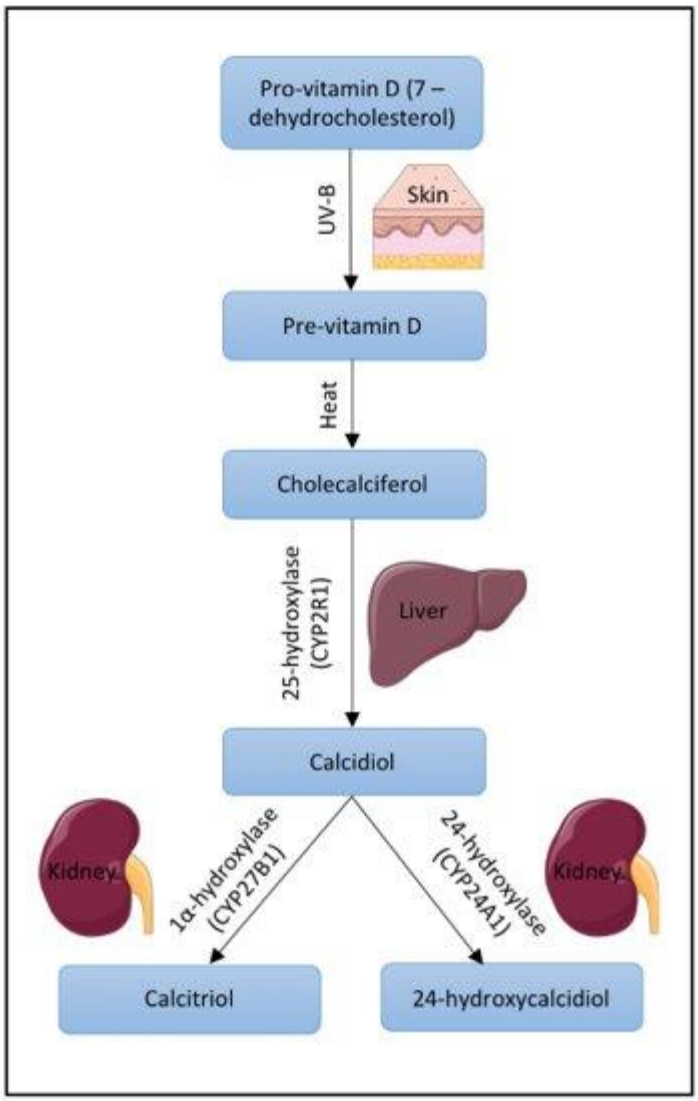

Figure 2.1. Vitamin D synthesis within the body including precursors, enzymes and body site. Images used within this figure were obtained from Smart Servier Medical Art and can be found at https://smart.servier.com.

Studies in both chicken and human skeletal muscle have identified the presence of the vitamin D receptor (VDR) within muscle cells thereby providing evidence for a direct effect of VD on muscle (Bischoff et al., 2001, Zanello et al., 1997). This has since been supported by human studies which have found that low serum 25(OH)D_3 concentrations in elderly individuals is associated with reduced muscle strength and an increased risk of falls (Ceglia and Harris, 2013). These effects of VD deficiency on muscle appear to be reversible with supplementation in the elderly population leading to beneficial outcomes such as increased strength, balance, and a decreased

risk of falls (Harwood et al., 2004). This effect is thought to be, at least in part, directly through the VDR present in muscle cells. VDR knockout mice have been found to have muscle fibres which are 20% smaller in size than controls as well as smaller body size, weight, and impaired motor co-ordination (Pojednic and Ceglia, 2014).

Within the literature, VDRs have been described in different cell locations, one as a nuclear hormone receptor and the other as a membrane receptor (Ceglia, 2008). The origin of the membrane receptor is unclear, some argue there is a distinct membrane receptor, however the majority of evidence points towards one VDR with the ability to translocate between the nucleus and membrane (Halfon et al., 2015).

It is well known that the VDR has a nuclear hormone receptor function, with the transcription of over 900 genes found to be affected upon treatment with active VD (Wang et al., 2005). $1,25(OH)_2D_3$, binds to the VDR which induces heterodimerisation with the retinoid X receptor (RXR). This complex is then able to bind to VD response elements (VDREs) to activate or repress transcription of target genes (Halfon et al., 2015). Expression of genes involved in myogenic proliferation and differentiation have been shown to change upon treatment with VD leading to the suggestion that VD may have a direct effect on myogenesis (Wiciński et al., 2019).

The aim of this systematic review is to summarise the current body of evidence on the effects of active VD on skeletal muscle cells in culture. There is conflicting evidence in this area, therefore this review aims to summarise, assess, and interpret the current body of evidence and identify areas where further investigation is required.

2.3 Materials and Methods

This review was constructed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2009).

2.3.1 Search and Selection Criteria

Relevant papers were identified through the computerised search database (PubMed (MEDLINE), Web of Science and Google Scholar). The search process followed the population (P), Intervention (I), Comparison (C) and outcome (O, PICO). The review population was *in vitro* models of muscle cells, the intervention was active VD treatment, comparison was controls not treated with VD and the measuring outcomes were the effects of active VD on muscle proliferation and differentiation. Specific search terms 'vitamin D OR 1,25Dihydroxyvitamin D_3 OR $1,25(OH)_2D_3$ OR calcitriol AND myogenesis OR muscle differentiation' were used to obtain relevant articles. To obtain the relevant articles, two independent reviewers (KHA & SVK) assessed the titles, abstract and full articles based on a strict inclusion and exclusion criteria and if any disagreements arose, these were resolved by discussion. Finally, the reference list of these were searched to find any additional papers.

2.3.2 Selected Articles Criteria

Articles were not restricted to any dates as there have been no previous systematic reviews conducted investigating the literature relating to active VD and myogenesis *in vitro*.

Inclusion Criteria

- Studies must have been written in English to avoid any translation errors.

- All articles must have described an *in vitro* model of muscle cells (primary or cell line).
- Any form of active VD can be considered ($1,25(OH)_2D_3$ or active VD analogues).
- Treatment of VD must be of known quantity and administered alone and not in combination with other drugs/vitamins/minerals.
- Must determine effects on proliferation/differentiation of muscle cells.

Exclusion Criteria

- Whole animal or human models.
- Systematic reviews or critical reviews.
- Studies investigating VD receptor and not VD.
- Studies investigating cancer or ageing.

2.3.3 Measured Outcomes

The primary measured outcomes of this review are markers of myogenesis such as level of DNA synthesis, mRNA and protein levels of myoD, myogenin, myosin/myosin heavy chain isoforms, creatine kinase activity and myotube size. No secondary outcomes were measured.

2.3.4 Data extraction

Using a standard extraction form, data from all studies were extracted and charted using Excel (Microsoft Excel, Washington, USA). Data extracted included title, author, publication year, muscle cell model used, exposure to VD and outcomes (DNA, myogenin, myoD, creatine kinase, myosin and myotube size). All key characteristics of the selected papers were expressed in tables. These included the

study design, model used, number of samples, outcome measures and doses of VD converted into moles for consistency.

2.3.5 Data Analysis

The significant effects ($p<0.05$) in response to VD were charted to compare across the articles reviewed, however some values were read from graphs where raw data was not provided so are best estimates. Changes in expression were used to generate bar graphs using Excel (Microsoft Excel, Washington, USA), all changes were converted to fold-change for consistency.

Meta-analysis could not be carried out due to variation in methods between papers. Differences in cell type, time points used and concentrations of active vitamin D used meant that direct comparisons in the form of a meta-analysis was not possible.

2.3.6 Quality Assessment

The quality assessment method used in this review is a modified version of the Risk of Bias (RoB) 2 tool from the Cochrane database to assess risk of bias in randomised trials. This assessment tool has been modified to be appropriate for cell culture experiments such as those included within this review (Supplementary Data 3.1). Responses in green indicate potential markers for a low risk of bias, orange indicates moderate risk and red indicates potential markers for a high risk of bias. (Y = yes, PY = probably yes, PN = probably no, N = no, NI = no information given or not applicable). Questions starting with 1 relate to risk of bias from treatment allocation. Questions starting with 2 relate to risk of bias in measurement of the data. Questions starting with 3 relate to risk of bias in selection of the reported result. Three or four questions were used to assess each section and an overall risk of bias

was decided upon. There are three options for overall risk of bias judgement: low risk, high risk or some concerns.

2.4 Results

2.4.1 Eligibility of Studies

Using electronic database (PubMed (MEDLINE), Web of Science and Google Scholar), we identified 349 articles between 1978 and 2020. The removal of duplicates and initial title screen left 301 articles for detailed assessment. Of these 25 were evaluated against the inclusion/exclusion criteria. 10 of these were animal studies and 3 focused on cancer cells, ageing, and VD receptor. This left 12 articles eligible for inclusion within this review (Figure 2.2). A detailed list of excluded studies with reasoning for exclusion can be found in Supplementary Data 2.2.

2.4.2 Quality Assessment

All 12 papers received a score of 'low risk' when assessed against the quality assessment criteria outlined in Supplementary Data 2.1. For three of the studies (Capiati et al., 1999, Girgis et al., 2014, Olsson et al., 2016) no information could be found regarding replicates and/or repeats therefore it was assumed that this was adequate when giving a low overall bias score (Table 2.1).

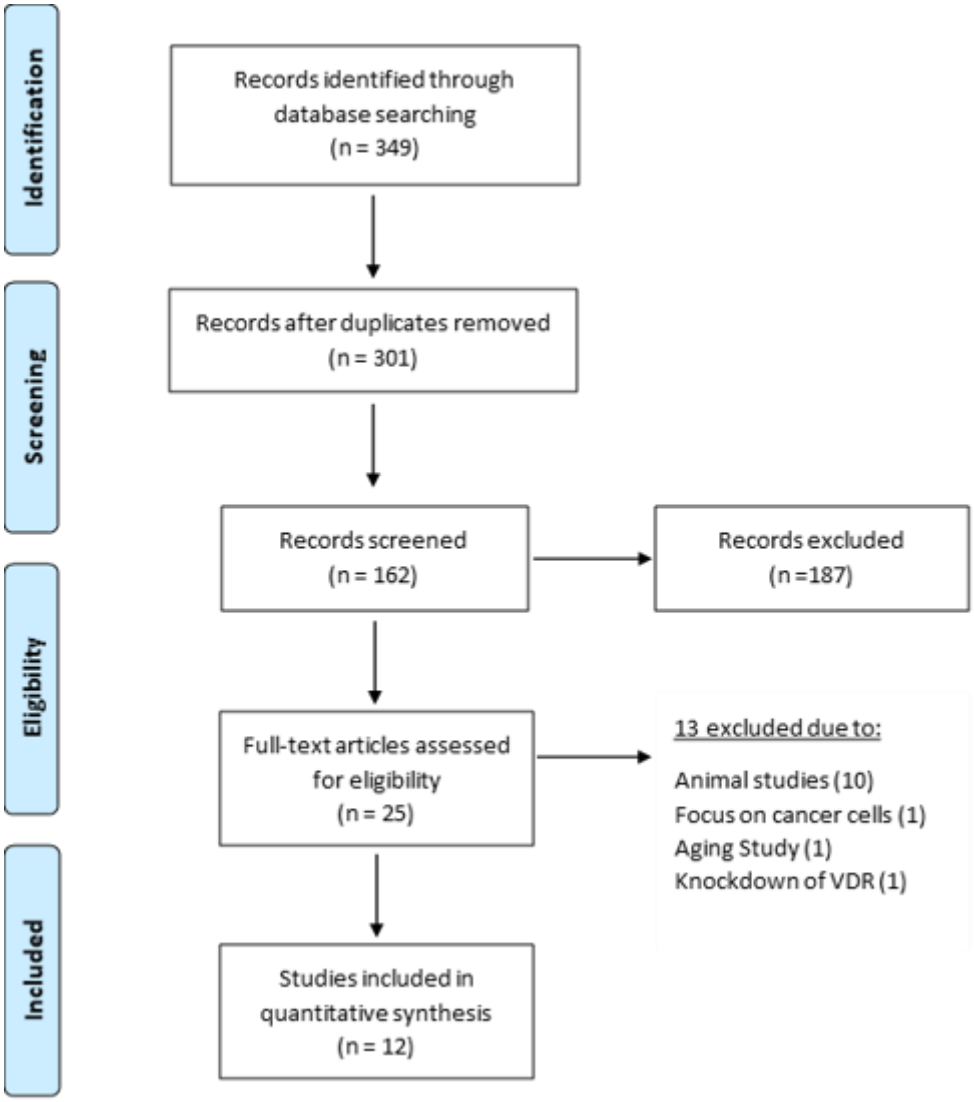

Figure 2.2. Selection and exclusion of studies in accordance with PRISMA guidelines (Moher at al., 2009)

2.4.3 Study Characteristics

All studies included within this review used the biologically active form of VD ($1,25(OH)_2D_3$) apart from one (Saito et al., 2017) where an analogue of the active form of VD called Eldecalcitol was used. Four different cell types were used across the studies (C2C12, primary human myoblasts, primary mouse satellite cells and primary chick myoblasts) and active VD concentration ranged from 10^{-5}M to 10^{-13} (Table 2.2).

Table 2.1. Summary of quality asessment of included studies

	Question										
Study, year	1.1	1.2	1.3	2.1	2.2	2.3	3.1	3.2	3.3	3.4	Rating
Braga, 2017	●	●	●	●	●	●	●	●	●	●	Low
Capiati, 1999	●	●	●	●	●	●	●	●	●	●	Low
Garcia, 2011	●	●	●	●	●	●	●	●	●	●	Low
Gili, 2016	●	●	●	●	●	●	●	●	●	●	Low
Girgis, 2014	●	●	●	●	●	●	●	●	●	●	Low
Okuno, 2012	●	●	●	●	●	●	●	●	●	●	Low
Olsson, 2016	●	●	●	●	●	●	●	●	●	●	Low
Romeu Montenegro, 2019	●	●	●	●	●	●	●	●	●	●	Low
Ryan, 2013	●	●	●	●	●	●	●	●	●	●	Low
Saini, 2019	●	●	●	●	●	●	●	●	●	●	Low
Saito, 2017	●	●	●	●	●	●	●	●	●	●	Low
Van der Meijden, 2016	●	●	●	●	●	●	●	●	●	●	Low
●low risk of bias, ●moderate risk of bias, ●high risk of bias, ●no information given/not applicable											

Table 2.2. Summary of study characteristics

Author, year	Cell type	Form of vitamin D	Concentration	Outcomes measured
Braga et al, 2017	Mouse skeletal muscle satellite cells	$1,25(OH)_2D_3$	10^{-7}M	MyoD, myogenin
Capiati et al, 1999	Chick myoblasts (obtained from 12-day-old embryo breast tissue	$1,25(OH)_2D_3$	10^{-9}M	Proliferation, creatine kinase, myosin
Garcia et al, 2011	C2C12	$1,25(OH)_2D_3$	10^{-7}M	MyoD, myogenin, myotube size
Gili et al, 2016	C2C12	$1,25(OH)_2D_3$	10^{-9}M	Myogenin, creatine kinase, myosin, myotube size
Girgis et al, 2014	C2C12	$1,25(OH)_2D_3$	10^{-7}M	Myogenin, myotube size
Okuno et al, 2012	C2C12	$1,25(OH)_2D_3$	10^{-7}M, 10^{-8}M and 10^{-9}M	Myogenin, myosin
Olsson et al, 2016	Human skeletal muscle myoblasts	$1,25(OH)_2D_3$	10^{-7}M	Proliferation, myoD, myogenin, myosin
Romeu Montenegro et al, 2019	Human skeletal muscle myoblasts	$1,25(OH)_2D_3$	10^{-7}M	Proliferation, myogenin, myosin, myotube size
Ryan et al, 2013	C2C12	$1,25(OH)_2D_3$	10^{-5}M, 10^{-7}M, 10^{-9}M, 10^{-11}M and 10^{-13}M	Myogenin, creatine kinase
Saini et al, 2019	Human skeletal muscle myoblasts	$1,25(OH)_2D_3$	10^{-7}M, 10^{-9}M and 10^{-11}M	Proliferation
Saito et al, 2017	C2C12	Eldecalcitol	10^{-7}M, 10^{-8}M and 10^{-9}M	MyoD, myosin
Van der Meijden et al, 2016	C2C12	$1,25(OH)_2D_3$	10^{-7}M	MyoD, myogenin, myosin, myotube size

2.4.4 Effects on Proliferation

From the relevant articles, eight (Capiati et al., 1999, Garcia et al., 2011, Girgis et al., 2014, Okuno et al., 2012, Olsson et al., 2016, Romeu Montenegro et al., 2019, Saini et al., 2019, van der Meijden et al., 2016) studied the effects of $1,25(OH)_2D_3$ on proliferation and all reported an inhibitory effect. Of these, four (Capiati et al., 1999, Olsson et al., 2016, Romeu Montenegro et al., 2019, Saini et al., 2019) quantified DNA content as a marker of proliferation, Figure 2.3. Interestingly, one study (Capiati et al., 1999) reported an initial short stimulatory effect of $1,25(OH)_2D_3$ treatment on DNA synthesis on day 1 (1.5-fold increase) however, this was followed by an inhibitory effect on day 4 (0.7-fold). The remaining three studies (Olsson et al., 2016, Romeu Montenegro et al., 2019, Saini et al., 2019) all revealed a decrease in DNA content of different magnitude (0.5 to 0.95-fold), Figure 2.3.

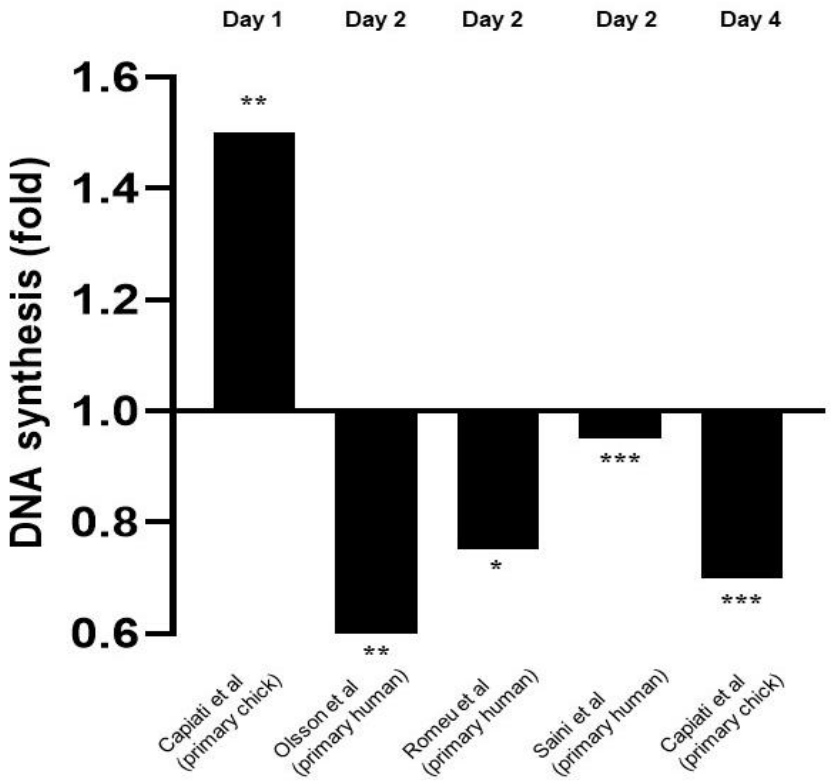

Figure 2.3. Effect of $1,25(OH)_2D_3$ treatment on DNA synthesis compared to untreated cells. The active form of vitamin D ($1,25(OH)_2D_3$) at 10^{-7}M was used in all studies. *p<0.05, **p<0.01, ***p<0.001

The other four studies measured proliferation in various ways, Table 2.3. One study showed an increase in p21 and p27 mRNA (Okuno et al., 2012) whilst three studies revealed a decrease in cyclin mRNAs (Girgis et al., 2014, Olsson et al., 2016, Saini et al., 2019). A decrease in proliferation was also shown by an increase in number of cells in the quiescent phase (Girgis et al., 2014, Okuno et al., 2012, Romeu Montenegro et al., 2019), decreased levels of proliferating cell nuclear antigen (PCNA) at the protein level (Garcia et al., 2011) and decreases in DNA synthesis as previously reported. It is important to note that only two out of eight of these studies checked for differences in apoptosis between treated and control cells (Girgis et al., 2014, Olsson et al., 2016).

Table 2.3. Effects of $1,25(OH)_2D_3$ on proliferation

Reference (Cell type)	VitD form and concentration	Effect on proliferation	Apoptosis check?
Capiati et al, 2019 (Primary chick)	10^{-9}M $1,25(OH)_2D_3$	[^{3}H] thymide incorporation 1.5-fold on day 1, then 0.7-fold on day 4	
Garcia et al, 2011 (C2C12)	10^{-7}M $1,25(OH)_2D_3$	Proliferating cell nuclear antigen (PCNA) protein 0.25-fold on day 7	
Girgis et al, 2014 (C2C12)	10^{-7}M $1,25(OH)_2D_3$	Proliferation 0.4-fold on day 2 23% increase in cells in G_0/G_1 quiescent phase on day 2 Cyclin D1 mRNA 0.75-fold on day 2	✓
Okuno et al, 2012 (C2C12)	10^{-7}M $1,25(OH)_2D_3$	17% increase in cells in G_0/G_1 quiescent phase on day 3 P21 mRNA 2-fold on day 3 P27 mRNA 3-fold on day 3	
Olsson et al, 2016 (Primary human)	10^{-7}M $1,25(OH)_2D_3$	BrdU incorporation 0.5-fold on day 2 Cyclin D2 mRNA down regulated 3-fold	✓
Romeu Montenegro et al, 2019 (Primary human)	10^{-7}M $1,25(OH)_2D_3$	BrdU incorporation 0.7-fold on day 2 Decrease in number of cells in G_2/M phase on day 2	
Saini et al, 2019 (Primary human)	10^{-7}M $1,25(OH)_2D_3$	EdU incorporation 0.95-fold on day 2 Down regulation of cyclin A2 and D1 mRNA after 24hr	
Van der Meijden et al, 2016 (C2C12)	10^{-7}M $1,25(OH)_2D_3$	27.6% fewer viable cells on day 4	
		All values reported are significant ($p<0.05$)	

2.4.5 Effects on differentiation

Differentiation of muscle cells was determined in all but one (Saini et al., 2019) of the final twelve studies. Markers of differentiation included expression of mRNA or protein for myoD (early differentiation), myogenin (early-mid stage), myosin/myosin heavy chain isoforms (late stage) or the measurement of creatine kinase activity (mid-stage). However, it should be noted that the mRNA expression of myogenin and myosin heavy chain isoforms have been shown to change during the time course of differentiation in C2C12 cells (Brown et al., 2012) indicating that the time point at which these markers are measured is important.

2.4.5.1 Effects of vitamin D on early-stage myogenic differentiation

Five studies measured myoD expression (Braga et al., 2017, Garcia et al., 2011, Olsson et al., 2016, Saito et al., 2017, van der Meijden et al., 2016). Three of these studies measured expression on day 4 (Garcia et al., 2011, Saito et al., 2017, van der Meijden et al., 2016) whilst one measured expression on day 1 (Olsson et al., 2016) and another on day 7 (Braga et al., 2017), Figure 2.4. mRNA expression was measured in all cases except for one (Braga et al., 2017) where protein expression was measured. Four out of five studies (Garcia et al., 2011, Saito et al., 2017, van der Meijden et al., 2016, Braga et al., 2017) reported an increase in expression of myoD which ranged from 1.8-fold to 3-fold. However, one study (Olsson et al., 2016) reported a decrease in expression of 0.5-fold on day 1. These changes in myoD expression were in response to 10^{-7}M $1,25(OH)_2D_3$ for all cases apart from one (Saito et al., 2017) which used 10^{-7}M Eldecalcitol, an analogue of the active form of VD.

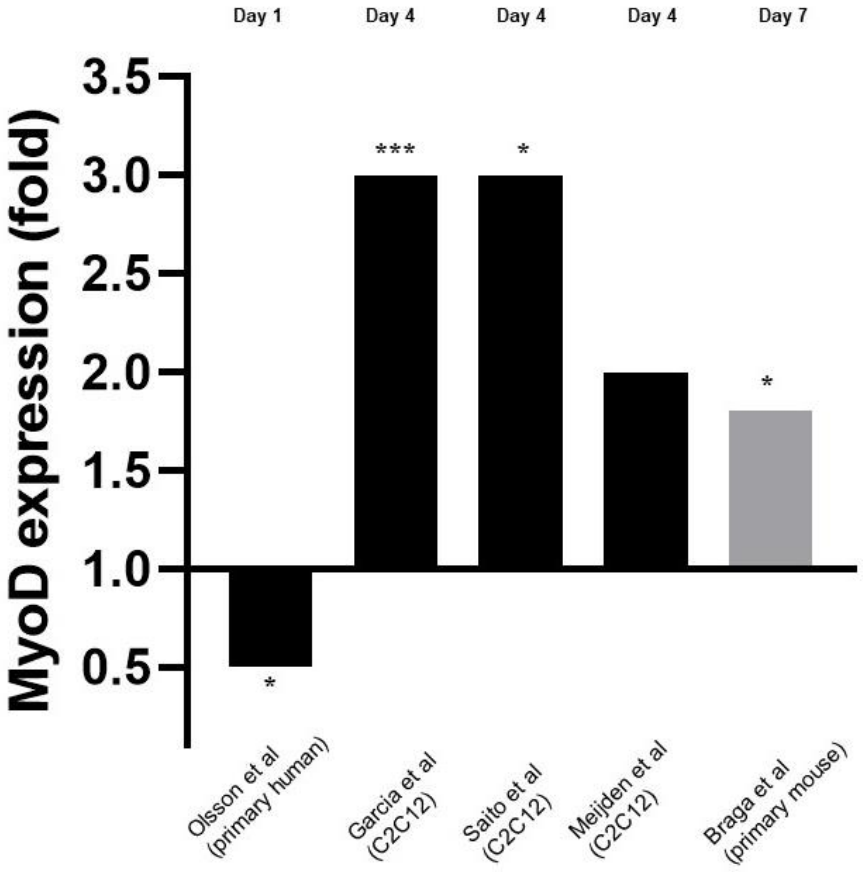

Figure 2.4. Effect of 1,25(OH)2D3 or analogue on MyoD mRNA expression. The active form of vitamin D (1,25(OH)2D3) at 10-7M was used in all studies apart from Saito et al, 2017 where Eldecalcitol (an analogue of the active form of vitamin D) was used. Black bars indicate mRNA expression whilst grey indicates protein expression. *p<0.05, ***p<0.001

2.4.5.2 Effects of vitamin D on early/mid-stage myogenic differentiation

Myogenin expression in response to $1,25(OH)_2D_3$ was investigated by nine of the twelve studies included within this review (Olsson et al., 2016, Gili et al., 2016, Garcia et al., 2011, Okuno et al., 2012, Ryan et al., 2013, van der Meijden et al., 2016, Braga et al., 2017, Girgis et al., 2014, Romeu Montenegro et al., 2019). The time points at which myogenin expression was measured varied from day 1 to day 7, Figure 2.5. For eight of the nine studies which measured myogenin, the concentration of $1,25(OH)_2D_3$ used was 10^{-7}M but one study (Gili et al., 2016) used 10^{-8}M $1,25(OH)_2D_3$. In most cases mRNA expression was measured but in two studies (Gili et al., 2016, Braga et al., 2017) protein expression was measured. Unlike myoD

expression, the level of agreement between studies relating to myogenin expression was low with five studies reporting a decrease in myogenin expression (Olsson et al., 2016, Okuno et al., 2012, Ryan et al., 2013, van der Meijden et al., 2016, Girgis et al., 2014) and four studies reporting an increase in expression (Gili et al., 2016, Garcia et al., 2011, Braga et al., 2017, Romeu Montenegro et al., 2019).

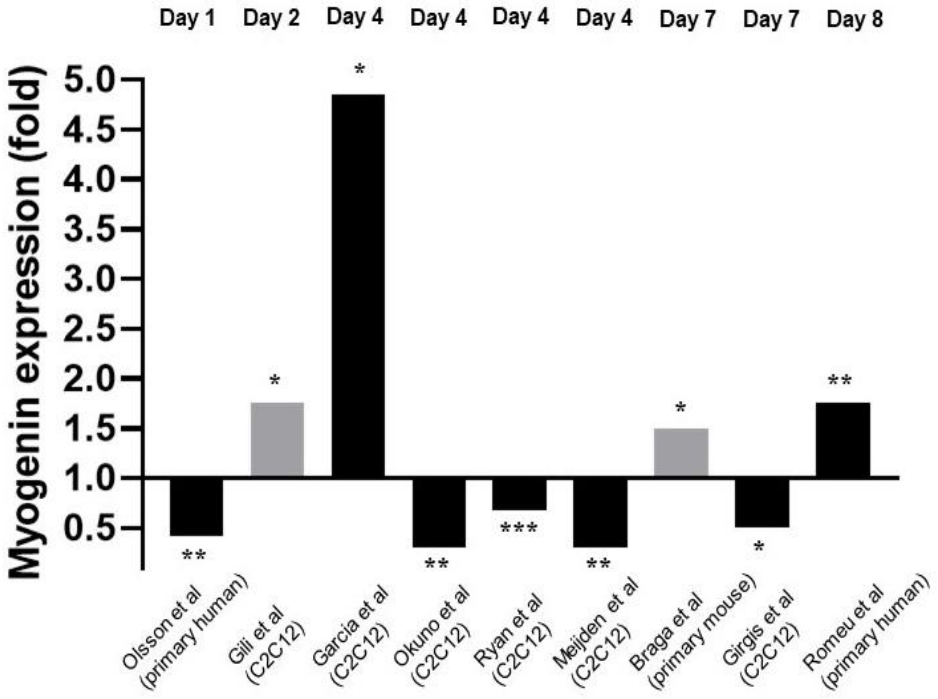

Figure 2.5. Effect of 1,25(OH)2D3 on Myogenin expression. The active form of vitamin D (1,25(OH)2D3) at 10-7M was used in all studies apart from Gili et al, 2016 where 10-8M was used. Black bars indicate mRNA expression whilst grey indicates protein expression. *p<0.05, **p<0.01, ***p<0.001.

Three studies measured creatine kinase activity as a marker of differentiation (Capiati et al., 1999, Gili et al., 2016, Ryan et al., 2013). Two of these studies reported their results as a time course (Capiati et al., 1999, Gili et al., 2016) whilst one reported results for day 4 only (Ryan et al., 2013). A variety of concentrations of $1,25(OH)_2D_3$ were used across the studies, Table 2.4. One study (Ryan et al., 2013) reported results for cells grown in either myogenic media or adipogenic media, but only the results for myogenic media have been included to allow comparison to the other studies. Two studies reported an increase in creatine kinase activity which

peaked on day 2 following 1,25$(OH)_2D_3$ treatment (Capiati et al., 1999, Gili et al., 2016) whilst the other study found that creatine kinase activity decreased across all 1,25$(OH)_2D_3$ concentrations on day 4 (Ryan et al., 2013).

Table 2.4. Effects of $1,25(OH)_2D_3$ on Creatine Kinase Activity

Reference (Cell type)	VitD form and concentration	CK activity	Significance
Capiati et al, 1999 (Primary chick)	10^{-9}M $1,25(OH)_2D_3$	-45% on day 1 +55% on day 2 +30% on day 3 + 15% on day 6	p<0.01 P<0.01 p<0.05 p<0.05
Gili et al, 2016 (C2C12)	10^{-7}M $1,25(OH)_2D_3$	1.7-fold on day 1 1.8-fold on day 2 1.3-fold on day 4	Individual p values not given. ANOVA interaction p<0.05
Ryan et al, 2013 (C2C12)	$1,25(OH)_2D_3$ for all: 10^{-13}M 10^{-11}M 10^{-9}M 10^{-7}M 10^{-5}M	Day 4 for all: Same as control 6% decrease 12.5% decrease 25% decrease 62.5% decrease	Individual p values not given. ANOVA interaction p<0.001

2.4.5.3 Effects of vitamin D on late-stage myogenic differentiation

A total of seven studies investigated the effects of $1,25(OH)_2D_3$ treatment on myosin protein or mRNA/protein levels of myosin heavy chain (MHC) isoforms, with the majority measuring the latter (Gili et al., 2016, Okuno et al., 2012, Olsson et al., 2016, Romeu Montenegro et al., 2019, Saito et al., 2017, van der Meijden et al., 2016). MHC neonatal (MHC neo) and type IIa (MHC IIa) were the most commonly studied isoforms across the papers. Time points of expression varied greatly between studies. In some cases, expression was measured as early as day 1 whereas others measured up to day 8, Table 2.5. Overall, one study found an increase in myosin protein (Capiati et al., 1999), one study found and increase in MHC protein (Gili et al., 2016), three studies reported an increase in expression of at least one MHC isoform (Gili et al., 2016, Okuno et al., 2012, Saito et al., 2017, van der Meijden et al., 2016) and two studies reported an decrease in MHC isoforms (MHC neo, MHC IIa and MHC II subtype unspecified) (Olsson et al., 2016, Romeu Montenegro et al., 2019).

Five studies measured the effects of $1,25(OH)_2D_3$ on myotube size (Romeu Montenegro et al., 2019, van der Meijden et al., 2016, Gili et al., 2016, Garcia et al., 2011, Girgis et al., 2014), Figure 2.6. This was also measured at varying time points from day 2 to day 10. For one study 10^{-9}M $1,25(OH)_2D_3$ was used (Gili et al., 2016) whilst 10^{-7}M $1,25(OH)_2D_3$ was used in the other four (Romeu Montenegro et al., 2019, van der Meijden et al., 2016, Garcia et al., 2011, Girgis et al., 2014). All five studies concluded that treatment with $1,25(OH)_2D_3$ resulted in an increase in myotube size which ranged from 1.1-fold to 2-fold.

Table 2.5. Effects of $1,25(OH)_2D_3$ or eldecalcitol on myosin or myosin heavy chain isoform expression

Reference (Cell type)	VitD form and concentration	Factor measured	Effect	Significance
Capiati et al, 1999 (Primary chick)	10^{-9}M $1,25(OH)_2D_3$	Myosin protein	+88% on day 2 + 31.5% on day 6	p<0.01 p<0.01
Gili et al, 2016 (C2C12)	10^{-9}M $1,25(OH)_2D_3$	MHC protein	1.2-fold on day 2 1.4-fold on day 4	p values not given
Okuno et al, 2012 (C2C12)	10^{-7}M $1,25(OH)_2D_3$	MHC neo mRNA MHCIIa mRNA	0.4-fold on day 4 2.5-fold on day 8	p<0.05 p<0.01
Olsson et al, 2016 (Primary human)	10^{-7}M $1,25(OH)_2D_3$	MHC neo mRNA MHCIIa mRNA	0.66-fold on day 1 0.73-fold on day 1	No p values given
Romeu Montenegro et al, 2019 (Primary human)	10^{-7}M $1,25(OH)_2D_3$	MHC2 mRNA	0.4-fold on day 5	p<0.01
Saito et al, 2017 (C2C12)	10^{-8}M eldecalcitol	MHC neo mRNA MHCIIa mRNA	1.4-fold on day 4 1.8-fold on day 4	Not significant p<0.01
Van der Meijden et al, 2016 (C2C12)	10^{-7}M $1,25(OH)_2D_3$	MHCIIa mRNA	2.5-fold on day 3	p value not given

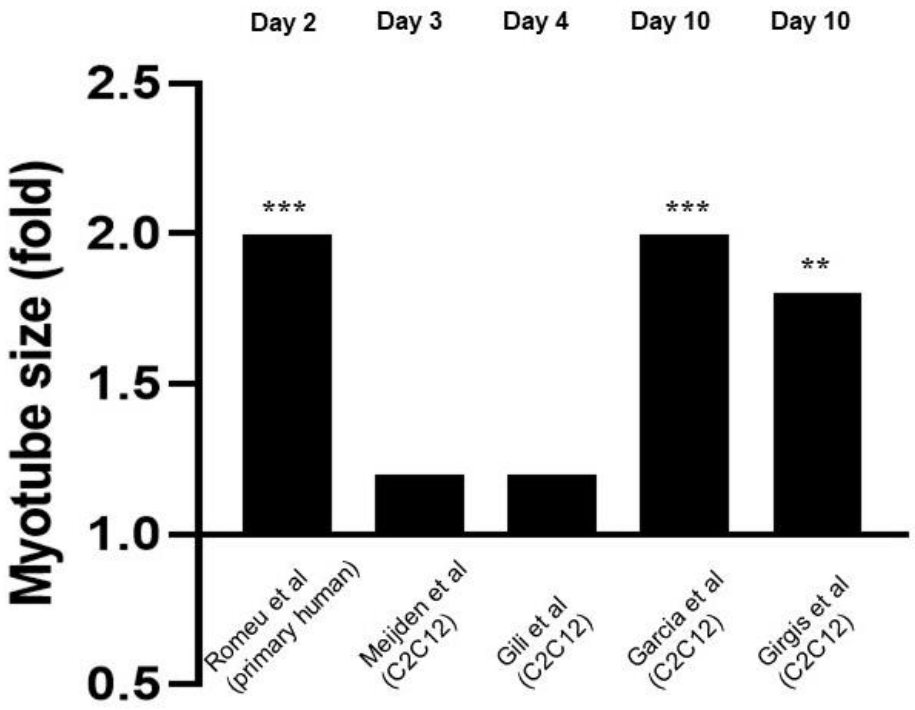

Figure 2.6. Effect of 1,25(OH)2D3 on myotube size. 5 studies investigated the effect of $1,25(OH)_2D_3$ on myotube size. Myotube size was measured at varying time points which ranged from day 3 to day 10. 1,25(OH)2D3 concentration was 10-7M for all cases apart from Gili et al, 2016 where 10-9M was used. **p<0.01, ***p<0.001.

2.5 Discussion

This review has shown good agreement across the different studies in terms of the active form of VD inhibiting muscle cell proliferation. However, the effects on differentiation, as determined by various markers, showed less consistency, probably due to a combination of different cell types and timepoints being used.

2.5.1 Treatment with vitamin D inhibits proliferation

Of the eight studies which investigated the effects of active VD on muscle cell proliferation (Capiati et al., 1999, Garcia et al., 2011, Girgis et al., 2014, Okuno et al., 2012, Olsson et al., 2016, Romeu Montenegro et al., 2019, Saini et al., 2019, van der Meijden et al., 2016), all of them found an inhibitory effect. One study observed a stimulatory effect on day 1, however this was followed by inhibition on day 4

(Capiati et al., 1999). It is worth noting that only two studies (Girgis et al., 2014, Olsson et al., 2016) checked for differences in apoptosis between treated and untreated groups therefore it cannot be ruled out that the decrease in DNA observed in some of the other studies was not due to apoptosis.

Importantly, Okuno et al., (2012), showed that active VD caused an increase in cell cycle arrest at G0/G1 which occurred in parallel with increased expression of p21 and p27. Both p21 and p27 are members of the Cip/Kip family and are able to bind to cyclin dependent kinases and inhibit their role in cell cycle progression (Bachs et al., 2018). In order for a cell to proliferate, expression of p21 must decrease to a level where it no longer forms a complex with p53 (Terzi et al., 2016). Additionally, cells must also reduce/eliminate p27 in order to progress through proliferation, which is achieved via translocation of p27 to the cytoplasm where it is degraded (Bachs et al., 2018). Both Okuno et al., (2012) and Olsson et al., (2016) reported increased expression of both p21 and p27 suggesting that active VD treatment leads to increased transcription of these factors which likely contributes to the inhibition of cell proliferation.

Two studies reported that active VD decreased expression of both cyclin A2 and cyclin D3 (Olsson et al., 2016, Saini et al., 2019). Cyclin A2 is able to bind to and activate two cyclin dependent kinases (CDKs) required for cell cycle progression: CDK4 as DNA synthesis begins during S phase and CDK1 during the transition from G2 to M phase (Pagano et al., 1992). The D cyclins activate CDK4/6 enabling entry into S phase and down-regulation of cyclin D3 specifically inhibits G1 to S transition (Bartkova et al., 1998). From this, it can be suggested that active VD represses transcription of at least two cyclins which leads to inhibition of cell cycle transition and therefore cell cycle arrest and inhibition of proliferation.

Overall, decreases in DNA synthesis, increases in expression of p21/p27 and decreases in expression of cyclin A2/D3 suggest that treatment with active VD has a strong anti-proliferative effect on muscle cells in culture. It is likely that the cumulative effect of all of these factors lead to an overall reduction in muscle cell proliferation. The process of this anti-proliferative effect of active VD is shown in figure 2.7.

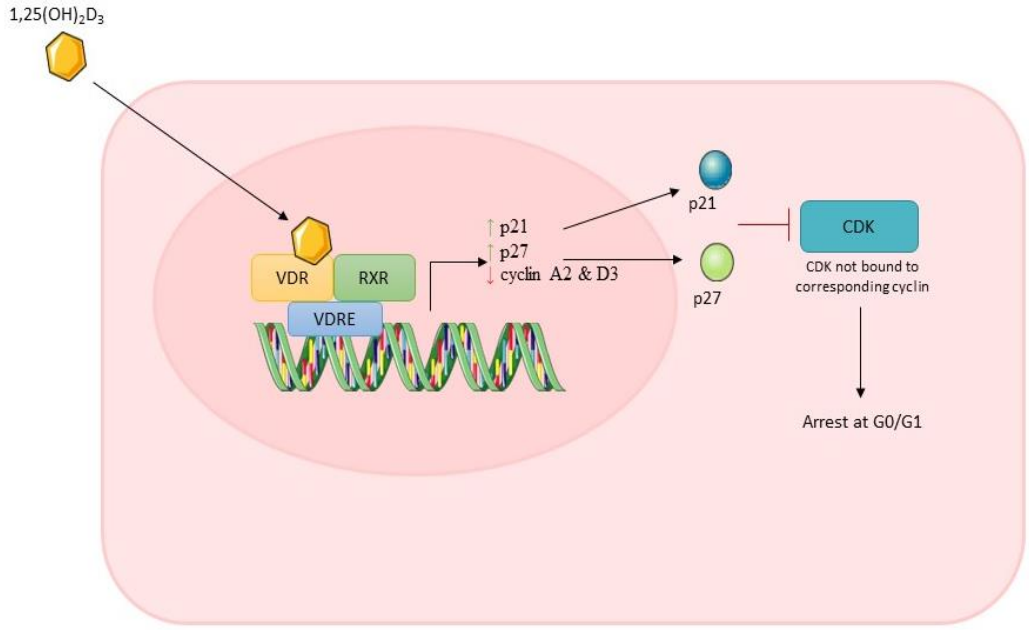

Figure 2.7. Effects of active vitamin D ($1,25(OH)_2D_3$) on myoblast proliferation. Images used within this figure were obtained from smart servier medical art and can be found at https://smart.servier.com

The potential result of this anti-proliferative effect for mature muscle is unclear. It is possible that a decrease in proliferation would result in a smaller number of muscle fibres within mature muscle. However, it is also possible that observed effects *in vitro* show an exaggeration of what would happen *in vivo*, especially due to the fact that there are many more external regulatory factors of proliferation *in vivo*.

2.5.2 Vitamin D appears to stimulate early-stage differentiation

Myogenesis is a highly ordered and sequential process, guided by several transcription factors at various stages. This process of myogenic differentiation, and the proposed effect of active vitamin D on this process, is shown in Figure 2.8.

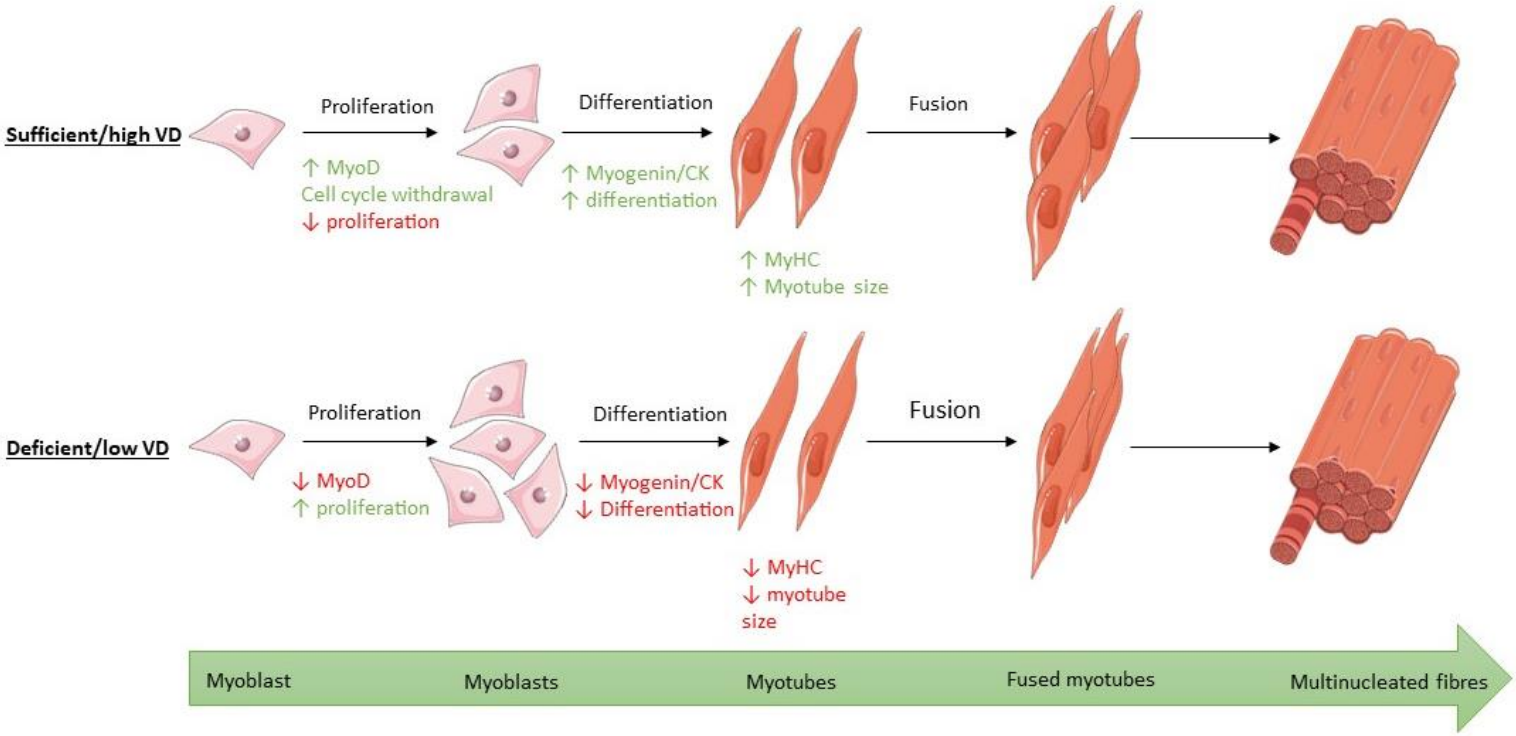

Figure 2.8. The process of differentiation from myoblasts to multinucleated muscle fibers showing the proposed effect of high/sufficient active vitamin D on various transcription factors within the process compared to low/deficient levels of vitamin D. Images used within this figure were obtained from smart servier medical art and can be found at https://smart.servier.com.

Following withdrawal from the cell cycle, as described previously, myoblast fusion occurs to form multinucleated myotubes (Garcia et al., 2011). MyoD is a transcription factor involved in the early stages of differentiation (Girgis et al., 2014). When subjected to culture conditions which should induce differentiation, myoD -/- cells have been shown to continue to proliferate suggesting that expression of myoD is essential for withdrawal from the cell cycle (Sabourin et al., 1999). However, we previously observed no change in myoD mRNA over the time course of differentiation in C2C12 cells (Brown et al., 2012).

Four of the five studies which measured myoD reported an increase in expression following treatment with active VD (Garcia et al., 2011, Saito et al., 2017, van der Meijden et al., 2016, Braga et al., 2017), although the increase in expression was not significant for one study (van der Meijden et al., 2016). Interestingly, the only study which found a decrease in myoD expression was also the only study which used primary human cells (Olsson et al., 2016). This suggests that the effects of active VD on differentiation may depend upon cell type and/or species. However, this study also measured myoD very early in the process (day 1) (Olsson et al., 2016) whereas the remaining four studies all reported increased myoD expression on either day 4 (Garcia et al., 2011, Saito et al., 2017, van der Meijden et al., 2016) or day 7 (Braga et al., 2017). The effects observed may depend on upon the cell type and/or time point.

There is evidence that inhibition of IGFII results in a decrease in expression of myoD target genes, suggesting that IGFII is a key regulator of myoD expression (Wilson and Rotwein, 2006). One of the studies which found a 1.8-fold increase in myoD expression (Braga et al., 2017) found that both IGFI and IGFII expression also increased at the same time point. Additionally, increased expression of the IGFs has

been shown to inhibit myostatin, the only known negative regulator of muscle mass (Retamales et al., 2015). These findings suggest that active VD may increase expression of myoD directly or possibly indirectly via effects on local expression of IGFI and/or IGFII.

2.5.3 Effects of vitamin D on mid-stage differentiation are cell type and time dependent.

Induction of myogenin expression precedes the fusion of myoblasts to form myotubes, then myogenin switches on transcription of various muscle-specific genes (e.g. creatine kinase and MHC isoforms) expressed by myotubes and muscle fibers (Bentzinger et al., 2012). Myogenin expression is therefore used as a marker of early to mid-stage differentiation and normally follows an increase in myoD expression (Hernández-Hernández et al., 2017). Indeed, myogenin knockout mice die immediately following birth, and whilst they have myoblasts present within the muscle, no muscle fibers are formed, resulting in the complete absence of functional skeletal muscle (Hernández-Hernández et al., 2017). Importantly, myogenin expression is completely blocked when the VD receptor is knocked down *in vitro* suggesting that VD has a direct effect on myogenin expression via the VDR (Gili et al., 2016).

However, the nature of this effect is controversial. As seen in Figure 2.5, five studies reported a decrease in myogenin expression (Olsson et al., 2016, Okuno et al., 2012, Ryan et al., 2013, van der Meijden et al., 2016, Girgis et al., 2014) whilst four reported an increase (Gili et al., 2016, Garcia et al., 2011, Braga et al., 2017, Romeu Montenegro et al., 2019). One possible explanation for this is the difference in methods between studies. Differentiation can be triggered via two mechanisms *in vitro:* 1. Serum starvation which leads to a decrease in mitogenic stimuli, withdrawal

from the cell cycle and increase in myogenin expression. 2. Prolonged confluence leading to a high cell density and increased cell-cell contacts, which leads to increased IGF expression and an increase in myogenin (Girgis et al., 2014). Garcia et al., (2011), who used the latter method, found that myogenin expression was increased at day 4 following active VD treatment of C2C12 cells. On the other hand, Girgis et al., (2014), used the serum deprivation method and reported a decrease in myogenin expression in C2C12 cells at day 7. It is important to note that we previously showed (Brown et al., 2012, Brearley et al., 2019) using multiple time points, that myogenin mRNA initially increases upon induction of differentiation (via serum starvation) of C2C12 cells, reaching a peak around day 2-3, then decreases again. Therefore, induction of differentiation would be associated with an increase in myogenin mRNA at early time points (days 0-3), but increased differentiation could also be associated with a more rapid decline in expression at later time points. It is also worth noting that C2C12 cells differentiate more rapidly than primary human myoblasts (Cheng et al., 2014) so are likely to have an earlier peak in myogenin expression. Hence, the discrepancies in the observed effects of active VD on myogenin expression could be due to the cell type used, the timepoints of measurement or a combination of the two. It is important that future studies should include measurements at several time points in order to make clear interpretations. It is also plausible that the two different methods of inducing differentiation may have different time frames, such that the rates of increase and decrease in expression as well as the peak of myogenin may be different. Certainly, there were large differences between studies in time points at which myogenin expression was measured, which likely contributed to the conflicting results.

Creatine kinase (CK) is a mid-stage marker of differenitation reported to peak around day 4 to 6 in both C2C12 (Brown et al., 2012) and primary chick myoblasts

(Capiati et al., 1999). Two studies (Capiati et al., 1999, Gili et al., 2016) reported an increase in CK activity following treatment with active VD both of which found expression to peak on day 2, earlier than the expected window of 4-6 days. The remaining study which looked at CK activity (Ryan et al., 2013) reported a decrease in activity in C2C12 cells on day 4 across all active VD concentrations studied. Once again this might relate to differences in the timing relative to the expected peak in expression, with an early increase and a later decrease in expression potentially indicating an increase in the rate of differentiation.

Overall, the data is conflicting for both markers of early to mid-stage differentiation (myogenin and creatine kinase), but this may be due to the varying time points that each marker was measured, the variation in cell type, the differing concentrations of active VD used or a combination of all three.

2.5.4 Vitamin D stimulates expression of late-stage markers of differentiation

Myosin and the myosin heavy chain (MHC) isoforms are muscle specific proteins that are often used as markers of mature, differentiated muscle cells (Gili et al., 2016) and together they form a significant proportion of the proteins present in differentiated muscle (Zammit, 2017). Five studies reported an increase in myosin or MHC isoforms following active VD treatment (Capiati et al., 1999, Gili et al., 2016, Okuno et al., 2012, Saito et al., 2017, van der Meijden et al., 2016) whilst two studies reported a decrease in expression (Olsson et al., 2016, Romeu Montenegro et al., 2019). We previously showed that the MHC isoforms are expressed in two distinct patterns during differentiation in C2C12 cells (Brown et al., 2012). Firstly with an increase then decrease, peaking around day 2-4, of mRNA for MHC embryonic (MHC emb), fetal (MHC neo) and slow type 1 (MHC I) isoforms (Brown et al., 2012). The

fast type II isoforms were all expressed later in differentiation, being induced around day 4 and continuing to increase through to day 8, in the order IIa > IIx > IIb (Brown et al., 2012). Hence, an increase in differentiation would always result in an increase in expression of the fast (type II) isoforms, but effects on the embryonic, fetal and slow (type I) isoforms would be time dependent, with an increase in expression at early time points, but a decrease in expression at later time points.

Okuno et al., (2012), found that MHC type IIa expression in C2C12 cells was increased by active VD at day 8 and they suggest that this indicated an anabolic effect in muscle. On the other hand, Olsson et al., (2016), found that both MHC neonatal and MHC IIa expression were reduced in C2C12 cells at day 1. Considering MHC is a marker of late stage differentiation (Gili et al., 2016) it is unclear why this study chose to measure MHC expression during the earlier stages of differentiation, possibly missing the timepoint where MHC expression may have increased.

The majority of *in vitro* evidence suggests that active VD stimulates the expression of MHC isoforms, suggesting that active VD stimulates differentiation. Additionally, one study showed that injection of active VD increased expression of MHC type IIa *in vivo (*Korn et al., 2013*)*. However, this could be due to effects on muscle fibre type rather than muscle cell differentiation. It is known that IGF1 can alter MHC isoform expression (Saito et al., 2017) so active VD may impact on muscle fibre type indirectly via induction of local IGF1 expression. Supporting this, Braga et al., (2017), reported an increase in expression of both IGF1 and IGF2 following active VD treatment in primary mouse muscle cells *in vitro*. However, some argue that non-genomic actions of active VD, such as increases in intracellular calcium concentrations, may be responsible for its effects on MHC expression (de Boland AR and RL, 1987).

2.5.5 Vitamin D increases myotube size

Of the five studies which measured myotube size, all five reported an increase in myotube size (Romeu Montenegro et al., 2019, van der Meijden et al., 2016, Gili et al., 2016, Garcia et al., 2011, Girgis et al., 2014), which suggests a stimulatory effect of active VD on differentiation. In addition, Garcia et al., (2011) found an increase in expression of Fst – the antagonist of myostatin, which is a negative regulator of muscle mass (Retamales et al., 2015). Therefore, active VD may increase myotube size directly via increasing levels of proteins such as MHC isoforms, and/or indirectly via increasing IFG1 or Fst expression, the latter then inhibits myostatin. Supporting this, Girgis et al., (2014) found myotube size was increased 1.8-fold on day 10 following a 10-fold decrease in myostatin expression on day 7.

2.5.6 Conclusions

There is reasonably strong evidence to suggest that active VD inhibits proliferation of myoblasts, stimulates differentiation and increases myotube size, although the effects on each stage of differentiation are not entirely consistent. Whilst it is not clear how an inhibition of proliferation leads to increased myotube size, one possibility could be increased levels of fusion by vitamin D, and future work should look at this. The inconsistencies between results in this review may relate to the use of different cell types and measurements at variable time points which makes interpretation more difficult. However, understanding the normal timecourse of expression during differentiation allows for some consistency across studies, but it clearly indicates that future studies should involve multiple time points. Also, only one study (Ryan et al., 2013) used concentrations of active VD within the physiological serum range (around 10^{-10}M) (Yi-Chou Hou et al., 2018) so future studies should also consider using concentrations of active VD which are more

physiologically relevent. However, it is worth noting that muscle cells do express 1α-hydroxylase (Mori et al., 2020) and therefore have the ability to locally convert inactive VD to the active form. As it is not possible to measure these local fluctuations in active VD, it cannot be ruled out that it is possible for intracellular physiological concentrations to reach levels used within the studies in this review (10^{-7}M). Due to this presence of 1α-hydroxylase within skeletal muscle cells future studies should also look at the effects of inactive VD on muscle cells to see whether this produces the same efffects as the active form.

It does appear that active VD has effects on skeletal muscle, particularly muscle cell proliferation and differentiation, indicating potential effects during embryonic development; when these processes mainly take place. VD deficiency has been shown to increase the risk of poor muscle strength and therefore falls, particularly in the elderly population (Garcia et al., 2011, Ceglia and Harris, 2013), but this review suggests that VD deficiency during embryonic and fetal development (i.e. during pregnancy and infancy) may also impact upon muscle development and function. Whilst VD supplementation in deficient individuals appears effective in increasing muscle strength and therefore decreasing fall risk in the elderly (Ryan et al., 2013), more research is needed to determine the impacts of supplementation during pregnancy/lactation and in young offspring on muscle cell differentiation.

2.6 Supplementary data

Supplementary data 2.1 – Quality Assessment tool to assess risk of bias to treatment allocation (1), risk of measurement bias (2) and risk of reported result bias (3).

Signalling questions	**Comments**	**Response options**
1.1 Was allocation to control/treated random?		**Y / PY / PN / N / NI**
1.2 Was the researcher aware of which cells were treated or control		**Y / PY / PN / N / NI**
1.3 Were cells in the same conditions before treatment allocation?		**Y / PY / PN / N / NI**
Risk of treatment allocation bias judgement		
2.1 Was the method measuring the outcome appropriate?		**Y / PY / PN / N / NI**
2.2 Were there any differences in measurement between treatment groups?		**Y / PY / PN / N / NI**
2.3 If yes to 2.2 could this have been due to knowledge of expected effects?		**Y / PY / PN / N / NI**
Risk of measurement bias judgement		
3.1 Was data analysed in accordance with a pre-specified analysis plan?		**Y / PY / PN / N / NI**
3.2 Are any results likely to have been selected from… multiple eligible outcome measurements?		**Y / PY / PN / N / NI**
3.3 …Multiple eligible analyses of the data?		**Y / PY / PN / N / NI**
3.4 Were adequate repeats of each experiment carried out?		**Y / PY / PN / N / NI**
Risk of selection of result bias judgement		
Key: Y = yes, PY = probably yes, PN = probably no, N = no, NI = no information given or not applicable.		

Supplementary data 2.2 – List of excluded studies and their reason for exclusion.

Reference	Reason for exclusion
(Abe et al., 1999)	This study looked at the effects of vitamin D on smooth muscle cells. Due to the focus of the review being skeletal muscle cells, this review was excluded.
(Akagawa et al., 2018)	This study administered vitamin D to live rats, so vitamin D treatment was *in vivo.* This was not in line with the inclusion criteria of *in vitro* studies.
(Alami-Durante et al., 2011)	This study administered vitamin D to sea bass larvae, so vitamin D treatment was *in vivo.* This was not in line with the inclusion criteria of *in vitro* studies.
(Belenchia et al., 2018)	This study administered vitamin D to live C57BL/6 J mice, so vitamin D treatment was *in vivo.* This was not in line with the inclusion criteria of *in vitro* studies.
(Camperi et al., 2017)	Muscle biopsies taken from cancer patients making it difficult to compare results to muscle cells from healthy patients/cell lines.
(Domingues-Faria et al., 2014)	This study administered vitamin D to live wistar rats, so vitamin D treatment was *in vivo.* This was not in line with the inclusion criteria of *in vitro* studies.
(Endo et al., 2003)	This study administered vitamin D to live mice, so vitamin D treatment was *in vivo.* This was not in line with the inclusion criteria of *in vitro* studies. The VDR was also knocked down so was not comparable to other studies.
(Hlaing et al., 2014)	This study looked at the effects of vitamin D on cardiomyocyte cells. Due to the focus of the review being skeletal muscle cells, this review was excluded.
(Hutton et al., 2014)	This study administered vitamin D to live broiler chickens, so vitamin D treatment was *in vivo.* This was not in line with the inclusion criteria of *in vitro* studies.
(Irazoqui et al., 2014)	This study used an *in vitro* model using C2C12 cells however, they knocked down the VDR using shRNA which makes it difficult to compare to the other studies within the review.
(Kinoshita et al., 2016)	This study administered vitamin D to live rats, so vitamin D treatment was *in vivo.* This was not in line with the inclusion criteria of *in vitro* studies.
(Max et al., 2014)	This study administered vitamin D to live rats, so vitamin D treatment was *in vivo.* This was not in line with the inclusion criteria of *in vitro* studies.
(Oku et al., 2016)	This study administered vitamin D to live rats, so vitamin D treatment was *in vivo.* This was not in line with the inclusion criteria of *in vitro* studies.

Chapter 3 Materials and Methods

3.1 *In vitro* experiments

3.1.1 C2C12 culture and differentiation

3.1.1.1 Cell culture

The C2C12 mouse myoblast cell line was grown in T75 flasks in growth medium consisting of Dulbecco's modified eagle's medium (DMEM; Sigma Aldrich, Poole, UK) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen, Paisley, UK) and 1% (v/v) penicillin and streptomycin (P/S; Invitrogen, Paisley, UK). Stock P/S solution contained 10,000 units of penicillin and 10,000 µg of streptomycin. Cells were incubated at 37°C and 5% (v/v) CO_2. In order to keep cells in a proliferating state, cell confluence was kept below 70% by passaging every 2 days. To stimulate cells to differentiate, the serum starvation method was used. The previous growth medium was replaced with differentiation medium (DMEM supplemented with 2% (v/v) horse serum (HS; Invitrogen, Paisley, UK) and 1% (v/v) P/S). Cells were cultured for up to 8 days and the differentiation medium was changed every 48 hours to ensure that pH levels were maintained, and nutrients were replenished.

3.1.1.2 Passaging cells

When keeping C2C12 stocks in T75 flasks, cells were passaged every 48 hours. When C2C12 cells reach a confluency of 70% and above, they make cell-cell contacts which stimulates exit from the cell cycle and terminal differentiation. Therefore, passaging every 48 hours ensured that cells remained in the proliferative state. A 1/10 dilution of cells was carried out at each passage. Briefly, media was removed from the flask and cells were washed with 10ml warm PBS (phosphate buffered saline, Sigma Aldrich, Saint Louis, USA). PBS stocks were made by dissolving 1 tablet per 200ml of

deionised water (to give 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4). PBS was then removed and 3ml of warm trypsin-EDTA (Sigma Aldrich, Saint Louis, USA) was added. Trypsin-Ethylenediaminetetraacetic acid (EDTA) contained 5g of porcine trypsin and 2g EDTA, 4Na per liter of 0.9% (w/v) sodium chloride. Cells were incubated for 5 minutes then 7ml of growth medium was added to the flask and then the entire volume was transferred to a 30ml tube and centrifuged for 5 minutes at 1000rpm (200 x g). The media was then removed and 10ml of fresh growth medium was added. A 10ml pipette was used to pipette up and down several times to resuspend the pellet and create a single cell suspension. From this, 1ml was added to a new T75 flask along with 12ml of fresh growth medium and incubated for 48 hours until the next passage. Passage number was noted down on each flask to keep track of cell age. For all cell experiments, cells between passage number 5 and 12 were used. Once passage 12 was reached, cells were discarded to prevent the use of old cells which can cause variability in differentiation (cells older than passage 12 tended to differentiate more slowly).

3.1.1.3 Counting and plating cells

Cells were counted using a haemocytometer. Cells underwent the passaging protocol described above until the step where there was 10ml of single cell suspension. From this, 20µl of cell suspension was pipetted under the coverslip on the haemocytometer. Cells in all 4 squares were counted and the average taken. The number of cells counted x 10^4 gave the number of cells per ml. The following calculation was then used:

(desired number of cells/ml / counted number of cells/ml) X total volume required

This gave the number in ml of cell suspension required. The appropriate volume of media was then added to make the volume up to the total volume required. For 6 well plates, cells were plated at $1.5x10^5$ cells per well, for 24 well plates, cells were plated at 30,000 cells per well and for 96 well plates, cells were plated at 5,000 cells per well.

3.1.1.4 Freezing cells

The normal protocol for passaging cells was carried out (see 2.1.1.2) until the 5-minute centrifugation stage to create a cell pellet. Whilst the cells were being centrifuged, 5ml of cryopreservation media was made which consisted of 90% (v/v) FBS and 10% (v/v) dimethyl sulfoxide (DMSO) by adding 4.5ml of cold FBS to 0.5ml of DMSO. After centrifugation, old media was removed and 3ml of cryopreservation media was added. The cell pellet was resuspended by pipetting up and down several times, being careful not to create bubbles. Cells were then counted using a haemocytometer (as above 2.1.1.3). More cryopreservation media was added if required, until the cell number was between $0.5 – 1 \times 10^6$ cells/ml. 1ml of cell solution was then added to each cryotube and tubes were labelled with cell type, passage number and date. The cryotubes were placed into a Mr Frosty™, containing isopropanol, and put into the -80°C freezer for 24 hours. After 24 hours, cryotubes were transferred to liquid nitrogen.

3.1.1.5 Vitamin D ($1,25(OH)_2D_3$) treatment

Cells undergoing vitamin D treatment were treated with one of four different concentrations (10^{-13}M, 10^{-11}M, 10^{-9}M and 10^{-7}M) of $1,25(OH)_2D_3$ dissolved in 0.1% (v/v) ethanol. Control cells were treated with either 0.1% (v/v) ethanol or normal differentiation media as described above. To keep ethanol at 0.1% (v/v) in all

treatments, 1/1000 dilutions in differentiation media were carried out from stocks of vitamin D in >99.9% ethanol. Tale 3.1 shows an example of this:

Table 3.1 – examples of vitamin D treatments made from stocks

Final $1,25(OH)_2D_3$ concentration required	**$1,25(OH)_2D_3$ stock concentration in ethanol**	**Volume of stock + differentiation media**
10^{-7}M	10^{-4}M	20µl stock + 20ml media
10^{-9}M	10^{-6}M	20µl stock + 20ml media
10^{-11}M	10^{-8}M	20µl stock + 20ml media
10^{-13}M	10^{-10}M	20µl stock + 20ml media

Initial experiments for training, practise and optimisation were carried out using 0.1% (v/v) DMSO as the vehicle for $1,25(OH)_2D_3$, however cells did not appear to proliferate very well, and cell death appeared to be high when checking cells using a light microscope. Therefore, a DMSO dose response experiment was carried out testing three concentrations of DMSO alongside a control containing just proliferation media (0%, 0.01%, 0.1% and 1% DMSO (v/v)). It was found that 0.1% (v/v) DMSO significantly decreased DNA levels by day 5 compared to the control ($p<0.05$, Figure 3.1). Following this, an additional dose response experiment was performed to test ethanol (0%, 0.01%, 0.1% and 1% (v/v)). It was found that 0.1% (v/v) EtOH did not cause a decrease in DNA content at any of the time points (Figure 3.2). Therefore, all final experiments were carried out using 0.1% (v/v) EtOH as the vehicle for $1,25(OH)_2D_3$.

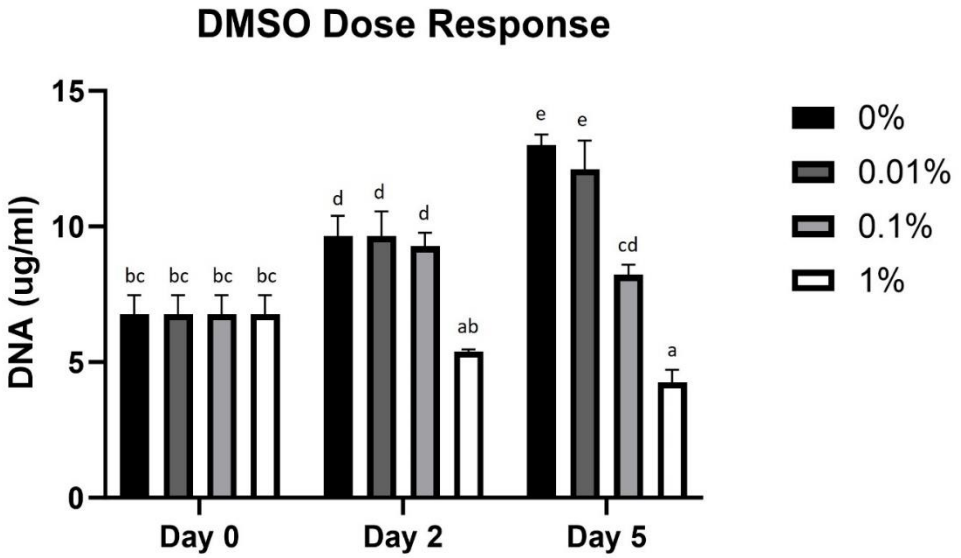

Figure 3.1. DMSO dose response for C2C12 cells grown on 96-well plates in proliferation media (10% FBS in DMEM, 1% pen strep) for 48 hours with 0%, 0.01%, 0.1% and 1% DMSO. Significant interaction between day*treatment ($p<0.001$). *Post hoc* Bonferroni shows that 1% DMSO significantly inhibits proliferation and decreases DNA (DNA used as an indication of cell number) over time, with significantly fewer cells on day 5 than day 0 ($p<0.05$), suggesting increased apoptosis. 0.1% DMSO does not affect DNA levels by day 2, but by day 5 there is significantly less DNA than the day 5 control ($p<0.05$), suggesting that 0.1% also has an inhibitory effect on proliferation.

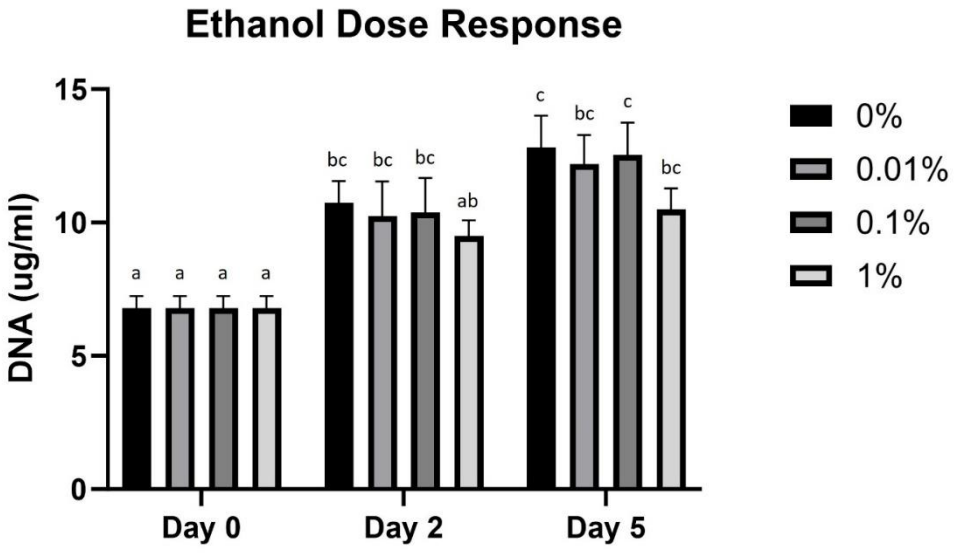

Figure 3.2. Ethanol (EtOH) dose response for C2C12 cells grown on 96 well plates in proliferation media (10% FBS in DMEM, 1% pen strep) for 48 hours with 0%, 0.01%, 0.1% and 1% EtOH. No significant interaction was found between day*treatment ($p=0.45$). Effect of treatment was not significant ($p=0.053$) although it was very close. Effect of time was significant ($p<0.001$). 0.1% EtOH did not cause a decrease in DNA content at any time point compared to the control.

3.1.1.6 Cell harvest

Media was removed immediately before harvesting for all cells. For proliferation studies (DNA assay) where cells were grown on a 96-well plate, cells were not harvested because the same plate was used for the assay (see section 2.1.2.1 below). For cells harvested for protein and creatine kinase (CK) assays, which were grown on 6-well plates (a DNA assay was also carried out on these cells for normalising protein and CK to), cells were washed in 1ml PBS, then scraped into 500ul tri-sodium citrate (0.05M, pH 6.8, 4°C), placed on ice whilst other samples were scraped, then stored at -20°C until use. For protein and creatine kinase assays, cells were harvested on days 0, 1, 2 and 5. For samples harvested for RT-qPCR, grown on 6-well plates, cells were scraped directly into 200µl of cold RNase-free PBS and stored at -80°C. For PCR work, cells were harvested on days 0, 1, 2, 4, 6 and 8. For transfection work, grown on 24-well plates, cells were not harvested, however media was removed and replaced with FluoroBrite DMEM colourless medium (Dulbecco's modified eagle's medium (DMEM), high D-glucose, 3.7g/L sodium bicarbonate, without L-glutamine, without phenol red; Fisher Scientific, Loughborough, UK). For transfection work, plates were measured on days 0, 1, 2, 3, 4, 5, 6, 7 and 8.

3.1.2 Assays

3.1.2.1 DNA assay

The DNA assay uses a fluorescent dye which binds to double stranded DNA to give a direct measurement of the amount of DNA in a sample and thus, an estimation of the number of cells (Bolger et al., 1997). For cells grown on 96 well plates (for proliferation studies), media was removed from each well using a pipette and cells were washed with 100µl warm PBS. The PBS was then removed and 100µl sterile

water was added. Plates were freeze/thawed twice to lyse cells to give 100µl of cell lysate per well. This cell lysate was then used for the DNA assay. For cells grown on 6 well plates (for differentiation studies on protein and CK), cells were washed in 500µl warm PBS and then scraped into 500µl cold tri-sodium citrate (0.05M, pH 6.8, 4°C) and sonicated on ice (15-30 seconds until samples were clear) at an amplitude of 10 microns. Cell lysates were stored in eppendorf tubes at -20°C until use. 50µl of each cell lysate mixed with 50µl dH_2O was used per well on a 96 well plate for DNA assays (following optimisation studies which tested various dilutions to ensure that samples fell within the standard curve). 10ml of working dye solution was made by adding 20µl of 1mg/ml Hoechst fluorescent dye to 10ml 2 x TNE (100mM Tris-HCl, 2M NaCl, 10mM EDTA, pH 7.4). This was enough for 1 X 96 well plate. A standard curve was generated using serial dilutions of calf thymus DNA (40, 20, 10, 5, 2.5, 1.25, 0.6µg/ml) which was used to quantify the DNA content in the samples. 100µl of working dye solution was added to each well (containing either 100µl cell lysate or DNA standard) and measured using a fluorescence plate reader (Fluostar Optima, BMG Labtech) at an excitation of 350 nm and emission of 460 nm. Samples and standards were carried out in triplicate.

3.1.2.2 Lowry protein assay

The Lowry protein assay quantifies total protein content within a sample. This can be used as an indirect marker of differentiation due to an increase in protein synthesis and cell size as cells differentiate from myoblasts to myotubes (Garrels, 1979). Cells were scraped into 500µl cold tri-sodium citrate, sonicated and stored as previously described. 50µl of sample (1:1 ratio of 25µl of cell lysate mixed with 25µl dH_2O) was pipetted in triplicate onto a 96 well plate. This quantity of cell lysate and dH_2O was optimised so that sample concentrations were roughly in the middle of the standard curve. Standards were made using serial dilutions of known concentrations of bovine

serum albumin (BSA). Standard concentrations were: 0, 0.2, 0.4, 0.6, 0.8, 1 and 1.2mg/ml. 50µl of each standard was added to the plate in triplicate. 150µl of 0.1M NaOH was added to all samples and standards to give a volume of 200µl in all wells. To each sample and standard, 50µl of reagent 1 was added (see table 3.2) and the plate was incubated at room temperature for 5 minutes. Then, 50µl of reagent 2 was added (see table 3.3) and incubated at room temperature for 20 minutes. Absorbance was then read using a plate reader at 655nm (BioRad 680XR Microplate reader).

Table 3.2 – Composition of Lowry reagent 1

Reagent	**Volume required (ml)**
2% Na_2CO_3 in 0.1M NaOH	5
1% $CuSO_4$	0.5
2% KNa tartrate	0.5

Table 3.3 – Composition of Lowry reagent 2

Reagent	**Volume required (ml)**
Folin and Ciocalteu's phenol reagent	0.5
0.1M NaOH	5

3.1.2.3 Creatine kinase assay

The Creatine Kinase (CK) assay measures activity of the Creatine Kinase enzyme. CK is a muscle specific protein which becomes expressed during myogenesis and remains highly expressed in mature muscle (Lyons et al., 1991). Therefore, CK activity is a good marker of differentiation. Cells were scraped into 500µl cold tri-sodium citrate and sonicated as described previously, but samples were used

immediately for CK assays because freeze/thawing can denature the protein and therefore affect enzyme activity. A CK kinetic assay kit was used (Thermo Electron Corporation, Fisher Scientific, Loughborough, UK). 10µl of each sample was loaded onto a 96 well plate then 200µl of reagent 1 from the kit was added to all samples and incubated at 37°C for 5 minutes. Following this, 50µl of reagent 2 from the kit was added to all samples. Absorbance was read at 340 nm (BioRad 680XR Microplate reader) every 30 seconds for a total of 5 minutes to determine the rate.

3.1.3 Gene Expression Analysis

3.1.3.1 RNA extraction from C2C12 cells

An RNA isolation kit (Roche high pure isolation kit) was used to extract RNA from samples. 400µl of lysis buffer was added to frozen samples and vortexed until defrosted, roughly 30 seconds. The sample containing the lysis buffer (approximately 600µl) was then pipetted into a high filter tube within a collection tube and centrifuged at 8000 x g for 15 seconds to sequester the RNA onto the membrane within the filter tube. After each centrifugation, the flow through was discarded. Each sample was then treated with 90µl DNase incubation buffer and 10µl DNase I and incubated for 15 minutes at room temperature to break down any DNA. 500µl of wash buffer I was added followed by 500µl of wash buffer II with centrifugation at 8000 x g for 15 seconds after each. Another 200µl of wash buffer II was then added and samples were centrifuged at 13000 x g for 2 minutes to ensure all contaminants were removed from the membrane. To elute the RNA, the filter tube was placed in a sterile eppendorf and 50µl elution buffer was added. This was centrifuged at 8000 x g for 1 minute.

RNA concentrations were determined using a Nanodrop ND-1000 (thermo Scientific, Wilmington, USA). RNA was considered pure if the 230/260nm ratio was between 2

and 2.2. If the ratio was lower than this, indicating possible contamination, an RNA 'clean-up' protocol was carried out (see section 2.1.3.2). Once pure, all samples were diluted to 50ng/µl. Extracted RNA was stored at -80°C until use.

3.1.3.2 RNA clean-up

To each RNA tube, 10% (v/v) RNase free 3M sodium acetate (NaAc) and 2.5x volume of 100% EtOH was added (e.g. for 40µl of RNA, 4µl of NaAc and 100µl of EtOH was added). Samples were mixed well by pipetting up and down and then spun down briefly before leaving in the -80°C freezer for 2 hours. Samples were then centrifuged at 18 000 x g for 15 minutes with the centrifuge set to 4°C. Supernatant was discarded and the pellet was washed by addition of 500µl of ice cold 75% (v/v) EtOH and vortexed well. Samples were centrifuged again at 18 000 x g for 5 minutes at 4°C. The supernatant was discarded, making sure to remove all traces using a P10 pipette, and RNA was re-suspended in 20µl of RNase free water. RNA concentrations and a re-check of RNA purity was carried out using a Nanodrop ND-1000 (thermo Scientific, Wilmington, USA).

3.1.3.3 cDNA synthesis

First strand cDNA synthesis was carried out on 50ng/µl samples using a Thermo Scientific RevertAid RT kit (Thermo Scientific, Vilnius, Lithuania) according to the manufacturer's protocol. 10µl RNA (500ng) was combined with 1µl random hexamer primers (0.2µg/µl) and 1µl RNase-free H_2O (12µl total volume) and incubated at 65°C for 5 min. Samples were immediately placed on ice and the following was added to each sample: 4µl 5x reaction buffer, 1µl RiboLock RNase inhibitor (20 U/µl), 2µl 10mM dNTP mix and 1µl RevertAid RT (200 U/µl). Total volume per well = 20µl. Samples were centrifuged briefly, then incubated for 5 mins at 25°C. Then,

samples were incubated for 60 mins at 42°C. Following this, samples were diluted 5-fold by adding 80µl RNase free water (total volume 100µl), and stored at -20°C.

3.1.3.4 Quantitative RT-PCR

A LightCycler® 480 (Roche, Burgess Hill, UK) was used to carry out quantitative real time PCR. Each well on a 384 well PCR plate contained the following: 5µl cDNA, 7.5µl SYBR Green I Master mix (Roche, Burgess Hill, UK), 0.45µl forward primer (10µM), 0.45µl reverse primer (10µM), 1.6µl RNase free H_2O (total volume = 15µl). The amplification protocol was as follows: pre-incubation at 95°C for 5 min followed by 45 amplification cycles (denaturation of strands at 95°C for 10 seconds, annealing at 60°C for 15 seconds, elongation at 72°C for 15 seconds). All reactions were carried out in triplicate.

Prior to measuring genes of interest, each cDNA sample was run using reference gene primers (TATA binding protein) to check that the crossing point (CP) value for each sample was within 2 standard deviations of the mean of all samples, thus they could be used for the cDNA pool. Once confirmed, a cDNA pool was created by taking a 5µl aliquot from every sample. This was used in serial dilutions (1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128) to create a standard curve which was used to determine relative transcript abundance. A linearity check was carried out for each standard curve to make sure that the slope gradient and PCR efficiency was as close to the optimum as possible (optimum slope gradient = -3.3, optimum efficiency = 2), Figure 3.3 C.

A list of genes measured, and their corresponding forward and reverse primers, can be viewed in table 3.4. All primers were tested to ensure that only one amplicon of the correct length was produced from each PCR reaction. Melt curve analysis was also used to ensure only one peak was present, indicating one amplicon product (Figure 3.3 D).

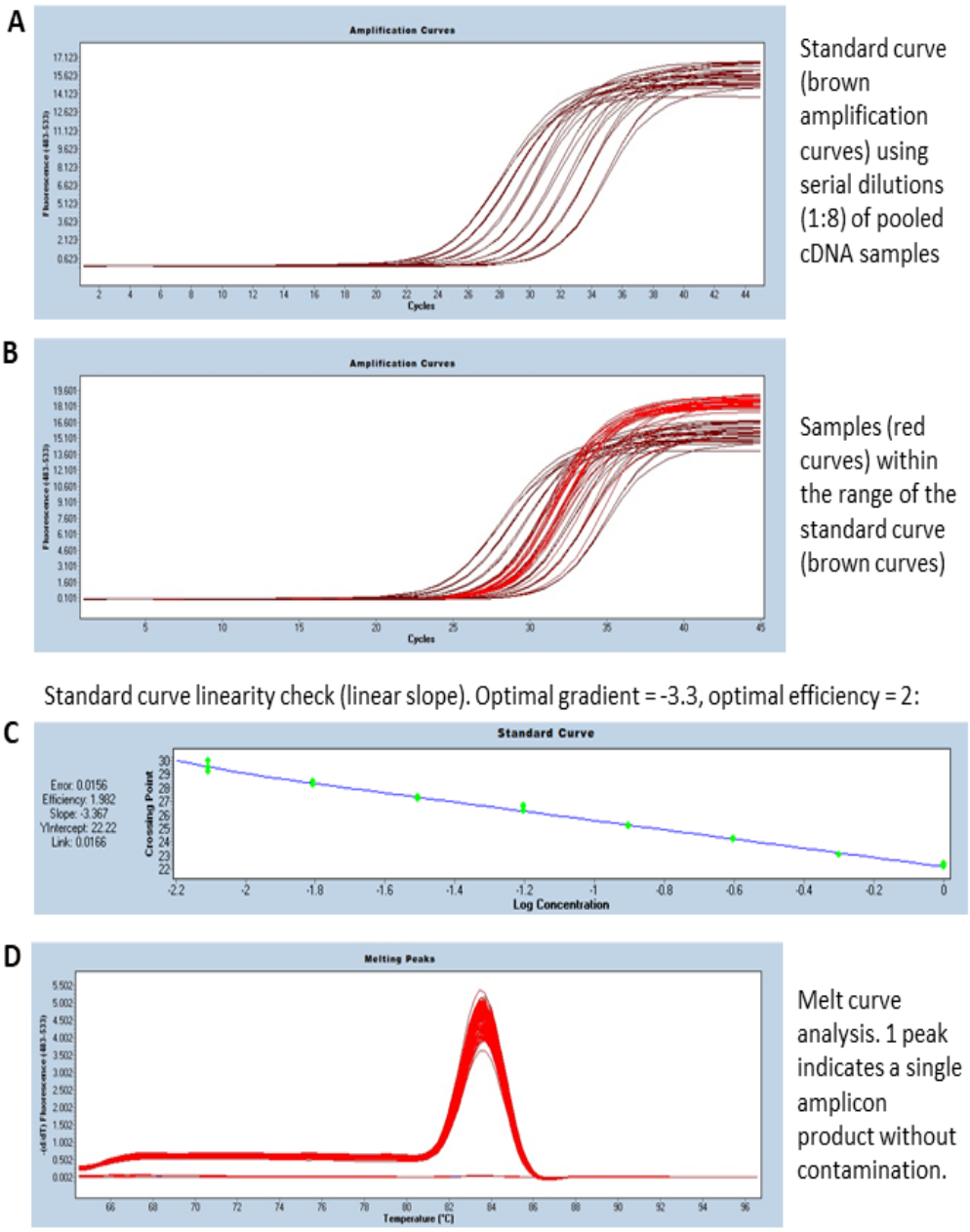

Figure 3.3. PCR outputs using LightCycler 480 software. Panel A shows the amplification curves for the standards in brown (made by 1:8 serial dilutions of the cDNA pool). B shows amplification curves of some of the samples (red) alongside the standards (brown) to show that the samples fit within the range of the standard curve. C shows the standard curve which confirms that the reaction was efficient (optimal efficiency = 2) and linear (optimal gradient = 3) which permits quantification of relative mRNA abundance. C shows melt curves where a single peak shows that

Table 3.4 – Genes measured and their corresponding primers

Gene name	*Forward primer sequence*	*Reverse primer sequence*	*Length of amplicon*
MyoD	5′ CGTGGCAGCGAGCACTAC 3′	5′ TGTAATCCATCATGCCATCAGA 3′	79bp
Myf5	5′ CAGCCCCACCTCCAACTG 3′	5′ GCAGCACATGCATTTGATACATC 3′	117bp
Myogenin	5′ CCCATGGTGCCCAGTGAA 3′	5′ GCAGATTGTGGGCGTCTGTA 3′	130bp
Myosin Heavy Chain I (MHCI)	5′ CTCAAGCTGCTCAGCAATCTA TTT 3′	5′ GGAGCGCAAGTTTGTCATAAGT 3′	152bp
Myosin Heavy Chain neonatal (MHC neo)	5′ CAGGAGCAGGAATGATGCTCT GAG 3′	5′ AGTTCCTCAAACTTTCAGCAGCC AA 3′	113bp
Myosin Heavy Chain embryonic (MHC emb)	5′ TCCGACAACGCCTACCAGTT 3′	5′ CCCGGATTCTCCGGTGAT 3′	72bp
Myosin Heavy Chain IIa	5′ AGGCGGCTGAGGAGCACGTA 3′	5′ GCGGCACAAGCAGCGTTGG 3′	130bp
Myosin Heavy Chain IIx	5′ GAGGGACAGTTCATCGATAG CAA 3′	5′ GGGCCAACTTGTCATCTCTCAT 3′	154bp
Myosin Heavy Chain IIb	5′ CAATCAGGAACCTTCGGAACAC 3′	5′ GTCCTGGCCTCTGAGAGCAT 3′	80bp

3.1.3.5 Oligreen quantification of total cDNA

Due to the time course studied, and large cell changes taking place from a proliferative to a differentiated state, a reference gene could not be found that did not change over time. Therefore, DNA content was quantified using Oligreen for all genes.

An Oligreen ssDNA Assay kit (Invitrogen, Eugene, Oregon) was used to quantify cDNA content on a 384 well PCR plate. A working reagent was made by diluting Oligreen ssDNA reagent 1:200 in 1 x Tris-EDTA (TE) buffer (i.e. 5µl Oligreen + 1ml 1 x TE). 5µl of working reagent was added to 5µl cDNA in triplicate. Fluorescence was quantified using a LightCycler® 480 (Roche, Burgess Hill, UK) with increasing temperature at a continuous ramp rate of 0.11°C/s until it reached 95°C. Fluorescence measurements at 80°C were plotted against a cDNA standard curve (as described above) to determine relative cDNA quantity.

3.1.4 Transfections

3.1.4.1 Generation of vectors

Vectors were generated externally by VectorBuilder (https://en.vectorbuilder.com/). To identify the myogenin promoter sequence, 'myogenin promoter' was searched on NCBI which gave a nucleotide sequence 4145 base pairs in length. The mouse genomic sequence was also found by searching the NCBI database, which gave the genome for CL57BL6 strain. The FASTA sequence for chromosome 1 (where myogenin is located) was downloaded. BLAST was used to align the myogenin promoter sequence to the mouse genome to check it matched. The myogenin promoter sequence aligned to the mouse genome starting from nucleotide 134,217,727 on chromosome 1. On chromosome view in Ensembl (Figure 3.4),

myogenin appears to start just after 134.2 million bp (circled in red), therefore the promoter sequence obtained for myogenin aligned where expected.

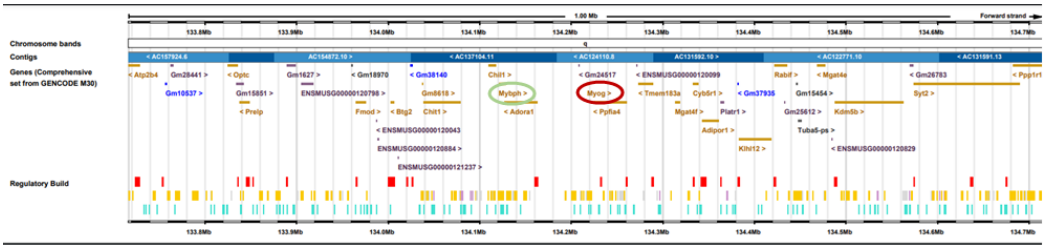

Figure 3.4. Chromosome view on Ensembl showing the mouse chromosome 1. Circled in red is the Myogenin gene which starts just after 134.2 million base pairs which confirmed that the myogenin promoter sequence had aligned in the correct position on chromosome 1 (BLAST alignment between the mouse chromosome 1 sequence and the mouse myogenin promoter sequence revealed that it aligned starting from nucleotide 134,217,727.

The annotated myogenin sequence (NCBI, accession M95800), including approximately 1.6kB of the promoter sequence, followed by the coding sequence is shown below. The transcriptional start site and start codon are highlighted in yellow, the stop site is highlighted in green, the coding sequence is highlighted in grey, transcription factor binding sites are highlighted in dark green and the putative VDRE is highlighted in blue. Exons are shown by different coloured text: exon 1 – **exon 2** – exon 3.

TCTAGAGTTGTATGACGCAGGCAAAGGTGATGACTCAGGCAGGAAGGAATAGAAGAGGCCAGC
CTGGTGGCCCAGGACAGACAAATGATGCAAAGGACTCTTTCTCCTTATCGACCCTTCTACAGA
AAGGAAAGAGTCAAAACGGTTCTAGTGCCAGAAGGCATTATTGAGGGGAAAGCACAGAAGAGA
TGATTAAGAGCATCAGACAGGGTCCATCCCATAATCAGCCACCAAACACAGACACTATTACAT
ATGCCAGCAAGATTTTGCTGAAAGAACCCTGATATAGCTGCCTCTTGTGAGGCTATGCAGTGC
VDRE
CTGGCAAATACAGAAGTGGATGCTCACAGTCAGCTATTGGATGGAACACAGGGCCCCCAATGG
AGGAGCTAGAGAAAGTACCCAAGGAACTGAAGGGGTCTGCAACCCTATAGGTGGAACAACAAT
ATGAACTAACCAGTACCCCCAGAGCTCATGTCTCTAGCTGCATATGTAGCAGAAGATGGCCTA
GTCGGCCATCATTGGGAAGAGAGGCCCCTTGGTATTGCAAACTATATGCCCCAGTACAGGGGA
ACGCCAGGGCCAAGAAGTGGGAATGAGTGGGTAGGGGAGCAGGGCGGGGGGAGGGGGGGGTAT
AGGGAACTTTTGGGATAGCATTTGAAATGTAAATGAAGAAAATATCTAATAAAAAATAATTTA
AAAAAGAGCGTCAGACAGGGGACTGAACAGCTCTTGCACTAGGGGAGAAGAAGGCAATGTAGA
GTAGTCTGTGAGTTCTAATCCTTGCTAAACACTGACTTCACCTGACCCCTACTACTTAAGGCC
CCCCCCCCTTACTTAAGAAGTCCCTGTGTTCTCTTACTTCAATCTACCCCCAACATCATGAGA
CCTGGTCAAAGAAGCTGTAGAAACCCAAAAGTTGAATCCATTTGCCCTTCTGGGTTTCTGTCT
TTGCCTCCATGGACGATAGGACACACACACACACACACACACACACACACACACACACACACA
CArG box
CACGCCCCAAATCTGGAGTGGTCCTGATGTGGTAGTGGTAGGTCTTTAGGGGTCTCATGGGAC
TGACATAGTACGGTTTAAGGTGCTGCTGCTGAGCAGGAAAGAGAAGGCTAAGTGGATTTTCAA
GACCCCTTCCCGTCCGTCCAAGACAACCCCTTTCTTGTTCCCTTCCTGCCCTGTCCACCAGCT
GCCTTGGACCATGGAGGAGAGAGTAGGCAGGAGGCCCGGGTAGGAGTAATTGAAAGGAGCAGA

TGAGACGGGGGAATGCACCCACCCCCACCTTCCCTGCCCCACAGGNTGTGGAGAAATGAAAAC
TAATCAAATTACAGCCGACGGCCTCCCGACCCGTGCACAGGAGCCGCCTGGGCCAGGGGCAGG

E box 2

CCTGCAGGGTGGGGTGGGGGCAAAAGGAGAGGGAAGGGGAATCACATGTAATCCACTGGAAAC

MEF2

GTCTTGATGTGCAGCAACAGCTTAGAGGGGGGCTCAGGTTTCTGTGGCGTTGGCTATATTTAT

NF1 **TATA box** **E box 1.** **TSS**

CTCTGGGTTCATGCCAGCAGGGAGGGTTTAAATGGCACCCAGCAGTTGGCGTGAGGGGCTGGG

Start codon

AGCTTGGGGGCCAGTGGCAGGAACAAGCCTTTTCCGACCTGATGGAGCTGTATGAGACATCCC
CCTATTTCTACCAGGAGCCCCACTTCTATGATGGGGAAAACTACCTTCCTGTCCACCTTCAGG
GCTTCGAGCCCCCGGGCTATGAGCGGACTGAGCTCAGCTTAAGCCCGGAAGCCCGAGGGCCCC
TGGAAGAAAAGGGACTGGGGACCCCTGAGCATTGTCCAGGCCAGTGCCTGCCGTGGGCATGTA
AGGTGTGTAAGAGGAAGTCTGTGTCGGTGGACCGGAGGAGGGCAGCCACACTGAGGGAGAAGC
GCAGGCTCAAGAAAGTGAATGAGGCCTTCGAGGCCCTGAAGAGGAGCACCCTGCTCAACCCCA
ACCAGCGGCTGCCTAAAGTGGAGATCCTGCGCAGCGCCATCCAGTACATTGAGCGCCTACAGG
CCTTGCTCAGCTCCCTCAACCAGGAGGAGCGCGATCTCCGCTACAGAGGCGGGGGCGGGCCCC
AGCCCATGGTAAGTGGCTAGTACACCAGATCCAGGGATAGGGCCAGAGGGAGGTGCCTGGTCA
GCCCCGGGCCACCAGAGCTAGAACAGGTGCAAAGCAGGGCCCTTGGGATCCCTGGACCCCTTT
CCTTGTCGGCTAGACTGTCCAGTGGCCTTCTGGGCTCATACTGTTTCCAAAATACAGATGGGG
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TGAGCCTAGGTGAAAATGAGGGAAAAGTGGCCTAAGAAACCTGCTTTGTCCAAGTCCACAGCC
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AAAATACTGTGGTTTATCACCCTCACCGCCGAAATGAAAGCAAAGTTCTCATCCTAGAAGCTA
GGAAAGACCTAGAAGTAGGATGTCTATGGTAACATTCTGCAGTAGACTTGACCTTGGACCTTG
GCCTTGTCTTGCATGCAG**GTGCCCAGTGAATGCAACTCCCACAGCGCCTCCTGCAGTCCGGAG**
TGGGGCAATGCACTGGAGTTCGGTCCCAACCCAGGAGGTAAGTGAATCCTGACCCTGGTAACA
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CAGCTTTTCTAGGAAATTGCTGACCTGAGGGCCCCAAATGGTGGGCTGGAGACCCTGTGCCTC
CCGGTAGCCTTGACCAGCTAAAGAGTAGTCAGAGCCTCCAGCAACTTCCCTGCTGGTCTCTGA
CCTCAGAGAGAAGAGCCTGCTCCTGCCCCAGGGGTCCCTGGGGATCACTCAGTCAGTGTTGTA
AAACTGTGCCCTGTGCCCTAGGATATCCTGGCTTCACCTAGGGATAGCCACTTTTAAAACCCC
ACAAGCACACACACAGCATGGGGAGTACTAGTGTCTTGGGGGCGGGGAACACTCCTGAGTCTT
CCTTACCCATTTTGTCCTGGACCAGTTGCCCAGGAAACATTCTCCCACTTTTTCTTGCAGATC
ATTTGCTCGCGGCTGACCCTACAGACGCCCACAATCTGCACTCCCTTACGTCCATCGTGGACA
GCATCACGGTGGAGGATATGTCTGTTGCCTTCCCAGACGAAACCATGCCCAACTGAGATTGTC
TGTCAGGCTGGGTGTGCATGTGAGCCCCCAAGTTGGTGTCAAAAGCCATCACTTCTGTAGCAG
GGGGCTTTTAAGTGGGGCTGTCCTGATGTCCAGAAAACAGCCCTGGGCTGCCACAAGCCAGAC
TCCCCACTCCCCATTCACATAAGGCTAACACCCAGCCCAGCGAGGGAATTTAGCTGACTCCTT
AAAGCAGAGAGCATCCTCTTCTGAGGAGAGAAAGATGGAGTCCAGAGAGCCCCCTTGTTAATG
TCCCTCAGTGGGGCAAACTCAGGAGCTTCTTTTTTGTTTATCATAATATGCCTCGAATTCCAC
CCTCCACCTCCAAAATGAAACCGTTTGAGAGACATGAGTGCCCTGACCTGGACAAGTGTGCAC
ATCTGTTCTAGTCTCTTCCTGAAGCCAGTGGCTGGGCTGGGCCTGCCCTGAGTTGAGAGAGAA
GGGGGAGGAGCTATCCGGTTCCAAAGCCTCTGGGGGCCAAGCATTTGCAGTGGATCTTGGGAA
CCTTCCAGTGCTTTGTGTATTGTTTATTGTTTTGTGTGTTGTTTGTAAAGCTGCCGTCTGCCA
AGGTCTCCTGTGCTGATGATACCGGGAACAGGCAGGCAAGGGGGTGGGGGCTCTTGGGGTGAC
TTCTTTTGTTAACTAAGCATTGTGTGGTTTTGCCAATTTTTTTTCTTTTGTAATTCTTTTGCT
AACTTATTTGGATTTCCTTTTTTAAAAAATGAATAAAGACTGGTTGCTATCAGAGCATCTGCA
ATGATATTATCAAGANGGGGAGCCACCAAAACTCTTGACACCTTTACC

To identify the VDRE on the myogenin promoter, the typical VDRE sequence (taken from Tsoumpra et al., (2020)) was aligned to the myogenin promoter sequence described above using Clustal. 12/15 bases (80% match) aligned at -1260 to -1246 from the TSS on the myogenin promoter, which was in a similar region to the VDRE identified on the Dtna1 gene at -1474 to -1461 (Tsoumpra et al., 2020).

A regular vector (for promoter testing) was used from Vector Builder. For the 'MyoG-VDRE-GFP' vector, Figure 3.5, the myogenin promoter sequence was added from the start of the sequence obtained on NCBI (-1603bp from TSS) to +32bp from the TATA box. A Turbo GFP reporter was added from the reporter database on Vector Builder. The 'MyoG-mutVDRE-GFP' vector is identical to this, Figure 3.6, apart from 3 bases which were changed in positions 2, 3 and 4 on the VDRE sequence from GAA to AGG (this matched the mutation used in Tsoumpra et al., (2020) and was also the only mutation option which would not generate a sequence that was part of another coding sequence or a start codon).

Due to the expectation that myogenin would not be expressed until mid-stage differentiation, and so cells may not appear green until a few days after transfection, a red fluorescent vector was used to check for transfection efficiency 24 hours after transfection. The red vector (pDsRed-Express-N1, Figure 3.7) contained a CMV promoter followed by the pDsRed-Express gene (a variant of *Discosoma sp*. red fluorescent protein) and was generated by a previous PhD student (Maddy Brearley).

Vector summary and map for MyoG-VDRE-GFP:

Vector Summary

Vector ID	VB221011-1168bfv
Vector Name	pRP[Pro]-{Myogenin incl normal VDRE}>TurboGFP
Vector Size	4896 bp
Vector Type	Mammalian Promoter-Testing Vector (In Vitro)
Inserted Promoter	{Myogenin incl normal VDRE}
Inserted Reporter	TurboGFP
Plasmid Copy Number	High
Antibiotic Resistance	Ampicillin
Cloning Host	VB UltraStable (or alternative strain)

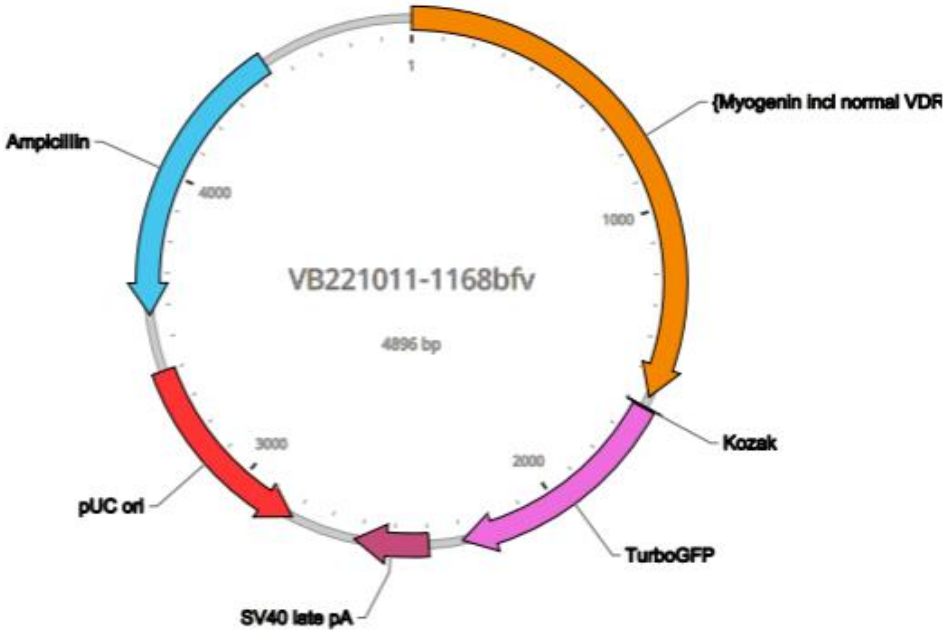

Figure 3.5. Vector summary and vector map for MyoG-VDRE-GFP made by Vector Builder.

Vector summary and map for MyoG-mutVDRE-GFP:

Vector Summary

Vector ID	VB221011-1171xeh
Vector Name	pRP[Pro]-{Myogenin incl mutated VDRE}>TurboGFP
Vector Size	4896 bp
Vector Type	Mammalian Promoter-Testing Vector (In Vitro)
Inserted Promoter	{Myogenin incl mutated VDRE}
Inserted Reporter	TurboGFP
Plasmid Copy Number	High
Antibiotic Resistance	Ampicillin
Cloning Host	VB UltraStable (or alternative strain)

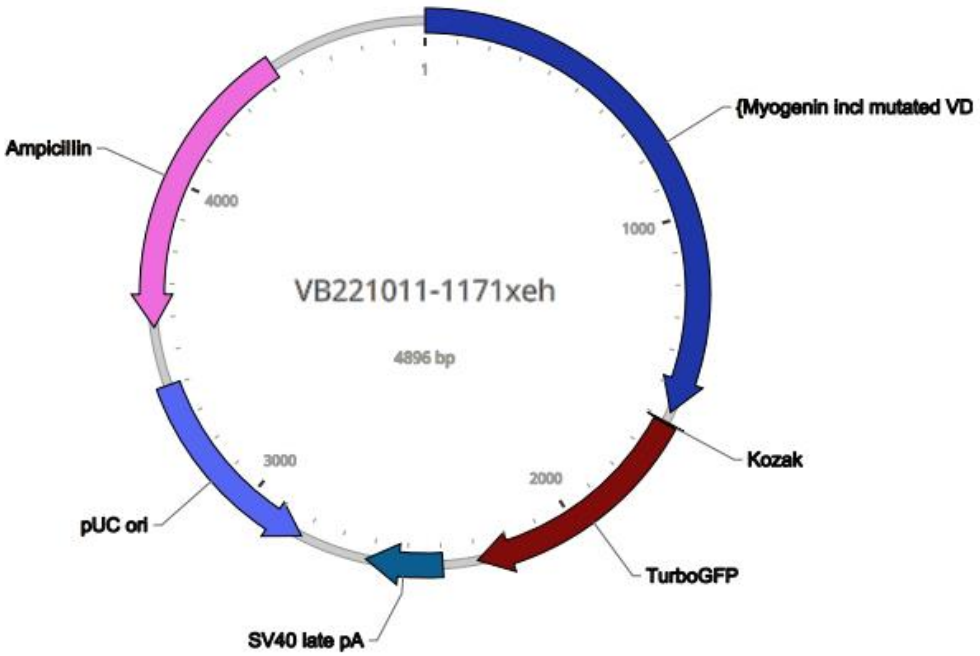

Figure 3.6. Vector summary and vector map for MyoG-mutVDRE-GFP made by Vector Builder.

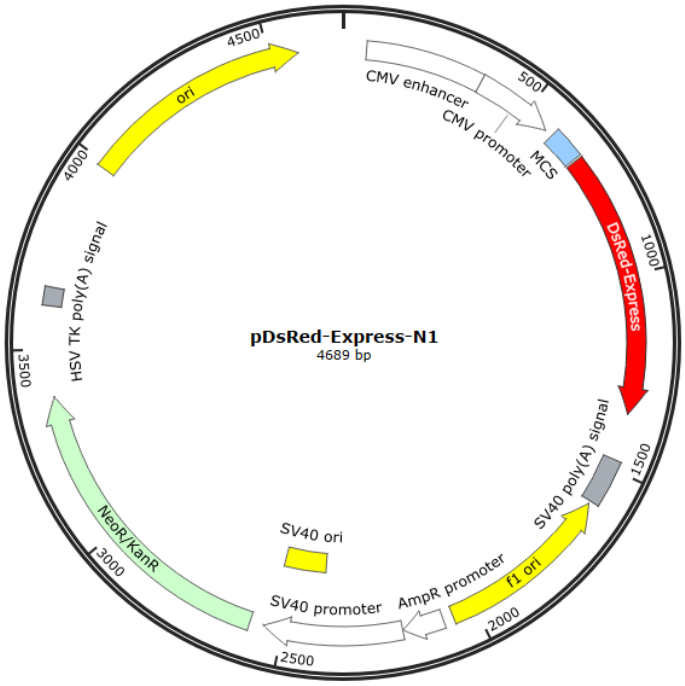

Figure 3.7. Vector map for pDsRed-Express-N1

3.1.4.2 Vector preparation

E-coli containing the required vector were obtained as glycerol stocks and were smeared out onto agar plates. Briefly, 500ml of agar was made by adding 7.5g of BACTO AGAR to 500ml of LB media (see below for composition) which was then autoclaved. Once cooled, 500µl of 1000x Ampicillin was added and then the liquid was poured into plates and left to set. E-coli smears were incubated overnight at 37°C, then stored at 5°C for no longer than a week. One day prior to mini-prep, an inoculation loop was used to pick one singular colony from the plate which was then transferred to 5ml LB media in a falcon tube (1L of LB media was made by combining 950ml dH_2O, 10g Bacto-Tryptone, 5g Bacto yeast extract and 10g NaCl, then topping up to 1L with dH2O). 5µl of ampicillin (100mg/ml) was added, and the mixture was incubated overnight in a shaking incubator at 37°C.

Mini-preps were carried out using a Promega PureYield Plasmid Miniprep kit (Madison, Wisconsin, USA). 600µl of culture media was transferred to a 1.5ml eppendorf and 100µl of lysis buffer was added, then mixed by inversion, resulting in a change of colour from clear to blue (indicating complete lysis). 350µl of cold neutalisation buffer was then added (within 2 minutes to prevent excessive lysis and damage to vector DNA) causing a yellow precipitate to form. A yellow precipitate indicated complete neutralisation. Samples were centrifuged at max speed (20,000 x g) for 3 minutes and the supernatant transferred to a PureYield mini column and centrifuged again for 15 seconds. Following this, 200µl of endotoxin removal wash was added and tubes were centrifuged at maximum speed for 15 seconds. Then, 400µl of column wash solution was added and tubes were centrifuged at max speed for 30 seconds. Finally, the PureYield mini column was transferred to a clean 1.5ml eppendorf, 30µl of elution buffer was added and samples were left to stand at room temperature for 1 minute. Tubes were centrifuged at max speed for 1 minute to elute the vector DNA. Vector concentrations were measured using a nanodrop® ND-1000 (Thermo Scientific, Wilmington, USA) and were stored at -20°C.

3.1.4.3 Transfection

C2C12 cells were plated on 24-well plates in growth medium. Optimisation experiments were carried out to test seeding densities of 20,000, 30,000, 40,000 and 50,000 cells/well. Seeding densities of 40,000 and 50,000 cells/well resulted in cell loss by day 5 due to large sections of cells lifting from the plate as the cells differentiated and over-populated the well. Spontaneous contraction of differentiated myotubes may have also contributed to this. Cell loss did not occur at 20,000 or 30,000 cells/well. 30,000 cells/well gave the highest level of fluorescence with a low level of cell loss and therefore was chosen for subsequent transfection experiments (Figure 3.8).

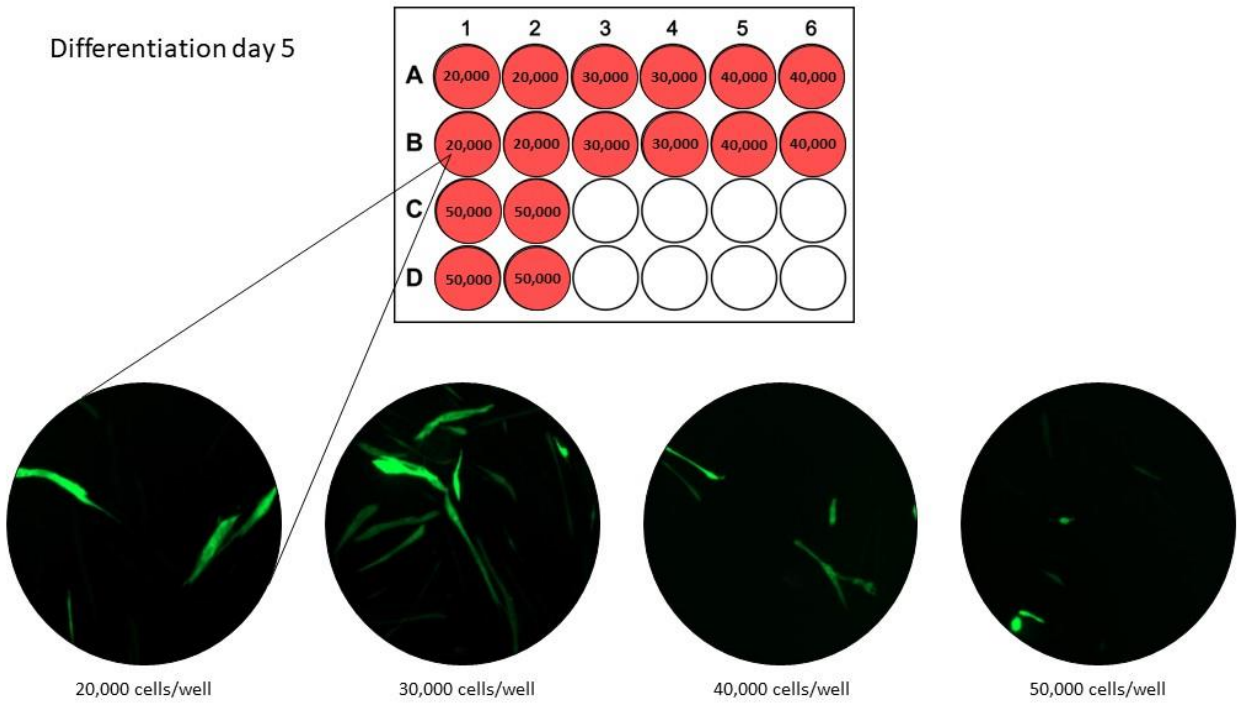

Figure 3.8. Figure to show plate set-up for optimisation of seeding density for transfection experiments. Cells were plated at 20,000, 30,000, 40,000 and 50,000 cells/well in proliferation media and transfected 24 hours later, then 24 hours after transfection, media was switched to differentiation media. By differentiation day 5, large amounts of cells were lost in the 40,000 and 50,000 wells due to cells peeling off (sheets of cells were visible floating in the media). 30,000 cells/well showed the highest level of fluorescent cells without cell loss due to peeling.

All cells were plated in growth medium, then 24 hours after plating (day -1), when in the log phase of growth, cells were transfected using Lipofectamine 2000 (Invitrogen, California, USA). 50µl of Opti-MEM (Gibco, Paisley, UK) containing 500ng vector (400ng MyoG-VDRE-GFP plasmid OR 400ng MyoG-mutVDRE-GFP + 100ng DS Red plasmid) and 2.5µl lipofectamine (following optimisation experiments for lipofectamine quantity) was added to each well. 24 hours after transfection (day 0) media was switched to differentiation medium. One plate was stopped every 24 hours to take cell images and measure fluorescence.

3.1.4.4 NucBlue staining

At each time point, media was removed and replaced with 500µl Fluorobrite media (Fisher Scientific, Loughborough, UK) containing 2 drops per ml of NucBlue (Hoechst33342) live cell stain (Fisher Scientific, Loughborough, UK)). Plates were incubated at 37°C, 5% CO_2 (v/v) for 30 minutes before cell images and fluorescence measurements were taken.

3.1.4.5 Measuring Fluorescence

Cell images were captured using an Evos FL microscope. Contrast and brightness settings were kept constant across all time points and all images were taken at the centre of each well.

Fluorescence was measured using a fluorescent plate reader (Fluostar Optima, BMG Labtech) on the matrix scan setting. For each well, 30 x 30 measurements were taken over a 1.5cm^2 area, and average well fluorescence was automatically calculated. GFP was measured at an excitation of 485nm and emission of 520nm, NucBlue was measured at an excitation of 355nm and emission of 460nm and RFP was measured at an excitation of 584nm and emission of 620nm. All presented GFP data was normalised to NucBlue (i.e. promoter activity was normalised to DNA).

3.1.4.6 Myotube diameters

Myotube diameters were measured for cells transfected with MyoG-VDRE-GFP using image J. Four images (one image per well) at the centre of the well at 10x magnification were captured. Images were then blinded to avoid bias. Five myotubes were selected from each image, one from the centre of each of the following areas: top left, top right, bottom left, bottom right and middle. Therefore, a total of 20 myotubes were measured per treatment. Using Image J software, a line was drawn across each myotube (perpendicular to its length – estimated by eye) at 25%, 50% and 75% of its length which gave each diameter in number of pixels. A 400μm scale bar was measured in pixels to convert diameters from pixels to μm. Myotube diameter was calculated by averaging the length at 25, 50 and 75% for each myotube measured, see Figure 3.9.

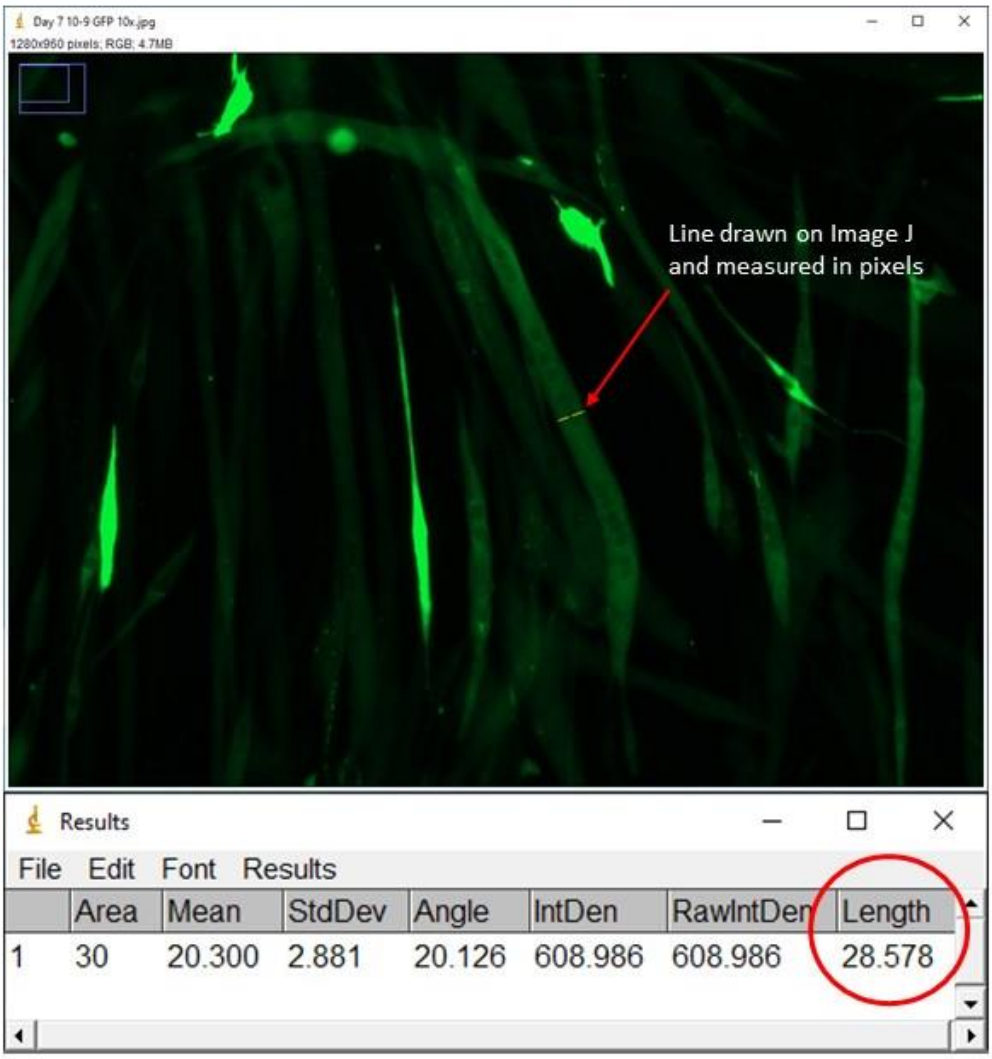

Figure 3.9. Print screen of Image J software with a day 7 myotube photo open. Yellow line represents a line drawn by KA from one side of a myotube to the other. The length of the line is given in number of pixels (circled in red). A 400µm scale bar was measured in pixels to convert number of pixels to µm.

3.1.5 Chromatin Immunoprecipitation (ChIP)

3.1.5.1 Cell culture and cross linking

Chromatin Immunoprecipitation (ChIP) was carried out using a Pierce™ Agarose ChIP kit (Thermo Scientific, Rockford, USA). Unless otherwise stated, all reagents used for ChIP came with the kit. C2C12 cells were plated in 6 well plates at 1.5 x 10^5 cells per well in 2ml of growth medium, then 24 hours after plating, growth medium was changed for differentiation medium. Cross linking was carried out on day 6 of differentiation by adding 125µl of 16% (v/v) formaldehyde to give a final concentration of 1% (v/v) formaldehyde. Plates were swirled gently and incubated at room temperature for 10 minutes. To quench any free formaldehyde, 200µl of 10X glycine solution (to give a final concentration of 1% (v/v)) was added and plates were incubated at room temperature for 5 minutes. Media was then removed, and cells were washed twice with 1ml PBS. 1ml ice cold PBS containing 10µl Halt Cocktail (containing protease inhibitors) was then added to each well and cells were scraped into a 1.5ml Eppendorf, then centrifuged at 3000 x g to form a cell pellet. Supernatant was removed and cell pellets were stored at -80°C.

3.1.5.2 Lysis and Micrococcal Nuclease (MNase) Digestion

Cell pellets were thawed on ice and 100µl of lysis buffer 1 (1µl of Halt cocktail + 100µl of membrane extraction buffer) was added. The cell pellet was broken up by pipetting up and down several times and then incubated on ice for 10 minutes. Tubes were then centrifuged at 9000 x g for 3 minutes to form a nuclei pellet and the supernatant was discarded.

Nuclei were resuspended in 100µl of MNase digestion buffer working solution (0.1µl of 1M dithiothreitol (DTT) + 100µl of MNase digestion buffer), then 0.25µl of ChIP grade micrococcal nuclease (10 U/µl) was added. Tubes were vortexed briefly, then

incubated at 37°C in a water bath for 15 minutes, mixing by inversion every 5 minutes. Following incubation, 10µl of MNase stop solution was added to stop the reaction and tubes were incubated on ice for 5 minutes. Tubes were centrifuged at 9000 x g for 5 minutes to recover the nuclei and the supernatant was discarded. The nuclei pellet was resuspended in 50µl lysis buffer 2 (0.5µl Halt cocktail + 50µl nuclear extraction buffer) and incubated on ice for 15 minutes, vortexing every 5 minutes. Finally, tubes were centrifuged at 9000 x g for 5 minutes. The supernatant, containing the digested chromatin, was transferred to a new 1.5ml Eppendorf and samples were stored at -80°C.

3.1.5.3 Checking fragment size

Ideally chromatin fragments should be 200-700 base pairs so digested chromatin was run on a 2% (w/v) agarose gel to check the size of the fragments. 2g agarose (Sigma-Aldrich, St. Louis, USA) was added to 200ml of 1x TAE (Tris Acetate-EDTA) buffer (1x TAE buffer was made by adding 40ml of 50x TAE (Fisher Scientific, Fair Lawn, NJ) to 1960ml of dH_2O). This was microwaved on full power (900W) until all the agarose was fully dissolved. The solution was cooled to between 50 - 60°C before pouring into the gel cast and left at room temperature for 30 minutes to set. Once set, 1 x TAE buffer was poured over the gel and the gel was stored overnight in the fridge. The following morning, the cold gel was added to an electrophoresis chamber and 1 x TAE buffer was added to the fill line. 5µl of sample and 1µl of 6 x loading dye was added to each well alongside a 1000bp DNA ladder (Sigma-Aldrich Saint Louis, USA). The gel was run at 100V for 15-30 minutes, or until the dye had almost reached the bottom of the gel, using a BioRad Power Pac 200. The gel was then imaged using an Azure Biosystems c200 imager.

3.1.5.4 Immunoprecipitation (IP)

450µl of 1 x IP dilution buffer (100µl of 5 x IP wash buffer, 5µl Halt cocktail and 395µl nuclease free water) was added to each digested chromatin sample to give a total volume of 500µl. All 500µl of diluted lysate was then added to a plugged spin column and primary antibody was added (see table 3.5). The anti-VDR primary antibody (Abcam, Cambridge, UK) had an estimated concentration between 1 – 10mg/ml and the ChIP kit recommended 1 - 10µg of target specific antibody. Therefore, it was decided to add 2µl of target specific antibody.

Table 3.5 – ChIP primary antibodies

Antibody	Volume added (µl)
Anti-RNA polymerase II antibody (positive control, included in kit)	10
Normal rabbit IgG antibody (negative control, included in kit)	1.5
Anti-VDR antibody	2

IP reactions were incubated overnight at 4°C on a rocking platform. Following incubation, 20µl of agarose resin (ChIP grade protein A/G plus agarose) was added to each sample and incubated for 1 hour at 4°C on a rocking platform. Samples were then centrifuged, within a collection tube, at 3000 x g for 30 seconds and flow-through discarded.

0.5ml of wash buffer 1 (100µl of 5 X IP wash buffer + 400µl of nuclease free water) was added and samples were incubated on a rocking platform at 5°C for 5 minutes, then samples were centrifuged at 3000 x g for 30 seconds and flow-through discarded. This process was then repeated twice using wash buffer 2 (200µl of 5 X IP wash buffer + 70µl of 5M sodium chloride + 730µl of nuclease free water) and once

using wash buffer 3 (100µl of 5 X wash buffer 3 + 400µl nuclease free water). Samples were then centrifuged at 3000 x g for 1 minute to remove any residual wash buffer.

3.1.5.5 IP Elution

A plug was fitted to the column and 150µl of 1 X IP elution buffer (75µl of 2 X IP elution buffer + 75µl nuclease free water) was added. Samples were incubated at 65°C for 30 minutes. As a thermomixer was unavailable, samples were incubated on a heat block and beads were re-suspended by flicking the tube every 5 minutes. During incubation, a new 1.5ml Eppendorf was prepared for each sample containing 6µl of 5M NaCl and 2µl of 20mg/ml proteinase K. Following incubation, the plug was removed from each column and columns were added to the previously prepared Eppendorfs. Samples were centrifuged at 6000 x g for 1 minute. Following this, samples were vortexed, then incubated in a heat block at 65°C for 1.5 hours.

3.1.5.6 DNA recovery

750µl of DNA binding buffer was added to each sample. 500µl of this was then transferred to a DNA clean up column inserted into a 2ml collection tube. Columns were centrifuged at 10,000 x g for 1 minute and flow through discarded. This was repeated for the remainder of the sample.

Next, 750µl of DNA column wash buffer was added, then samples were centrifuged at 10,000 x g for 1 minute and flow through discarded. To remove any residual wash buffer, columns were then centrifuged at 10,000 x g for 2 minutes.

Columns were placed into a new, sterile 1.5ml Eppendorf and 50µl of DNA column elution solution was pipetted directly into the centre of each column. Samples were centrifuged at 10,000 x g for 1 minute. DNA samples were stored at -80°C.

3.1.5.7 Primer design for ChIP qPCR

Primers were designed using primer express software to amplify the region around the VDRE. 3 sets of primers were designed. Primer sequences can be seen in table 3.6.

Table 3.6 – Primers to amplify genomic DNA containing the VDRE for ChIP experiments.

Primer name	**Sequence**	**Amplicon length**	**Description**
VDRE primer 1 fwd	5' CCCAATGGAGGAGCTAGAGAAA 3'	138bp	Starts on last 2 bp of VDRE
VDRE primer 2 rvr	5' CGACTAGGCCATCTTCTGCTACA 3'		Starts +113bp from end of VDRE
VDRE primer 2 fwd	5' GAACACAGGGCCCCCAAT 3'	62bp	Starts on 2nd bp of VDRE
VDRE primer 2 rvr	5' TGCAGACCCCTTCAGTTCCTT 3'		Starts +25 bp from end of VDRE
VDRE primer 3 fwd	5' CACAGAAGAGATGATTAAGAG CATCAG 3'	82bp	Starts -179 from start of VDRE
VDRE primer 3 rvr	5' GCTGGCATATGTAATAGTGTC TGTGTT 3'		Starts -124 from start of VDRE

3.1.5.8 qPCR

A LightCycler® 480 (Roche, Burgess Hill, UK) was used to carry out quantitative real time PCR. Each well on a 384 well PCR plate contained the following: 5µl genomic DNA (isolated from section 2.5.6), 7.5µl SYBR Green I Master mix (Roche, Burgess Hill, UK), 0.45µl forward primer (10µM), 0.45µl reverse primer (10µM), 1.6µl RNase free H_2O (total volume = 15µl). The amplification protocol was as follows: pre-

incubation at 95°C for 5 min followed by 45 amplification cycles (denaturation of strands at 95°C for 10 seconds, annealing at 60°C for 15 seconds, elongation at 72°C for 15 seconds). All reactions were carried out in triplicate.

Positive control primers (GAPDH) were provided within the ChIP kit and were used in wells containing the positive control samples. VDRE primers 1, 2 and 3 were used on the negative control and VDR samples (table 3.6).

3.1.5.9 Checking PCR product size

To check that the amplicons were of the correct length, PCR products were run on a 2.5% (w/v) Metaphor Agarose gel. 7.5g of Metaphor Agarose (Lonza, Rockland, ME, USA) was sprinkled into 300ml of cold 1 x TAE buffer whilst being stirred using a magnetic stirrer until dissolved. The solution was then left to stand for 15 minutes at room temperature to prevent foaming. Next, the solution was heated in a microwave on medium power (600W) for 2 minutes, then gently swirled to incorporate any agarose on the side of the beaker. Then, the solution was heated on high power (900W) until boiling and then held at boiling for 1 minute, or until all of the agarose was completely dissolved. The solution was cooled to between 50 - 60°C before pouring into the gel cast and leaving at room temperature for 30 minutes to set. Once set, 1 x TAE buffer was poured over the gel and the gel was stored overnight in the fridge.

The following morning, the cold gel was added to an electrophoresis chamber and 1 X TAE buffer was added to the fill line. To each well, a mixture of 5µl of sample, 1µl of 6 x loading dye and 1µl SYBR green was added. A 25bp and a 50bp ladder (Sigma-Aldrich Saint Louis, USA) were also run alongside the samples. The gel was run at 100V for 3 hours using a BioRad Power Pac 200. Finally, the gel was imaged using an Azure Biosystems c200 imager.

3.1.5.10 Statistical Analysis

Four biological replicates (i.e. wells) were used throughout all assays, PCR, and transfection experiments. Assays and PCR experiments were carried out in triplicate for each biological replicate, then an average of each triplicate was taken to give a mean value for each biological replicate (n = 4). Statistical analyses were carried out using GenStat 22nd edition. Assays, PCR experiments and some transfection experiments were analysed by 2-way ANOVA where a time x treatment interaction was tested for. For the second transfection experiment, where two different vectors were used (MyoG-VDRE-GFP and MyoG-mutVDRE-GFP), a 3-way ANOVA was used to measure the time x treatment x vector interaction. Significance was deemed at $p < 0.05$. *Post hoc* Bonferroni tests were carried out where appropriate. All data were tested for normality using a Shapiro Wilk test and a QQ scatterplot. All data were normally distributed ($p < 0.05$). A QQ scatterplot can be seen in figure 3.10 for PCR data.

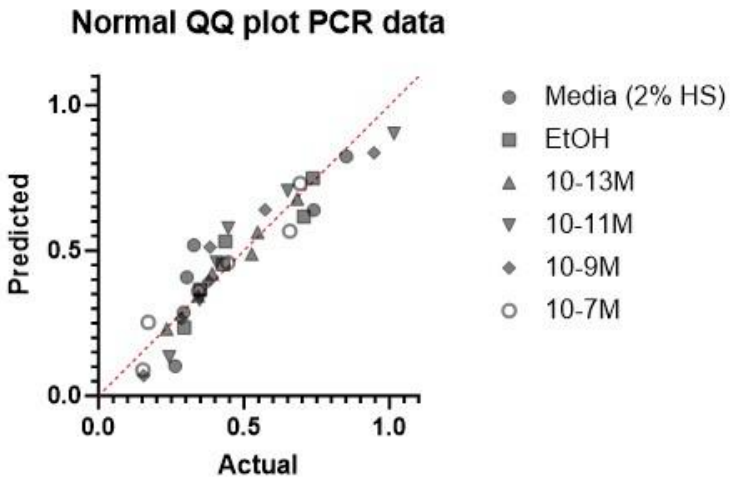

Figure 3.10. Example of QQ scatterplot for PCR data to test for normality of the dataset.

Animal Experiments

All experiments were carried out under the UK Animals (Scientific Procedures) Act 1986, under the project licence PP6562343. All animal procedures were approved by the University of Nottingham Local Ethical Review Committee. The trial was carried out at the Bio Support Unit (BSU), Queens Medical Centre, Nottingham.

3.1.6 Housing, diet and care

3-week-old virgin female C57BL/6 mice were purchased from Charles River, around 14-16g bodyweight, and were housed in same sex pairs until mating. Female mice were purchased in 3 batches, around 3 weeks apart, to allow for staggering of mating and offspring testing. 12-week-old male C57BL/6 mice were also purchased from Charles River for breeding, these were singularly housed until mating. All mice were housed in ventilated animal research cages under incandescent lighting and the lighting was on a 12-hour light cycle (on 7am to 7pm, off 7pm to 7am). Temperature was maintained at 21 ± 1°C. Female mice were randomly allocated to either the control (n = 20) or vitamin D deficient (VDD, n = 22) diet group. Both diets were purchased from Inotiv (TD.89123 and TD.89124, Leicester, UK) with the control diet containing 2.2 IU/g of vitamin D and the VDD diet containing 0 IU/g vitamin D. All male mice were fed the control diet. Diet stocks were stored at 4°C. Female mice were placed on their respective diet for 8 weeks prior to mating, throughout pregnancy and during lactation. All mice had *ad libitum* access to food and water (with the exception of the fasting period before insulin tolerance testing where food was removed) and were weighed twice weekly (Monday and Friday), with food topped up to 250g every week (Friday). Body weight and food intake was recorded for analysis every week (Friday).

3.1.7 Breeding

After the female mice had been on their respective diets for 8 weeks, and were around 12 weeks old, the mating process began. For mating, one male and one female were housed together for up to 2 weeks. During this time, the male was switched onto the female's respective diet. Mating was confirmed by the presence of a mucus plug and the male was removed from the cage at this point.

3.1.8 Pregnancy, Lactation and Weaning

During pregnancy, female mice were singularly housed. After birth, dams and their litters were left alone for 6 days to avoid maternal rejection and unnecessary stress on the litter/dam. From day 7 to day 21, litters were weighed as a whole, and litter weight was divided by number of pups, so that weighing could be carried out quickly to reduce stress. Maternal food intake was not measured during this time (food was topped up to fill the hopper prior to birth to ensure that it would last throughout this period), again to reduce the stress on the dam/litter. In instances where the litter was not gaining weight as expected, or if the dam was losing weight, the feed was made into 'mash' (crushed food to make a powder, mixed with water to make a paste) and left in the bottom of the cage. Litters were weaned at 21 days old and pups were put onto the same diet as their mother until the end of the study. Offspring mice were housed in same sex pairs, ideally with a pup from a different litter, and were matched as closely as possible for body weight. If the total number of pups due to be weaned within a treatment group was odd, then one cage would be made where 3 mice were housed together. Pups remained in these pairs/groups from weaning until 12 weeks old.

3.1.9 Insulin Tolerance Testing

Insulin tolerance testing (ITT) was carried out on offspring mice at 6 and 12 weeks of age. ITT was not possible at 3 weeks, as originally planned, because many of the mice had a body weight of less than 10g. In line with NC3R guidelines, mice weighing less than 10g were not suitable for ITT due to the fasting period required prior to ITT, the volume of blood taken during the test and the stress placed on the mice. Mice were fasted for 6 hours (8am – 2pm) prior to ITT by removing the food hopper and any free food from the cage. A 6-hour fast was deemed appropriate with regard to animal welfare because it applied the shortest possible fasting period in order to provoke a catabolic state. Overnight fasting has previously been shown to lead to a reduction in body mass of up to 15% (Andrikopoulos et al., 2008) which can have a negative impact on animal welfare and increase risk of hypoglycaemia, hence a 6-hour fasting period was used.

For best practice, ITT was carried out based on the method outlined in Benedé-Ubieto et al, (2020). Mice were weighed at the start of the fasting period, at 8am, and again at 2pm prior to ITT, and weight was rounded to the nearest 0.5g. 30 minutes prior to ITT, Emla cream (5% Lidocaine), was applied to the tail to minimise discomfort. An initial blood glucose measurement was taken from the tail vein, via a tail vein prick, using a 26-gauge hypodermic needle and a blood glucose monitor (Zoetis AlphaTrack). Mice were then injected with 0.75 IU/kg of insulin via intraperitoneal (IP) injection (a 1:400 insulin stock was made prior to each ITT by adding 25µl of 100 IU/ml insulin (ActRapid®, Novo Nordisk A/S) to 9975µl saline and stored at 4°C. Insulin was warmed to room temperature before injecting). Bodyweight (g) at 2pm x 3 was used to calculate the volume (µl) for insulin injection. Blood glucose measurements were then taken at 15-, 45- and 90-minutes post IP injection by warming the tail and massaging the prick site to encourage blood flow. If

blood glucose measurements dropped below 1mmol/L or mice showed signs of hypoglycaemia (e.g. tremor, hunched posture, starry coat, severe lethargy), 200µl of 10% glucose in saline (w/v) solution was injected via IP injection and data from the mouse was excluded, in line with the method stated in Benedé-Ubieto et al (2020). After the 90-minute measurement, food was returned to the cage along with mash (powdered food pellet mixed with water). Mice were monitored hourly for the first 6 hours following insulin injection and then again 24 hours later to assess food intake, bodyweight, and adverse behaviours (lack of grooming, lack of fresh faeces, lack of drinking, hunched posture, starry coat). Week 12 mice were culled following the 90-minute time point to collect tissues (fat, muscle, liver) which could be affected by insulin and/or glucose levels.

3.1.10 Muscle function (Grip Strength)

Grip strength was measured using a Bioseb BIO-GS3 grip strength monitor (BIOSEB, France) set to measure force in Newtons. The bar attachment was added to the monitor which measures forelimb grip strength. Each mouse was removed from its cage by gripping the base of the tail between the thumb and forefinger and the mouse was suspended above the bar to encourage it to reach down and grab hold of the bar. Once holding the bar, the mouse was gently pulled away in a horizontal position at an even speed for up to 5 seconds, until the mouse let go. Peak force (i.e. the maximum force exerted by the mouse) was measured and recorded. Each mouse performed 6 tests (2 acclimation tests followed by 4 tests, with an average taken of the last 4 tests) with a 60 second interval between each test where the mouse was returned to its cage. The same person (KA) carried out every grip strength test across the study for consistency and to try to reduce variation from the handler. At each time point, grip strength mice were culled immediately after

testing, for tissue collection, to reduce any stress caused to the animals from tail pulling.

3.1.11 Mazes

The same sub-set of mice were used for both mazes at 3, 6 and 12 weeks to reduce the total number of animals required. The maze tests utilised within the study are widely used within the university and have been refined to give optimum results. The maze tests were always carried out on different days (plus maze on a Monday and Y maze on a Friday) so that mice had sufficient time to recover between tests. Maze tests were carried out between 9am-12pm, during the light phase of the light/dark cycle, and mice were not handled before the test to reduce stress. For both mazes, one mouse was tested at a time.

3.1.11.1 Elevated Plus Maze

The main purpose of the elevated plus maze (EPM) was to assess innate anxiety-like behaviour. However, total distance moved was also recorded in order to analyse physical activity as an indicator of muscle function. The EPM consisted of two opposing closed arms, which were enclosed with high walls, and two opposing open arms with no walls. All arms were L36 x W6cm and extended from a central platform which was 6 x 6cm and the maze was elevated 89cm above the floor, Figure 3.11. Soft mats were placed below the open arms to prevent injury if a mouse fell from the maze. Prior to testing, mice were acclimatised to the room for a minimum of 30 minutes. Each mouse was placed individually into the central platform, facing the same closed arm each time, and was allowed to explore the maze freely for 5 minutes. A camera suspended above the maze recorded the activity for each mouse and activity analysis was carried out using a computerised tracking system (Ethovision 9, Noldus IT, The Netherlands). Total distance moved, as well as time

spent in each section of the maze was recorded. After 5 minutes, the mouse was returned to its cage and the maze was cleaned with 70% ethanol followed by distilled water before the next mouse was tested.

Figure 3.11. Illustration of the elevated plus maze taken from https://stoeltingco.com/Neuroscience/Elevated-Plus-Maze~9853

3.1.11.2 Y maze

The main purpose of the Y maze was to assess spontaneous alternation behaviour / memory, however total distance measured was again recorded to analyse physical activity as an indication of muscle function. The Y maze was constructed of clear, colourless Plexiglas with 3 identical arms (L40 x W15cm with walls 30cm in height) which were all at equal 120° angles from each other to form an equilateral triangular central area, Figure 3.12. Prior to testing, mice were acclimatised to the room for a minimum of 30 minutes. Mice were placed into the central area, always facing the same arm, and were allowed to explore the maze freely for 5 minutes. A camera suspended above the maze recorded the activity for each mouse and activity analysis was carried out using a computerised tracking system (Ethovision 9, Noldus IT, The Netherlands). Total distance moved, time spent in each arm and alternation

were recorded. After 5 minutes, the mouse was returned to its cage and the maze was cleaned with 70% ethanol followed by distilled water before the next mouse was tested.

Figure 3.12. Illustration of the Y maze taken from https://stoeltingco.com/Neuroscience/Y-Maze~9850

3.1.12 Culling and tissue collection

Mice were culled under terminal anaesthesia by placing them into an induction chamber and exposing them to 5% (v/v) isoflurane gas. Once under anaesthesia, mice were transferred to a mask which supplied the same gas. Cardiac puncture was carried out to collect approximately 0.5ml of blood in heparin lithium microtubes (Sarstedt, Germany) followed by cervical dislocation whilst still under anaesthesia. Blood samples were centrifuged at 18,000 x g for 10 minutes to separate the plasma. Following this, plasma was collected using a pipette and snap frozen on dry ice. Tissues were collected at room temperature, weighed and immediately transferred to dry ice to snap freeze. Full dissection for each mouse took no longer than 10 minutes. Tissues collected were brain, liver, both kidneys, uterus (mothers only),

muscle (gastrocnemius and soleus from both back legs), interscapular brown adipose tissue (BAT), abdominal white adipose tissue (AB WAT) and interscapular white adipose tissue (IS WAT). Half of the brains were snap frozen on dry ice (as above) whilst half were placed into 4% (v/v) paraformaldehyde and were left to fix for 24 hours before being transferred to a 30% sucrose (w/v) solution for a further 24 hours. After this, brains were placed in PBS and stored at 4°C. All other tissues and plasma samples were transferred from dry ice to -80°C following each cull for subsequent analysis.

3.1.13 Enzyme-linked immunosorbent assay (ELISA)

A 25-Hydroxy vitamin D ELISA kit (Immunodiagnostic Systems Ltd, Boldon, UK) was used to measure total vitamin D (25(OH)D and other hydroxylated VD metabolites) in the serum samples from all mice (mothers and offspring). 25µl of each sample, control (control sample provided in kit with known concentration of 25(OH)D_3) or calibrator (CAL 0 = 0 nmol/l, CAL 1 = 18.1 nmol/l, CAL 2 = 30.5 nmol/l, CAL 3 = 51.1 nmol/l, CAL 4 = 85.1 nmol/l, CAL 5 = 132 nmol/l, CAL 6 = 252 nmol/l 25(OH)D_3 also provided in kit) was added to 1ml of 50x 25-D biotin solution and vortexed for 10 seconds (the 50x biotin solution contained a reagent for dissociating 25(OH)D from any binding proteins). 200µl of each sample/control/calibrator was then pipetted into a 96-well antibody coated plate in duplicate. The plate was covered with an adhesive plate sealer and incubated at room temperature for 2 hours to allow the antibodies within the wells of the plate to bind to the antigen (i.e. 25(OH)D). The contents of the plate were then removed by inverting the plate firmly. All wells were then washed with 250µl of wash buffer three times. After the final wash, the plate was tapped firmly onto absorbent tissue to remove any residual wash buffer. Following this, 200µl of enzyme conjugate (PBS containing avidin linked to

horseradish peroxidase as well as protein and enzyme stabilisers) was added to each well, the plate was sealed using an adhesive plate sealer and then incubated for 30 minutes at room temperature. The enzyme conjugate was then removed, and the plate washed three times as above. 200µl of TMB substrate (aqueous tetramethylbenzidine and hydrogen peroxide) was then added to each well and the plate was covered and incubated for 30 minutes at room temperature. Finally, 100µl of stop solution (0.5M HCl) was added to each well. Absorbance at 450nm was read for each well immediately after the last step using a plate reader (Fluostar Optima, BMG Labtech).

3.1.14 Statistics

Power calculations and statistical analysis was carried out using GenStat 22nd edition. Power calculations were based on standard deviations obtained from previous pilot data (unpublished) with probability of a type I error (a) set at 0.05 and a type II error (p) set at a maximum of 0.20 to give a power of > 0.80. Based on previous pilot data, 8 mice/sex were needed for both behavioural tests and for insulin tolerance testing whilst 6 mice/sex were needed for tissue collection to measure gene expression. A total of 136 offspring were required (34 males and 34 females for both control and VDD). Based on the assumption that 2 male and 2 female offspring would be obtained from each litter, this gave a total of 34 mice required for mating (17 per treatment group). However, due to natural variation in number of offspring, chance of unsuccessful pregnancies and chance of postnatal offspring loss, 42 female mice were utilised within the study for mating (20 control and 22 VDD).

Data was analysed by 1-, 2- or 3-way ANOVA (age*treatment*sex) with a *Post* hoc Bonferroni test where appropriate (i.e. if an effect or interaction was significant). Significance was deemed at $p<0.05$. All data were tested for normality using a

Shapiro Wilk test and a QQ scatterplot. All data were normally distributed ($p < 0.05$).

An example of a QQ scatterplot for offspring bodyweight can be seen in figure 3.13.

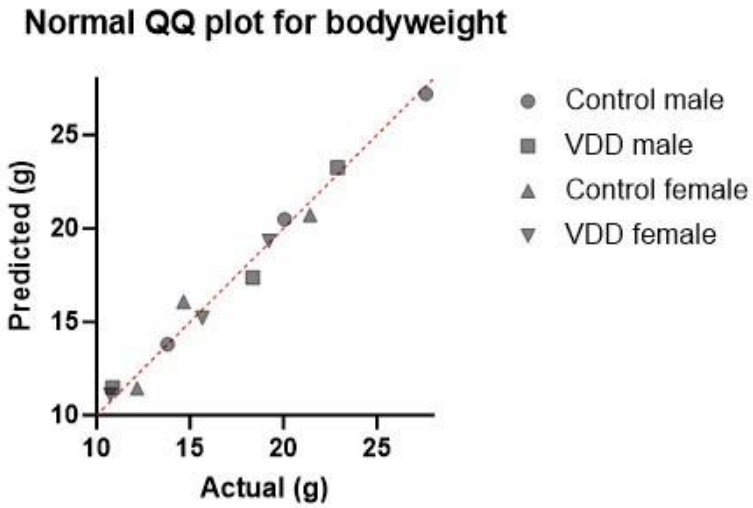

Figure 3.10. Example of QQ scatterplot for offspring bodyweight data to test for normality of the dataset.

Chapter 4 Manuscript 2

Alliband, K. H., Parr, T., Jethwa, P. H. & Brameld, J. M. 2023. Active vitamin D increases myogenic differentiation in C2C12 cells via a vitamin D response element on the myogenin promoter. *Front Physiol,* 14, 1322677.

4.1 Abstract

Skeletal muscle development during embryogenesis depends on proliferation of myoblasts followed by differentiation into myotubes/multinucleated myofibers. Vitamin D (VD) has been shown to affect these processes, but there is conflicting evidence within the current literature on the exact nature of these effects due to a lack of time course data. With 20-40% of pregnant women worldwide being VD deficient, it is crucial that a clearer understanding of the impact of VD on myogenesis is gained.

A detailed 8-day differentiation time course was used where C2C12 cells were differentiated in control differentiation media (2% horse serum) or with different concentrations of active VD, $1,25(OH)_2D_3$ (10^{-13}M, 10^{-11}M, 10^{-9}M or 10^{-7}M), and measurements were taken at 6 time points. DNA, creatine kinase and protein assays were carried out as well as quantitative PCR to determine expression of Myf5, MyoD, myogenin, MHC I, MHC neonatal, MHC embryonic, MHC IIa, MHC IIx and MHC IIb mRNAs. Transfections were carried out using one vector containing the myogenin promoter and another containing the same promoter with a 3-base mutation within a putative vitamin D response element (VDRE) to determine effects of $1,25(OH)_2D_3$ on myogenin transcription. Finally, a ChIP assay was performed to determine whether the VD receptor (VDR) binds to the putative VDRE.

$1,25(OH)_2D_3$ caused an inhibition of proliferation and an increase in differentiation in C2C12 cells. Myogenin, MHC I, MHC neonatal, MHC embryonic, MHC IIa, MHC IIx

and MHC IIb mRNA expression were all increased by 1,25(OH)$_2$D$_3$, but MyoD was not. Myotube size was also increased by VD. When the putative VDRE on the myogenin promoter was mutated, the increase in expression by VD was lost. ChIP analysis revealed that the VDR does bind to the putative VDRE on the myogenin promoter.

Active VD directly increases myogenin transcription via a functional VDRE on the myogenin promoter, resulting in increased myogenic differentiation, increased expression of both the early and late MHC isoforms, and also increased myotube size. These results highlight the importance of VD status during pregnancy for normal myogenesis to occur, but further *in vivo* work is needed.

4.2 Introduction

It is well documented that vitamin D (VD) deficiency, defined in the UK by serum levels of inactive VD (25(OH)D$_3$) falling below 25 nmol/L (Griffin et al., 2021), is associated with muscle weakness, and that this can be reversible with dietary supplementation (Braga et al., 2017), indicating a link between serum 25(OH)D$_3$ status and muscle function. There is also evidence of vitamin D receptor (VDR) expression in human muscle cells, as well as effects of VD treatment on muscle cells in culture, suggesting that the effects on muscle may be direct and mediated through the VDR (Olsson et al., 2016). Furthermore, VDR knockout mice have smaller muscle fibers than wild type mice at 3 weeks of age, which becomes even more prominent by 8 weeks (Endo et al., 2003). Collectively, these findings suggest that VD and the VDR play an important role in the regulation of skeletal muscle function and development.

The biologically active form of VD, 1,25(OH)$_2$D$_3$ (also called calcitriol), has the highest affinity for the VDR with a k_D of 0.1nM (Carlberg, 2019). The VDR belongs to the zinc

finger class of transcription factors (Molnár, 2014), which can be activated by low (nanomolar) concentrations of $1,25(OH)_2D_3$, and interacts in a sequence specific manner with genomic DNA (Carlberg and Campbell, 2013). Upon binding of $1,25(OH)_2D_3$ to the VDR, a conformational change occurs allowing the VDR to bind to other proteins to form a dimer (Thompson et al., 1998). Hence, the VDR either forms a homodimer with another VDR, or a heterodimer by binding with a retinoic acid receptor (RAR) or a retinoid X receptor (RXR), with the latter being the predominant heterodimer formed (Carlberg and Molnár, 2015). Once formed, the homo- or heterodimer binds to a VD response element (VDRE) on a target gene, which are typically comprised of two conserved hexameric repeats separated by a 3-nucleotide spacer (Tsoumpra et al., 2020). In order for successful activation of transcription, the activated VDR needs to cause efficient dissociation of co-repressors as well as efficient association of co-activators (Carlberg and Molnár, 2015). Primarily, p160 coactivators and steroid receptor activators 1, 2 and 3 are recruited, which have histone acetylase activity resulting in the unwinding of DNA from the histones and allowing access for the transcriptional machinery (Christakos et al., 2016). In total, over 900 genes have been found to be regulated by VD (Wang et al., 2005).

Within the genes that have been shown to be regulated by VD, some genes relating to myogenesis have been identified (Girgis et al., 2014). Our recent systematic review (Alliband et al., 2021), evaluating the effects of active VD on muscle differentiation and expression of myogenic genes, showed a consistent anti-proliferative effect of active VD on muscle cells in culture, as well as an increase in myotube size. However, the effects on differentiation and the mRNA expression of myogenic regulatory factors (MRFs) and myosin heavy chain (MHC) isoforms were inconsistent. The main issue across the studies appeared to be a lack of time course data, with most only measuring gene expression at one or two time points, with

large inconsistencies as to which day of differentiation this was done on. There was also variability in the concentrations of active VD used.

Hence, the aim of this study was to determine the effects of $1,25(OH)_2D_3$ on muscle cell proliferation and differentiation, using the C2C12 muscle cell line, with the effects on differentiation being via a detailed time course for mRNA expression of the MRFs and MHC isoforms (Figure 4.1). This builds on our previous work which mapped the time course of mRNA expression of these genes during normal C2C12 differentiation (Brown et al., 2012). We used four different concentrations of $1,25(OH)_2D_3$ which ranged from below physiological to above physiological concentrations (physiological range for $1,25(OH)_2D_3$ = 4.3 $x10^{-11}$M to 1.9 $x10^{-10}$M) (Pagana et al., 2019) to determine whether any observed effects were dose dependent, and further investigated the mechanism for the observed effects of $1,25(OH)_2D_3$ on myogenic differentiation. We hypothesise that VD will stimulate expression of one or more of the myogenic regulatory factors and that this could be direct stimulation via a VD response element on the stimulated MRFs. We also hypothesis that VD will increase myotube size.

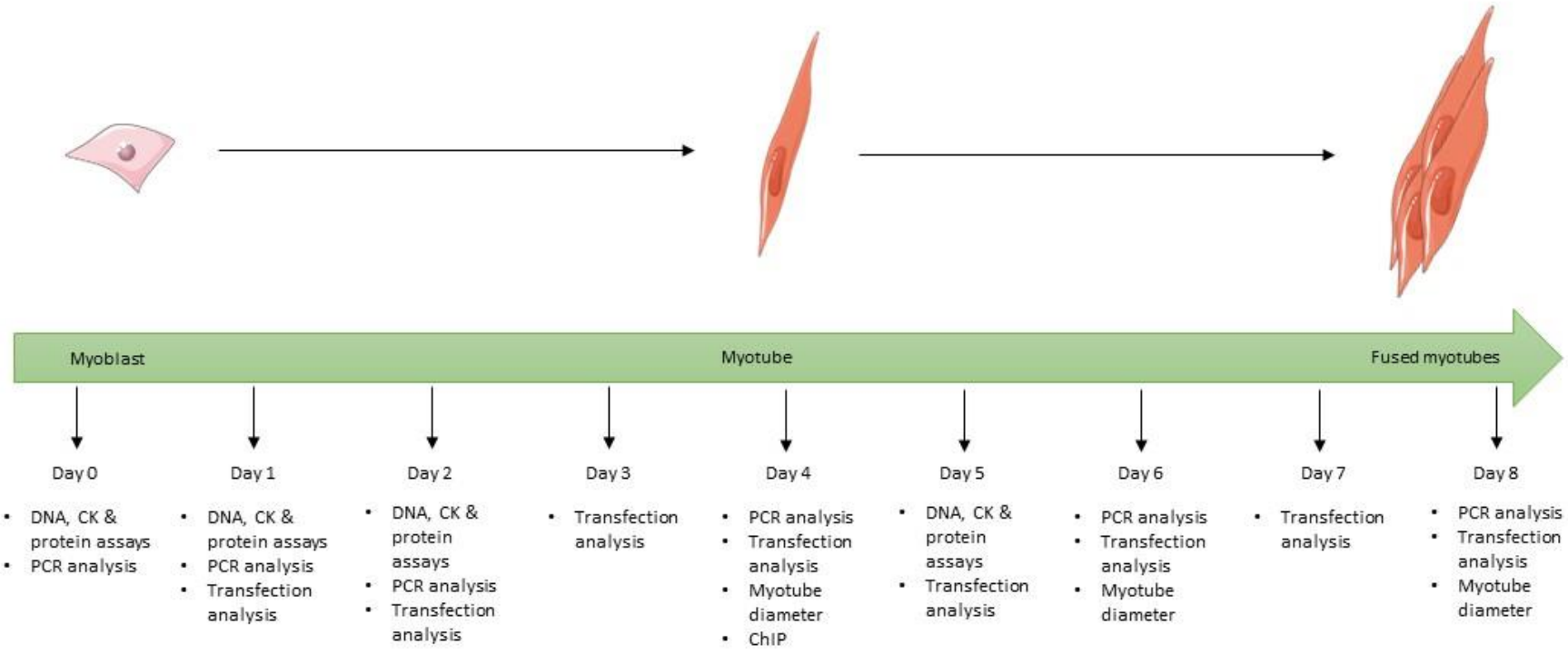

Figure 4.1. Schematic showing the full 8-day time course used for the differentiation of C2C12 cells during the study and at which time point each experiment was undertaken for all cells grown in differentiation media. Images used within this figure were obtained from smart servier medical art and can be found at https://smart.servier.com

4.3 Materials and Methods

4.3.1 Cell Culture

The C2C12 mouse myoblast cell line was obtained from the European Collection of Authenticated Cell Cultures (ECACC). C2C12 myoblasts were grown in growth medium consisting of Dulbecco's modified eagle's medium (DMEM; Sigma Aldrich, Poole, UK) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen, Paisley, UK) and 1% (v/v) 100 X penicillin streptomycin (P/S; Invitrogen, Paisley, UK) and incubated at 37°C and 5% CO_2. Cells were treated with different concentrations of $1,25(OH)_2D_3$ (either 10^{-13}M, 10^{-11}M, 10^{-9}M or 10^{-7}M in 0.1% ethanol (v/v)). Two controls were used, one treated with 0.1% (v/v) ethanol and the second with normal differentiation media to determine whether the vehicle was causing any of the effects.

For the proliferation experiment, cells were plated onto 96-well plates (at 5,000 cells/well) prior to $1,25(OH)_2D_3$ treatments being added 24 hours later. Plates were stopped at t=0 (24 hours after plating, before addition of treatments) and at 24 hours and 48 hours post $1,25(OH)_2D_3$ treatment by removing media and washing with phosphate buffered saline (PBS). 100µl of dH_2O was then added to each well and plates were frozen at -20°C prior to freeze-thawing and DNA assays.

For differentiation studies, cells were plated onto 6-well plates ($1.5x10^5$ cells/well). On day 0 (approximately 70% confluence) cells underwent serum starvation to encourage differentiation by switching to differentiation media consisting of DMEM supplemented with 2% (v/v) horse serum (HS; Invitrogen, Paisley, UK) and 1% (v/v) 100 X P/S. VD treatments were also added at this point. Cells were cultured for up to 8 days and media was replaced every 48 hours. Cells for protein and creatine kinase assays were harvested on days 0, 1, 2 and 5 (Figure 4.1) by scraping into 500µl of tri-

sodium citrate (0.05M, pH 6.8, 4°C) and placed on ice, then stored at -20°C. Cells for gene expression analysis were harvested on days 0, 1, 2, 4, 6 and 8 (Figure 4.1) by scraping into 200µl of ice cold RNase free PBS and stored at -80°C prior to RNA extraction (see below).

4.3.2 DNA, Protein and Creatine Kinase Assays

To lyse the cells prior to DNA assays, plates underwent two freeze/thaw cycles. DNA contents were determined using the Hoechst fluorescent dye method as previously described (Hurley et al., 2006, Rago et al., 1990). In brief, 100µl of working dye solution was added to 100µl cell lysates on the 96-well plates. A standard curve of known concentrations of calf thymus DNA was run alongside the samples. Fluorescence was measured using a fluorescent plate reader (Fluostar Optima, BMG Labtech) at excitation of 350 nm and emission 460nm.

For protein and creatine kinase assays, cells in tri-sodium citrate buffer were thawed on ice and then sonicated (Soniprep150, MSE, UK) using 10µm amplitude for roughly 30 seconds, or until the sample was clear. Cell lysates were used immediately after sonication for the creatine kinase assay and were stored at -20°C again prior to protein and DNA assays.

Creatine kinase activity (U/l) was determined as previously described (Brown et al., 2012, Hurley et al., 2006) using a CK kinetic assay kit (Thermo Electron Corporation, Fisher Scientific, Loughborough, UK). Samples were run on a 96-well plate and absorbance at 340nm was read every 30 seconds for a total of 5 minutes at 37°C to determine enzyme activity (BioRad 680XR Microplate reader).

Protein contents were determined using the Lowry method as previously described (Brown et al., 2012, Hurley et al., 2006). Samples were run on a 96-well plate

alongside a standard curve of known concentrations of bovine serum albumin and absorbance was measured at 655nm (BioRad 680XR Microplate reader).

A DNA assay was also carried out (as above) on these samples to normalise protein and CK activity against.

4.3.3 Gene Expression Analysis via Quantitative RT-qPCR

An RNA isolation kit (ROCHE high pure isolation kit) was used according to the manufacturer's protocol to extract total RNA from cells previously scraped into 200µl RNase free PBS, after thawing on ice. Total RNA concentrations were determined using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, USA). All samples were diluted to 50ng/µl and stored at -80°C. First strand cDNA synthesis was carried out using a Thermo Scientific RevertAid RT kit (Thermo Scientific, Vilnius, Lithuania) according to manufacturer's protocol using 500ng total RNA per sample. First strand cDNA samples were then diluted 5-fold using RNase free water and stored at -20°C.

A LightCycler® 480 (Roche, Burgess Hill, UK) was used to carry out RT-qPCR analysis. Each well on a 384 well PCR plate contained the following: 5µl first strand cDNA, 7.5µl SYBR Green I Master mix (Roche, Burgess Hill, UK), 0.45µl forward primer (10µM), 0.45µl reverse primer (10µM), 1.6µl RNase free H_2O. The PCR amplification protocol was as follows: pre-incubation at 95°C for 5 min followed by 45 amplification cycles (denaturation of strands at 95°C for 10 seconds, annealing at 60°C for 15 seconds, elongation at 72°C for 15 seconds) (Brown et al., 2012). All reactions were carried out in triplicate.

Initially, qPCR analysis was run on every cDNA sample using primers for a potential reference gene (TATA binding protein) to check that the crossing point (CP) for each sample was within 2 standard deviations of the mean of all samples. Once this was

confirmed, a cDNA pool was made by combining 10µl aliquots from every sample. This pool was then used in serial dilutions to create a standard curve to determine the PCR efficiencies for all primer pairs/genes and subsequently the transcript concentrations of the samples. Melt curve analysis was used to ensure only one peak was present (indicating a single amplicon product) for all primers in all samples.

Supplementary data 4.1 includes a list of genes measured and their corresponding forward and reverse primers. All PCR primers were designed to cross exon-exon boundaries and working primers were diluted from stock primers.

4.3.4 Oligreen Quantification of Total cDNA

As in our previous study (Brown et al., 2012), we were unable to find a reference gene that did not significantly change across the time course studied (data not shown). Therefore, an Oligreen ssDNA Assay kit (Invitrogen, Eugene, Oregon) was used to quantify cDNA content for each sample on a 384 well PCR plate according to manufacturer's protocol. Fluorescence was quantified using the LightCycler® 480 (Roche, Burgess Hill, UK) with increasing temperature at a continuous ramp rate of 0.11°C/s until it reached 95°C. Fluorescence measurements at 80°C were plotted against a cDNA standard curve (as described above) to determine relative cDNA quantity in each sample and this was subsequently used for normalisation of all genes.

4.3.5 Generation of Myogenin Promoter Plasmids

The mouse myogenin promoter sequence was obtained from the NCBI database (accession M95800). The typical VDRE sequence obtained from (Tsoumpra et al., 2020) was aligned to the myogenin promoter sequence using Clustal Omega, which identified a potential VDRE (12/15 bases, 80% match) on the myogenin promoter at -

1260 to -1246 from the transcription start site (TSS).

A vector containing the mouse myogenin promoter sequence, including the potential VDRE, linked to a Turbo-green fluorescent protein (GFP) reporter (MyoG-VDRE-GFP) was purchased from VectorBuilder. Another vector with a mutated VDRE (MyoG-mutVDRE-GFP) was also purchased (VectorBuilder) which was identical in sequence apart from 3 bases in positions 2, 3 and 4 of the VDRE sequence (-1261 to -1264) which were changed from GAA to AGG. Full vector components and sequences are shown in Supplementary Data 4.2. Plasmids were prepared using a Promega PureYield Plasmid Miniprep kit (Madison, Wisconsin, USA) according to manufacturer's protocol. Plasmid concentrations were measured using a nanodrop® ND-1000 (Thermo Scientific, Wilmington, USA) and were stored at -20°C.

4.3.6 Myogenin Promoter Transfection Studies

Transfection of C2C12 cells with the different vectors was carried out at 60-70% confluency on day -1 (i.e. 24 hours before switching to differentiation media). C2C12 cells were cultured in 24-well plates, at 30,000 cells/well, and were transfected 24 hours after plating (whilst in the log phase of growth) using Lipofectamine 2000 (Invitrogen, California, USA) according to manufacturer's protocol. Each well contained 500ng vector (400ng MyoG-GFP vector plus 100ng pDsRed-Express-NI vector with a CMV promoter followed by red fluorescence protein (RFP) (Brown et al., 2014). This was to check for transfection efficiency after 24 hours due to myogenin not being expressed until mid-stage differentiation and so the MyoG-GFP cells did not start to appear green until at least day 2. 2.5µl lipofectamine in Opti-MEM medium (Gibco, Paisley, UK) was used per well. 30 minutes prior to cell imaging, media was replaced with Fluorobrite DMEM (Fisher Scientific, Loughborough, UK) with 2 drops per ml of NucBlue Hoechst nuclear stain (Fisher

Scientific, Loughborough, UK) and incubated at 37°C, 5% CO_2. Cell images were taken on a fluorescent microscope (Evos FL) and fluorescence was measured every 24 hours over 8 days with a fluorescent plate reader (Fluostar Optima, BMG Labtech) using the matrix scan setting. This is where 30 X 30 measurements are taken over a 1.5cm^2 area for each well, and the average for each well calculated. GFP was measured at an excitation of 485nm and emission of 520nm and NucBlue was measured at an excitation of 355nm and emission of 460nm. All GFP data was normalised to NucBlue to account for any differences in cell number.

Images of GFP transfected myotubes were analysed for myotube diameter using Image J as described previously (Brown et al., 2018, Brearley et al., 2021). Briefly, four images at 10x magnification were used for each treatment at each time point and five myotubes were analysed per image, a total of 20 myotubes per treatment at each time point. Myotube diameter was measured at 25%, 50% and 75% of total myotube length to provide an average diameter per myotube. Length in number of pixels was converted to µM by measuring the length of a 400µM scale bar in pixels.

4.3.7 Chromatin Immunoprecipitation (ChIP) Assay

Cells were plated on 6 well plates at 1.5 x 10^5 cells/well and cultured in growth medium for 48 hours until 70% confluent. Media was then removed and replaced with differentiation media supplemented with either 0.1% (v/v) EtOH, 10^{-11}M or 10^{-7}M $1,25(OH)_2D_3$ for 4 days, with media changed every 48 hours. 4 days after switching to differentiation media, cells were cross linked using 1% (v/v) formaldehyde. ChIP assay was carried out using a Pierce Agarose ChIP kit (Pierce, Thermo Scientific, Wilmington, USA) according to manufacturer's protocol. After digestion, genomic DNA fragment size was checked by running on a 1% (w/v) agarose gel to ensure fragments were between 200-1000bp. Anti-vitamin D receptor

antibody (ab3508 Abcam, UK) was used to isolate genomic DNA fragments containing a VDRE. The myogenin promoter VDRE primers (Supplementary data 4.1) used for PCR were designed using Primer Express. Each PCR reaction contained the following: 5µl genomic DNA, 7.5µl SYBR Green I Master mix (Roche, Burgess Hill, UK), 0.45µl forward primer (10µM), 0.45µl reverse primer (10µM), 1.6µl RNase free H_2O and PCR conditions were the same as described above. PCR products were run on a 2.5% (w/v) Metaphor Agarose gel in 1 x TAE buffer at 100V to confirm fragment size.

4.3.8 Statistical Analysis

Four biological replicates (i.e. wells) were used throughout. Assays and PCR analyses were carried out in triplicate for each sample. Statistical analyses were carried out using GenStat 22nd edition. Most data were analysed by 2-way ANOVA (day x treatment) with the exception of the second promoter transfection experiment where two different vectors were used so a 3-way ANOVA (day x treatment x vector) was carried out. *Post hoc* Bonferroni tests were then used to identify groups that were significantly different. Results were deemed significant if $p<0.05$.

4.4 Results

4.4.1 Vitamin D decreases proliferation and increases differentiation of C2C12 cells

In order for myoblasts to undergo myogenic differentiation they must first exit from the cell cycle so that they can switch from a proliferative to a differentiative state (Kitzmann and Fernandez, 2001). DNA content of C2C12 cells was measured as a marker of cell proliferation with a decrease in DNA content indicating a decrease in cell number. A significant day x treatment interaction was observed for DNA content ($p=0.001$) for cells in proliferation media, Figure 4.2 A. The two highest

concentrations of $1,25(OH)_2D_3$ resulted in significantly lower levels of DNA at 48 hours compared to controls ($p<0.05$) but were similar to DNA levels at the time 0 point. For cells in differentiation media a significant day x treatment interaction was also observed for DNA content ($p<0.001$), Figure 4.2 B. By day 1 (24 hours post switching to differentiation media) cells treated with 10^{-7}M $1,25(OH)_2D_3$ had significantly lower DNA content than controls ($p<0.05$) indicating decreased proliferation. By day 5, both 10^{-7}M and 10^{-9}M $1,25(OH)_2D_3$ had significantly lower DNA content than day 5 controls ($p<0.05$).

Within differentiating muscle cells in culture, creatine kinase (CK) begins to be expressed after proliferating myoblasts exit from the cell cycle and begin to express differentiation specific factors (Tai et al., 2011). Therefore, CK enzyme activity was measured as a marker of mid stage differentiation (Figure 4.2 C). There was a significant day x treatment interaction ($p<0.001$) for CK activity. Activity did not increase between day 0 and 2 for all treatment groups, but CK enzyme activity for both 10^{-9}M and 10^{-7}M $1,25(OH)_2D_3$ was significantly higher than controls by day 5 ($p<0.05$).

A significant day x treatment interaction was also seen for the protein/DNA ratio in response to $1,25(OH)_2D_3$ treatment ($p<0.01$, Figure 4.2 D). Again, there were no differences until day 5, when both 10^{-7}M and 10^{-9}M $1,25(OH)_2D_3$ resulted in significantly higher protein contents than controls ($p<0.05$). The increases in both CK enzyme activity and protein contents, relative to DNA, indicate a significant increase in differentiation with both 10^{-7}M and 10^{-9}M $1,25(OH)_2D_3$ compared to the controls.

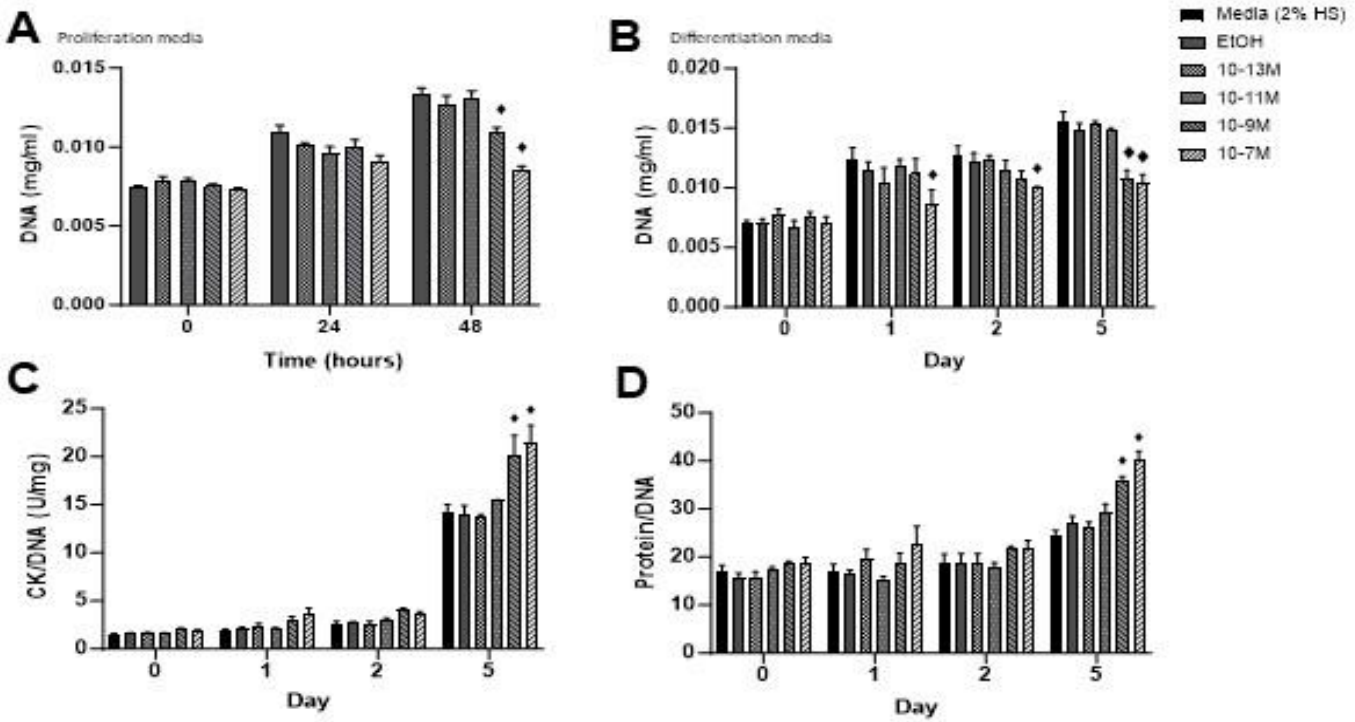

Figure 4.2. Effects of $1,25(OH)_2D_3$ treatment in C2C12 cells on DNA, creatine kinase and protein. **A.** DNA (mg/ml) of C2C12 cells grown in proliferation media for 48 hours with varying concentrations of $1,25(OH)_2D_3$ in 0.1% (v/v) ethanol. **B-D.** C2C12 cells grown in differentiation media for 5 days with varying concentrations of $1,25(OH)_2D_3$ in 0.1% (v/v) ethanol. **B.** DNA (mg/ml) **C.** Creatine Kinase activity normalised to DNA (U/L) **D.** Protein content normalised to DNA. ♦ indicates where a bar is significantly different ($p<0.05$) to both media and ethanol controls (media (2% HS) and EtOH) within the same time point. Error bars show standard deviation. 4 biological replicates (wells) were used throughout and all assays were carried out in triplicate. Statistical analysis was carried out via a 2-way ANOVA with a *post hoc* Bonferroni test where time*treatment interaction was significant ($p<0.05$).

4.4.2 Vitamin D influences mRNA expression of two myogenic regulatory factors

Myf5 and MyoD are myogenic regulatory factors (MRFs) responsible for cell commitment to the myogenic lineage, exit from the cell cycle and the initiation of terminal differentiation (Montenegro et al., 2019) and therefore are markers of early stage differentiation. For MyoD mRNA expression (Figure 4.3 A) there was no significant day x treatment interaction ($p=0.49$) and no significant effect of treatment ($p=0.12$). As expected, there was a significant effect of time ($p<0.001$) with mRNA expression decreasing from day 0 to day 1 ($p<0.05$) and from day 1 to day 2 ($p<0.05$). For Myf5 mRNA expression (Figure 4.3 B) there was a significant day x treatment interaction ($p<0.001$). As expected, Myf5 mRNA expression decreased over time for all treatment groups, but all $1,25(OH)_2D_3$ treatment groups significantly decreased on day 1 compared to day 0 ($p<0.05$) whereas controls did not decrease until day 2 ($p<0.05$).

Myogenin is an MRF which is expressed following MyoD and Myf5 expression, and is necessary for the terminal differentiation of myoblasts to myotubes (Montenegro et al., 2019), so is often used as the main marker of myogenic differentiation. There was a significant day x treatment interaction ($p<0.001$) for myogenin mRNA expression (Figure 4.3 C). There were no significant differences in expression from day 0 to day 4, but there was an increase in expression for the 3 highest doses of $1,25(OH)_2D_3$ on day 6 compared to day 4, although only 10^{-11}M and 10^{-9}M $1,25(OH)_2D_3$ were significant ($p<0.05$). Myogenin mRNA expression did not significantly increase until day 8 for controls ($p<0.05$), at which point, expression for 10^{-9}M and 10^{-7}M $1,25(OH)_2D_3$ was significantly lower than controls ($p<0.05$). Hence,

there was an earlier peak for myogenin expression with 1,25$(OH)_2D_3$ treatment indicating that the differentiation process had been induced sooner.

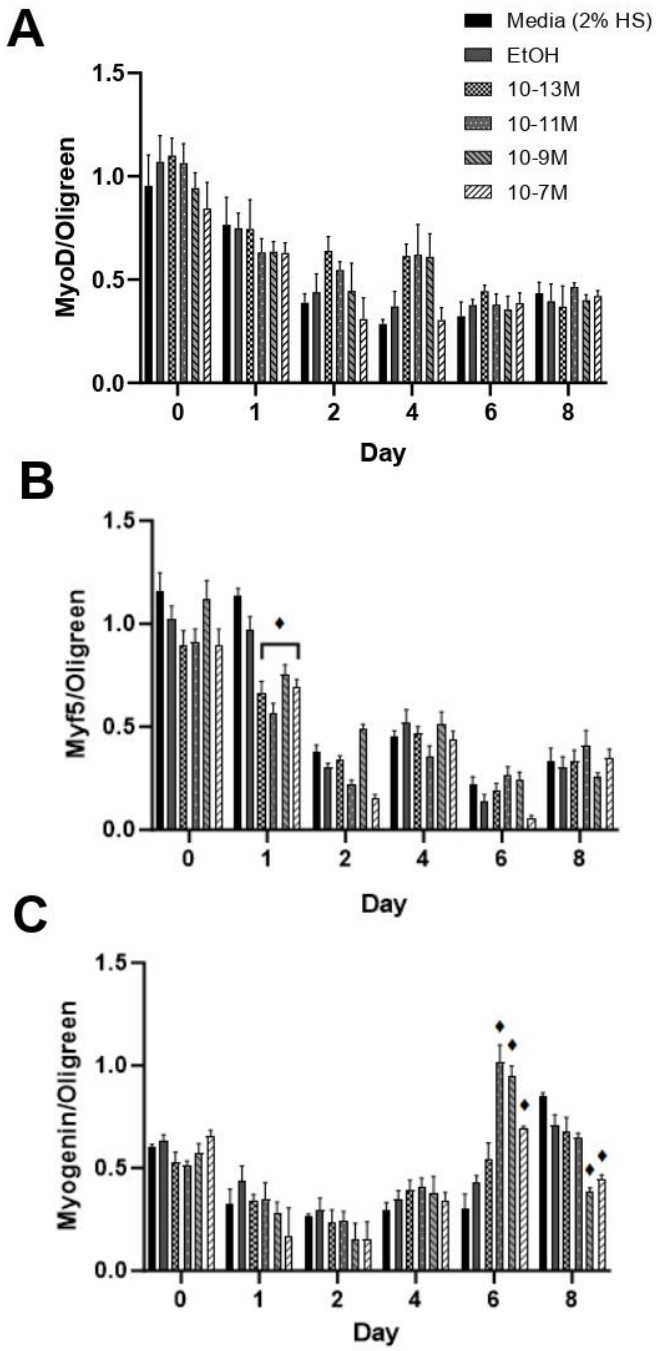

Figure 4.3. Effects of 1,25$(OH)_2D_3$ on C2C12 cells over 8 days on myogenic regulatory factor gene expression. **A.** MyoD **B.** Myf5 and **C.** Myogenin mRNA expression normalised to Oligreen during C2C12 differentiation over an 8-day time course with varying concentrations of 1,25$(OH)_2D_3$ dissolved in 0.1% (v/v) ethanol. ♦ symbol indicates where a bar is significantly different ($p<0.05$) to both media and ethanol controls (media (2% HS) and EtOH) within the same time point. Error bars show standard deviation. 4 biological replicates (wells) were used throughout and all PCR experiments were carried out in triplicate. Statistical analysis was carried out via a 2-way ANOVA with a *post hoc* Bonferroni test where time*treatment interaction was significant ($p<0.05$).

4.4.3 Vitamin D influences mRNA expression of myosin heavy chain (MHC) isoforms

Brown et al (2012) showed that expression of the myosin heavy chain (MHC) isoforms can be categorised into early or late during C2C12 muscle cell differentiation. The MHC type I, neonatal and embryonic isoforms are expressed earlier during differentiation. For all 3 of these isoforms, mRNA expression remained low from day 0 to day 4, peaked on day 6, then fell on day 8 (Figure 4.4 A, B, C). Importantly, on day 6, mRNA expression of both MHCI and MHC embryonic was significantly higher for the 3 highest concentrations of $1,25(OH)_2D_3$ compared to controls ($p<0.05$), whereas only 10^{-9}M $1,25(OH)_2D_3$ was significantly higher than controls for MHC neonatal mRNA on day 6 ($p<0.05$).

Expression of all three early MHC isoforms was positively correlated with myogenin mRNA expression (Supplementary Data 4.3) with expression peaking on day 6. The strongest correlation was observed between myogenin and MHCI with an R^2 value of 0.59 ($p<0.05$), followed by MHC neonatal ($R^2 = 0.55$, $p<0.05$) then MHC embryonic ($R^2 = 0.49$, $p<0.05$).

The late MHC isoforms are expressed after the early isoforms in the order MHC IIa, IIx, IIb towards the latter stages of C2C12 differentiation (Brown et al., 2012), hence an increase in mRNA expression was only seen on days 6 and 8 (Figure 4.4 D, E, F). MHC IIa mRNA expression was significantly increased by 10^{-7}M $1,25(OH)_2D_3$ ($p<0.05$), whilst MHC IIx expression was significantly increased by both 10^{-9}M and 10^{-7}M $1,25(OH)_2D_3$ ($p<0.05$), and MHC IIb expression was significantly increased by 10^{-11}M, 10^{-9}M and 10^{-7}M $1,25(OH)_2D_3$ ($p<0.05$) on day 6 compared to controls. Importantly, expression of all 3 late MHC isoforms did not change from day 0 to day 6 in the

control cells, and only significantly increased on day 8 ($p<0.05$) indicating an earlier induction of differentiation with $1,25(OH)_2D_3$ treatment.

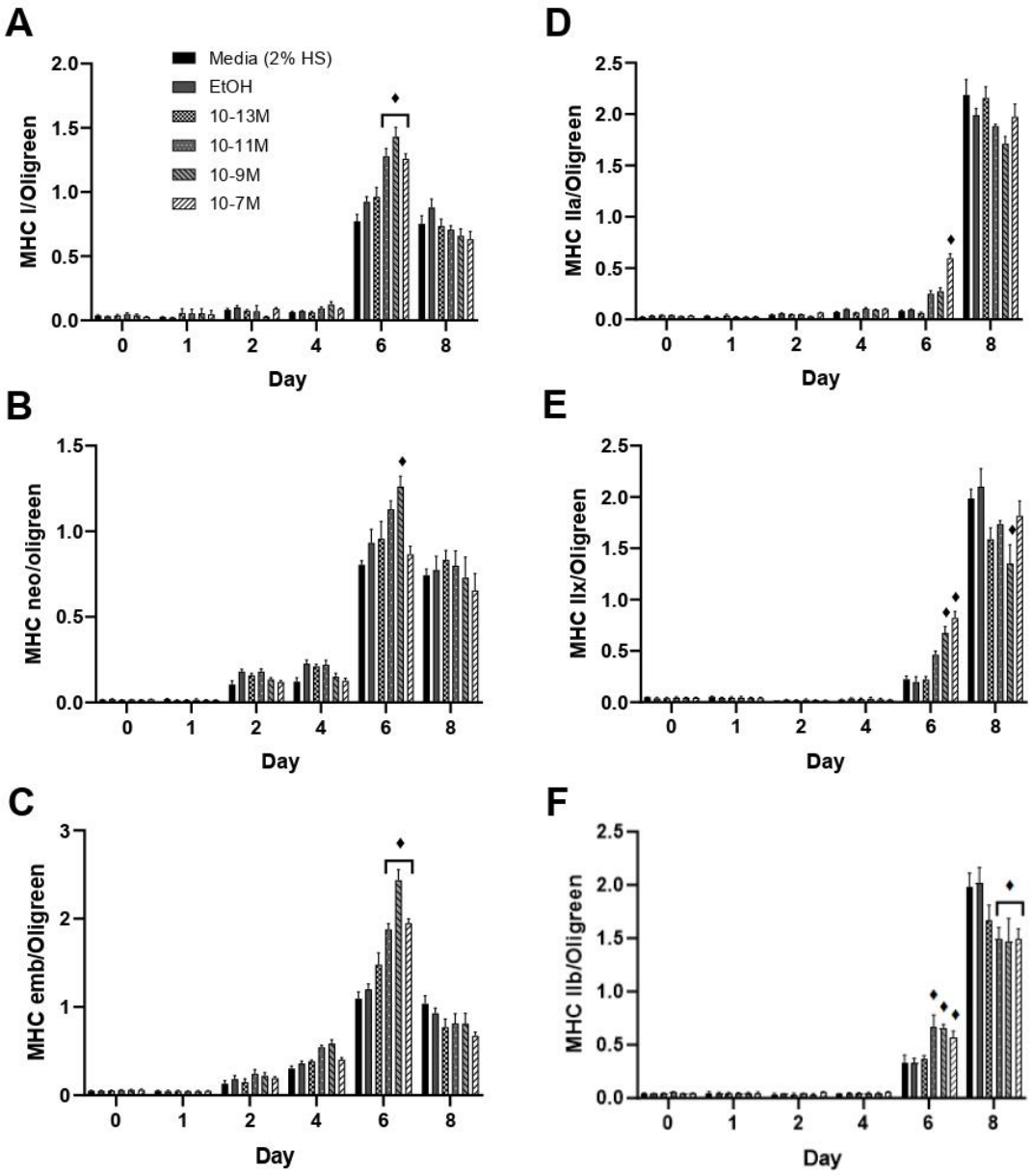

Figure 4.4. Effects of $1,25(OH)_2D_3$ on C2C12 cells over 8 days on myosin heavy chain gene expression. **A** MHC I **B** MHC neonatal **C** MHC embryonic **D** MHC IIa **E** MHC IIx **F** MHC IIb mRNA expression normalised to Oligreen during C2C12 differentiation over an 8-day time course with varying concentrations of $1,25(OH)_2D_3$ dissolved in 0.1% (v/v) ethanol. ♦ symbol indicates where a bar is significantly different ($p<0.05$) to both media and ethanol controls (media (2% HS) and EtOH) within the same time point. Error bars show standard deviation. 4 biological replicates (wells) were used throughout and all PCR experiments were carried out in triplicate. Statistical analysis was carried out via a 2-way ANOVA with a *post hoc* Bonferroni test where time*treatment interaction was significant ($p<0.05$).

4.4.4 Vitamin D increases myogenin expression via a functional VDRE on the gene promoter

Since myogenin is a transcription factor known to induce expression of many of the genes associated with myogenic differentiation, including the MHC isoforms, we hypothesised that $1,25(OH)_2D_3$ effects on differentiation might be via increases in mRNA myogenin expression, which subsequently increases the expression of other genes. This was backed up by the observed positive correlations between myogenin and MHC early isoform mRNA expression. We therefore investigated whether the myogenin promoter contained a putative VDRE, which was identified at -1260 to -1246 base pairs from the transcriptional start site, and then tested its responsiveness to $1,25(OH)_2D_3$ by performing transfection studies using a vector containing the myogenin promoter sequence (with the putative VDRE) upstream of a GFP reporter sequence (MyoG-VDRE-GFP, Supplementary data 4.2). Over the 8-day time course, there was a significant day x treatment interaction for myogenin promoter activity ($p<0.001$, Figure 4.5 A). On day 4, GFP fluorescence was significantly higher with 10^{-7}M and 10^{-11}M $1,25(OH)_2D_3$ compared to controls ($p<0.05$). By day 5, all 3 $1,25(OH)_2D_3$ treatment groups were significantly higher than controls ($p<0.05$). The largest effects were seen on day 6, with GFP fluorescence for 10^{-7}M, 10^{-9}M and 10^{-11}M $1,25(OH)_2D_3$ being 15%, 20% and 15% higher than the EtOH control ($p<0.05$).

Transfections were then repeated using the myogenin promoter sequence with a 3-base mutation within the putative VDRE at -1261 to -1264 (MyoG-mutVDRE-GFP), with the MyoG-VDRE-GFP run alongside for comparison. Due to consistent results at the 3 timepoints used (days 4, 5 and 6), the day x treatment x vector interaction was not significant ($p=0.769$, Figure 4.5 B) but there was a significant treatment x vector interaction ($p<0.001$). As previously observed, in cells treated with the normal VDRE

construct (MyoG-VDRE-GFP), both 10^{-7}M and 10^{-11}M $1,25(OH)_2D_3$ caused a significant increase in fluorescence compared to controls on days 4, 5 and 6 ($p<0.05$). However, there was no effect of $1,25(OH)_2D_3$ treatment in cells transfected with that mutated VDRE construct (MyoG-mutVDRE-GFP), indicating that responsiveness to $1,25(OH)_2D_3$ was lost when the putative myogenin VDRE site was mutated.

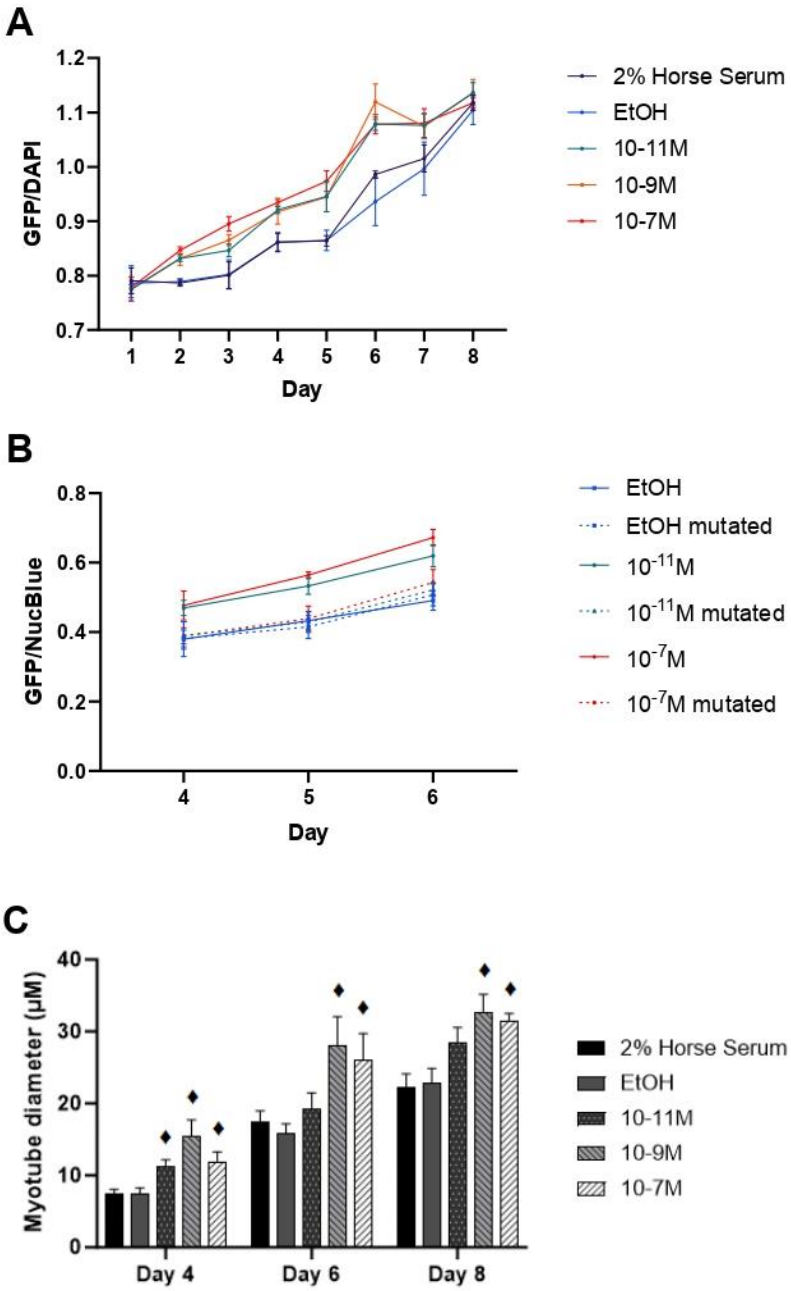

Figure 4.5. Effects of $1,25(OH)_2D_3$ on C2C12 cells transfected with wild-type or mutated mouse myogenin GFP vectors. **A** GFP fluorescence (normalised to NucBlue) using a vector containing the wild type myogenin promoter sequence 5' to a turbo GFP sequence during C2C12 differentiation over an 8-day time course with varying concentrations of $1,25(OH)_2D_3$ dissolved in 0.1% (v/v) ethanol. **B** GFP fluorescence (normalised to NucBlue) using both the wild type myogenin promoter sequence as well as the myogenin promoter sequence with a 3-base mutation in positions 2,3 and 4 on the VDRE sequence using a 0.1% (v/v) ethanol control and two $1,25(OH)_2D_3$ treatment concentrations (10^{-7}M and 10^{-11}M in 0.1% (v/v) ethanol) based on results from A on days 4, 5 and 6. **C** Myotube diameter (µM) of C2C12 cells, transfected with the wild type myogenin promoter vector 5' to a turbo GFP sequence, grown in differentiation media with varying concentrations of $1,25(OH)_2D_3$ dissolved in 0.1% (v/v) ethanol on days 4, 6 and 8. ♦ symbol indicates where a bar is significantly different ($p<0.05$) to both media and ethanol controls (media (2% HS) and EtOH) within the same time point. Error bars show standard deviation and 4 biological replicates (wells) were used throughout. Statistical analysis was carried out via a 2-way ANOVA for A and C and a 3-way ANOVA for B, with a *post hoc* Bonferroni test where time*treatment interaction was significant ($p<0.05$).

Myotube diameter was measured in fluorescent myotubes and was also affected by $1,25(OH)_2D_3$ treatment (Figure 4.5 C). The time x treatment interaction was not significant ($p=0.8$), but there was a significant effect of treatment ($p<0.001$), with 10^{-7}M, 10^{-9}M and 10^{-11}M $1,25(OH)_2D_3$ resulting in larger myotube diameters compared to controls on day 4 ($p<0.05$), and 10^{-7}M and 10^{-9}M $1,25(OH)_2D_3$ resulting in larger myotube diameters compared to controls on days 6 and 8 ($p<0.05$). Cell images can be seen in Supplementary Data 4.4.

To further confirm that the identified VDRE sequence was functional, and that the VDR binds to this region, a ChIP assay was performed. Following the pull-down of genomic DNA fragments using a VDR-specific antibody, 3 different primer pairs were used for PCR to amplify slightly different regions of DNA around the VDRE. Amplicon lengths for primer pairs 1, 2 and 3 were 138bp, 62bp and 82bp respectively (Supplementary Data 4.1). Amplification curves are shown Figure 5.6 A and a single PCR amplicon was produced for all 3 primer pairs and melt curve analysis also showed a single peak, again indicating a single product. The PCR products were run on a 2.5% metaphor agarose gel to confirm that they were the correct length (Figure 4.6 B, Supplementary Data 4.5). For primer pairs 1 and 2 (which both spanned the VDRE), the Cp values for $1,25(OH)_2D_3$ treated cells were significantly lower than the control ($p<0.05$, Figure 4.6 C) indicating an increase in binding of the VDR to the VDRE with $1,25(OH)_2D_3$ treatment. However, Cp values for primer pair 3 were not significantly different (Figure 4.6 C) likely because they bind ~97bp upstream of the VDRE (Figure 4.6 D) and therefore some DNA fragments containing the VDRE were not detected by this primer pair.

When the newly identified mouse myogenin VDRE sequence was aligned to the human myogenin genomic sequence (Accession AF050501.1) using Clustal Omega, it

revealed an 11/15 (73%) base pair match (Figure 4.6 E). This could indicate that this VDRE sequence on the myogenin promoter is conserved across species. However, alignment with cow, pig and sheep myogenin sequences was attempted but not possible due to the available sequences not containing enough of the 3' promoter sequence.

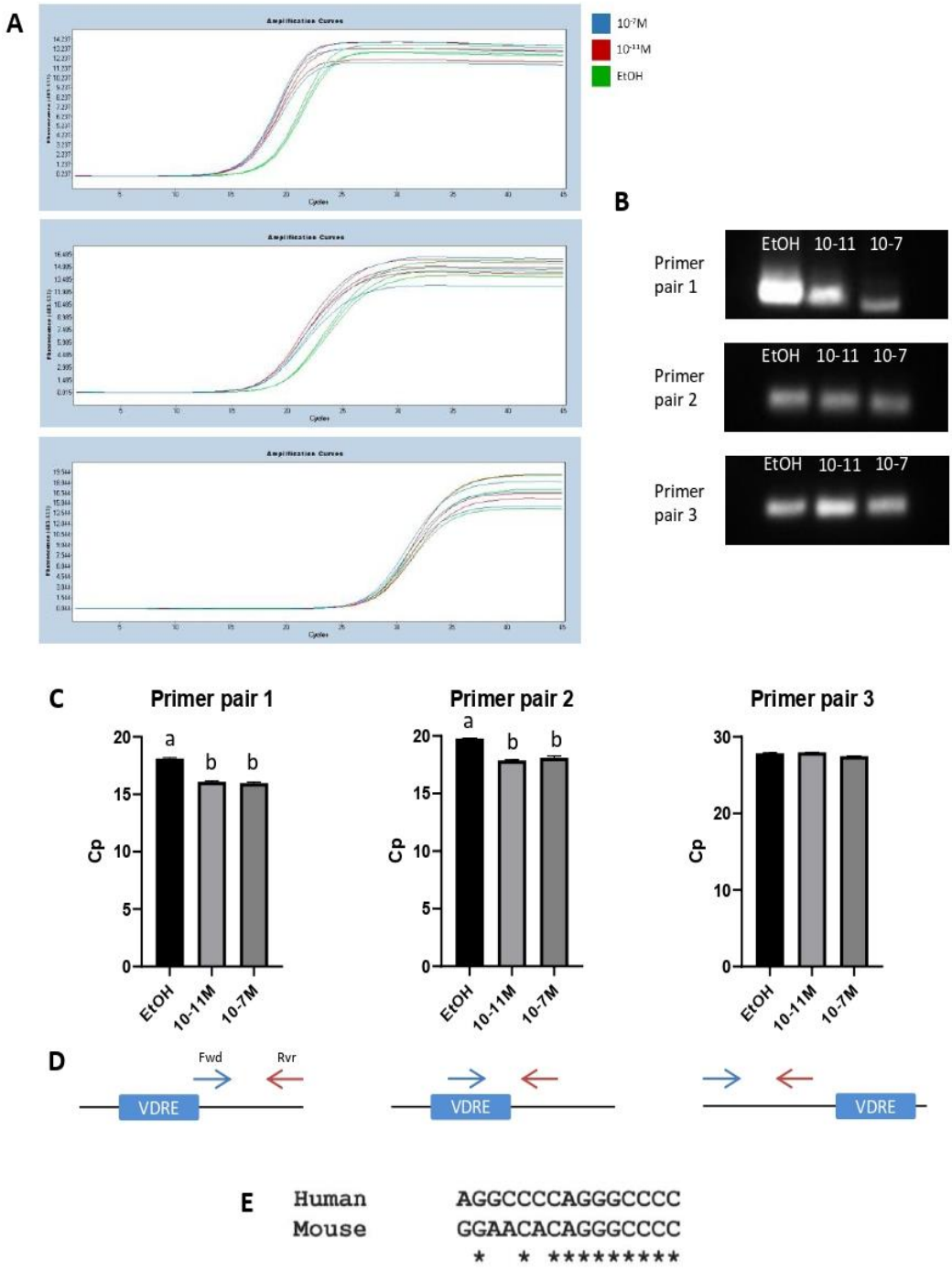

Figure 4.6. PCR products from ChIP assays for C2C12 cells treated with $1,25(OH)_2D_3$. **A.** PCR amplification curves for ethanol (EtOH), 10^{-11}M and 10^{-7}M $1,25(OH)_2D_3$ generated by LightCycler 480 software following a ChIP assay using an anti-VDR antibody and primers to amplify a region of DNA around the VDRE sequence (-1260 to -1246). **B.** PCR products from **A** run on a 2.5% (w/v) metaphor agarose gel to confirm fragment size and that a single PCR product was produced. Positive and negative controls were also run (for full gel image including ladder see Supplementary Data 4.5). **C.** Average Cp values for ethanol, 10^{-11}M and 10^{-7}M $1,25(OH)_2D_3$. Error bars show standard deviation and samples were run in triplicate. a is significantly different to b ($p<0.05$) determined by one way ANOVA and post hoc Bonferroni. **D.** Diagram to show forward (Fwd) and reverse (Rvr) primer positions in relation to the VDRE. Primer pair 1: Fwd primer starts on the last base pair of the VDRE sequence (+13 from VDRE start site), Rvr primer position is +128 from start of VDRE. Primer pair 2: Fwd primer position is +1 from start of VDRE, Rvr primer position is +41 from start of VDRE. Primer pair 3: Fwd primer position is -179 from start of VDRE, Rvr primer position is -124 from start of VDRE. **E.** Sequence alignment using Clustal Omega between the human myogenin promoter sequence (Accession AF050501.1) and the newly identified mouse myogenin VDRE sequence.

4.5 Discussion

For the first time, this detailed C2C12 differentiation time course study has shown a clear stimulatory effect of active VD ($1,25(OH)_2D_3$) on myogenic differentiation which appears to be via a functional VDRE on the myogenin gene promoter.

Firstly, we showed that $1,25(OH)_2D_3$ inhibits cell proliferation, inducing a switch towards a differentiative state. At 48 hours, DNA content for the highest two $1,25(OH)_2D_3$ concentrations were similar to day 0 indicating that the reduced level of DNA compared to controls was due to an inhibition of proliferation rather than an increase in apoptosis. This agrees with previous work in C2C12 cells showing a 75% decrease in proliferating cell nuclear antigen (PCNA), a marker of proliferation, after 7 days in $1,25(OH)_2D_3$ treated cells (Garcia et al., 2011), and also agrees with work in chick myoblasts where proliferation was significantly decreased by $1,25(OH)_2D_3$ on days 4 and 6 compared to controls (Capiati et al., 1999). It has been reported that myoblasts treated with active VD have a higher percentage of cells in the G_0/G_1 (quiescent) phase and a lower percentage of cells in the S/M (active) phase compared to controls (Girgis et al., 2014) suggesting that this anti-proliferative effect of VD is associated with cell cycle arrest. Our results show that creatine kinase (CK) activity, which is expressed after myoblasts exit from the cell cycle and begin to differentiate (Tai et al., 2011), is significantly higher for $1,25(OH)_2D_3$ treated cells than controls. Again, this agrees with Capiati et al (Capiati et al., 1999) who showed that CK activity was increased in $1,25(OH)_2D_3$ treated chick myoblasts on days 2, 4 and 6 compared to controls, which suggests that active VD inhibits proliferation and stimulates cells to differentiate.

The MRFs are basic helix-loop-helix transcription factors that recognise and bind to E box sequences (CANNTG) on target genes (Adhikari et al., 2021). They possess

potent signalling effects, such that inducing expression of any of the MRFs on a variety of tissue culture cells results in conversion to muscle-type cells (Hasty et al., 1993). Myf5 is the first MRF to be expressed, followed closely by MyoD and then myogenin (Zammit, 2017). The myogenin promoter contains E box sequences both within the proximal promoter sequence (-130 to +18bp) and in enhancer regions -4.5kb and -6.5kb upstream of the coding sequence, which become bound by MyoD/E-protein heterodimers to activate transcription during differentiation (Faralli and Dilworth, 2012). Our results show that Myf5 mRNA expression is significantly lower in $1,25(OH)_2D_3$ treated cells than controls on day 1, possibly indicating a faster down regulation of early differentiation markers, which may suggest that cells are differentiating more quickly. These results are in agreement with previous work which found a decrease in Myf5 gene expression just 6 hours post $1,25(OH)_2D_3$ injection in chick embryos (Chen et al., 2021). In addition, work in C2C12 cells showed that both inactive ($25(OH)D_3$) and active VD treatment reduced Myf5 mRNA expression (Girgis et al., 2014). MyoD mRNA expression was not found to be affected by $1,25(OH)_2D_3$ in this study. The current literature reports inconsistent effects of active VD on MyoD mRNA expression *in vitro* with some finding increased expression (Garcia et al., 2011, Saito et al., 2017), one study finding decreased expression (Olsson et al., 2016) and another finding no significant effect (van der Meijden et al., 2016). It is worth noting that there is large variability between these studies on the day that MyoD expression was measured, often at only a single time point, which ranged from day 1 to day 7. Despite this study finding no change in MyoD mRNA expression, it is worth noting that MyoD increases transcription of p21 which inhibits various cyclin dependent kinases (CDKs), this leading to cell cycle arrest, allowing myoblasts to enter a differentiative state (Christ and Brand-Saberi, 2002). Previous studies have shown an increase in p21 mRNA expression in response

to vitamin D (Okuno et al., 2012, Olsson et al., 2016), therefore it cannot be ruled out that vitamin D could also be acting via cell cycle regulatory factors to stimulate cells to enter the differentiation phase more quickly. As far as we are aware, this study has used the most detailed time course to measure the effects of $1,25(OH)_2D_3$ on MRF expression, measuring at 6 different time points across differentiation, and shows that $1,25(OH)_2D_3$ causes an earlier increase in myogenin mRNA expression compared to controls. There are also inconsistencies within the literature on the effect of active VD on myogenin expression *in vitro* with some studies finding a decrease in mRNA expression (Olsson et al., 2016, Okuno et al., 2012, Ryan et al., 2013, van der Meijden et al., 2016, Girgis et al., 2014) and others finding an increase in mRNA and protein expression (Gili et al., 2016, Garcia et al., 2011, Braga et al., 2017, Montenegro et al., 2019), and protein expression. Again, most of these studies measure expression at one or two time points, rather than using time courses, with variation on day of measurement. *In vivo*, dietary VD ($25(OH)D_3$) has been shown to increase myogenin expression in piglets from mothers fed a high VD diet, with higher levels of myogenin mRNA observed in the longissimus dorsi and psoas major muscles compared to controls (Zhou et al., 2016).

Myogenin is an essential component of the regulatory pathway that leads to skeletal muscle formation during embryogenesis. In myogenin knockout mice, pups survive fetal development but are born immobile and die shortly after birth with a lack of differentiated skeletal muscle, despite normal levels of MyoD (Hasty et al., 1993). In contrast, knockout of either MyoD or Myf5 result in normal muscle formation (Asfour et al., 2018), but knockout of both Myf5 and MyoD results in complete lack of skeletal muscle (Rudnicki et al., 1993), with mice being born alive but immobile, and dying soon after birth. A previous study has shown that myogenin mRNA expression is completely blocked when the VDR is knocked down *in vitro* (Gili et al.,

2016) suggesting that VD is essential for myogenin expression. The VDRE sequence that we have found is, to our knowledge, the first time that a functional VDRE has been identified on the myogenin promoter. Our results show that when this VDRE sequence is mutated, the increase in myogenin mRNA expression previously seen in $1,25(OH)_2D_3$ treated cells becomes abolished, suggesting that this sequence is essential for the upregulation of myogenin gene transcription by $1,25(OH)_2D_3$. In addition to this, ChIP analysis showed that the VDR does in fact bind to the sequence identified suggesting that the effects of $1,25(OH)_2D_3$ on myogenin mRNA expression are direct and VDR mediated.

A recent study has shown that depleting myogenin levels via shRNA in C2C12 cells resulted in a significant reduction in myosin heavy chain protein levels (Adhikari et al., 2021). In addition, over expression of rat myogenin during mouse fetal development results in increased levels of oxidative enzymes within skeletal muscle (Hughes et al., 1999). Both of these suggest that myogenin plays a role in regulating expression of the slow twitch, or early, MHC isoforms which are oxidative in nature. In agreement with this, our results show a positive correlation between myogenin and early MHC isoform mRNA expression. Therefore, the increase in mRNA expression of the early MHC isoforms observed in response to $1,25(OH)_2D_3$ is likely to be due, at least in part, to the increase in myogenin expression.

Our previous work has found that during C2C12 differentiation the MHC fast isoforms are expressed in the order IIa, IIx, IIb (Brown et al., 2012). Our results show an earlier increase in expression of all three of the fast isoforms with $1,25(OH)_2D_3$ compared to controls, suggesting that treatment causes cells to move through differentiation more quickly. In agreement, other work has found that eldecalcitol (a VD analogue) increased mRNA expression of all three fast isoforms in C2C12 cells

(Saito et al., 2017). In addition to this, one study found that 1,25$(OH)_2D_3$ caused an increase both MHC IIa mRNA and protein expression in already differentiated C2C12 cells (Okuno et al., 2012) suggesting that the effects of VD on MHC expression may not be isolated to embryogenesis, but may also have effects in differentiated muscle.

Finally, we show that 1,25$(OH)_2D_3$ causes a significant increase in myotube diameter. In our recent systematic review (Alliband et al., 2021) there was strong agreement across the literature with all 5 *in vitro* studies that measured myotube size finding a significant increase with 1,25$(OH)_2D_3$. This is reflected *in vivo* since rat pups from VD deficient mothers had smaller muscle fibers and larger interfibrillar spaces within the muscle (Max et al., 2014). Cluster gene analysis showed that VD sufficient pups showed an upregulation of genes involved in cell differentiation and a down regulation of genes involved in protein catabolism compared to VD deficient pups (Max et al., 2014). In addition, piglets born to mothers fed a high VD diet show a larger muscle fiber cross sectional area in the psoas major and longissimus dorsi muscles (Zhou et al., 2016). Further supporting the effects of VD on muscle mass, previous work has also shown that active vitamin D down regulates the expression of myostatin, a negative regulator of muscle mass, both *in vitro* (Garcia et al., 2011) and *in vivo* (McPherron et al., 1997). Hence, there appears to be a clear effect of VD on muscle fiber size both *in vitro* and *in vivo*. Due to the findings of the effects of VD on myogenin, one possible explanation for this could be due to increased levels of fusion resulting in larger myotubes.

Overall, the results presented show that active VD directly increases myogenin transcription via a functional VDRE on the myogenin promoter, resulting in increased myogenic differentiation, increased mRNA expression of both the early and late

MHC isoforms, as well as increased myotube size. However, we cannot rule out that $1,25(OH)_2D_3$ may also be exerting direct effects on expression of the MHC isoforms. Future work could investigate the effects of VD at the protein level to check that the effects on gene expression found in this study correlate with effects at the protein level. It has previously been shown that whilst mRNA and it's corresponding protein levels do correlate, this is not always a strong correlation, with around 40% of variation at the protein level being accounted for by changes in mRNA and around 60% being due to post-transcriptional regulation (Vogel and Marcotte, 2012). Therefore, it is important to study the genes within this study at the protein level before strong conclusions can be drawn. Future work should also investigate the impact of VD deficiency during embryogenesis *in vivo.* Whilst *in vitro* data can provide a good insight into the effects of treatment and the mechanisms behind this, cells *in vivo* behave very differently to *in vitro*. For example, unlike *in vitro*, myogenic precursor cells *in vivo* do not all differentiate into myotubes, some will enter a quiescent state and become satellite cells; committed but undifferentiated cells which remain primed for activation in response to injury (Dumont et al., 2015). *In vivo* work could also investigate the long-term impact on postnatal muscle growth and body composition. Finally, an 11/15 base pair match was found between the mouse myogenin VDRE sequence and a potential VDRE sequence on the human myogenin genomic sequence. Therefore, further work to investigate the impact of $1,25(OH)_2D_3$ on human myogenesis is needed. Currently, it is estimated that between 20-40% of pregnant women worldwide are vitamin D insufficient or deficient (Urrutia-Pereira and Solé, 2015), therefore it is crucial that a clearer understanding of the impact of maternal VD deficiency on offspring development and health is gained.

4.6 Supplementary data

Supplementary data 4.1 – table to show a list of forward and reverse primer sequences, and amplicon length, for all PCR primers to measure gene expression and to amplify DNA fragments from ChIP.

Target Transcript	*Forward primer sequence*	*Reverse primer sequence*	*Length of amplicon*
MyoD	5′ CGTGGCAGCGAGCACTAC 3′	5′TGTAATCCATCATGCCATCA GA 3′	79bp
Myf5	5′ CAGCCCCACCTCCAACTG 3′	5′GCAGCACATGCATTTGATAC ATC 3′	117bp
Myogenin	5′ CCCATGGTGCCCAGTGAA 3′	5′GCAGATTGTGGGCGTCTGTA 3′	130bp
Myosin Heavy Chain I (MHCI)	5′ CTCAAGCTGCTCAGCAATCTA TTT 3′	5′GGAGCGCAAGTTTGTCATAA GT 3′	152bp
Myosin Heavy Chain neonatal (MHC neo)	5′ CAGGAGCAGGAATGATGCTCT GAG 3′	5′AGTTCCTCAAACTTTCAGCA GCCAA 3′	113bp
Myosin Heavy Chain embryonic (MHC emb)	5′ TCCGACAACGCCTACCAGTT 3′	5′ CCCGGATTCTCCGGTGAT 3′	72bp
Myosin Heavy Chain IIa	5′ AGGCGGCTGAGGAGCACGTA 3′	5′ GCGGCACAAGCAGCGTTGG 3′	130bp
Myosin Heavy Chain IIx	5′ GAGGGACAGTTCATCGATAGC AA 3′	5′GGGCCAACTTGTCATCTCTC AT 3′	154bp
Myosin Heavy Chain IIb	5′ CAATCAGGAACCTTCGGAACA C 3′	5′GTCCTGGCCTCTGAGAGCAT 3′	80bp
VDRE ChIP primer 1	5′ CCCAATGGAGGAGCTAGAGAA A 3′	5′CGACTAGGCCATCTTCTGCT ACA 3′	138bp
VDRE ChIP primer 2	5′ GAACACAGGGCCCCCAAT 3′	5′TGCAGACCCCTTCAGTTCCTT 3′	62bp
VDRE ChIP primer 3	5′CACAGAAGAGATGATTAAG AGCATCAG 3′	5′GCTGGCATATGTAATAGTGT CTGTGTT 3′	82bp

Supplementary data 4.2 - breakdown of components and the full genetic sequence for both vectors used in the transfection experiments (MyoG-VDRE-GFP and MyoG-mutVDRE-GFP). The VDRE sequence is highlighted in yellow and the 3 base mutation is shown in red. The rest of the sequence is colour coded (underlined) according to the corresponding vector table.

Vector name – **MyoG-VDRE-GFP**

Name	Position	Size (bp)	Type	Description	Application notes
Myogenin promoter including normal VDRE	1-1577	1577	Promoter	Promoter sequence for mouse gene Myogenin	Region of DNA upstream from the Myogenin coding sequence where relevant proteins bind to initiate or repress transcription of the gene.
Kozak	1608-1613	6	Miscellaneous	Kozak translation initiation sequence	Facilitates translation initiation of ATG start codon downstream of the Kozak sequence.
TurboGFP	1614-2297	684	ORF	Also known as maxGFP; green fluorescent protein from maxillopoda	Fast maturation; ranked high in brightness, photostability and pH stability among all fluorescent proteins.
SV40 late pA	2398-2619	222	PolyA_signal	Simian virus 40 late polyadenylation signal	Allows transcription termination and polyadenylation of mRNA transcribed by Pol II RNA polymerase.
pUC ori	complement (2815-3403)	589	Rep_origin	pUC origin of replication	Facilitates plasmid replication in E. coli; regulates high-copy plasmid number (500-700).
Ampicillin	complement (3574-4434)	861	ORF	Ampicillin resistance gene	Allows E. coli to be resistant to ampicillin.

Sequence:

```
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641  TGGGATAGCA TTTGAAATGT AAATGAAGAA AATATCTAAT AAAAAATAAT TTAAAAAAGA GCGTCAGACA GGGGACTGAA
721  CAGCTCTTGC ACTAGGGGAG AAGAAGGCAA TGTAGAGTAG TCTGTGAGTT CTAATCCTTG CTAAACACTG ACTTCACCTG
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961  ATAGGACACA CACACACACA CACACACACA CACACACACA CACACACACA CGCCCCAAAT CTGGAGTGGT CCTGATGTGG
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3921 GTATGGCTTC ATTCAGCTCC GGTTCCCAAC GATCAAGGCG AGTTACATGA TCCCCCATGT TGTGCAAAAA AGCGGTTAGC

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4561 ACCTGACGTC TAAGAAACCA TTATTATCAT GACATTAACC TATAAAAATA GGCGTATCAC GAGGCCCTTT CGTCGGCGCG

4641 CCGCGGCCGC CAACTTTGTA TAGAAAAGTT GCTGTGCCTT CTAGTTGCCA GCCATCTGTT GTTTGCCCCT CCCCCGTGCC

4721 TTCCTTGACC CTGGAAGGTG CCACTCCCAC TGTCCTTTCC TAATAAAATG AGGAAATTGC ATCGCATTGT CTGAGTAGGT

4801 GTCATTCTAT TCTGGGGGGT GGGGTGGGGC AGGACAGCAA GGGGGAGGAT TGGGAAGACA ATAGCAGGCA TGCTGGGGAT

4881 GCGGTGGGCT CTATGG

Vector name – MyoG-mutVDRE-GFP

Name	Position	Size (bp)	Type	Description	Application notes
Myogenin promoter including mutated VDRE	1-1577	1577	Promoter	Promoter sequence for mouse gene Myogenin	Region of DNA upstream from the Myogenin coding sequence where relevant proteins bind to initiate or repress transcription of the gene.
Kozak	1608-1613	6	Miscellaneous	Kozak translation initiation sequence	Facilitates translation initiation of ATG start codon downstream of the Kozak sequence.
TurboGFP	1614-2297	684	ORF	Also known as maxGFP; green fluorescent protein from maxillopoda	Fast maturation; ranked high in brightness, photostability and pH stability among all fluorescent proteins.
SV40 late pA	2398-2619	222	PolyA_signal	Simian virus 40 late polyadenylation signal	Allows transcription termination and polyadenylation of mRNA transcribed by Pol II RNA polymerase.
pUC ori	complement (2815-3403)	589	Rep_origin	pUC origin of replication	Facilitates plasmid replication in E. coli; regulates high-copy plasmid number (500-700).
Ampicillin	complement (3574-4434)	861	ORF	Ampicillin resistance gene	Allows E. coli to be resistant to ampicillin.

Sequence:

```
1    TCTAGAGTTG TATGACGCAG GCAAAGGTGA TGACTCAGGC AGGAAGGAAT AGAAGAGGCC AGCCTGGTGG CCCAGGACAG
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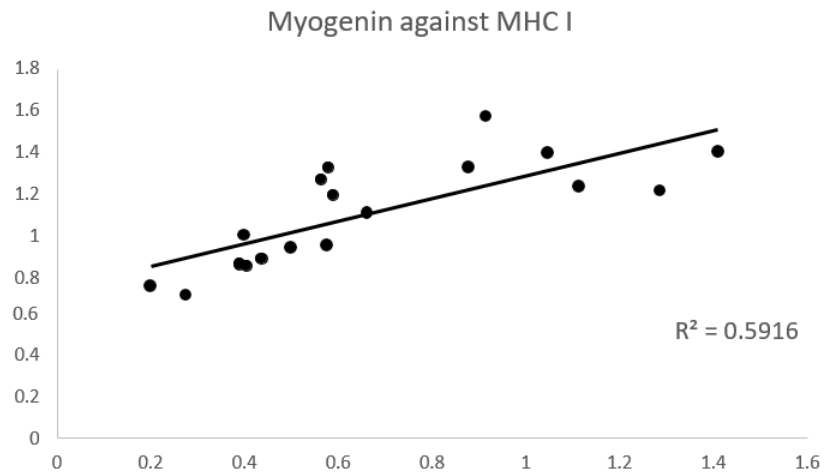
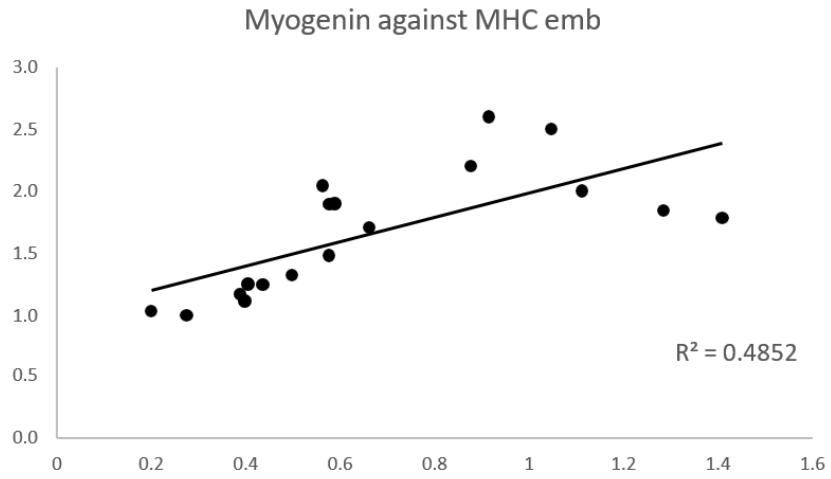
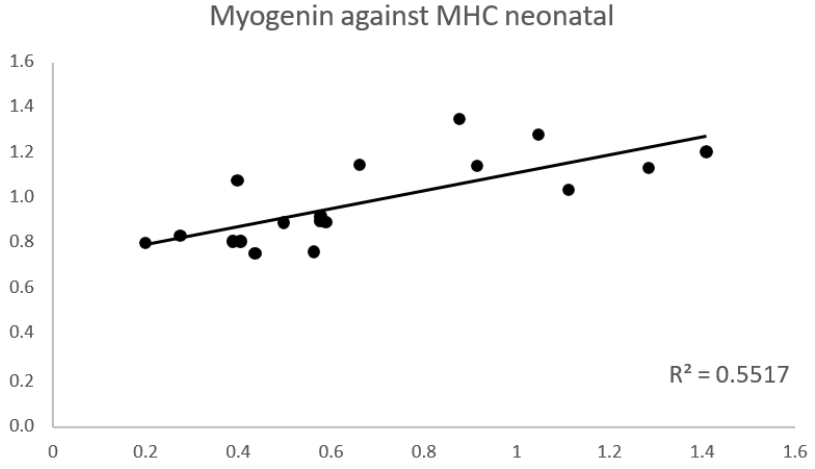
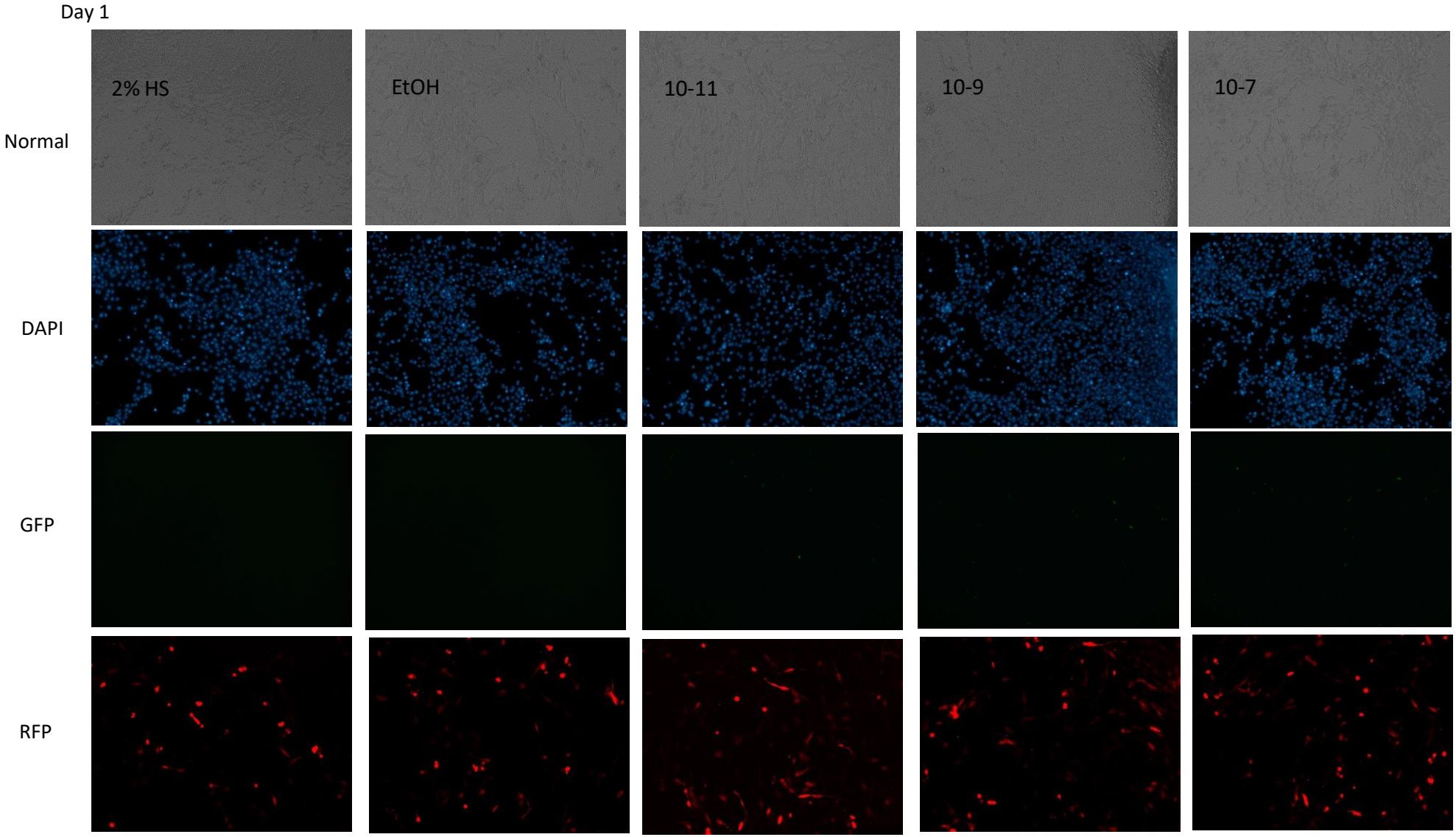
Supplementary data 4.3 - correlation graphs for myogenin mRNA levels plotted against MHC I, MHC embryonic and MHC neonatal mRNA levels on day 6 and day 8 including the R^2 values which can be seen on the graphs. This includes all vitamin D treatment groups and controls.

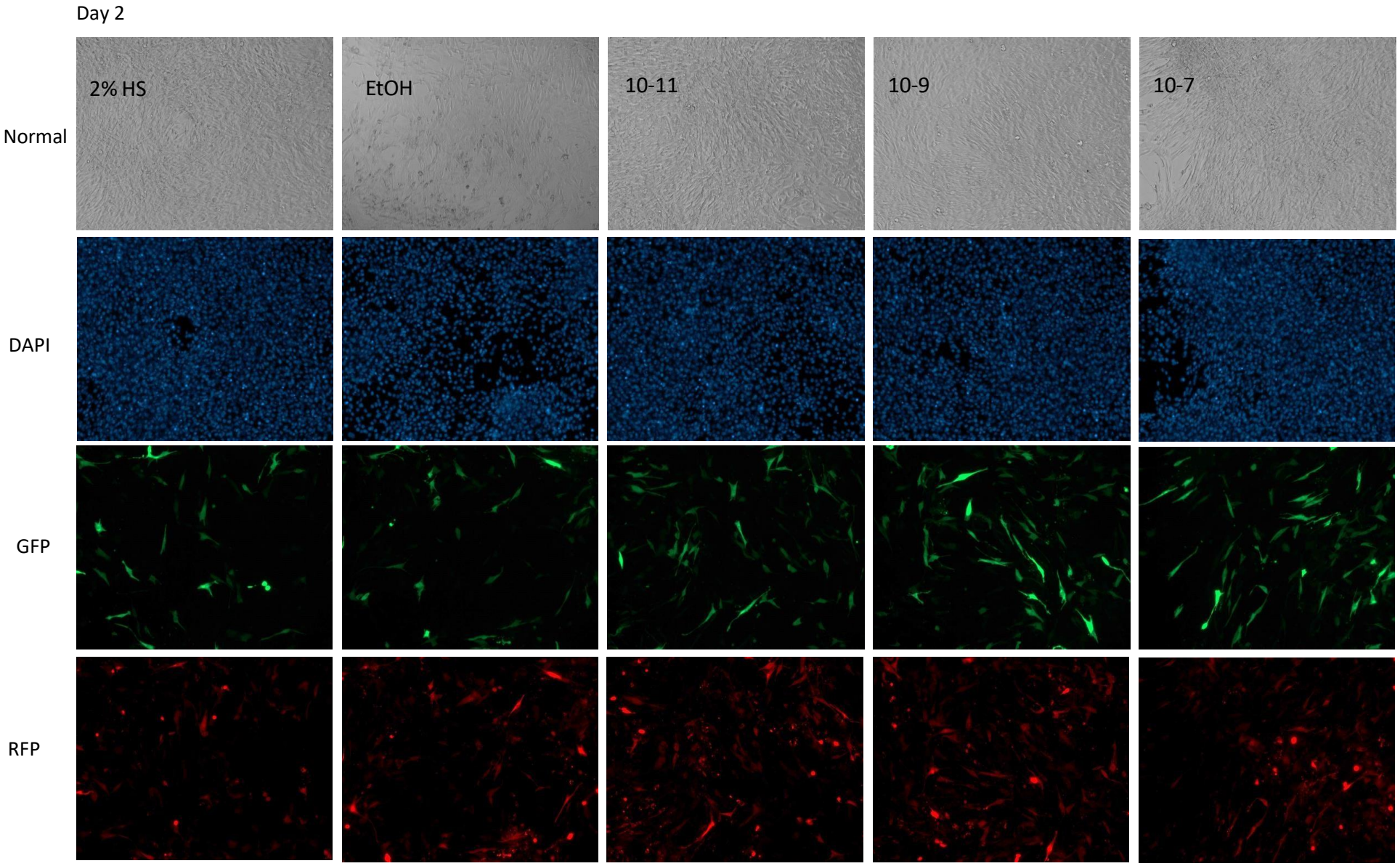




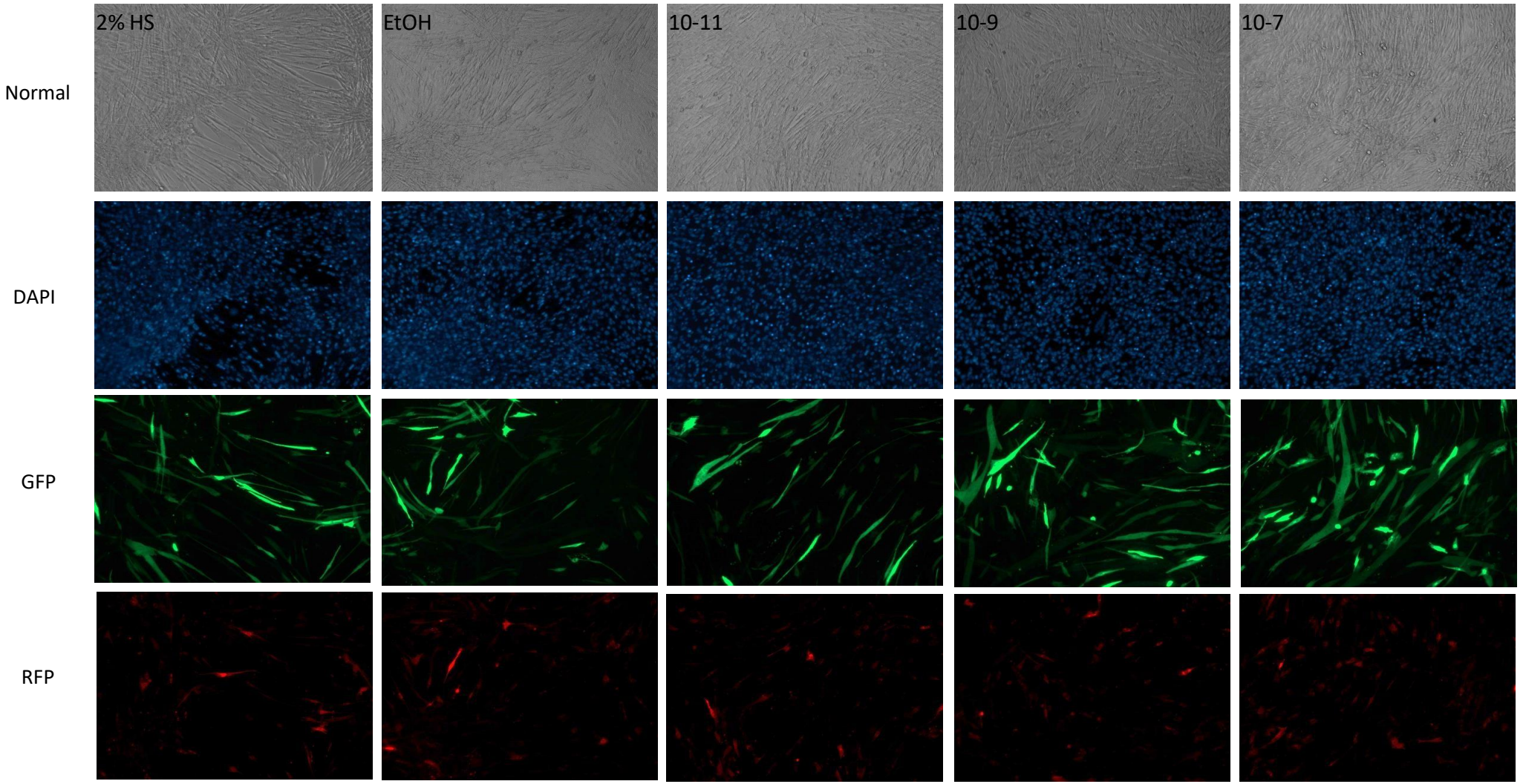


Supplementary data 4.4 - cell images (at 10X magnification) of transfected C2C12 cells on days 1, 2, 5 and 8 (representing the beginning, early, mid and late stages of differentiation) taken with a fluorescent microscope. GFP (green cells) are transfected with the MyoG-VDRE-GFP vector which can be seen in supplementary data 4.2. Blue cells are stained with DAPI to give an indication of cell number. All GFP fluorescence results within this study were normalised to DAPI. RFP (red) cells were transfected with a vector containing a constitutively active CMV promoter followed by RFP which becomes expressed within a few hours of the vector being within the cells. This was to check for transfection efficiency after 24 hours. Due to myogenin not being expressed until later in differentiation, cells did not appear green within 24 hours post transfection, therefore the RFP vector was used to confirm that cells had transfected 24 hours post transfection.

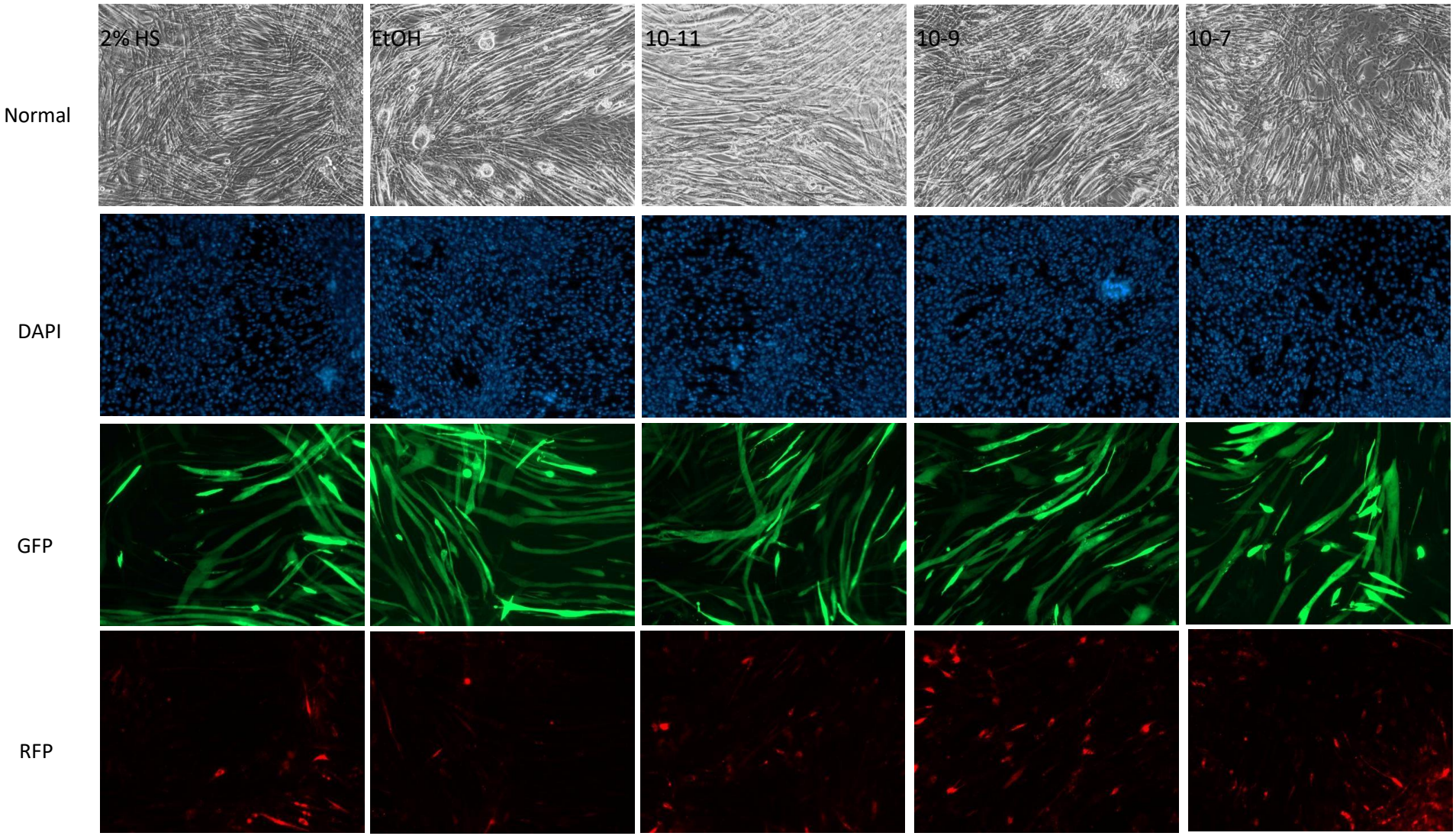

Day 1
2% HS
EtOH
10-11
10-9
10-7
Normal
DAPI
GFP
RFP


Day 2
2% HS
EtOH
10-11
10-9
10-7
Normal
DAPI
GFP
RFP

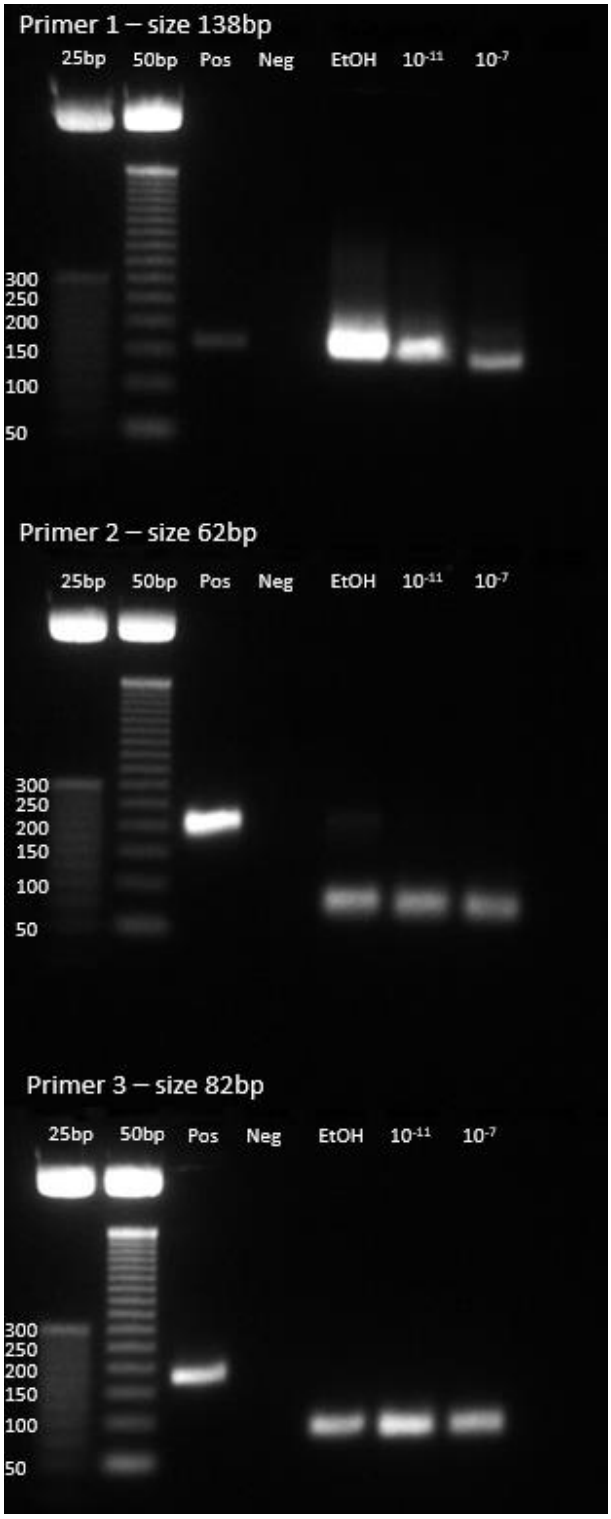
Day 5



Day 8



Supplementary data 4.5 - gel image of PCR products from the ChIP experiment run on a 2.5% metaphor agarose gel alongside a 25bp and 50bp ladder. Positive control was an RNA pol II antibody with GAPDH primers (provided in Chip kit) and negative control was a normal rabbit IgG antibody run with the VDRE primers (primer sequences can be viewed in supplementary data 4.1).



Chapter 5 Manuscript 3

Effects of maternal and infant vitamin D deficiency on offspring growth, metabolic health, strength, and physical activity. Not published.

5.1 Abstract

Vitamin D deficiency is a major health concern affecting between 18-84% of the population worldwide. During pregnancy, the fetus is exposed to environmental cues which can be influenced by maternal nutrition and can impact offspring health. There is emerging evidence that maternal vitamin D status may be an independent risk factor for offspring health.

3-week-old virgin female C57BL/6 mice were placed onto a control or vitamin D deficient (VDD) diet for 8 weeks prior to mating, during pregnancy and lactation. Offspring mice were weaned onto the same diet as their mothers. Vitamin D status, body weight, food intake, tissue weights, insulin tolerance, grip strength and physical activity were measured in offspring mice at 3, 6 and 12 weeks old.

Birth weight was not affected by vitamin D but bodyweight from 2 – 4 weeks old was lower in VDD offspring than controls ($p < 0.05$). VDD offspring showed insulin resistance compared to controls at 6 weeks in female mice only (blood glucose 13.6 and 7.2mmol/L respectively, $p < 0.05$). At 12 weeks there was no effect of sex, with both male and female mice showing insulin resistance compared to controls (10.5 and 8mmol/L, $p < 0.001$). There was no difference in tissue weights between control and VDD females whereas several VDD male tissues weighed significantly less than control males, although this due to a smaller overall bodyweight in most cases. Grip strength was also lower in VDD males compared to controls (78.7 N and 100.6 N respectively, $p < 0.05$), again due to lower bodyweight. Finally, physical activity was lower in VDD offspring than controls ($p = 0.002$).

For the first time, this study has shown the effect of maternal VD deficiency followed by infant vitamin D deficiency on offspring health. Vitamin D deficiency appears to have the greatest impact on male offspring, resulting in decreased body and tissue weights, and decreased grip strength whereas insulin resistance and decreased physical activity was seen in both male and female VDD offspring. The mechanism behind this insulin resistance is unknown but is possibly due to lower levels of adipose tissue and muscle. Insulin resistance is a characteristic feature of many metabolic diseases, so it is vital that a clearer understanding of maternal VD status on offspring metabolic health is gained.

5.2 Introduction

Vitamin D (VD) deficiency amongst pregnant women is a major health concern, with a prevalence ranging from 18-84% depending on the country and reporting body (Vandevijvere et al., 2012). It has been reported that VD deficiency could be an independent risk factor for complications during pregnancy and birth as well as offspring health (Bodnar et al., 2007). Reported complications in offspring from VD deficient mothers include low birth weight, decreased postnatal weight gain, hypocalcaemia, impaired development, and rickets. In the mother, VD deficiency has been linked to increased risk of needing a caesarean section, bacterial vaginosis, osteomalacia, gestational diabetes, hypertension, and pre-eclampsia (Belenchia et al., 2018). Currently, there is no recommended dose for VD supplementation that is specific for pregnant women (Palacios et al., 2019).

Fetal programming, also known as developmental programming or the 'Barker hypothesis' refers to the process where the fetus is exposed to environmental cues during development which can have long-term effects on physiology, metabolism and health status of the child (Reichetzeder et al., 2014). The Barker hypothesis

originates from epidemiological data which shows that low birth weight due to maternal malnutrition has long-term negative health outcomes in the offspring which continue into adulthood (Barker et al., 2002). Maternal nutrition affects the intrauterine environment and therefore can influence the environmental cues that the fetus is exposed to. To date, most studies have looked at the effects of macronutrient deficiency or total energy deficiency, however, it is becoming increasingly apparent that deficiency in micronutrients such as VD may have a significant impact on fetal programming (Belenchia et al., 2018). It is currently estimated that VD has the ability to modulate around 10% of the human genome and it plays a major role in the developmental process (Morris and Anderson, 2010).

It is well established that VD affects the process of skeletal muscle formation (myogenesis) but the exact nature of these effects, as well as the mechanisms behind them, remain unclear (Alliband et al., 2021). Few studies have investigated the effect of maternal VD deficiency *in vivo* on offspring muscle development, but one study published in 2016 showed that pigs fed a high VD diet during pregnancy produced offspring with an increased number of muscle fibers in the longissimus dorsi and increased muscle fiber size in the longissimus dorsi and the psoas major at birth and weaning (Zhou et al., 2016). The number of muscle fibers within each muscle is set at around the time of birth in most mammals (with rodents being the exception to this where it is around weaning). Postnatal muscle growth therefore occurs as a result of increases in fiber size (hypertrophy) rather than fiber number, hence myogenesis predominantly occurs *in utero* (Brameld et al., 2003b). During fetal development, the primary muscle fibers are formed during the embryonic stage (early pregnancy), but because only a small number of fibers are formed during this stage, maternal nutrition has little impact on the size or number of these fibers. Later in pregnancy, during the fetal stage, secondary fibers are formed. The number

of fibers formed during this stage is dependent on the number of available myogenic precursor cells, the proliferation of which is highly sensitive to nutrients and maternal nutrition (Yan et al., 2013).

Observational human studies give rise to inconclusive results due to variables such as ethnicity, definition of VD deficiency, extent of confounder adjustment and methodology of measuring VD levels (Mao et al., 2023), therefore more *in vivo* studies are required to investigate the impact of VD deficiency during pregnancy on health of the offspring and muscle development and function. Hence, the aim of this study is to evaluate the effects of maternal VD deficiency on offspring body weight, tissue weight, muscle weight, grip strength, insulin tolerance and physical activity from birth through to adulthood using mice as a model. We hypothesise that maternal VD deficiency will result in stunted offspring growth, smaller muscles with reduced muscle strength and that VD deficiency will have a negative impact on insulin sensitivity.

5.3 Materials and Methods

All experiments were carried out under the UK Animals (Scientific Procedures) Act 1986 and all procedures were approved by the University of Nottingham Local Ethical Review Committee.

5.3.1 Animals and Diet

3-week-old virgin female C57BL/6 mice were purchased from Charles River and were randomly assigned to either the control (n = 20) or vitamin D deficient (VDD, n = 22) treatment group. Mice were housed under incandescent lighting on a 12-hour light cycle and temperature was maintained at 21 ± 1°C with *ad libitum* access to either control diet (TD.89123) containing 2.2 IU/g of vitamin D or VDD diet (TD.89124) containing 0 IU/g vitamin D (Inotiv, Leicester, UK). Dams were fed their respective

diet for approximately 14 weeks (8 weeks prior to mating, 3 weeks during pregnancy and 3 weeks during lactation). At weaning, offspring were placed onto the same diet

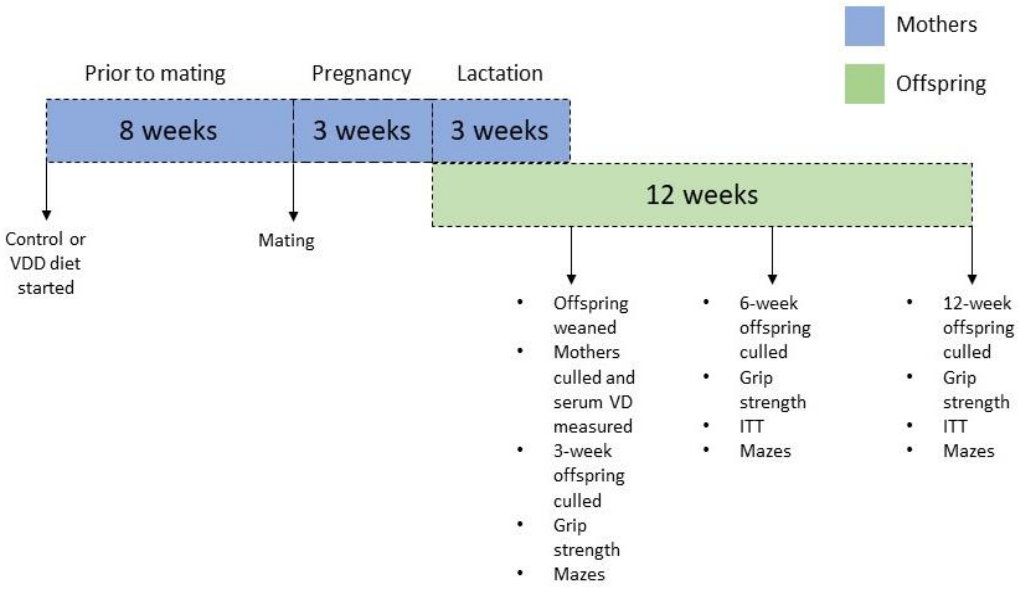


Figure 5.1. Schematic to show duration of study and measurements taken throughout. All virgin female mice were placed on either the control or vitamin D deficient (VDD) diet for 8 weeks prior to mating and remained on their respective diet throughout pregnancy and lactation. Offspring mice went onto the same diet as their mothers and experiments were conducted at 3, 6 and 12 weeks of age.

as their mother until the end of the study. Bodyweight and food intake were measured weekly in offspring mice (for weeks 1 – 3 bodyweight was calculated by weighing the whole litter and dividing by number of pups). Measurements in offspring mice were taken at 3, 6 and 12 weeks old (Figure 5.1).

5.3.2 Tissue and Blood Collection

Dams were euthanised at weaning and a sub-set of the offspring at 3, 6 and 12 weeks. All mice were euthanised under terminal anaesthesia using 5% (v/v) isoflurane gas. Cardiac puncture was performed to collect approximately 0.5ml of blood in heparin lithium microtubes (Sarstedt, Germany) followed by cervical dislocation. Plasma was separated by centrifugation, frozen and stored for subsequent measurement of vitamin D levels. Tissues collected were brain, liver,

kidneys (both kidneys combined for reported tissue weight), gastrocnemius muscle and soleus muscle (muscles were taken from both the right and left leg and the average muscle weight is reported), interscapular brown adipose tissue (BAT), abdominal white adipose tissue (AB WAT) and interscapular white adipose tissue (IS WAT). Each tissue was weighed then snap frozen on dry ice and then transferred to -80°C for subsequent analysis.

5.3.3 Insulin Tolerance Testing (ITT)

ITT was carried out on offspring at 6 and 12 weeks. It was not possible to carry out ITT on mice at 3 weeks old due to their size (<10g), according to NC3R guidelines. Mice were fasted for 6 hours then an initial blood glucose measurement was taken from the tail vein using a blood glucose monitor (Zoetis Alphatrack). Mice were then injected with 0.75 IU/kg of insulin (ActRapid®, Novo Nordisk A/S) via intraperitoneal (IP) injection. Blood glucose measurements were taken at 15-, 45- and 90-minutes post insulin injection. Any mice that experienced hypoglycaemia were given 200µl of 10% glucose in saline solution via IP injection and all results from those mice were excluded from any graphs and analysis.

5.3.4 Muscle Function (Grip Strength)

Grip strength was measured using a Bioseb BIO-GS3 grip strength monitor (BIOSEB, France). Mice were removed from their cage by gripping the base of the tail and then each mouse was lowered towards the bar to encourage them to grab onto the bar with their forelimbs. Once holding the bar, each mouse was pulled away from the bar at an even speed for 5 seconds, until the mouse let go. Peak force, in Newtons, was recorded. Each mouse performed 6 tests, 2 acclimation tests followed by 4 experimental tests, with a 60 second break between each one.

5.3.5 Physical Activity

The elevated plus maze (EPM) and the Y maze were used to assess physical activity. The same sub-set of mice were used for both the EPM and Y maze at 3, 6 and 12 weeks, with a minimum of three days between each test. Prior to assessment, all mice were acclimatised to the room for a minimum of 30 minutes. Each mouse was placed individually into the central platform of the maze, facing the same way, and activity was recorded for 5 minutes using a computerised tracking system (Ethovision 9, Moldus IT, The Netherlands). Total distance moved as well as time spent in each section of the maze was recorded.

5.3.6 Enzyme-Linked Immunosorbent Assay (ELISA)

A 25-Hydroxy vitamin D ELISA kit (Immunodiagnostic Systems Ltd, Boldon, UK) was used to measure total vitamin D (25(OH)D and other hydroxylated VD metabolites) in serum samples from all mice (mothers and offspring). Briefly, 25µl of sample/control/calibrator was added to 1ml of 50 x 25-D biotin solution, then 200µl of this was transferred to a 96 well antibody coated plate and incubated for 2 hours at room temperature. All wells were washed with the wash buffer provided 3 times, then 200µl of enzyme conjugate was added and plates were incubated for 30 minutes at room temperature. Wells were washed again 3 times and 200µl of TMB substrate was added before incubating the plate for a further 30 minutes at room temperature. Finally, 100µl of stop solution was added and absorbance at 450nm was read immediately using a plate reader (Fluostar Optima, BMG Labtech).

5.3.7 Statistics

Power calculations and statistical analyses were carried out using GenStat 22nd edition. Power calculations were based on standard deviations obtained from

previous pilot data (unpublished), with probably of a type I error (a) set at 0.05 and a type II error (p) set at a maximum of 0.20 to give a power of > 0.80. A total of 136 offspring were needed to achieve this, so 34 female mice (mothers) were required (based on 2 male and 2 female offspring per litter). However, to account for any mice lost during the study, or mice having an unsuccessful pregnancy, 42 female mice were utilised.

Most data were analysed by 3-way ANOVA (age*treatment*sex) with a *Post hoc* Bonferroni test where appropriate (i.e. if an effect or interaction was significant). Significance was deemed at $p < 0.05$.

5.4 Results

5.4.1 Maternal Impact of Vitamin D Deficiency

A total of 20 control and 22 VDD dams were mated with successful pregnancies in 12 control and 20 VDD dams (Table 5.1). Over the 7 weeks prior to mating, body weight and food intake were measured weekly (Figure 5.2). Whilst there was no significant time*treatment interaction ($p = 0.19$), there was a significant effect of both time ($p < 0.001$) and treatment ($p = 0.02$) on pre-pregnancy bodyweight with VDD mice weighing more than controls (Figure 5.2, A). There was also no significant time*treatment interaction for cumulative food intake ($p = 0.997$) and no difference between control and VDD mice ($p = 0.274$, Figure 5.2, B). Serum VD status of the mothers, measured at offspring weaning, showed that levels were significantly lower in mice from the VDD group than controls ($p < 0.001$, Figure 5.2, C). Average serum VD for control mice was 53.8nmol/L whilst for VDD mice it was 16.6nmol/L.

Table 5.1 – Numbers of pregnant and offspring mice and survival rates

	Control	**VDD**
Total no. of mothers	20	22
No. of mothers with confirmed pregnancy	12	20
Total no. of pups born	72	143
Births/mother	6	7.1
Range in litter size	1 – 9	3 – 9
No. of offspring for experiments	70	102
% survival	97.2%	71.3%

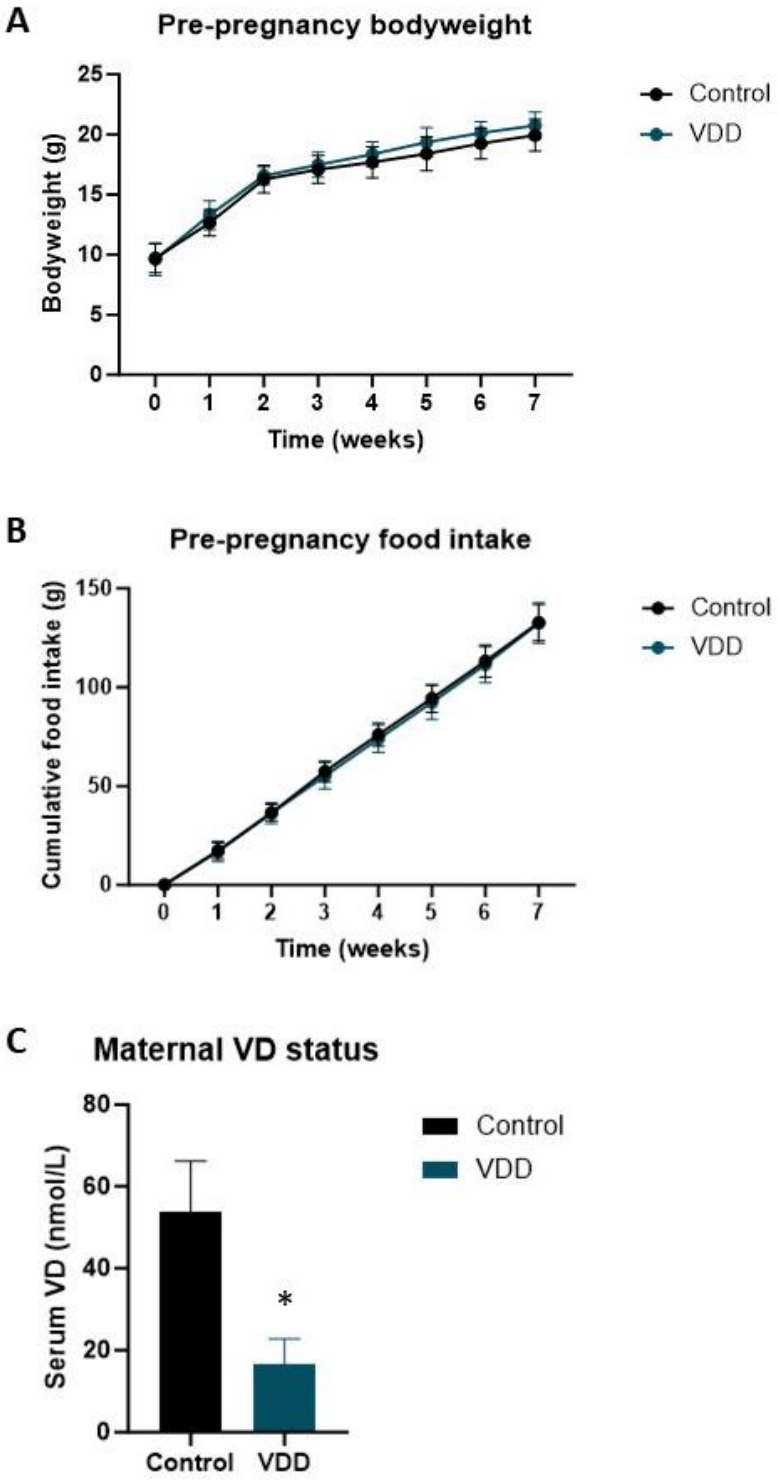


Figure 5.2. Impact of vitamin D deficiency on mothers. A. Bodyweight (g) of virgin female mice prior to mating. A significant effect of time ($p < 0.001$) and treatment ($p = 0.02$) was observed with VDD mice weighing more than controls. **B.** Cumulative food intake (g) of virgin female mice prior to mating. No difference in food intake was observed. **C.** Serum VD levels ($25(OH)D_3$) of mothers at offspring weaning. VDD mice had significantly lower levels of serum VD than controls (average = 53.8 nmol/L and 16.6 nmol/L respectively, * $p < 0.001$). Error bars show standard deviation. N = 12 control and 20 VDD. Data analysed by 2-way ANOVA for A and B and 1-way ANOVA for C.

5.4.2 Offspring Bodyweight, Food Intake and VD Status

Survival of offspring in the control group was 97.2% whilst in the VDD group it was 71.3% (Table 5.1). In most cases where offspring did not survive, they were cannibalised by the mother or found dead, but in a small number of cases (n = 4 for VDD and n = 2 for control) pups were culled due to ill health.

There was an age*treatment*sex interaction ($p < 0.001$, Figure 5.3, A) for offspring bodyweight. At week 1 there was no difference in bodyweight between the mice but from weeks 2 to 4, VDD males were significantly smaller than control males and VDD females were significantly smaller than control females ($p < 0.05$). VDD female mice then appeared to have catch up growth whereby from 5 to 12 weeks there was no difference in bodyweight between VDD and control females. However, VDD males remained significantly smaller than control males from week 2 to week 12 ($p < 0.05$).

No age*treatment*sex interaction was found for food intake ($p = 0.098$, Figure 5.3, B), but treatment*sex ($p < 0.001$) and a treatment*age ($p < 0.001$) interactions were found. *Post hoc* analysis on treatment*sex showed that there was no difference in food intake between control and VDD females, but that control males consumed significantly more than VDD males. Analysis on treatment*age showed that during weeks 3 to 5, there was no difference in food intake between control and VDD mice, but from week 6 to 12, control mice consumed significantly more than VDD mice ($p < 0.05$).

Serum samples were taken from culled mice at 3, 6 and 12 weeks to determine VD (25(OH)D) status (Figure 5.3, C). There was no age*treatment*sex interaction for offspring serum VD status, however there was a significant age*treatment interaction ($p = 0.002$). In control mice, average VD status remained similar between 3 and 6 weeks (44.2 and 44.7 nmol/L), but then significantly increased by week 12

(52.4 nmol/L, $p < 0.05$). In VDD mice, average VD status was significantly lower than the controls at all timepoints, with similar levels at weeks 3 and 6 (16.7 and 18 nmol/L), but then decreased further by week 12 (13.8 nmol/L, $p < 0.05$).

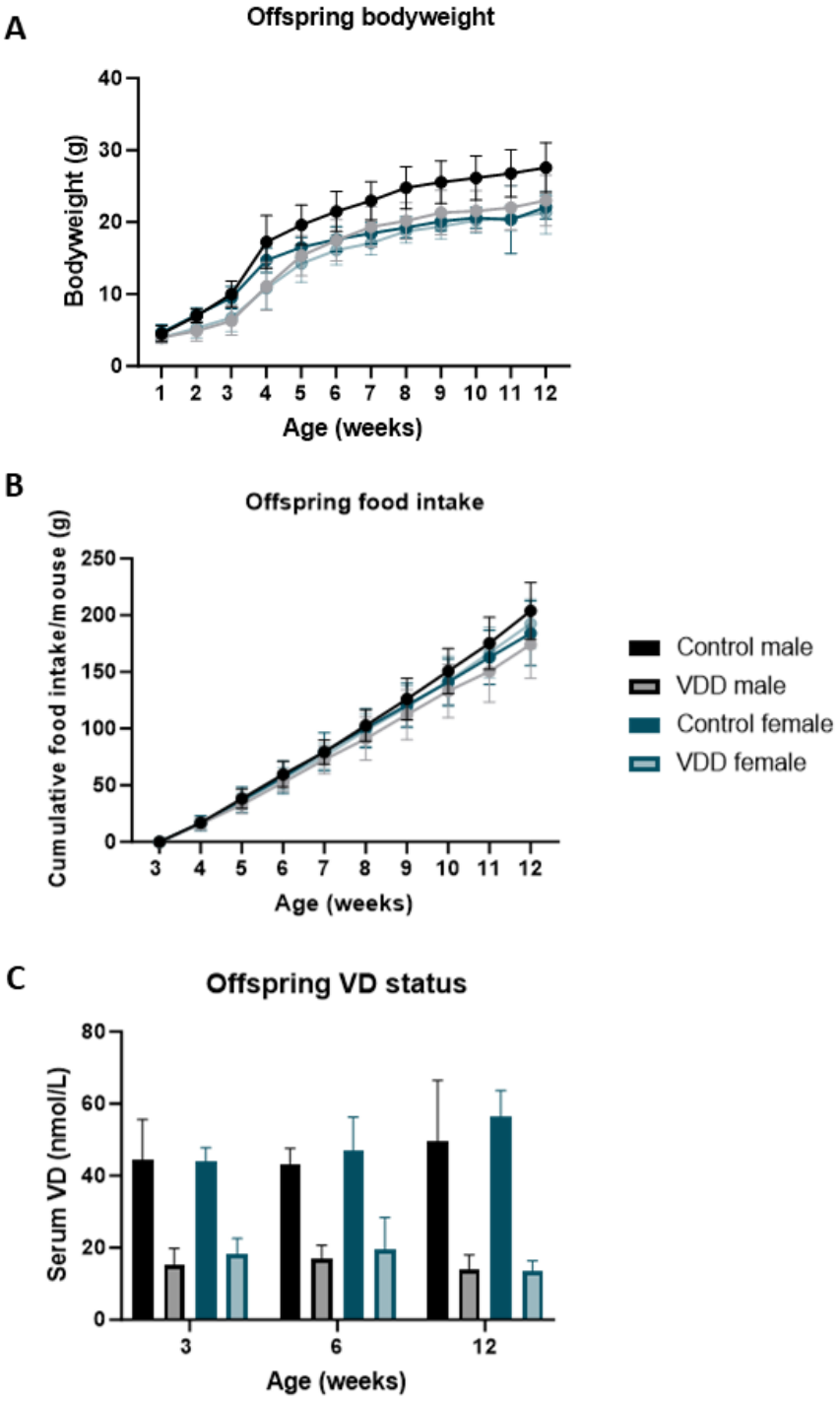


Figure 5.3. Offspring bodyweight, food intake and serum vitamin D status. A. Bodyweight (g) measured weekly (for weeks 1 – 3 the whole litter was weighed and divided by number of mice. An age*treatment*sex interaction was found ($p < 0.001$). B. Cumulative food intake (g) per mouse, measured weekly (total food intake per cage was divided by number of mice per cage, which was 2 in most cases, but 3 in a small number of cases). Both age*treatment ($p < 0.001$) and treatment*sex ($p < 0.001$) interactions were found. C. Serum VD status (25(OH)D) measured from blood samples taken at culling at 3, 6 and 12 weeks. A significant age*treatment interaction was observed ($p = 0.002$) with VD status remaining similar for each group from week 3 to 6, but then increasing in controls from week 6 to 12 and decreasing in VDDs from week 6 to 12. Average VD status overall was significantly lower in VDD mice (16nmol/L) than control mice (48.1nmol/L), $p < 0.001$. Error bars show standard deviation. N = 173 (70 control and 103 VDD).

5.4.3 Effects on Offspring Brain, Liver and Kidneys

Offspring tissues were collected at 3, 6 and 12 weeks old and included: brain, liver, kidney, interscapular brown adipose tissue (BAT), interscapular white adipose tissue (IS WAT), abdominal white adipose tissue (AB WAT), gastrocnemius muscle, and soleus muscle.

There was no age*treatment*sex interaction for brain weight ($p = 0.44$) however there was a treatment*sex interaction ($p = 0.03$, Figure 5.4, A). *Post hoc* analysis showed that brain weight for control and VDD females did not differ (429 and 423mg respectively) but that control males had a significantly higher brain weight (440mg) than VDD males (416mg, $p < 0.05$). There was also a treatment*age interaction ($p = 0.037$) where brain weight for control mice was significantly higher than VDD mice at 3 weeks ($p < 0.05$) but not at 6 or 12 weeks. When brain weight was adjusted to bodyweight (Figure 5.4, B), there was still a significant treatment*age interaction ($p = 0.018$), but the opposite effect was seen at 3 weeks where this time, VDD mice had a higher brain/BW than controls ($p < 0.05$) but no difference at 6 or 12 weeks.

For liver weight, there was no age*treatment*sex interaction ($p = 0.323$), but there was a treatment*sex interaction ($p = 0.001$) where control females and VDD females were not significantly different (812 and 887mg respectively), but control males had significantly higher liver weight (1147mg) than VDD males (947mg, $p < 0.05$, Figure 5.4, C). There was also a significant effect of age ($p < 0.001$) where liver weight increased with age in all treatment groups. When adjusted for bodyweight (Figure 5.4, D) the treatment*sex interaction was no longer present ($p = 0.118$), and there was no effect of treatment or sex individually ($p = 0.09$ and $p = 0.95$ respectively).

There was still a significant effect of age, but this time liver weight/BW decreased with age ($p = 0.01$).

For kidney weight, again there was no age*treatment*sex interaction ($p = 0.955$, Figure 5.4, E) but there was a treatment*sex interaction ($p = 0.013$) where control females and VDD females were not significantly different (206 and 211mg respectively), but control males had a significantly higher kidney weight than VDD males (280 and 235mg respectively, $p < 0.05$). There was also a treatment*age interaction ($p = 0.016$) where control mice had a significantly higher kidney weight than VDD mice at 3 weeks (197 and 147mg respectively, $p < 0.05$), but there was no difference at 6 and 12 weeks. When adjusted for bodyweight, both the treatment*sex and treatment*age interactions are lost ($p = 0.34$ and $p = 0.58$ respectively, Figure 5.4, F) and there was no effect of treatment or sex individually. There was a significant effect of age ($p = 0.03$) where kidney/BW decreased as age increased.

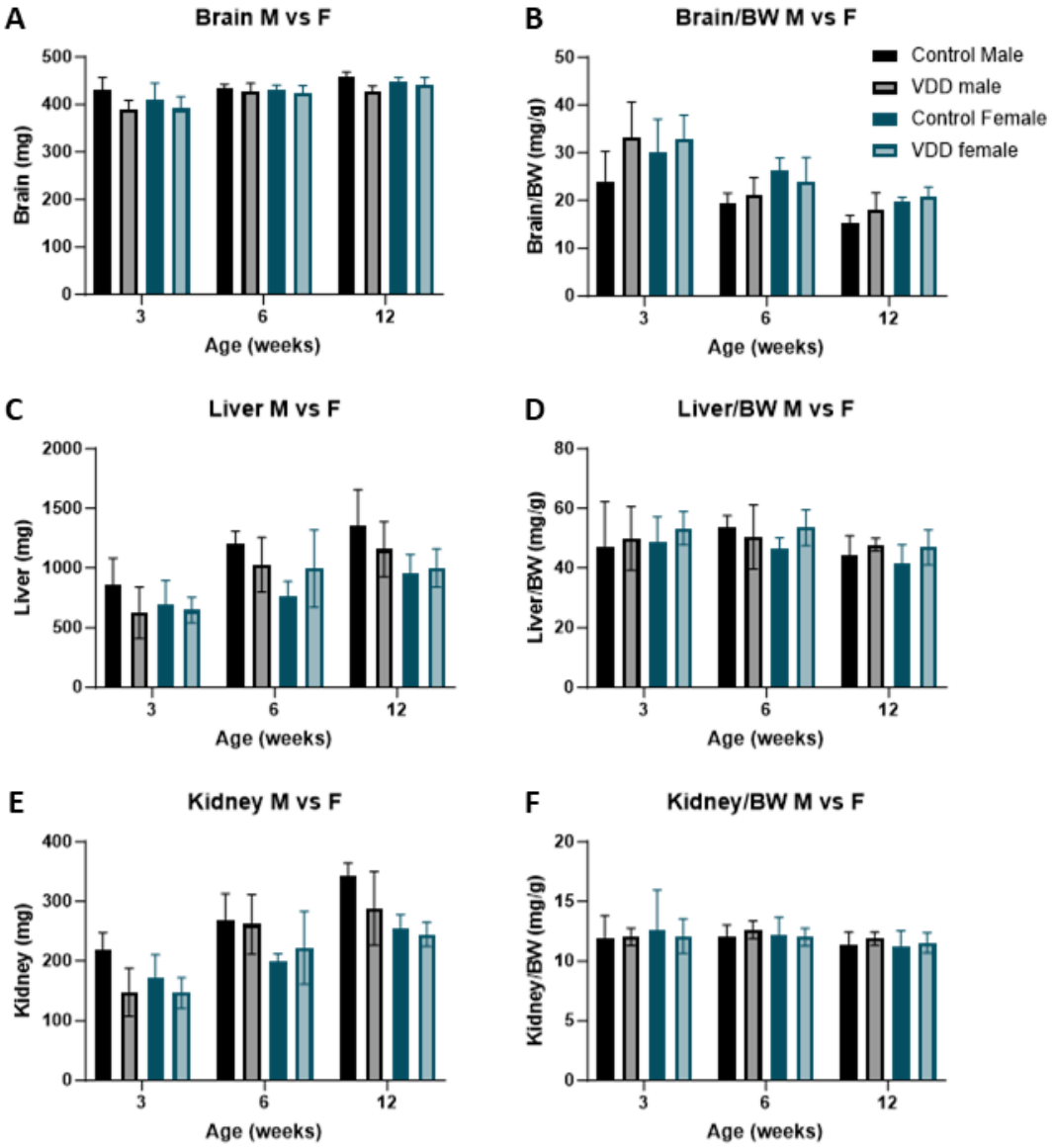


Figure 5.4. Offspring brain, liver, and kidney weight (mg) and weight corrected to bodyweight (mg/g). A. Brain weight (mg) at 3, 6 and 12 weeks. Significant treatment*sex ($p = 0.03$) and treatment*age ($p = 0.037$) interactions were found. B. Brain weight divided by bodyweight (mg/g). Significant treatment*sex ($p = 0.022$) and treatment*age ($p = 0.018$) interactions were found. C. Liver weight (mg) at 3, 6 and 12 weeks. A significant treatment*sex interaction ($p = 0.001$) was found as well as a significant effect of age ($p < 0.001$). D. Liver weight divided by bodyweight (mg/g). Only a significant effect of age ($p = 0.01$) was found. E. Kidney weight (mg) at 3, 6 and 12 weeks. Significant treatment*sex ($p = 0.013$) and treatment*age ($p = 0.016$) effects were found. F. Kidney weight divided by bodyweight (mg/g). Only a significant effect of age was found ($p = 0.03$). At 3 weeks n = 36 (8 control male, 7 control female, 11 VDD male, 10 VDD female). At 6 weeks n = 36 (8 control males, 6 control females, 16 VDD males, 6 VDD females). At 12 weeks n = 40 (8 control males, 6 control females, 9 VDD males, 17 VDD females). Error bars show standard deviation. Results analysed by 3-way ANOVA with *Post hoc* Bonferroni tests where appropriate (i.e. if an effect or interaction was significant). Significance was deemed at $p < 0.05$.

5.4.4 Effects on Offspring Adipose Tissue

For interscapular brown adipose tissue (BAT) weight, although there was no age*treatment*sex interaction, there was a treatment*sex interaction ($p = 0.013$), an age*sex interaction ($p < 0.001$) and an age*treatment interaction ($p = 0.05$, Figure 5.5, A). *Post hoc* analysis on treatment*sex showed that control and VDD females had similar BAT weights (89.8 and 86.9mg respectively) but control males had significantly more BAT than VDD males (113.5 and 78.6mg respectively, $p < 0.05$). *Post hoc* analysis on age*sex showed that at 3 and 6 weeks there was no difference between males and females, but at 12 weeks females had significantly more BAT than males (127 and 92mg respectively). Finally, *Post hoc* analysis on age*treatment revealed no difference between control and VDD mice at 3 or 6 weeks but at 12 weeks, control mice had significantly more BAT than VDD mice (137.5 and 94mg respectively, $p < 0.05$). When adjusted for bodyweight, only an age*sex interaction was seen ($p < 0.001$, Figure 5.5, B) with no difference between males and females at 3 and 12 weeks, but at 6 weeks females had significantly more BAT than males ($p < 0.05$). There was no effect of treatment on BAT/BW ($p = 0.269$).

For interscapular white adipose tissue (IS WAT), there were no interactions between treatment, age and sex (Figure 5.5, C). There was a significant effect of age with IS WAT weight increasing as age increased ($p < 0.001$) and a significant effect of treatment with control mice having more IS WAT than VDD mice (183.2 and 137.1mg, $p = 0.008$). When adjusted for bodyweight (Figure 5.5, D), again no interactions were observed, but there were significant effects of sex ($p < 0.001$), age ($p = 0.006$) and treatment ($p = 0.038$). On average, females had more IS WAT than males (9 and 6.5mg/g), controls had more IS WAT than VDD (8.6 and 7.1mg/g) and as age increased, IS WAT also increased (6.7, 7.4 and 8.7mg/g at 3, 6 and 12 weeks).

There was also no age*treatment*sex interaction for abdominal white adipose tissue (AB WAT), but there was a treatment*sex ($p = 0.019$) and a treatment*age ($p = 0.003$) interaction (Figure 5.5, E). *Post hoc* analysis revealed that control females and VDD females were not significantly different (355 and 306mg respectively) but that control males had significantly more AB WAT than VDD males (674 and 365mg respectively, $p < 0.05$). In addition, control and VDD mice had similar AB WAT weights at 3 and 6 weeks, but control mice had significantly more AB WAT than VDD mice at 12 weeks (966 and 505mg respectively, $p < 0.05$). When adjusted for bodyweight (Figure 5.5, F), the treatment*sex interaction was lost ($p = 0.09$) but the treatment*age interaction remained ($p = 0.003$), again there was no difference between control and VDD at 3 or 6 weeks, but at 12 weeks control mice had significantly more AB WAT than VDD mice (34 and 21mg/g respectively, $p < 0.05$). There was also an effect of sex on WAT AB/BW ($p = 0.02$), with males having higher levels than females (19.5 and 16.6mg/g respectively).

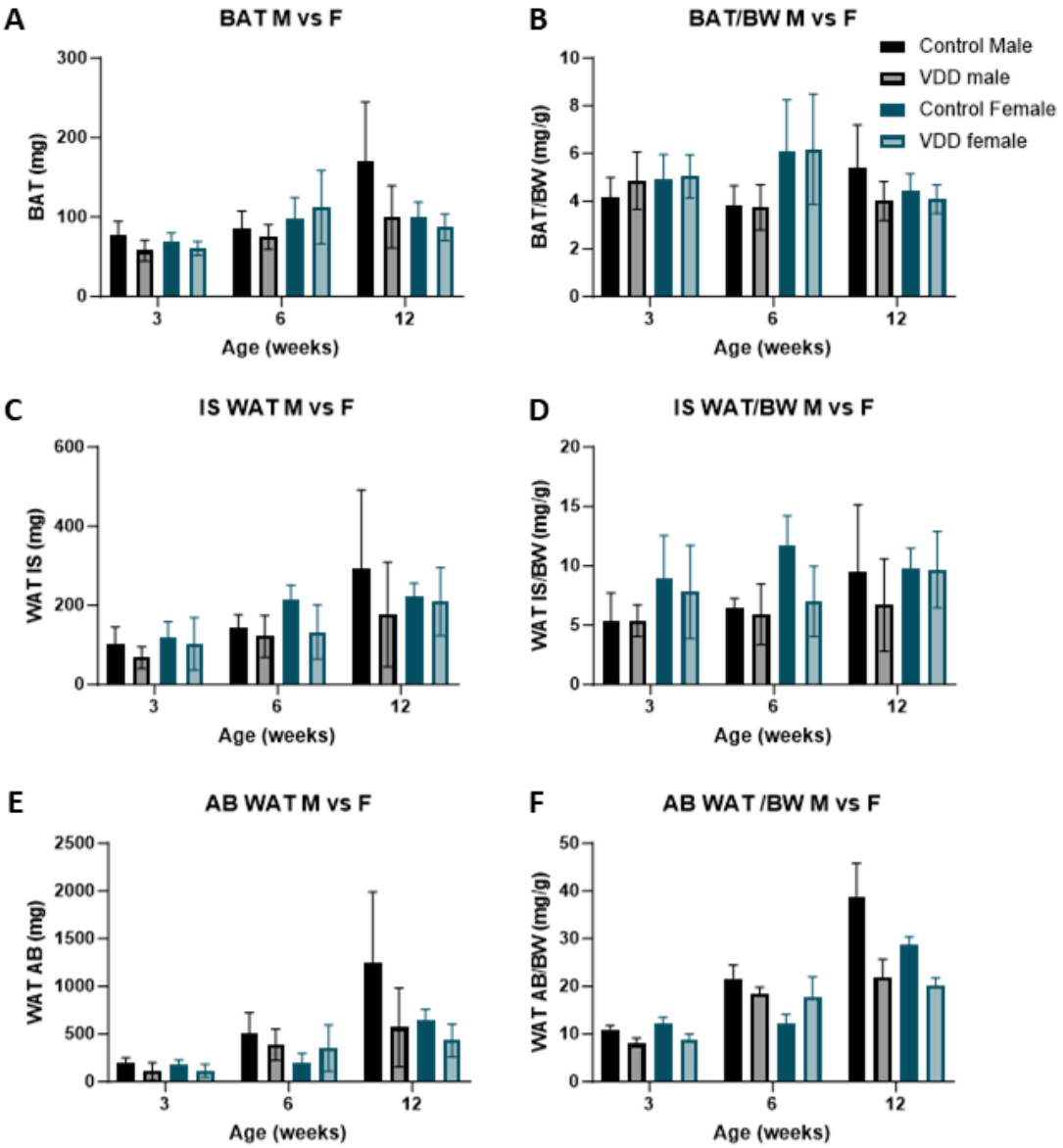


Figure 5.5. Offspring brown adipose tissue (BAT), interscapular white adipose tissue (IS WAT) and abdominal white adipose tissue (AB WAT) weight (mg) and weight corrected to bodyweight (mg/g). A. BAT weight (mg) at 3, 6 and 12 weeks. Treatment*sex ($p = 0.013$), age*sex ($p < 0.001$) and age*treatment ($p = 0.05$) interactions were found. B. BAT weight divided by bodyweight (mg/g). A significant age*sex interaction was found. C. IS WAT weight (mg) weight at 3, 6 and 12 weeks. No interactions were found but a significant effect of both age ($p < 0.001$) and treatment ($p = 0.008$) were found. D. IS WAT divided by bodyweight (mg/g). Significant effects of sex ($p < 0.001$), age ($p = 0.006$) and treatment ($p = 0.038$) were found. E. AB WAT weight (mg) at 3, 6 and 12 weeks. Both treatment*sex ($p = 0.019$) and treatment*age ($p = 0.003$) interactions were found. F. AB WAT divided by bodyweight (mg/g). Only a treatment*age interaction was found ($p = 0.003$). At 3 weeks n = 36 (8 control male, 7 control female, 11 VDD male, 10 VDD female). At 6 weeks n = 36 (8 control males, 6 control females, 16 VDD males, 6 VDD females). At 12 weeks n = 40 (8 control males, 6 control females, 9 VDD males, 17 VDD females). Error bars show standard deviation. Results analysed by 3-way ANOVA with *Post hoc* Bonferroni tests where appropriate (i.e. if an effect or interaction was significant). Significance was deemed at $p < 0.05$.

5.4.5 Effects on Offspring Muscle

Gastrocnemius (gastroc) and soleus muscles were taken from both hind legs (reported weight is the average for the right and left leg). For the gastroc muscle, no age*treatment*sex interaction was observed ($p = 0.776$) but both treatment*sex ($p = 0.001$) and treatment*age ($p = 0.018$) interactions were seen (Figure 5.6, A). *Post hoc* analysis on treatment*sex showed that control and VDD females were similar (83 and 86.9mg respectively) but that control males had significantly larger gastroc muscles than VDD males (120 and 91.9mg, $p < 0.05$). *Post hoc* analysis on treatment*age showed that control mice had significantly larger gastroc muscles than VDD mice at 3 weeks (72 and 49.8mg respectively, $p < 0.05$), but there was no difference between control and VDD at 6 or 12 weeks. When adjusted for bodyweight (Figure 5.6, B), both treatment*sex ($p = 0.254$) and treatment*age ($p = 0.225$) interactions were lost, and this time an age*sex interaction was seen ($p = 0.004$). *Post hoc* analysis revealed that there was no difference between males and females at 3 and 12 weeks, but at 6 weeks males had significantly larger gastroc muscles than females (5.1 and 4.6mg/g, $p < 0.05$). There was no significant effect of treatment when gastroc weight was corrected for bodyweight ($p = 0.08$), however it was close to significance with controls having larger gastroc muscles than VDD (4.9 and 4.6mg/g respectively).

For soleus muscle weight, again there was no significant age*treatment*sex interaction ($p = 0.64$, Figure 5.6, C), but there were significant interactions for treatment*sex ($p = 0.005$) and age*sex ($p = 0.015$). *Post hoc* analysis on treatment*sex showed that there was no difference in soleus weight between control and VDD females (5.3 and 4.3mg) but control male soleus muscles weighed significantly more than VDD males (7.3 and 4.9mg, $p < 0.05$). *Post hoc* analysis on

age*sex revealed that males had significantly heavier soleus muscles than females at 6 weeks (6.1 and 3.9mg, $p < 0.05$), but there was no difference between males and females at 3 or 12 weeks. When adjusted for bodyweight (Figure 5.6, D), the age*sex interaction remained ($p < 0.001$) with males having larger soleus/BW than females at 6 weeks but not at 3 or 12 weeks ($p < 0.05$). A treatment*age interaction ($p = 0.008$) was also seen for soleus/BW with control mice being higher than VDD mice at both 3 weeks and at 6 weeks ($p < 0.05$), but not at 12 weeks.

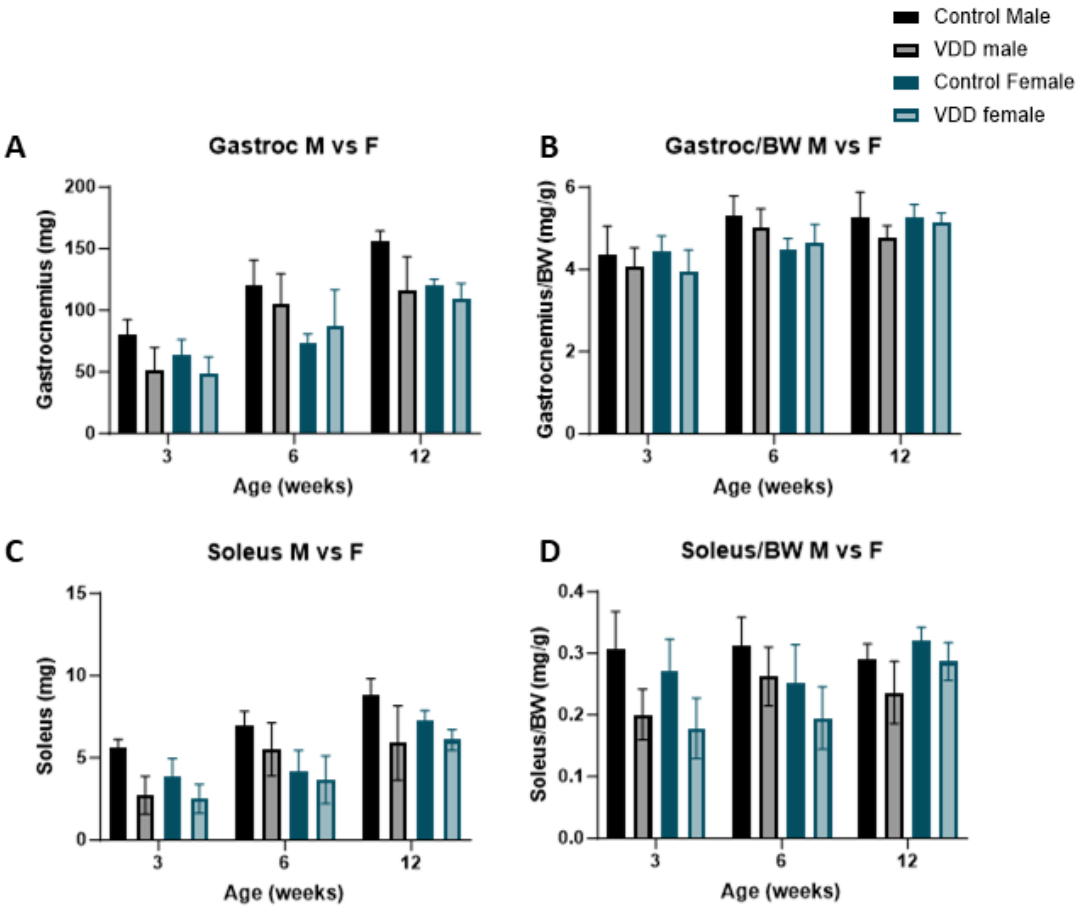


Figure 5.6. Offspring gastrocnemius (gastroc) muscle and soleus muscle weight (mg) and weight corrected to bodyweight (mg/g). A. Gastroc weight (mg) at 3, 6 and 12 weeks. Treatment*sex ($p = 0.001$) and treatment*age ($p = 0.018$) interactions were found. B. Gastroc weight divided by bodyweight (mg/g). An age*sex interaction was found ($p = 0.004$). C. Soleus weight (mg) at 3, 6 and 12 weeks. Treatment*sex ($p = 0.005$) and age*sex ($p = 0.015$) interactions were found. D. Soleus weight divided by bodyweight (mg/g). Both age*sex ($p < 0.001$) and treatment*age ($p = 0.008$) interactions were found. At 3 weeks n = 36 (8 control male, 7 control female, 11 VDD male, 10 VDD female). At 6 weeks n = 36 (8 control males, 6 control females, 16 VDD males, 6 VDD females). At 12 weeks n = 40 (8 control males, 6 control females, 9 VDD males, 17 VDD females). Error bars show standard deviation. Results analysed by 3-way ANOVA with *Post hoc* Bonferroni tests where appropriate (i.e. if an effect or interaction was significant). Significance was deemed at $p < 0.05$.

5.4.6 Effects on Offspring Insulin Tolerance

At 6 weeks, there was no treatment*sex interaction for fasting glucose levels ($p = 0.54$) and no effect of treatment ($p = 0.52$, Figure 5.7, A). There was a significant effect of sex ($p = 0.02$) with male mice having higher fasting glucose levels than females. At 12 weeks, there was also no treatment*sex interaction ($p = 0.23$, Figure 5.7, B) and no effect of treatment or sex individually ($p = 0.41$, $p = 0.5$ respectively). At 6 weeks (Figure 5.7, B), there was no time*treatment*sex interaction ($p = 0.09$) but there were both time*sex ($p < 0.001$) and treatment*sex ($p < 0.001$) interactions. *Post hoc* analysis on time*sex showed that there was no difference between male and female mice at 0, 15 or 45 minutes, but at 90 minutes female mice had a significantly higher blood glucose level than males ($p < 0.05$). *Post hoc* analysis on treatment*sex showed that control males and VDD males had a similar blood glucose level (average 8.4 and 9.8mmol/L respectively) but that control females had significantly lower blood glucose levels on average than VDD females (7.2 and 13.6mmol/L respectively, $p < 0.05$). At 12 weeks (Figure 5.7, C), no interactions between time, treatment and sex were observed. As expected, there was a significant effect of time ($p < 0.001$) with glucose levels decreasing over time, and there was also a significant effect of treatment ($p < 0.001$), with average blood glucose levels being significantly lower for control mice than VDD mice (8 and 10.5mmol/L). No effect of sex was observed. The number of mice for each treatment group that experienced hypoglycaemia can be seen in table 5.2.

Table 5.2 – N numbers and numbers of hypoglycaemic mice for insulin tolerance testing.

6 weeks				
	CON male	**VDD male**	**CON female**	**VDD female**
N number	8	8	6	8
No. of hypoglycaemia	1	1	2	4
% hypoglycaemia	12.5%	12.5%	33%	50%
12 weeks				
	CON male	**VDD male**	**CON female**	**VDD female**
N number	7	7	6	8
No. of hypoglycaemia	3	1	1	0
% hypoglycaemia	43%	14%	17%	0%

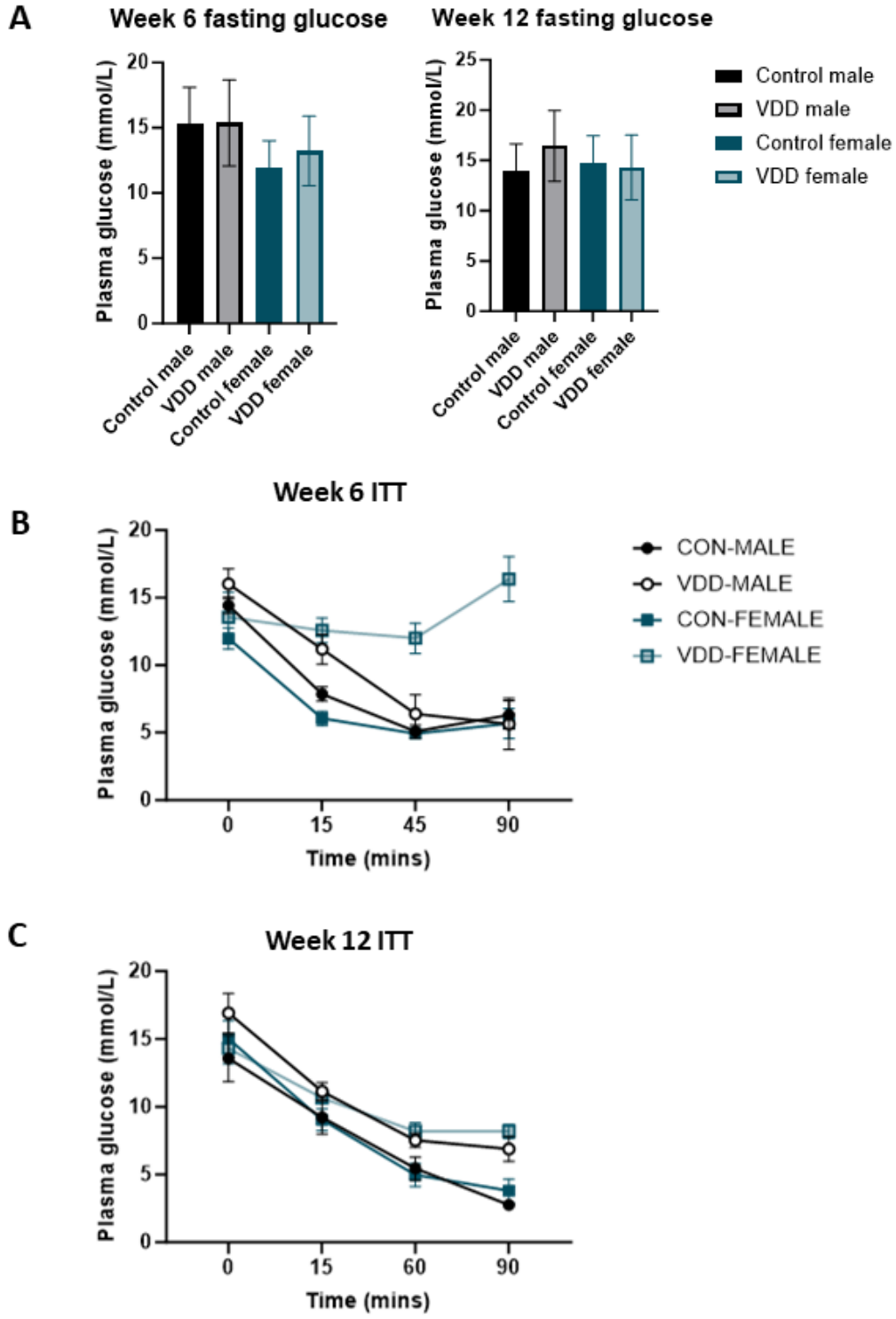


Figure 5.7. Offspring insulin tolerance testing (ITT). Offspring mice at 6 and 12 weeks were dosed with 0.75IU/kg of insulin via intraperitoneal injection and blood glucose measured at 0 minutes (prior to insulin injection) then at 15-, 45- and 90-minutes post injection. A. Fasting blood glucose levels at 0 minutes (including all mice) at 6 and 12 weeks. B. ITT at 6 weeks excluding mice which had hypoglycaemia. Time*sex ($p < 0.001$) and treatment*sex ($p < 0.001$) interactions were found. C. ITT at 12 weeks excluding mice which had hypoglycaemia. No interactions between time, treatment and sex were found but there was a significant effect of time ($p < 0.001$) and treatment ($p < 0.001$). Error bars show standard deviation. A repeated measures design was used, and results analysed by 3-way ANOVA with *Post hoc* Bonferroni tests where appropriate (i.e. if an effect or interaction was significant). Significance was deemed at $p < 0.05$. N numbers can be seen in table 5.2.

5.4.7 Effects on Offspring Grip Strength

Grip strength was measured in offspring mice at 3, 6 and 12 weeks and peak force (N) was recorded. Whilst there was no age*treatment*sex interaction for grip strength ($p = 0.213$) there was a treatment*sex interaction ($p = 0.005$, Figure 5.8, A). *Post hoc* analysis showed that control males had significantly higher grip strength than VDD males, control females and VDD females ($p < 0.05$). There was no difference in grip strength between control and VDD females. There was also a significant effect of age ($p < 0.001$), with grip strength increasing as age increased. When adjusted for bodyweight (Figure 5.8, B), no interactions between age, treatment and sex were observed. There was still a significant effect of age ($p < 0.001$), but no effect of treatment ($p = 0.79$) or sex ($p = 0.77$). When gastroc weight was plotted against grip strength at 3, 6 and 12 weeks, a positive correlation was found ($R^2 = 0.67$, $p < 0.005$, Figure 5.8, C).

5.4.1 Effect on Offspring Physical Activity

For the plus maze (Figure 5.9, A), there was no age*treatment*sex interaction ($p = 0.78$) but there was an age*treatment interaction ($p = 0.05$). *Post hoc* analysis showed that at 3 weeks, total distance moved by control mice was significantly higher than VDD mice ($p < 0.05$) but there was no difference at 6 and 12 weeks. For the Y maze (Figure 5.9, B), there were no significant interactions between age, treatment, and sex but a significant effect of treatment ($p = 0.002$) was found with control mice moving significantly further than VDD mice.

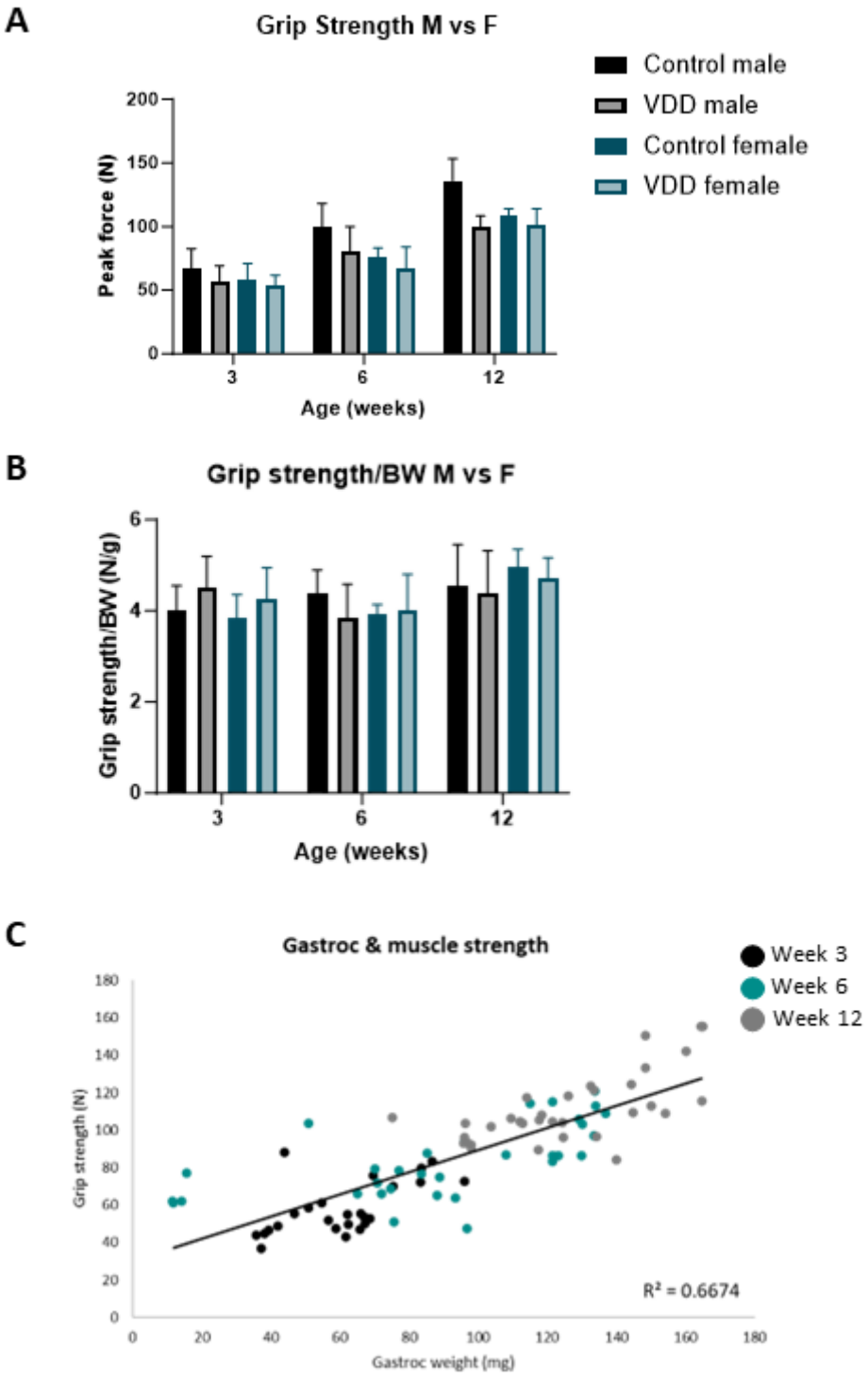


Figure 5.8. Offspring grip strength. Grip strength was measured at 3, 6 and 12 weeks and peak force recorded. A. Grip strength (N). A treatment*sex interaction ($p = 0.005$) was found as well as a significant effect of age. B. Grip strength divided by bodyweight (N/g). No interactions were observed however there was still a significant effect of age ($p < 0.001$). C. Gastroc weight and grip strength correlation. A positive correlation was found, $R^2 = 0.67$, $p < 0.005$. N numbers = 18 controls (8 males and 10 females) and 25 VDD (12 males and 13 females). Error bars show standard deviation. Results analysed by 3-way ANOVA with *Post hoc* Bonferroni tests where appropriate (i.e. if an effect or interaction was significant). Significance was deemed at $p < 0.05$.

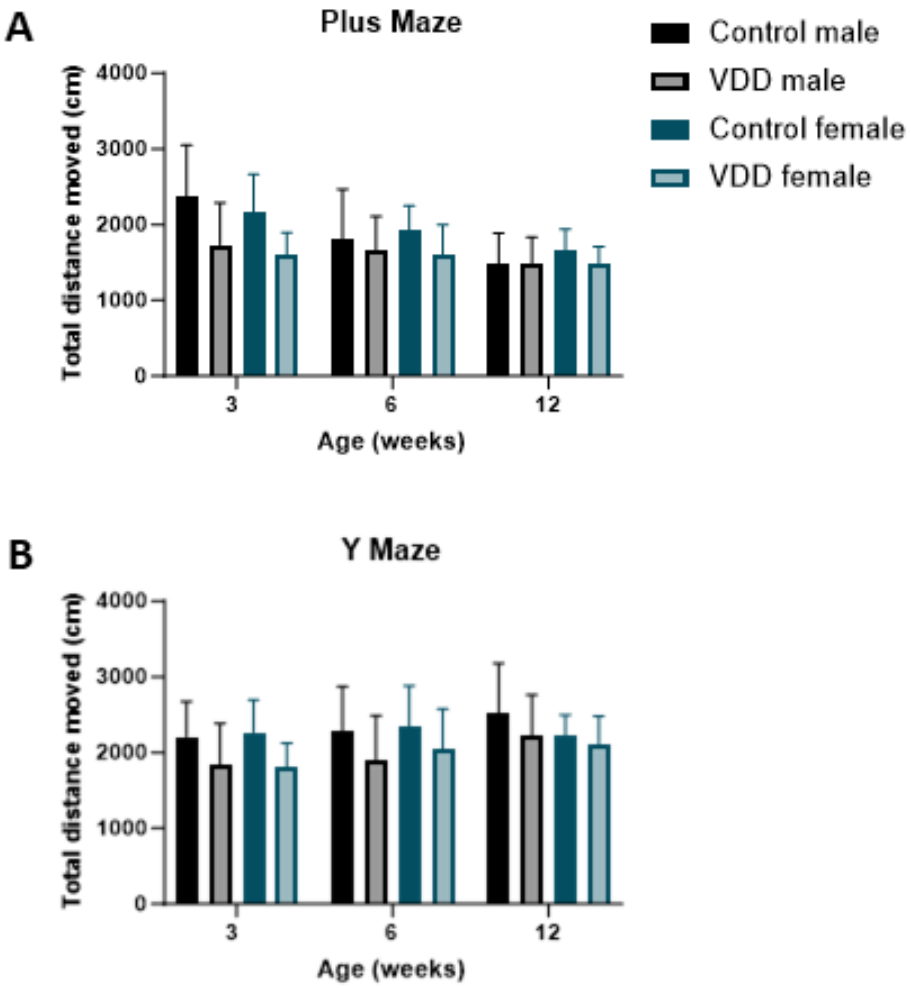


Figure 5.9. Offspring mazes. Total distance moved in the plus maze and Y maze measured at 3, 6 and 12 weeks. A. Plus maze total distance moved (cm). An age*treatment interaction was found ($p = 0.05$). B. Y maze total distance moved (cm). A significant effect of treatment was found ($p = 0.002$). N numbers = 17 controls (9 male, 8 female) and 16 VDD (8 male, 8 female). Error bars show standard deviation. Results analysed by 3-way ANOVA with *Post hoc* Bonferroni tests where appropriate (i.e. if an effect or interaction was significant). Significance was deemed at $p < 0.05$.

5.5 Discussion

For the first time, this study has provided preliminary data for the effects of maternal VD deficiency, followed by offspring VD deficiency from infancy into early adulthood. It appears that maternal and infant VD deficiency does not affect birth weight, but causes stunted growth, and reduces the size of certain tissues in proportion to bodyweight, particularly in male offspring. Maternal VD deficiency also causes offspring insulin resistance and a decrease in physical activity.

5.5.1 Body and Tissue Weight

Maternal VD deficiency did not affect offspring bodyweight at 1 week old, but from weeks 2 to 4 both VDD males and VDD females were smaller than their controls, despite no differences in food intake post weaning (weeks 3-4), possibly indicating an effect of VD on metabolism. This is similar to findings from other rodent studies which also find no difference in birth weight between pups born to control or VDD mothers (Reichetzeder et al., 2014, Nascimento et al., 2012, Zhang et al., 2014). Interestingly, female offspring appeared to have catch up growth from week 5 as there were no differences in bodyweight between control and VDD females from weeks 5 to 12, whereas VDD males remained smaller than control males throughout. Similarly, Reichetzeder et al (2014), found that offspring growth from VDD dams was stunted compared to controls from age 4 weeks onwards but this study did not separate mice by sex. This stunted growth was despite VDD pups being weaned onto a control diet (Reichetzeder et al., 2014), suggesting that exposure to VD deficiency during development may have an irreversible impact on offspring growth. In contrast, many studies in humans show that maternal VD deficiency is associated with low birth weight (Aghajafari et al., 2013), but maternal VD deficiency also

increases risk of pre-term birth so it is not clear whether this data is skewed due to a higher prevalence of pre-term births.

It was found that brain, liver, kidney, BAT, AB WAT, gastroc and soleus weight were all affected by VD deficiency in males only with control males having higher tissue weights than VDD males, but no difference between control and VDD females. After adjusting for bodyweight, the tissue weights which were affected by VD deficiency were brain, IS WAT, AB WAT and the soleus muscle. VDD offspring were found to have a larger brain/BW than controls at 3 weeks, but not at 6 or 12 weeks. Control offspring were found to have a higher level of IS WAT/BW than VDD offspring across all ages suggesting that IS WAT may be influenced by maternal VD status because effects were seen at weaning. On the other hand, AB WAT/BW was found to be higher in control offspring than VDD offspring at 12 weeks, but not different at 3 or 6 weeks, which could indicate that AB WAT weight was influenced more by offspring diet than maternal VD status. Likewise, in a study where male rats were put onto a VDD diet at 3 weeks old, VDD rats were found to have lower visceral fat and lower total body fat than controls (Bhat et al., 2014). This was found to be reversible with supplementation, however, unlike this study, these rats were not exposed to maternal VD deficiency during fetal development so whether effects are reversible after exposure to VD deficiency during fetal development remains unclear. In contrast, another study in mice, which did subject mothers to either a control or VDD diet during pregnancy then weaned pups onto a high fat diet, found that there was no difference in adiposity for female offspring, but that male pups from VDD mothers had a higher adiposity index than controls (Haroun et al., 2022). In humans, newborns of women with VD deficiency are reported to have higher levels of subcutaneous adipose tissue at 2 weeks old compared to newborns from VD sufficient mothers, but no further measurements are reported after 2 weeks

(Tint et al., 2018). However, many studies have shown that obese pregnant women tend to have a lower VD status than non-obese pregnant women, due to a larger volume of adipose tissue resulting in increased VD storage (Alhomaid et al., 2021). Therefore, it is not clear whether the higher levels of subcutaneous adipose tissue seen in offspring from VD deficient mothers in human studies is a direct effect of VD status or due to the increased likelihood of maternal obesity.

This study finds that control offspring had a larger soleus/BW, (slow-twitch, predominantly type I fibers), compared to VDD offspring at 3 and 6 weeks but no difference was found at 12 weeks. Whilst control offspring also had larger gastrocnemius muscles, (fast twitch, predominantly type II fibers), than VDDs, the effect of treatment was not quite significant ($p = 0.08$). A recent study in rats (Reis et al., 2022), which fed a VDD diet to mothers during pregnancy and lactation, but weaned pups onto a control diet, found that male offspring from VDD mothers had smaller extensor digitorum longus (EDL, type II, fast twitch), soleus and gastrocnemius muscles at 3 weeks old compared to controls. No difference was observed for female offspring (Reis et al., 2022). Our study did also find that control males had significantly larger soleus muscles than VDD males, with no difference between females, but this sex effect was lost after accounting for bodyweight. Interestingly, in Reis et al (2022), the EDL for males from VDD mothers showed a 20% decrease in number of muscle fibers but a 17% increase in fiber cross sectional area, increased levels of MHC IIa, IIx and IIb fibers (measured by immunostaining), a two-fold increase in intramuscular calcitriol levels and a 58% increase in CYP27B1 protein expression (the enzyme which converts inactive VD to active VD). Serum levels of VD were significantly lower in VDD rats compared to control rats in this study (Reis et al., 2022). The authors of this study concluded that in male offspring from VDD mothers, there is a localised over-compensation of intracellular VD levels

within fast twitch muscle, which leads to this increase in levels of MHC type 2 fibers. On the other hand, a study in rats which fed dams a control or VDD diet prior to and during pregnancy, found that newborn rats from VDD mothers had smaller muscle fiber diameters in the gastrocnemius muscle compared to offspring from control dams (Max et al., 2014). In another study, where pigs were fed a high dose VD diet from mating to weaning, both *the* longissimus (LM) and psoas major (PM) were larger in offspring piglets from the high dose VD mothers at weaning, despite no difference in bodyweight (Zhou et al., 2016). Overall, it appears that maternal VD status is positively associated with offspring muscle weight for both fast and slow twitch muscles, but the effects on muscle fiber size and type seem less clear. Due to time constraints, it was not possible to carry out fiber typing and cross-sectional analysis on muscle samples in this study, but future work should include this.

5.5.2 Insulin Tolerance

For the first time, this study investigated the effects of maternal and infant VD deficiency on offspring insulin tolerance. It was found that VDD female mice were insulin resistant at 6 weeks old, and at 12 weeks old both male and female VDD mice were insulin resistant compared to controls. However, at both 6 and 12 weeks, VD deficiency did not have any effect on fasting blood glucose levels. At 6 weeks, although control males did have a lower average blood glucose level across the test than VDD males (8.4 vs 9.8mmol/L), it was not statistically significant and the difference between control and VDD females was much larger (7.2 and 13.6mmol/L, $p < 0.05$). At 6 weeks, control females had more interscapular white adipose tissue (IS WAT) than VDD females, whereas control and VDD males had similar amounts (Figure 5.5, D). GLUT4 receptors are insulin regulated glucose transporters and are present within adipose and muscle tissue to a high extent, removing glucose from

the blood into adipose/muscle tissue in response to insulin (James et al., 1988). It is possible that VDD females could have been less sensitive to insulin at 6 weeks because they had less adipose tissue/BW and so a lower ability to remove glucose from the blood. Also at 6 weeks, male mice had larger gastroc/BW and soleus/BW compared to females, which could further explain the reduced the level of insulin sensitivity in VDD female mice. However, of the 8 VDD female mice used for ITT at 6 weeks, 4 experienced hypoglycaemia and were removed from all data analysis. It could be argued that these VDD female mice were extremely sensitive to insulin, resulting in hypoglycaemia, opposing the results for the VDD females which were included. Therefore, no clear conclusions can be drawn for the effects of insulin tolerance in VDD mice, particularly females, at 6 weeks so ITT data from 6 week old mice should be interpreted with caution and ideally repeated in future.

At 12 weeks, both male and female VDD mice showed insulin resistance compared to controls, and this time, there were no differences between males and females. Again, this could be due to differences in abdominal tissue with control mice having higher levels of IS WAT across all ages and higher levels of AB WAT at 12 weeks compared to VDD mice. As no difference in muscle mass was found at 12 weeks in this study (control mice did have larger soleus/BW than VDD at 3 and 6 weeks, but not at 12 weeks), it is unlikely that differences in insulin tolerance at week 12 are as a result of muscle mass. However, muscle fiber type could play a role in the insulin response, which was not investigated due to time constraints, so cannot be ruled out.

These findings align with findings from other rodent studies where glucose tolerance tests have been carried out. In these studies, dams were exposed to a VDD diet during pregnancy and pups were weaned onto a control diet, but in all cases, offspring from VDD mothers had higher blood glucose levels during a glucose

tolerance test when compared to offspring from control mothers (Reichetzeder et al., 2014, Chen et al., 2023, Zhang et al., 2014). Interestingly, in Zhang et al (2014), it was found that at 15 weeks old, offspring from VDD mothers had significantly higher fasting insulin levels compared to offspring from control mothers, despite no difference in fasting blood glucose levels. This suggests that maternal VD deficiency decreases offspring insulin sensitivity and therefore higher insulin levels are required to maintain the same blood glucose levels. Additionally, a recent study in adult pre-diabetic rats found that VD supplementation improved fasting blood glucose levels which was associated with increased expression of PPAR-Y within adipose tissue (Krisnamurti et al., 2023). It is well established that levels of PPAR-Y, expressed in tissues such as fat and muscle, is positively associated with insulin sensitivity (Spiegelman, 1997), and that activation of PPAR-Y improves insulin sensitivity in both rodents and humans (Tontonoz and Spiegelman, 2008). It is not fully understood how PPAR-Y improves insulin sensitivity but it is known that PPRAR-Y binds to the retinoid X receptor (RXR) to form a heterodimer which can bind to DNA and regulate transcription of genes involved in cellular differentiation, glucose and lipid metabolism and lipid storage (Fürnsinn and Waldhäusl, 2002). One theory, called the lipid steal hypothesis, suggests that PPAR-Y increases the capacity of adipose tissue to store lipids, sequestering lipids into adipose tissue and away from muscle tissue, resulting in lower levels of lipid accumulation in muscle and thus, increased insulin sensitivity (Tontonoz and Spiegelman, 2008). In humans, VD has been shown to improve insulin sensitivity in pre-diabetic adults which was associated with an increased number of insulin receptors in muscle and increased expression of adipogenic PPAR-Y (Hoseini et al., 2013). Due to lack of time in this study, gene and protein expression of the insulin receptor and PPAR-Y within tissues could not be carried out, but future work should investigate this.

5.5.3 Muscle strength and physical activity

In this study, control males had significantly higher grip strength than VDD males but there was no difference between control and VDD females. When bodyweight was adjusted for, there was no effect of treatment on grip strength suggesting that the increased grip strength seen in control males was as a direct effect of larger muscles and a larger bodyweight in this group. This was also supported by a strong positive correlation found between gastrocnemius weight and grip strength, again suggesting that increased grip strength in control males compared to VDD males was due to larger muscles. Other studies have investigated the impact of VD on grip strength; however, this has usually been done in adult mice rather than looking at maternal VD deficiency and the effects on the offspring. Studies in VDR knockout mice show that when VDR is absent, grip strength is significantly reduced (Girgis et al., 2015, Girgis et al., 2019, Nakamura et al., 2020). Additionally, the Southampton women's survey, which included 678 children, found that maternal VD status was positively correlated with offspring grip strength at 4 years old (Harvey et al., 2014). On the other hand, a randomised control trial in humans where mothers were either given a high dose VD supplement (28,000 IU/week) or a placebo during pregnancy showed that there was no difference in offspring grip strength at 4 years old between the two groups, despite the women in the placebo group being classed as VD deficient at the end of pregnancy (O'Callaghan et al., 2022). Therefore, whilst results of rodent studies do tend to show that VD increases grip strength, potentially as a result of increased body/muscle weight, results in humans are less clear.

This study also found that physical activity was higher in control offspring compared to VDD. Mice moved a greater distance in the plus maze at 3 weeks, but not 6 or 12, and a greater distance in the Y maze at all ages. No other studies could be found where physical activity was measured in offspring from VD deficient mothers,

however one study in VDR knockout mice did measure endurance and found that endurance was reduced when VDR was absent (Girgis et al., 2015). The difference in results seen between the two mazes in this study could be due to other factors influencing physical activity such as brain activity and anxiety-like behaviours (which was measured in a separate study using the same mice). In the Y maze, every arm is identical and has walls on both sides, so anxiety-like behaviours are less likely to affect the total distance moved. However, in the plus maze, two arms were 'closed' (had walls on either side) and two arms were 'open'. A significant difference was observed between control and VDD mice where VDD mice spent significantly more time in the closed arms compared to controls ($p < 0.05$). This suggests that VD may have influenced brain activity and anxiety in these mice which could have influenced total distance moved.

Unlike other studies, this study investigated maternal VD deficiency followed by continued VD deficiency from infancy through to early adulthood which may be more physiologically relevant considering that offspring from VD deficient mothers are likely to also be VD deficient throughout childhood (Dawodu and Wagner, 2007). The range of measurements taken during this study, including insulin tolerance testing, gives an indication of offspring metabolic heath as well as body and tissue weights. Due to time limitations, it was not possible to carry out analysis such as muscle fiber typing and gene and protein expression in the tissues, but future work should do this to investigate the mechanisms behind the effects seen. It was also not possible, due to the increased length of time that the study would have taken, to swap offspring diets so that offspring from VDD mothers were split into two groups; one receiving a diet containing VD and another which was VDD, and vice versa for controls. A study of this design would have provided a greater insight into whether

the effects seen here were due to fetal programming, infant VD deficiency, or a combination of the two.

5.5.4 Conclusions

For the first time, this study has provided preliminary data on the effect of maternal VD deficiency followed by infant VD deficiency on offspring health. Maternal VD deficiency appears to have a greater impact on body weight in male than in female offspring with females appearing to have catch up growth between weeks 4 and 6 whilst males do not. VDD male offspring also had smaller tissue weights for almost all tissues measured, whilst there was no difference between control and VDD females, but in most cases these differences were lost when bodyweight was accounted for. This study also shows that maternal and infant VD deficiency increases offspring insulin resistance at 12 weeks, however the mechanism behind this is unclear, but is possibly due to lower levels of adipose tissue and muscle. Insulin resistance is a characteristic feature of many metabolic disorders including type 2 diabetes, non-alcoholic fatty liver disease, polycystic ovarian syndrome and obesity (Puttabyatappa et al., 2020), therefore it is vital that a clearer understanding of the effects of maternal and infant VD deficiency on offspring metabolic health is gained. Future work should look at effects of swapping the offspring diet to replenish VD in pups from deficient mothers, as well as investigating the effects of VD on metabolic rate and repeating ITT tests.

Chapter 6 General Discussion

The research presented within this thesis contributes to a greater understanding of the effects of vitamin D on myogenic differentiation and a mechanism behind these effects. This research also shows the impact of maternal and infant vitamin D deficiency on offspring health including effects on body weight, tissues, insulin tolerance, strength, and physical activity using an *in vivo* model. This research shows that there is a clear effect of vitamin D deficiency on offspring health including impacts on growth, muscle development and strength. It also shows a potential relationship between vitamin D status and metabolic health which requires further investigation.

6.1 Pattern of gene expression during myogenic differentiation, effects of vitamin D and problems within the current literature.

A previous study carried out in C2C12 cells showed the pattern of mRNA expression for the myogenic regulatory factors (myoD, myf5 and myogenin) and the myosin heavy chain isoforms (MHC emb, MHC neo, MHC I, MHC IIa, MHC IIx and MHC IIb) during differentiation (Brown et al., 2012). By using a detailed time course (measuring gene expression every 24 to 48 hours) this work showed that expression for these genes increased, peaked, then decreased during differentiation and that the myosin heavy chain isoforms could be divided into two categories: early and late. mRNA expression for the early isoforms (MHC neo, emb and I) peaked around day 4 which was then followed by the late isoforms (MHC IIa, IIx and IIb) which increased around day 8. The study ended on day 8, so a decrease in expression was not seen for the late, type II isoforms, however type II isoforms are expressed in

adult skeletal muscle so expression generally remains high (Resnicow et al., 2010). **Manuscript 1** reviewed the current body of literature which investigates the effects of vitamin D (VD) on myogenic differentiation *in vitro*. There was strong agreement between the studies that VD has an inhibitory effect on proliferation and increases myotube size, but the effect on MRF and MHC isoform expression at both the gene and protein level was unclear. Most of the studies within **manuscript 1** measured gene or protein expression at one or two time points. As mentioned, MRFs and MHC isoforms, particularly MHC neo and emb, tend to increase then decrease in expression during differentiation to create a curve shaped pattern of expression. Many factors such as the cell type used, the seeding density of cells, the method used to stimulate differentiation and treatments such as dose of VD could cause differentiation to happen more quickly or more slowly resulting in a shift of this curve to the left or right. Therefore, measuring gene or protein expression at one or two time points during differentiation does not give a true picture of the pattern of expression, and the peak in expression can easily be missed. This is likely to explain the disagreement between the studies included within **manuscript 1** because different studies measured gene/protein expression on different days.

When a detailed time course was carried out in **manuscript 2** measuring mRNA expression during C2C12 differentiation every 24 to 48 hours over 8 days, a clearer understanding of the effects of VD on differentiation could be seen. The effect of VD on proliferation agreed with all of the other studies which measured proliferation in **manuscript 1** (Capiati et al., 1999, Garcia et al., 2011, Girgis et al., 2014, Okuno et al., 2012, Olsson et al., 2016, Romeu Montenegro et al., 2019, Saini et al., 2019, van der Meijden et al., 2016) finding that both 10^{-9}M and 10^{-7}M VD decreased DNA levels compared to controls suggesting that these doses of VD had an inhibitory effect on proliferation. Although markers of apoptosis were not measured, DNA levels

remained similar from day 0 to day 5 in the two highest dose VD treatment groups, indicating that cells were not lost due to cell death during this time. Creatine Kinase (CK) is expressed shortly after cells exit the cell cycle and begin to differentiate (Tai et al., 2011), therefore if VD is causing cells to switch from a proliferative to a differentiative state, an increase in CK activity would be expected shortly after the inhibition of proliferation. On day 5, CK activity was significantly higher for cells treated with the highest two VD concentrations which further strengthened the hypothesis that VD stimulates cells to move from a proliferative to a differentiative state more quickly.

As mentioned, the effects of VD on MRF and MHC isoform expression reviewed in **manuscript 1** were unclear and there was a lack of time course data. Therefore, for the first time, a detailed time course, along with four different concentrations of VD were used in **manuscript 2**, to gain a clearer understanding of the pattern of gene expression for the MRFs and the MHC isoforms. In manuscript 2, we addressed our hypothesis that VD would increase expression of the MRFs by measuring Myf5, MyoD and myogenin expression in response to these four concentrations of VD. For MRF expression, no effect of VD was found for MyoD and a decrease in expression for Myf5 was found on day 1, suggesting that VD treated cells were moving through differentiation more quickly. For myogenin, VD appeared to shift the peak in expression to the left with mRNA expression peaking on day 6 for the highest 3 VD concentrations, then decreasing on day 8 whereas mRNA expression for control cells didn't peak until day 8. If myogenin expression had been measured only on days 0 to 4, where no difference was found between control and VD treated cells, it would have been assumed that VD had no impact on myogenin gene expression. If myogenin gene expression would have been measured only on day 8, where expression for the highest two VD concentrations was lower than controls, it would

have been assumed that VD caused an inhibition of myogenin gene expression. This highlights the importance of time course data so that the full pattern of expression over the time course can be seen.

In agreement with Brown et al., (2012), we also found that the MHC isoforms were expressed in two cohorts with MHC I, neo and emb being expressed first, peaking on day 6 in our study, and MHC IIa, IIx and IIb being expressed after this, increasing significantly on day 8 in our study. For the early isoforms, VD did not appear to shift gene expression to the left, with mRNA expression for controls and VD treated cells all peaking on day 6, but the peak in expression was higher for VD treated cells than controls. However, for the late isoforms, VD did shift gene expression to the left. Although the highest mRNA expression was seen on day 8 for both control and VD treated cells, VD treatment significantly increased expression from day 6 whereas for control cells, expression did not significantly increase until day 8.

These findings led to the question of whether the effects of VD seen for MRF and MHC isoform expression are direct effects of VD/the VDR, or whether other pathways are involved. Although it has been established for a period of time that VD has effects on myogenic differentiation, the nature of these effects and the mechanisms behind them remain unknown. We hypothesised that these effects on expression are via a VDRE on the myogenin promoter which led to the bioinformatics and transfection work, which manipulated the myogenin promoter sequence, to test whether the effects seen on myogenin mRNA expression were direct effects of VD/VDR.

6.2 Direct effects of vitamin D on myogenin gene expression

Nutritional genomics, often referred to as nutrigenomics, describes the relationship between nutrients consumed within food, and how the genome reacts in response

to them (Carlberg, 2019). VD, which is produced endogenously but also consumed in foods such as oily fish and egg yolks, is metabolised through a range of steps to produce $1,25(OH)_2D_3$ (active VD) which acts as a high affinity ligand for the vitamin D receptor (VDR), a nuclear hormone receptor (Carlberg, 2017). Via binding to vitamin D response elements (VDREs) on target genes, the VDR has been shown to modulate transcription of over 900 genes (Wang et al., 2005).

Bioinformatic work in **manuscript 2** revealed a putative vitamin D response element (VDRE) on the myogenin gene promoter (-1261 to -1264) in a similar region to the VDRE identified on the Dtna1 gene promoter in Tsoumpra et al., (2020). Responsiveness of the myogenin promoter to VD was tested using green fluorescent protein and it was found that fluorescence was significantly higher with VD treatment compared to controls, indicating that VD increases myogenin promoter activity. When the putative VDRE sequence was mutated, this increase in fluorescence with VD treatment was lost, which suggested that the VDRE sequence identified was essential for the effects of VD on the myogenin promoter. Finally, ChIP analysis proved that the VDR does bind to the identified VDRE sequence on the myogenin promoter. For the first time, this work has revealed a mechanism behind the effects of VD on myogenesis showing that VD directly increases transcription of the myogenin gene via the VDR which binds to a VDRE on the myogenin promoter.

In **manuscript 2**, a larger increase in MHC slow mRNA expression and an earlier increase in MHC fast isoform mRNA expression for VD treated cells was observed. A positive correlation was also found between myogenin and early MHC isoform mRNA expression, both peaking on day 6 and both increased in VD treated cells. Studies in mice do show a down regulation of MHC, measured by immunostaining, in the trunk and limb muscles when myogenin is absent (Rawls et al., 1995, Venuti et

al., 1995) and myogenin mRNA expression has been found to be correlated with MHC I protein expression in rats (Mozdziak et al., 1998). Furthermore, ChIP-seq data in C2C12 cells and primary human myoblasts has shown that myogenin binds to the MYH7 enhancer region which is a gene encoding the slow MHC sub-type (MHC I) (Long et al., 2022). Combined, these findings strongly suggest a role of myogenin in regulating MHC expression. Therefore, it is likely that the increase in mRNA expression for the MHC isoforms seen in **manuscript 2**, particularly MHC I, is at least partially due to an increase in myogenin mRNA expression by VD. However, it is plausible that a VDRE could also be present within the enhancer regions of the MHC isoform genes and that VD could directly manipulate their expression in the same way as myogenin. It is also possible that VD could increase expression of other MHC enhancers which could also be contributing to increased MHC isoform expression. Future work could investigate whether VDRE sequences can be identified on MHC isoform promoters and whether the VDR is able to bind to them, via bioinformatic work and ChIP, to establish a mechanism behind this.

There is good evidence that myogenin positively regulates muscle fiber size. Depletion of myogenin in adult mouse skeletal muscle results in muscle fibers which are smaller in size than controls (Moresi et al., 2010). It is difficult to investigate the effects of myogenin depletion during development on muscle fiber size in mammals considering that myogenin knockout mice are born with unfused myoblasts and a lack of muscle fibers, and die shortly after birth (Hasty et al., 1993). In zebrafish however, myogenin knockouts are born with small, 'thin' muscle fibers (Ganassi et al., 2018). Results from **manuscript 2** agree with this finding that increases in myogenin mRNA expression by VD was also associated with an increase in fiber size. One way in which myogenin is likely to regulate muscle fiber size is through its regulation of muscle fiber fusion. Myogenin acts as a transcription factor and is able

to bind to E box sequences on the promoters of target genes including myomaker (MYMK), myomixer (MYMX) and jam3b (JAM3B) which are fusogenic proteins enabling myotubes to fuse and form multinucleated fibers (Ganassi et al., 2018). Supporting this, in zebrafish, myogenin knockouts have myofibers with a severely reduced number of nuclei suggesting a low level of fusion in the absence of myogenin (Ganassi et al., 2020) and as mentioned, myogenin knockout mice are born with unfused myoblasts (Hasty et al., 1993). Therefore, the increase in muscle fiber size seen in **manuscript 2** may be, at least in part, because of increased myogenin expression by VD leading to increased fusion. However, it should be noted that increased levels of fusion do not always directly equate to larger myotubes if the myotubes fusing together both have a low number of nuclei, therefore other factors are also likely to be important.

6.3 Effects of vitamin D deficiency during fetal development and infancy; fetal programming or infant deficiency?

The 'Barker hypothesis' or 'fetal origins' hypothesis is the theory that inadequate *in utero* nutrition exposes the fetus to environmental cues which can program the offspring to become predisposed to future disease (Barker, 1992). Current research supporting the Barker hypothesis suggests that inadequate maternal nutrition during fetal development is linked to offspring diseases in adulthood including obesity, heart disease and type 2 diabetes (Almond and Currie, 2011). VD status in the mother is essential for the fetus with the level of 25(OH)D in cord blood corresponding to around 80% of the value of the mother, so if the mother is deficient, the same occurs in the fetus (Treiber et al., 2020). There are many negative offspring health outcomes associated with maternal VD deficiency which include low birth weight, childhood growth restriction, obesity, type 2 diabetes (Wu

et al., 2023), type 1 diabetes (Infante et al., 2019), decreased immune function (Liu et al., 2006) and asthma (Wolsk et al., 2017).

In **manuscript 3**, the effects of maternal and infant VD deficiency appeared to affect male offspring to a greater extent than female offspring. We hypothesised that maternal VD deficiency would result in stunted offspring growth. Although there was no difference in birth weight between any groups, from weeks 2 to 4 both VDD male and VDD female offspring were smaller than their controls. Then, VDD females appeared to have catch up growth whereby there was no difference between VDD and control female body weight from weeks 5 to 12, whilst VDD males remained smaller than control males throughout. For almost all tissues measured (brain, liver, kidneys, interscapular brown adipose tissue (BAT), abdominal white adipose tissue (AB WAT), gastrocnemius (gastroc) muscle and soleus muscle) there was no difference between control and VDD females, but control male tissues were significantly larger than VDD males. Control males also had higher grip strength than VDD males, with no difference between females. Therefore, our hypothesis that maternal VD deficiency would result in smaller offspring tissues, in particular muscle, and decreased muscle strength was true for male offspring, but not female. This was likely due to a larger bodyweight/muscle weights because this difference was lost when bodyweight was accounted for, and a significant positive correlation was found between gastroc muscle size and grip strength. It should be noted that the gastroc muscle was taken from the back leg, and forelimb grip strength was measured, but the gastroc is a predominantly fast twitch muscle used for short, explosive movements, so is a good representation for the muscles used during the grip strength test. Studies in humans show conflicting results for the effect of VD on muscle strength. In a systematic review, 7 of the 15 studies found that VD supplementation increased grip strength whilst the rest found no effect (Rejnmark,

2011). However, the majority of studies within the literature are carried out in elderly adults and evidence for effects of maternal VD deficiency on offspring muscle strength is sparse. Of the two studies in humans which do investigate offspring grip strength in children from VD deficient mothers, one study found that maternal VD status was positively correlated with offspring grip strength (Harvey et al., 2014) whilst the other study found no relationship (O'Callaghan et al., 2022). **Manuscript 3** appears to be the first study which investigates both muscle strength and physical activity in response to maternal and infant VD deficiency in mice.

Although VD deficiency affected body and tissue weights in male offspring to a greater extent than female offspring, effects on insulin sensitivity were found in both male and female VDD offspring. As outlined in **manuscript 3**, only VDD females were insulin resistant at 6 weeks but 4 VDD female mice experienced hypoglycaemia and were excluded from analysis, whilst the 4 that remained showed insulin resistance, making it difficult to interpret this data. However, at 12 weeks both male and female VDD mice were insulin resistant compared to controls and only one mouse (male) experienced hypoglycaemia from the VDD group. These findings proved our hypothesis that maternal VD deficiency would have negative effects on offspring insulin sensitivity. Both muscle and adipose tissue contain GLUT4 which are insulin-sensitive glucose transporters (Leguisamo et al., 2012), therefore both of these tissues play a significant role in regulation of blood glucose levels in response to insulin. In terms of explaining the effects of muscle and adipose tissue mass on insulin sensitivity, tissue weight corrected to bodyweight was used rather than raw tissue weight. This is because the dose of insulin given was based on the bodyweight of each mouse.

Although the increased level of gastroc/BW in control mice was not significant compared to VDD, this may have been one of the contributing factors which led to increased insulin sensitivity in control mice. An increased number of milligrams of muscle tissue per gram of bodyweight would increase the capacity to remove glucose from the blood into muscle tissues. Differences in muscle fiber type could also have contributed to insulin sensitivity. The gastroc muscle is considered to be a fast twitch muscle, containing around 50% type I muscle fibers compared to around 80% type I muscle fibers in the soleus, a slow twitch muscle (Gollnick et al., 1974). Whilst controls did have a larger soleus/BW at 3 and 6 weeks, no difference was found at 12 weeks. A potential link between muscle fiber type and metabolic health has been suggested due to several studies finding that individuals with type 2 diabetes have a lower percentage of type I muscle fibers (Zierath and Hawley, 2004). Studies using human muscle biopsies from the vastus lateralis (predominantly fast, type II) have found that percentage of type I fibers within this muscle was positively associated with insulin sensitivity whilst the percentage of type II fibers was negatively associated with insulin sensitivity (Fisher et al., 2017). This is also in line with rodent studies which find increased insulin-mediated glucose uptake in type I fibers compared to type II fibers (James et al., 1985, Ploug et al., 1987). A study in rats found that maternal VD deficiency resulted in a higher percentage of type II fibers in the EDL muscle (fast twitch) compared to offspring from control rats (Reis et al., 2022). Therefore, if VD deficiency does affect fiber type in fast twitch muscles by shifting muscles towards having an even greater proportion of type II fibers, this could reduce insulin sensitivity of muscles due to the lower ability of type II fibers to uptake glucose compared to type I. In **manuscript 3**, it was also found that control mice had greater physical activity compared to VDD mice. This may suggest that control mice did have a higher percentage of type I fibers considering that type I

fibers are fatigue resistant so can function for longer (Wang and Pessin, 2013). Unfortunately, due to time constraints, muscle fiber typing was not possible, however future work should investigate this using the tissues from **manuscript 3**.

Another explanation for the differences seen in insulin sensitivity could be due to levels of white adipose tissue. Control mice had more IS WAT/BW and AB WAT/BW compared to VDD. Not only does adipose tissue have the capacity to remove glucose from the blood via GLUT4 transporters (James et al., 1988), but PPAR-Y is expressed to a high extent within adipose tissue which is strongly positively associated with insulin sensitivity (Spiegelman, 1997). Studies in both rodents and humans have demonstrated this with mutations in PPAR-Y resulting in insulin resistance (Barroso et al., 1999). Unfortunately, due to time constraints, PPAR-Y expression could not be analysed within tissues, but it is plausible that if VDD offspring had smaller, under-developed adipose tissues, their level of PPAR-Y could also be lower, especially considering PPAR-Y mRNA expression has been found to be positively correlated with body mass index (BMI) (Darwish et al., 2022). One theory behind the mechanism for PPAR-Y improving insulin sensitivity is via the 'lipid steal' hypothesis (Tontonoz and Spiegelman, 2008). This theory suggests that PPAR-Y increases the capacity of adipose tissue to store lipids which results in fatty acids being stored in adipose tissues, sequestering them away from muscles, thus increasing the ability of muscle tissue to respond to insulin. It is known that accumulation of lipids within muscle is associated with insulin resistance, particularly diacylglycerol accumulation which has been shown to impact activation of the muscle insulin receptor (Samuel et al., 2010). However, evidence investigating the relationship between PPAR-Y levels and adipose tissue levels is sparse and the limitation for the association between PPAR-Y and BMI is that BMI does not distinguish between adipose and muscle weight, so it cannot be concluded that PPAR-Y levels are directly associated with

adipose tissue levels. Additionally, the mechanism behind how PPAR-Y improves insulin sensitivity is poorly understood so much more research in this area is needed.

In addition, a case-control study in humans found that maternal VD deficiency led to impaired pancreatic β-cell function and higher fasting glucose levels (in the mothers) as well as a higher incidence of gestational diabetes (Chen et al., 2020). It is known that gestational diabetes is associated with offspring insulin resistance (Moon and Jang, 2022), so it is unclear whether maternal VD deficiency has direct effects on the fetus which influences insulin sensitivity, or whether it has effects on maternal insulin sensitivity which in turn affects fetal insulin sensitivity, or both.

The significant difference in bodyweight between control and VDD offspring between weeks 2 and 4 is likely to have been due to maternal VD deficiency because pups were not weaned until 3 weeks old. There were also three tissues (brain, kidney, gastrocnemius) where the weight was higher in control offspring than VDD offspring at 3 weeks but not different at 6 or 12 weeks. For these tissues, maternal factors may have been responsible for these differences, and for tissues where differences were not seen until 12 weeks (BAT and AB WAT), the infant VDD diet may have played more of a role than maternal factors. However, the exact effects and mechanisms behind fetal programming are not well understood and fetal programming may predispose offspring to certain outcomes which do not present until late childhood / early adulthood. Additionally, outcomes such as insulin resistance are likely to be multifactorial with genetics, fetal environment and offspring environment all playing a role (Moon and Jang, 2022). The only way to measure whether the effects seen in **manuscript 3** were due to fetal programming, offspring diet or a combination of both would be to swap offspring diets so that a sub-group of pups from both control and VDD mothers were weaned onto the

control diet and another sub-group onto the VDD diet, which was not possible due to the length of time this study would have taken.

6.4 Strengths and Limitations and Future Work

As outlined in **manuscript 1**, there was a severe lack of time course data within the literature when investigating effects of vitamin D on myogenic gene expression *in vitro*. This led to conflicting results so the effects of VD on MRF and MHC mRNA expression remained inconclusive. The main strength of the systematic review carried out in **manuscript 1** was that it highlighted the need for time course data going forwards. Therefore, **manuscript 2** provided a detailed time course measuring gene expression at 6 time points over 8 days which enabled the full pattern of gene expression during myogenic differentiation in response to VD to be established. In addition to this, four different concentrations of $1,25(OH)_2D_3$ were used which ranged from below to above physiological serum concentrations and therefore was physiologically relevant. Gene expression data showed that VD caused an upregulation in myogenin expression which was further confirmed by transfection work which showed that VD increased myogenin gene promoter activity. Although mutating the putative VDRE provided good evidence that the sequence identified was a VDRE, ChIP analysis confirmed that the VDR does bind to this sequence. Overall, this combination of results provided strong evidence that the sequence identified in **manuscript 2** was a VDRE, and also provided a mechanism behind VD increasing myogenic differentiation. Carrying out this work in other muscle cell lines, such as L6 cells, and/or primary human muscle cells would have provided stronger evidence for this mechanism across other species, and protein expression for the genes measured would have also been useful to confirm that the effects seen on mRNA expression are also consistent at the protein level. Finally, studies which used

inactive VD would also have been useful to test whether the same effects were seen. The major limitation for all cell work is that single cell type populations do not exist in mammals, instead tissues and bodily fluids are made up of a mix of many different types of cells which often interact with each other. Therefore, there is only a certain level of extrapolation that is possible from *in vitro* work. However, the work in **manuscript 2** provided good evidence that VD has an impact on myogenic differentiation which warranted further *in vivo* work.

The animal trial in **manuscript 3** provided the first study which has looked at the effect of maternal VD deficiency followed by offspring VD deficiency on offspring growth, strength, and metabolic health. Unlike other studies, this study weaned offspring from VDD mothers onto a VDD diet, rather than a control diet, which may be more relevant to public health considering that children born to VD deficient mothers are likely to be VD deficient during childhood (Dawodu and Wagner, 2007). The animal trial also investigated effects through to adulthood (considered to be around 12 weeks old in mice) as well as taking measurements at two other time points during offspring growth. Additionally, many different factors were investigated including offspring overall growth, growth of several tissues, strength, physical activity, and metabolic health. The biggest limitation of the mouse trial in **manuscript 3** was time. The trial started 6 months prior to KA's hand in date (an extension was later granted, but this was not known until 3 months into the trial) which meant that a longer study where offspring diets were swapped could not be carried out. Ideally, offspring from both control and VDD mothers would have been split into two groups with one group being weaned onto a control diet and the other onto the VDD diet. This would have provided a greater insight into whether effects were due to fetal programming (by comparing offspring from VDD mothers weaned onto the control diet to offspring weaned onto the VDD diet) or whether any effects

were due to infant VD deficiency, or both. Additionally, again due to time constraints, tissue analysis could not be carried out to investigate fiber typing in muscle samples and gene expression in the tissues. There is a limitation in extrapolating findings from mice to humans however, in human studies there are a large number of variables which are very difficult to control (such as lifestyle, diet, compliance) whereas the mouse trial provided data from a highly controlled study where mice within the same treatment group consumed exactly the same diet, all mice were housed in the same conditions and were the same age.

Overall, both the cell work and the animal trial were carried out in mouse cell lines / mice which leads to difficulties in extrapolating this data to other species. Future *in vitro* work should be carried out in different cell types from a range of species, look at both gene and protein expression and should involve analysis of the MHC isoform promoters to see whether VDRE sequences can be identified. Future *in vivo* work should involve a longer study where offspring diets are swapped and studies in different species should be carried out. Studies in animals which do not have litters, such as sheep, would be especially useful for comparison to humans. Additionally, tissue analysis should be carried out on the tissues from **manuscript 3**, in particular muscle fiber typing and gene expression for MRFs, MHCs, glucose and insulin receptors, and PPAR-Y.

6.5 Conclusions

Overall, the work in this thesis finds clear effects of active VD on myogenesis with an earlier increase in myogenic regulatory factor mRNA and increased myosin heavy chain slow and fast isoform mRNA. One mechanism for this is a direct effect of VD on myogenin mRNA expression via the VDR binding to a functional VDRE on the myogenin promoter, which is increased in the presence of VD. In mice, maternal VD

deficiency appears to affect body and tissue growth in male offspring to a greater extent than female offspring. Although maternal VD deficiency does cause stunted growth in both males and females around weaning age, females appear to have catch up growth following this whilst males do not. Likewise, maternal and infant VD deficiency results in decreased muscle strength in males. Finally, maternal and infant VD deficiency causes insulin resistance and decreased physical activity in both male and female offspring.

The prevalence of maternal VD deficiency (ranging from 18-84% worldwide, (Vandevijvere et al., 2012)) highlights the importance of the work carried out in this thesis, especially due to the emerging evidence that maternal VD status may be in independent risk factor for birth complications and offspring health (Bodnar et al., 2007). Stunted childhood growth has been linked to high blood glucose concentrations and harmful lipid profiles in adulthood (Victora et al., 2008) and childhood insulin resistance has been shown to increase risk of insulin resistance and type II diabetes in adulthood, independent of BMI (Chiarelli and Marcovecchio, 2008). There is currently no recommended dose for VD supplementation specifically during pregnancy (Palacios et al., 2019). Therefore, it is vital that more work is carried out in this area to further confirm the results found within this thesis and to highlight the importance of the need to re-evaluate vitamin D supplementation guidelines for pregnant women.

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Appendix A Impact Log

This log outlines the difficulties faced throughout this thesis. In summary, labs were closed for 6 months during the Covid-19 pandemic in 2020, then again for 3 months during the move to a new lab in 2021. We faced great difficulties in getting approval for the mouse trial which delayed the start of the trial by around 1 year.

March - August 2020:
2 weeks into my last rotation labs were closed due to COVID-19 lockdown restrictions. This happened very quickly – we only found out at 11am that labs were closing at 3pm that same day. We were told to stop all experiments, collect our belongings, and leave. I was half-way through a differentiation experiment at this point but had to kill the cells, so I was not able to get any results/data from this. From March to the end of August we had to work from home. During this time, I wrote my systematic review so that I could focus on lab work upon returning to labs. This was a difficult period of time because I was living in a student house, so my desk was in my bedroom, which meant spending most of my time in the same room, with very little opportunity to leave the house due to COVID restrictions.

September 2020:
Labs re-opened and first year students were advised to go back to basics after doing no lab work for almost 6 months. I was given some old samples to practise protein and DNA assays on. At this point labs were only allowed to be open at 10% capacity which meant 1 person per lab so I was only able to go in a couple of times per week, for 2 hours at a time so that everybody got their fair share of lab time.

October 2020 – March 2021:
Labs were allowed to open at 25% capacity which meant that 2 people were allowed per lab and I was able to go into labs 3 to 4 times per week. Lab slots increased to 4 hours, so I was able to spend around 12-16 hours in labs per week. I was able to do a few C2C12 differentiation experiments during this time followed by DNA, Creatine Kinase and Protein Assays and get some data. I was still not able to be in labs as often as pre-COVID (especially because equipment such as plate readers could not be shared – so if you needed the plate reader but one other person was already booked onto it, you could not use it) but I was grateful for the lab time I was able to get and I was still able to get some data during this time.

March – June 2021:
Labs closed for lab move (Friday 26th March). For the 2 weeks leading up to lab close we were instructed to wind down lab work / sort through and throw out old samples/reagents and only keep essential things, so no new experiments could be started from 12th March. From this time, we had to pack up everything from the labs and move it all over to a storage room in the North Lab building.
Predicated date of North lab opening – week commencing Monday 10th May.
Despite North lab being newly refurbished, the lab got flooded after heavy rain. Repairs needed to be carried out so the anticipated re-opening date was moved to the first week in June.
At this point it was proposed by mine and Sofia's supervisors that we should do an

animal trial. Sofia and I completed our personal animal licences which consisted of teaching, practical handing lessons and an exam.
North lab remained closed until 6th June as a result of needing repair work, lab inductions were carried out on 7th June. Because we were not allowed to start new experiments in the old lab from 12th March, and North Lab inductions did not take place until 7th June, this has meant that we had a further 3 months out of labs, on top of the 6 months that labs were closed last year due to Covid.

July 2021 – March 2022:
During this time lab work was able to commence. Labs were still not at full capacity for the rest of 2021, however I was able to book a slot in the lab most days. During this time, Preeti Jethwa wrote the project licence for our mouse trial and sent it to the University of Nottingham ethical review board. We had major issues with getting approval/feedback on the project licence. We would receive changes to be made and then have to wait for 2-3 months for them to look at it again, then they would send more changes and we would have to wait another 2-3 months to hear back. During this time, it was very difficult to plan in new experiments because we did not know when the animal trial might start. I also needed to book in a PIP but again, I was concerned about taking 3 months off in case the project licence became approved and the mouse trial started.

April – June 2022:
By the start of April 2022, the project licence had still not been approved by the University and I was advised by my supervisors to book my PIP. Luckily, I was able to go on my PIP straight away, so I started my PIP in April 2022 (I had Covid until 10th April so started on the 11th) and finished on Friday 1st July.

July 2022 – April 2023:
I started back in the lab on Monday 4th July following my PIP. At this point there were still issues with getting the project licence approved so I focused on my cell work and carried out my bioinformatics, transfection and ChIP work. Towards the end of 2022, the project licence was finally approved and sent to the Home Office. By November 2022, everything was in place to start the animal trial.
In December 2022 the animal unit at the University of Nottingham announced that it was closing down, and no new animal trials could start. This was a huge shock to everyone; academics were in tears in meetings over the situation. We had ordered our diet so had to cancel this and began to search for alternative options such as contacting Nottingham Trent University to discuss prices for them to carry out the work. 2 weeks later, the University announced that the animal unit was not in fact closing, and that work could carry on as normal. We re-ordered the diet and planned to start in January 2023. Unfortunately, there was confusion with the diet company because we had ordered, cancelled then re-ordered, and our second order was not processed properly which led to a 3-month delay in receiving the diet. The animal unit do not allow mice to be purchased until the diet is on site, so we could not order the mice until March 2023 when we finally received the diet. Finally, in April 2023, around 1 year later than originally planned, the animal trial started. However, the predicted end date for the trial at this point was the end of October 2023 which was only 3 weeks before my thesis hand in date. Extensions could not be applied for until August.

April – November 2023:

From April to November the mouse trial was carried out. Due to problems with mating and mice taking longer to mate than anticipated, the trial went on for 3 weeks longer than planned. Thankfully, a 12-week extension was granted in August so that my new hand in date would be 23rd February 2024.

Due to only having 12 weeks from the end of the mouse trial to my hand-in date, I was not able to carry out tissue analysis. The problems we faced in getting approval for the animal trial were immense and the trial was never supposed to be right at the very end of my PhD. It was a shame that I did not have at least a year in the lab following the trial, as originally planned, to carry out all of the analysis that I would have liked to.

Appendix B Manuscript 1

ALLIBAND, K. H., KOZHEVNIKOVA, S. V., PARR, T., JETHWA, P. H. & BRAMELD, J. M. 2021. Effects of Biologically Active Vitamin D on Myogenesis: A Systematic Review. Front Physiol, 12, 736708.

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SYSTEMATIC REVIEW
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In vitro Effects of Biologically Active Vitamin D on Myogenesis: A Systematic Review

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Vitamin D (VD) deficiency is associated with muscle weakness. A reduction in the incidence of falls in the elderly following VD supplementation and identification of the VD receptor within muscle cells suggests a direct effect of VD on muscle, but little is known about the underlying mechanisms. Here we systematically searched the literature to identify effects of active VD [1,25(OH)2D3] on skeletal muscle myogenesis *in vitro*, with no restriction on year of publication. Eligibility was assessed by strict inclusion/exclusion criteria and agreed by two independent investigators. Twelve relevant papers were identified using four different cell types (C2C12, primary mouse satellite cells, primary chick myoblasts, and primary human myoblasts) and a range of myogenic markers (myoD, myogenin, creatine kinase, myosin heavy chain, and myotube size). A clear inhibitory effect of 1,25(OH)2D3 on proliferation was reported, while the effects on the different stages of differentiation were less consistent probably due to variation in cell type, time points and doses of 1,25(OH)2D3 used. However, myotube size was consistently increased by 1,25(OH)2D3. Overall, the evidence suggests that 1,25(OH)2D3 inhibits proliferation and promotes differentiation of myoblasts, but future studies should use time courses to gain a clearer understanding.

Keywords: vitamin D, 25-dihydroxyvitamin D3, 25-hydroxyvitamin D2, myogenesis, myogenin, differentiation, MyoD, systematic review

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INTRODUCTION

The link between vitamin D (VD) and bone health has been studied extensively, but recent evidence points toward a relationship between VD and skeletal muscle function (Wiciński et al., 2019). Muscle biopsies from VD deficient individuals show muscle wasting (mostly type II fibre atrophy), large interfibrillar spaces, and fat infiltration within the muscle (Ceglia, 2008). In general, deficiency occurs when levels of 25(OH)D3 (inactive vitamin D) fall below 25 nmol/L, however this cut- off point can vary within the literature (Halfon et al., 2015). In the elderly population, vitamin D deficiency has been linked to an increased risk of falls which is thought to be partly due to muscle weakness and wasting (Garcia et al., 2011).
Around 80–90% of VD is obtained via UV-B induced synthesis in the skin in humans, whilst 10–20% comes from dietary intake (Halfon et al., 2015). In the skin, 7-dehydrocholesterol is converted to pre-vitamin D upon UV-B radiation. This is then converted to cholecalciferol which becomes bound to VD binding globulin and this complex is transported to the liver where it undergoes

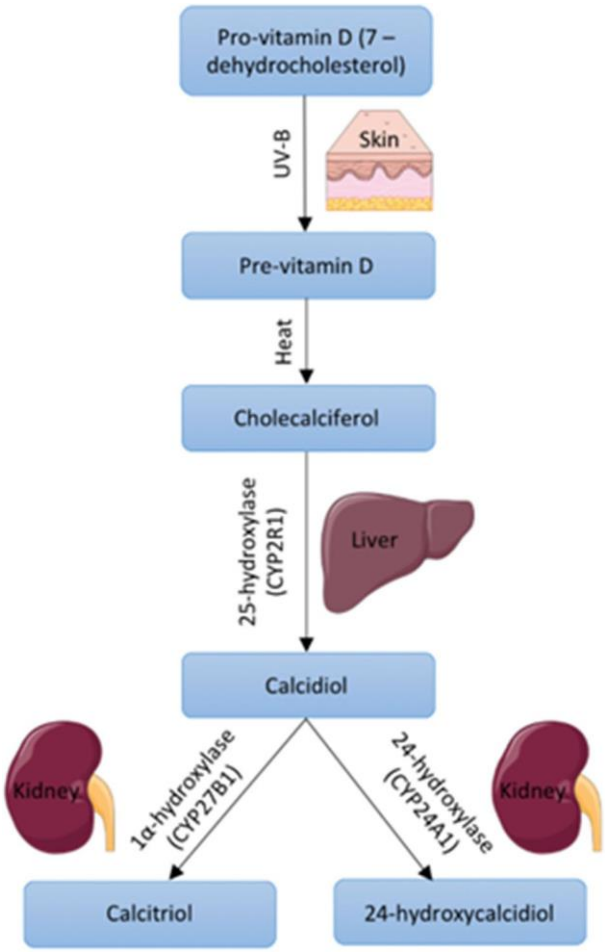

FIGURE 1 | Vitamin D synthesis within the body including precursors, enzymes, and body site. Images used within this figure were obtained from smart servier medical art and can be found at https://smart.servier.com.

hydroxylation by 25-hydroxylase to form 25(OH)D3 or calcidiol (Hamilton, 2010). 25(OH)D3 is the major circulating form of VD and is measured as a marker of VD status (Pojednic and Ceglia, 2014). A final step, to produce the biologically active form of VD, involves hydroxylation by 1α-hydroxylase to produce 1,25(OH)2D3 otherwise known as calcitriol (Hamilton, 2010; **Figure 1**). 1α-hydroxylase is expressed largely in the kidney, which contributes to active VD in the circulation, however the enzyme is also expressed within other tissues such as muscle, which allows local conversion of inactive to active VD (Ceglia, 2008).

Studies in both chicken and human skeletal muscle have identified the presence of the vitamin D receptor (VDR) within muscle cells thereby providing evidence for a direct effect of VD on muscle (Zanello et al., 1997; Bischoff et al., 2001). This has since been supported by human studies which have found that low serum 25(OH)D3 concentrations in elderly individuals is associated with reduced muscle strength and an increased risk of falls (Ceglia and Harris, 2013). These effects of VD deficiency on muscle appear to be reversible with supplementation in the elderly population leading to beneficial outcomes such as increased strength, balance, and a decreased risk of falls (Harwood et al., 2004). This effect is thought to be, at least in part, directly through the VDR present in muscle cells. VDR knockout mice have been found to have muscle fibres which are 20% smaller in size than controls as well as smaller body size, weight, and impaired motor co-ordination (Pojednic and Ceglia, 2014).

Within the literature, VDRs have been described in different cell locations, one as a nuclear hormone receptor and the other as a membrane receptor (Ceglia, 2008). The origin of the membrane receptor is unclear, some argue there is a distinct membrane receptor, however the majority of evidence points toward one VDR with the ability to translocate between the nucleus and membrane (Halfon et al., 2015).

It is well-known that the VDR has a nuclear hormone receptor function, with the transcription of over 900 genes found to be affected upon treatment with active VD (Wang et al., 2005). 1,25(OH)2D3, binds to the VDR which induces heterodimerisation with the retinoid X receptor (RXR). This complex is then able to bind to VD response elements (VDREs) to activate or repress transcription of target genes (Halfon et al., 2015). Expression of genes involved in myogenic proliferation and differentiation have been shown to change upon treatment with VD leading to the suggestion that VD may have a direct effect on myogenesis (Wiciński et al., 2019).

The aim of this systematic review is to summarise the current body of evidence on the effects of active VD on skeletal muscle cells in culture. There is conflicting evidence in this area, therefore this review aims to summarise, assess, and interpret the current body of evidence and identify areas where further investigation is required.

MATERIALS AND METHODS

This review was constructed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2009).

Search and Selection Criteria

Relevant papers were identified through the computerised search databases (PubMed (MEDLINE), Web of Science and Google Scholar). The search process followed the population (P), Intervention (I), Comparison (C), and outcome (O, PICO). The review population was *in vitro* models of muscle cells, the intervention was active VD treatment, comparison was controls not treated with VD and the measuring outcomes were the effects of active VD on muscle proliferation and differentiation. Specific search terms "vitamin D OR 1,25Dihydroxyvitamin D3 OR 1,25(OH)2D3 OR calcitriol AND myogenesis OR muscle differentiation" were used to obtain relevant articles. To obtain the relevant articles, two independent reviewers (KHA & SVK) assessed the titles, abstract and full articles based on a strict inclusion and exclusion criteria and if any disagreements arose, these were resolved by discussion. Finally, the reference list of these were searched to find any additional papers.

Selected Articles Criteria

Articles were not restricted to any dates as there have been no previous systematic reviews conducted investigating the literature relating to active VD and myogenesis *in vitro*.

Inclusion Criteria

- Studies must have been written in English to avoid any translation errors.
- All articles must have described an *in vitro* model of muscle cells (primary or cell line).
- Any form of active VD can be considered [1,25(OH)2D3 or active VD analogues].
- Treatment of VD must be of known quantity and administered alone and not in combination with other drugs/vitamins/minerals.
- Must determine effects on proliferation/differentiation of muscle cells.

Exclusion Criteria

- Whole animal or human models.
- Systematic reviews or critical reviews.
- Studies investigating VD receptor and not VD.
- Studies investigating cancer or ageing.

Measured Outcomes

The primary measured outcomes of this review are markers of myogenesis such as level of DNA synthesis, mRNA and protein levels of myoD, myogenin, myosin/myosin heavy chain isoforms, creatine kinase activity, and myotube size. There were no secondary measured outcomes.

Data Extraction

Using a standard extraction form, data from all studies were extracted and charted using Excel (Microsoft Excel, Washington, USA). Data extracted included title, author, publication year, muscle cell model used, exposure to VD, and outcomes (DNA, myogenin, myoD, creatine kinase, myosin, and myotube size).

All key characteristics of the selected papers were expressed in tables. These included the study design, model used, number of samples, outcome measures, and doses of VD converted to moles for consistency.

Data Analysis

The significant effects ($p < 0.05$) in response to VD were charted to compare across the articles reviewed, however some values were read from graphs where raw data was not provided so are best estimates. Changes in expression were used to generate bar graphs using Excel (Microsoft Excel, Washington, USA), all changes were converted to fold-change for consistency.

Meta-analysis could not be carried out due to variation in methods between papers. Differences in cell type, time points used and concentrations of VD used meant that direct comparisons in the form of a meta-analysis was not possible.

Quality Assessment

The quality assessment method used in this review is a modified version of Risk of Bias (RoB) 2 tool from the Cochrane database to assess risk of bias in randomised trials. This assessment tool has been modified to be appropriate for cell culture experiments such as those included within this review (**Supplementary Table 1**). Responses in green indicate potential markers for a low risk of bias, orange indicates moderate risk and red indicates potential markers for a high risk of bias (Y = yes, PY = probably yes, PN = probably no, N = no, NI = no information given or not applicable). Questions starting with 1 relate to risk of bias from treatment allocation. Questions starting with 2 relate to risk of bias in measurement of the data. Questions starting with 3 relate to risk of bias in selection of the reported result. Three or four questions were used to assess each section and an overall risk of bias was decided upon. There are three options for overall risk of bias judgement: low risk, high risk or some concerns.

RESULTS

Eligibility of Studies

Using electronic databases (PubMed (MEDLINE), Web of Science and Google Scholar), we identified 349 articles between 1978 and 2020. The removal of duplicates and initial title screen left 301 articles for detailed assessment. Of these 25 were evaluated against the inclusion/exclusion criteria. Ten of these were animal studies and 3 focused on cancer cells, ageing and VD receptor. This left 12 articles eligible for inclusion within this review (**Figure 2**). A detailed list of excluded studies with reasoning for exclusion can be found in **Supplementary Table 2**.

Quality Assessment

All 12 papers received a score of "low risk" when assessed against the quality assessment criteria previously outlined in **Supplementary Table 1**. For three of the studies (Capiati et al., 1999; Girgis et al., 2014; Olsson et al., 2016) no information could be found regarding replicates and/or repeats therefore it was assumed that this was adequate when giving a low overall bias score (**Table 1**).

Study Characteristics

All studies included within this review used the biologically active form of VD [1,25(OH)2D3] apart from Saito et al. (2017) where an analogue of the active form of VD called Eldecalcitol was used. Four different cell types were used across the studies (C2C12, primary human myoblasts, primary mouse satellite cells, and primary chick myoblasts) and active VD concentration ranged from 10–5 to 10–13M (**Table 2**).

Effects on Proliferation

From the relevant articles, eight (Capiati et al., 1999; Garcia et al., 2011; Okuno et al., 2012; Girgis et al., 2014; Olsson et al., 2016; van der Meijden et al., 2016; Romeu Montenegro et al., 2019; Saini et al., 2019) studied the effects of 1,25(OH)2D3 on proliferation and all reported an inhibitory effect. Of these, four (Capiati et al., 1999; Olsson et al., 2016; Romeu Montenegro et al., 2019; Saini et al., 2019) quantified DNA content as a marker of proliferation (**Figure 3**). Interestingly, one study (Capiati et al., 1999) reported an initial short stimulatory effect of 1,25(OH)2D3 treatment on DNA synthesis on day 1 (1.5-fold increase) however, this was followed by an inhibitory effect on

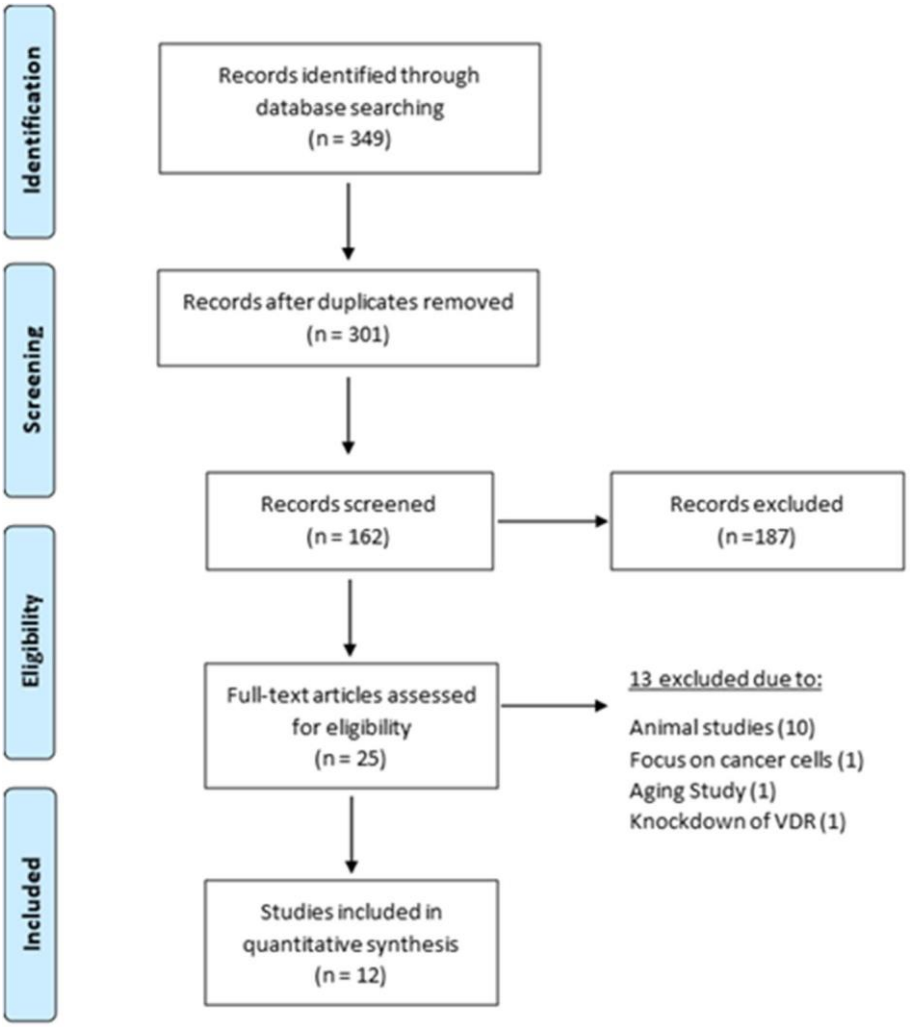

FIGURE 2 | Selection and exclusion of studies in accordance with PRISMA guidelines (Moher et al., 2009).

day 4 (0.7-fold). The remaining three studies (Olsson et al., 2016; Romeu Montenegro et al., 2019; Saini et al., 2019) all revealed a decrease in DNA content of different magnitude (0.5 to 0.95-fold) (**Figure 3**).

The other four studies measured proliferation in various ways (**Table 3**). One study showed an increase in p21 and p27 mRNA (Okuno et al., 2012) whilst three studies revealed a decrease in cyclin mRNAs (Girgis et al., 2014; Olsson et al., 2016; Saini et al., 2019). Decreases in proliferation was also shown by an increase in number of cells in the quiescent phase (Okuno et al., 2012; Girgis et al., 2014; Romeu Montenegro et al., 2019), decreased levels of proliferating cell nuclear antigen (PCNA) at the protein level (Garcia et al., 2011) and decreases in DNA synthesis as previously reported. It is important to note that only two out of eight of these studies checked for differences in apoptosis between treated and control cells (Girgis et al., 2014; Olsson et al., 2016).

Effects on Differentiation

Differentiation of muscle cells was determined in all but one (Saini et al., 2019) of the final twelve studies. Markers of differentiation included expression of mRNA or protein for myoD (early differentiation), myogenin (early-mid stage), myosin/myosin heavy chain isoforms (late stage), or the measurement of creatine kinase activity (mid-stage). However, it should be noted that the mRNA expression of myogenin and myosin heavy chain isoforms have been shown to change during the time course of differentiation in C2C12 cells (Brown et al., 2012) indicating that the time point at which these markers are measured is important.

Effects of Vitamin D on Early-Stage Myogenic Differentiation

Five studies measured myoD expression (Garcia et al., 2011; Olsson et al., 2016; van der Meijden et al., 2016; Braga et al., 2017; Saito et al., 2017). Three of these studies measured expression on day 4 (Garcia et al., 2011; van der Meijden et al., 2016; Saito et al., 2017) whilst one measured expression on day 1 (Olsson et al., 2016) and another on day 7 (Braga et al., 2017) (**Figure 4**). mRNA expression was measured in all cases except for one (Braga et al.,

TABLE 1 | Summary of quality assessment of included studies.

References	Question										Rating
	1.1	1.2	1.3	2.1	2.2	2.3	3.1	3.2	3.3	3.4	
Braga et al. (2017)	🟢	🟠	🟢	🟢	🟢	⚫	🟢	🟢	🟢	🟢	Low
Capiati et al. (1999)	🟢	🟠	🟢	🟢	🟢	⚫	🟢	🟢	🟢	⚫	Low
Garcia et al. (2011)	🟢	🟠	🟢	🟢	🟢	⚫	🟢	🟢	🟢	🟢	Low
Gili et al. (2016)	🟢	🟠	🟢	🟢	🟢	⚫	🟢	🟢	🟢	🟢	Low
Girgis et al. (2014)	🟢	🟠	🟢	🟢	🟢	⚫	🟢	🟢	🟢	⚫	Low
Okuno et al. (2012)	🟢	🟠	🟢	🟢	🟢	⚫	🟢	🟢	🟢	🟢	Low
Olsson et al. (2016)	🟢	🟠	🟢	🟢	🟢	⚫	🟢	🟢	🟢	⚫	Low
Romeu Montenegro et al. (2019)	🟢	🟠	🟢	🟢	🟢	⚫	🟢	🟢	🟢	🟢	Low
Ryan et al. (2013)	🟢	🟠	🟢	🟢	🟢	⚫	🟢	🟢	🟢	🟢	Low
Saini et al. (2019)	🟢	🟠	🟢	🟢	🟢	⚫	🟢	🟢	🟢	🟢	Low
Saito et al. (2017)	🟢	🟠	🟢	🟢	🟢	⚫	🟢	🟢	🟢	🟢	Low
van der Meijden et al. (2016)	🟢	🟠	🟢	🟢	🟢	⚫	🟢	🟢	🟢	🟢	Low

🟢 *low risk of bias,* 🟠 *moderate risk of bias,* ⚫ *high risk of bias,* 🔴 *no information given/not applicable.*

TABLE 2 | Summary of study characteristics.

References	Cell type	Form of vitamin D	Concentration	Outcomes measured
Braga et al. (2017)	Mouse skeletal muscle satellite cells	$1,25(OH)_2D_3$	10^{-7}M	MyoD, myogenin
Capiati et al. (1999)	Chick myoblasts (obtained from 12-day-old embryo breast tissue)	$1,25(OH)_2D_3$	10^{-9}M	Proliferation, creatine kinase, myosin
Garcia et al. (2011)	C2C12	$1,25(OH)_2D_3$	10^{-7}M	MyoD, myogenin, myotube size
Gili et al. (2016)	C2C12	$1,25(OH)_2D_3$	10^{-9}M	Myogenin, creatine kinase, myosin, myotube size
Girgis et al. (2014)	C2C12	$1,25(OH)_2D_3$	10^{-7}M	Myogenin, myotube size
Okuno et al. (2012)	C2C12	$1,25(OH)_2D_3$	10^{-7}M, 10^{-8}M, and 10^{-9}M	Myogenin, myosin
Olsson et al. (2016)	Human skeletal muscle myoblasts	$1,25(OH)_2D_3$	10^{-7}M	Proliferation, myoD, myogenin, myosin
Romeu Montenegro et al. (2019)	Human skeletal muscle myoblasts	$1,25(OH)_2D_3$	10^{-7}M	Proliferation, myogenin, myosin, myotube size
Ryan et al. (2013)	C2C12	$1,25(OH)_2D_3$	10^{-5}M, 10^{-7}M, 10^{-9}M, 10^{-11}M, and 10^{-13}M	Myogenin, creatine kinase
Saini et al. (2019)	Human skeletal muscle myoblasts	$1,25(OH)_2D_3$	10^{-7}M, 10^{-9}M, and 10^{-11}M	Proliferation
Saito et al. (2017)	C2C12	Eldecalcitol	10^{-7}M, 10^{-8}M, and 10^{-9}M	MyoD, myosin
Van der Meijden et al. (2016)	C2C12	$1,25(OH)_2D_3$	10^{-7}M	MyoD, myogenin, myosin, myotube size

2017) where protein expression was measured. Four out of five studies (Garcia et al., 2011; van der Meijden et al., 2016; Braga et al., 2017; Saito et al., 2017) reported an increase in expression of myoD which ranged from 1.8 to 3-fold. However, one study (Olsson et al., 2016) reported a decrease in expression of 0.5-fold on day 1. These changes in myoD expression were in response to 10–7M 1,25(OH)2D3 for all cases apart from one (Saito et al., 2017) which used 10–7M Eldecalcitol, an analogue of the active form of VD.

Effects of Vitamin D on Early/Mid-Stage Myogenic Differentiation

Myogenin expression in response to 1,25(OH)2D3 was investigated by nine of the twelve studies included within

this review (Garcia et al., 2011; Okuno et al., 2012; Ryan et al., 2013; Girgis et al., 2014; Gili et al., 2016; Olsson et al., 2016; van der Meijden et al., 2016; Braga et al., 2017; Romeu Montenegro et al., 2019). The time points at which myogenin expression was measured varied from day 1 to day 7. For eight of the nine studies which measured myogenin,

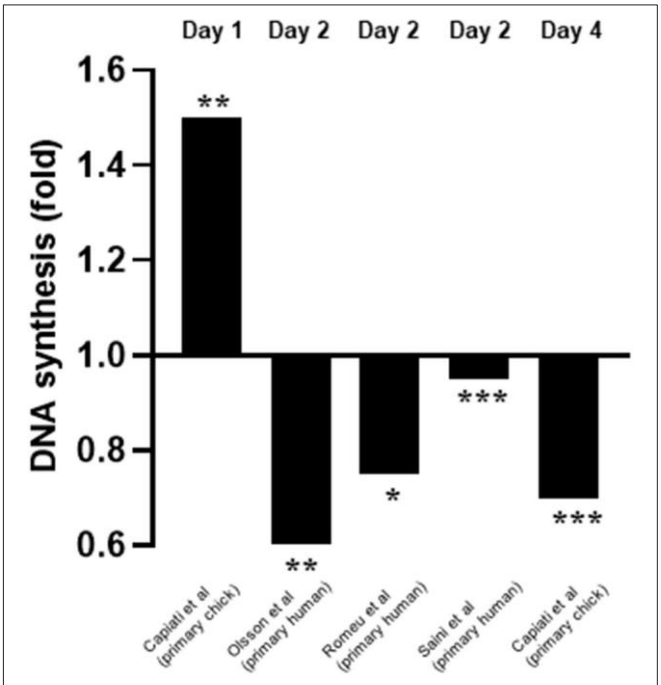

FIGURE 3 | Effect of $1,25(OH)_2D_3$ on DNA synthesis compared to untreated cells. The active form of vitamin D [$1,25(OH)_2D_3$] at 10^{-7}M was used in all studies. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

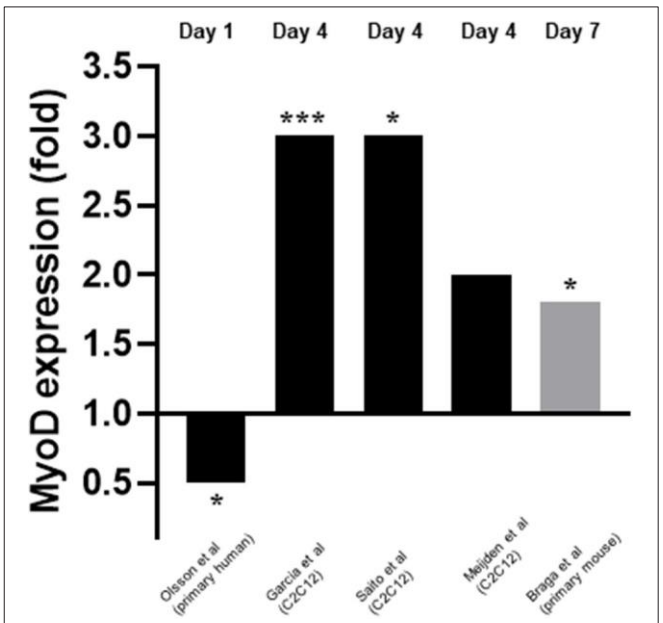

FIGURE 4 | Effect of $1,25(OH)_2D_3$ or analogue on MyoD mRNA expression. The active form of vitamin D [$1,25(OH)_2D_3$] at 10^{-7}M was used in all studies apart from Saito et al. (2017) where Eldecalcitol (an analogue of the active form of vitamin D) was used. Black bars indicate mRNA expression whilst grey indicates protein expression. *$p < 0.05$, ***$p < 0.001$.

TABLE 3 | Effects of $1,25(OH)_2D_3$ on proliferation.

Reference (cell type)	VitD form and concentration	Effect on proliferation	Checked for apoptosis?
Capiati et al. (1999) (Primary chick)	10^{-9}M $1,25(OH)_2D_3$	[^{3}H]thymide incorporation 1.5-fold on day 1, then 0.7-fold on day 4	
Garcia et al. (2011) (C2C12)	10^{-7}M $1,25(OH)_2D_3$	Proliferating cell nuclear antigen (PCNA) protein 0.25-fold on day 7	
Girgis et al. (2014) (C2C12)	10^{-7}M $1,25(OH)_2D_3$	Proliferation 0.4-fold on day 2 23% increase in cells in G_0/G_1 quiescent phase on day 2 Cyclin D1 mRNA 0.75-fold on day 2	✓
Okuno et al. (2012) (C2C12)	10^{-7}M $1,25(OH)_2D_3$	17% increase in cells in G_0/G_1 quiescent phase on day 3 P21 mRNA 2-fold on day 3 P27 mRNA 3-fold on day 3	
Olsson et al. (2016) (Primary human)	10^{-7}M $1,25(OH)_2D_3$	BrdU incorporation 0.5-fold on day 2 Cyclin D2 mRNA down regulated 3-fold	✓
Romeu Montenegro et al. (2019) (Primary human)	10^{-7}M $1,25(OH)_2D_3$	BrdU incorporation 0.7-fold on day 2 Decrease in number of cells in G_2/M phase on day 2	
Saini et al. (2019) (Primary human)	10^{-7}M $1,25(OH)_2D_3$	EdU incorporation 0.95-fold on day 2 Down regulation of cyclin A2 and D1 mRNA after 24 h	
van der Meijden et al. (2016) (C2C12)	10^{-7}M $1,25(OH)_2D_3$	27.6% fewer viable cells on day 4	

the concentration of 1,25(OH)2D3 used was 10–7M but one study (Gili et al., 2016) used 10–8M 1,25(OH)2D3. In most cases mRNA expression was measured but in two studies (Gili et al., 2016; Braga et al., 2017) protein expression was measured. Unlike myoD expression, the level of agreement between studies relating to myogenin expression was low with five studies reporting a decrease in myogenin expression (Okuno et al., 2012; Ryan et al., 2013; Girgis et al., 2014; Olsson et al., 2016; van der Meijden et al., 2016) and four studies reporting an increase in expression (Garcia et al., 2011; Gili et al., 2016; Braga et al., 2017; Romeu Montenegro et al., 2019; **Figure 5**).

Three studies measured creatine kinase activity as a marker of differentiation (Capiati et al., 1999; Ryan et al., 2013; Gili et al., 2016). Two of these studies reported their results as a time course (Capiati et al., 1999; Gili et al., 2016) whilst one reported results for day 4 only (Ryan et al., 2013). A variety of concentrations of 1,25(OH)2D3 were used across the studies (**Table 4**). One study (Ryan et al., 2013) reported results for cells grown in either myogenic media or adipogenic media, but only the results for myogenic media have been used to allow comparison to the other studies. Two studies reported an increase in creatine kinase activity which peaked on day 2 following 1,25(OH)2D3 treatment (Capiati et al., 1999; Gili et al., 2016) whilst the other study found that creatine kinase activity decreased across all 1,25(OH)2D3 concentrations on day 4 (Ryan et al., 2013).

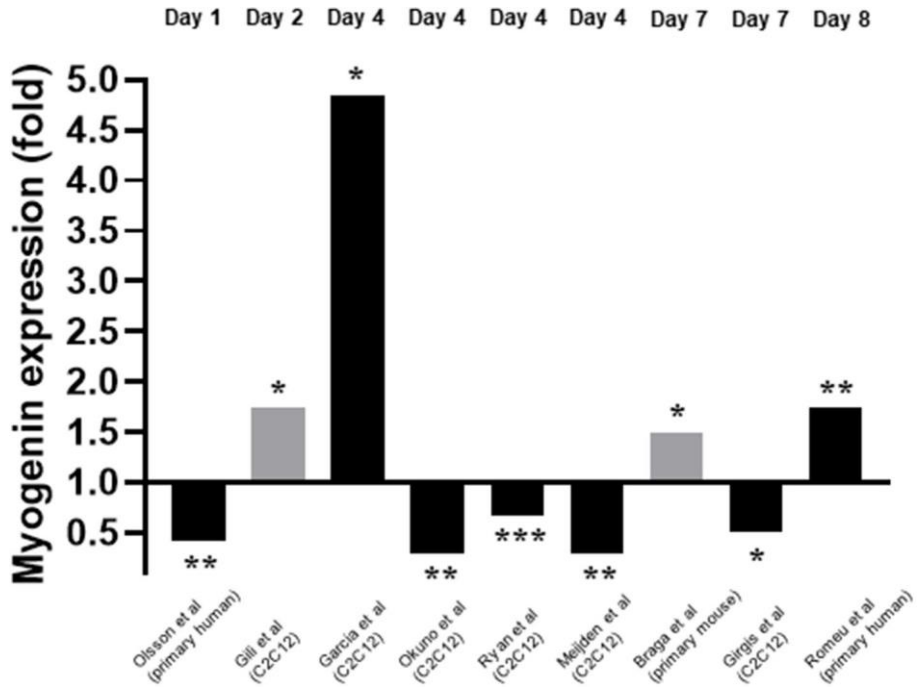

FIGURE 5 | Effect of 1,25(OH)$_2$D$_3$ of Myogenin expression. The active form of vitamin D [1,25(OH)$_2$D$_3$] at 10^{-7}M was used in all studies apart from Gili et al. (2016) where 10^{-8}M was used. Black bars indicate mRNA expression whilst grey indicates protein expression. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

TABLE 4 | Effects of 1,25(OH)$_2$D$_3$ on creatine kinase activity.

Reference (Cell type)	VitD form and concentration	CK activity	Significance
Capiati et al. (1999) (Primary chick)	10^{-9}M 1,25(OH)$_2$D$_3$	−45% on day 1 +55% on day 2 +30% on day 3 + 15% on day 6	$p < 0.01$ $P < 0.01$ $p < 0.05$ $p < 0.05$
Gili et al. (2016) (C2C12)	10^{-7}M 1,25(OH)$_2$D$_3$	1.7-fold on day 1 1.8-fold on day 2 1.3-fold on day 4	Individual *p*-values not given. ANOVA interaction $p < 0.05$
Ryan et al. (2013) (C2C12)	1,25(OH)$_2$D$_3$ for all: 10^{-13}M 10^{-11}M 10^{-9}M 10^{-7}M 10^{-5}M	Day 4 for all: Same as control 6% decrease 12.5% decrease 25% decrease 62.5% decrease	Individual *p*-values not given. ANOVA interaction $p < 0.001$

TABLE 5 | Effects of $1,25(OH)_2D_3$ or eldecalcitol on myosin or myosin heavy chain isoform expression.

Reference (Cell type)	VitD concentration and form	Factor measured	Effect	Significance
Capiati et al. (1999) (Primary chick)	10^{-9}M $1,25(OH)_2D_3$	Myosin protein	+88% on day 2 +31.5% on day 6	$p < 0.01$ $p < 0.01$
Gili et al. (2016) (C2C12)	10^{-9}M $1,25(OH)_2D_3$	MyHC protein	1.2-fold on day 2 1.4-fold on day 4	*p*-values not given
Okuno et al. (2012) (C2C12)	10^{-7}M $1,25(OH)_2D_3$	MyHC neo mRNA MyHCIIa mRNA	0.4-fold on day 4 2.5-fold on day 8	$p < 0.05$ $p < 0.01$
Olsson et al. (2016) (Primary human)	10^{-7}M $1,25(OH)_2D_3$	MyHC neo mRNA MyHCIIa mRNA	0.66-fold on day 1 0.73-fold on day 1	No *p*-values given
Romeu Montenegro et al. (2019) (Primary human)	10^{-7}M $1,25(OH)_2D_3$	MyHCII mRNA	0.4-fold on day 5	$p < 0.01$
Saito et al. (2017) (C2C12)	10^{-8}M eldecalcitol	MyHC neo mRNA MyHCIIa mRNA	1.4-fold on day 4 1.8-fold on day 4	Not significant $p < 0.01$
van der Meijden et al. (2016) (C2C12)	10^{-7}M $1,25(OH)_2D_3$	MyHCIIa mRNA	2.5-fold on day 3	*p*-value not given

Effects of Vitamin D on Late-Stage Myogenic Differentiation

A total of seven studies investigated the effects of 1,25(OH)2D3 treatment on myosin protein or mRNA/protein levels of myosin heavy chain (MyHC) isoforms, with the majority measuring the latter (Okuno et al., 2012; Gili et al., 2016; Olsson et al., 2016; van der Meijden et al., 2016; Saito et al., 2017; Romeu Montenegro et al., 2019). MyHC neonatal (MyHC neo) and type IIa (MyHCIIa) were the most commonly studied isoforms across the papers. Time points of expression varied greatly between studies. In some cases, expression was measured as early as day 1 whereas others measured up to day 8 (**Table 5**). Overall, one study found an increase in myosin protein (Capiati et al., 1999), one study found and increase in MyHC protein (Gili et al., 2016), three studies reported an increase in expression of at least one MyHC isoform (Okuno et al., 2012; Gili et al., 2016; van der Meijden et al., 2016; Saito et al., 2017) and two studies reported a decrease in MyHC isoforms (MyHC neo, MyHC IIa and MyHCII subtype unspecified) (Olsson et al., 2016; Romeu Montenegro et al., 2019).

Five studies measured the effects of 1,25(OH)2D3 on myotube size (Garcia et al., 2011; Girgis et al., 2014; Gili et al., 2016; van der Meijden et al., 2016; Romeu Montenegro et al., 2019; **Figure 6**). This was also measured at varying time points from day 2 to day 10. For one study 10–9M 1,25(OH)2D3 was used (Gili et al., 2016) whilst 10–7M 1,25(OH)2D3 was used in the other four (Garcia et al., 2011; Girgis et al., 2014; van der Meijden et al., 2016; Romeu Montenegro et al., 2019). All five studies concluded that treatment with 1,25(OH)2D3 resulted in an increase in myotube size which ranged from 1.1 to 2-fold.

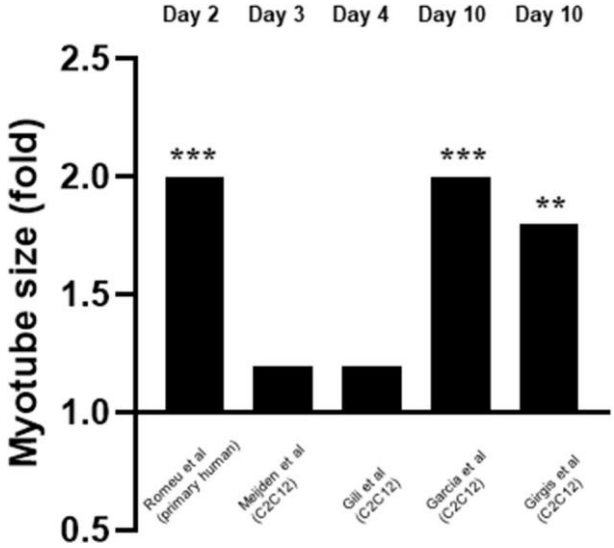

FIGURE 6 | Effect of $1,25(OH)_2D_3$ on myotube size. 5 studies investigated the effect of $1,25(OH)_2D_3$ on myotube size. Myotube size was measured at varying time points which ranged from day 3 to day 10. $1,25(OH)_2D_3$ concentration was 10^{-7}M for all cases apart from Gili et al. (2016) where 10^{-9}M was used. $^{**}p < 0.01$, $^{***}p < 0.001$.

DISCUSSION

This review has shown good agreement across the different studies in terms of the active form of VD inhibiting muscle cell proliferation. However, the effects on differentiation, as determined by various markers, showed less consistency, probably due to a combination of different cell types and time points being used.

Treatment With Vitamin D Inhibits Proliferation

Of the eight studies which investigated the effects of active VD on muscle cell proliferation (Capiati et al., 1999; Garcia et al., 2011; Okuno et al., 2012; Girgis et al., 2014; Olsson et al., 2016; van der Meijden et al., 2016; Romeu Montenegro et al., 2019; Saini et al., 2019), all of them found an inhibitory effect. One study observed a stimulatory effect on day 1, however this was

followed by inhibition on day 4 (Capiati et al., 1999). It is worth noting that only two studies (Girgis et al., 2014; Olsson et al., 2016) checked for differences in apoptosis between treated and untreated groups therefore it cannot be ruled out that the decrease in DNA observed in some of the other studies was not due to apoptosis.

Importantly, Okuno et al. (2012) showed that active VD caused an increase in cell cycle arrest at G0/G1 which occurred in parallel with increased expression of p21 and p27. Both p21 and p27 are members of the Cip/Kip family and are able to bind to cyclin dependent kinases and inhibit their role in cell cycle progression (Bachs et al., 2018). In order for a cell to proliferate, expression of p21 must decrease to a level where it no longer forms a complex with p53 (Terzi et al., 2016). Additionally, cells must also reduce/eliminate p27 to progress through proliferation, which is achieved via translocation of p27 to the cytoplasm where it is degraded (Bachs et al., 2018). Both Okuno et al. (2012) and Olsson et al. (2016) reported increased expression of both p21 and p27 suggesting that active VD treatment leads to increased transcription of these factors which likely contributes to the inhibition of cell proliferation.

Two studies reported that active VD decreased expression of both cyclin A2 and cyclin D3 (Olsson et al., 2016; Saini et al., 2019). Cyclin A2 is able to bind to and activate two cyclin dependent kinases (CDKs) required for cell cycle progression: CDK4 as DNA synthesis begins during S phase and CDK1 during the transition from G2 to M phase (Pagano et al., 1992). The D cyclins activate CDK4/6 enabling entry into S phase and down-regulation of cyclin D3 specifically inhibits G1 to S transition (Bartkova et al., 1998). From this, it can be suggested that active VD represses transcription of at least two cyclins which leads to inhibition of cell cycle transition and therefore cell cycle arrest and inhibition of proliferation.

Overall, decreases in DNA synthesis, increases in expression of p21/p27 and decreases in expression of cyclin A2/D3 suggest that treatment with active VD has a strong anti-proliferative effect on muscle cells in culture. It is likely that the cumulative effect of all of these factors lead to an overall reduction in muscle cell proliferation. The process of this anti-proliferative effect of active VD is shown in **Figure 7**.

Vitamin D Appears to Stimulate Early-Stage Differentiation

Myogenesis is a highly ordered and sequential process, guided by several transcription factors at various stages. This process of myogenic differentiation, and the proposed effect of active vitamin D on this process, is shown in **Figure 8**. Following withdrawal from the cell cycle, as described previously, myoblast fusion occurs to form multinucleated myotubes (Garcia et al., 2011). MyoD is a transcription factor involved in the early stages of differentiation (Girgis et al., 2014). When subjected to culture conditions which should induce differentiation, myoD$^{-/-}$ cells have been shown to continue to proliferate suggesting that expression of myoD is essential for withdrawal from the cell cycle (Sabourin et al., 1999). However, we previously observed no change in myoD mRNA over the time course of differentiation in C2C12 cells (Brown et al., 2012).

Four of the five studies which measured myoD reported an increase in expression following treatment with active VD (Garcia et al., 2011; van der Meijden et al., 2016; Braga et al., 2017; Saito et al., 2017), although the increase in expression was not significant for one study (van der Meijden et al., 2016). Interestingly, the only study which found a decrease in myoD expression was also the only study which used primary human cells (Olsson et al., 2016). This suggests that the effects of active VD on differentiation may depend upon cell type and/or species. However, this study also measured myoD very early in the process (day 1) (Olsson et al., 2016) whereas the remaining four studies all reported increased myoD expression on either day 4 (Garcia et al., 2011; van der Meijden et al., 2016; Saito et al., 2017) or day 7 (Braga et al., 2017). The effects observed may depend on upon the cell type and/or time point.

Evidence has shown that inhibition of IGFII results in a decrease in expression of myoD target genes, suggesting that IGFII is a key regulator of myoD expression (Wilson and Rotwein, 2006). One of the studies which found a 1.8-fold increase in myoD expression (Braga et al., 2017) found that both IGFI and IGFII expression also increased at the same time point. Additionally, increased expression of the IGFs has been shown to inhibit Myostatin, the only known negative regulator of muscle mass (Retamales et al., 2015). These findings suggest that active VD may increase expression of myoD directly or possibly indirectly via effects on local expression of IGFI and/or IGFII.

Effects of Vitamin D on Mid-Stage Differentiation Are Cell Type and Time Dependent

Induction of myogenin expression precedes the fusion of myoblasts to form myotubes, then myogenin switches on transcription of various muscle-specific genes (e.g., creatine kinase and MyHC isoforms) expressed by myotubes and muscle fibres (Bentzinger et al., 2012). Myogenin expression is therefore used as a marker of early to mid-stage differentiation and normally follows an increase in myoD expression (Hernández- Hernández et al., 2017). Indeed, myogenin knockout mice die immediately following birth, and whilst they have myoblasts present within the muscle, no muscle fibres are formed, resulting in the complete absence of functional skeletal muscle (Hernández-Hernández et al., 2017). Importantly, myogenin expression is completely blocked when the VD receptor is knocked down *in vitro* suggesting that VD has a direct effect on myogenin expression via the VDR (Gili et al., 2016).

However, the nature of this effect is controversial. As seen in **Figure 5**, five studies reported a decrease in myogenin expression (Okuno et al., 2012; Ryan et al., 2013; Girgis et al., 2014; Olsson et al., 2016; van der Meijden et al., 2016) whilst four reported an increase (Garcia et al., 2011; Gili et al., 2016; Braga et al., 2017; Romeu Montenegro et al., 2019). One possible explanation for this is the difference in methods between studies. Differentiation

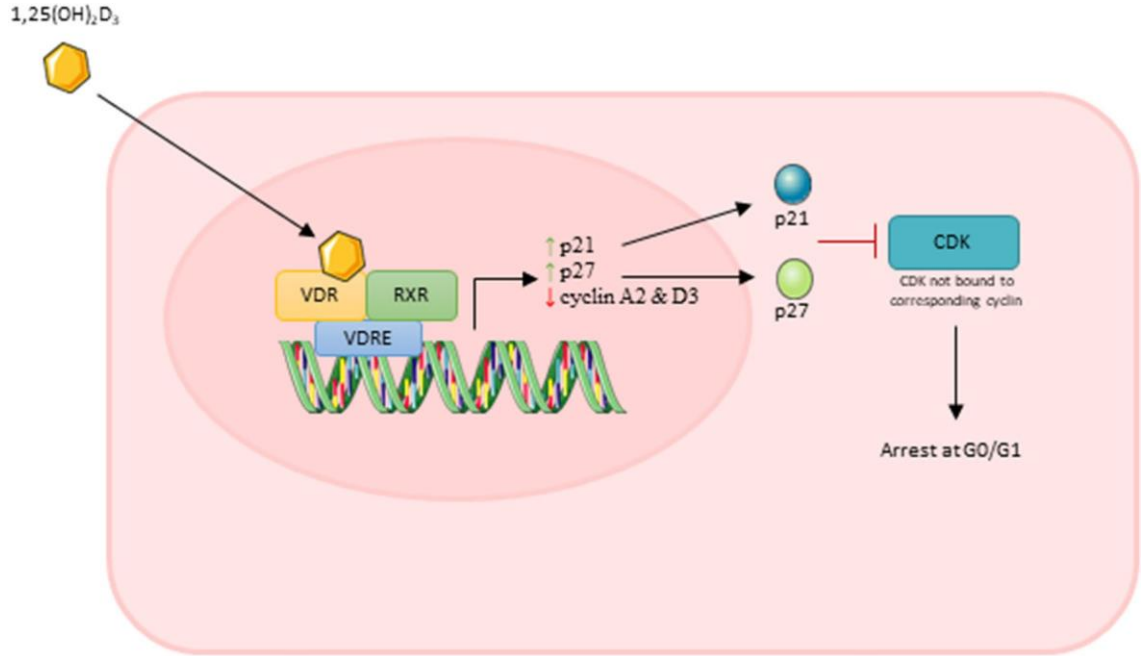

FIGURE 7 | Effect of active vitamin D [1,25(OH)$_2$D$_3$] on myoblast proliferation. Images used within this figure were obtained from smart servier medical art and can be found at https://smart.servier.com.

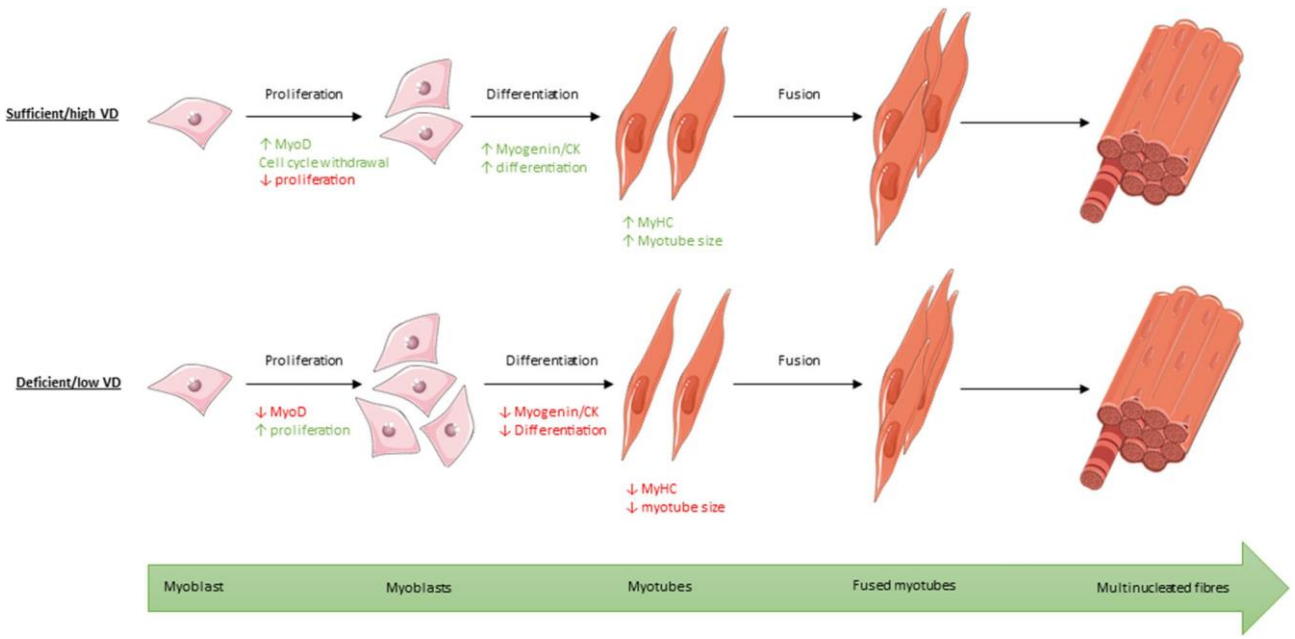

FIGURE 8 | Process of myogenic differentiation from myoblasts to multinucleated muscle fibres showing the effect of high/sufficient active vitamin D (VD) on various transcription factors within the process compared to low/deficient levels. Images used within this figure were obtained from smart servier medical art and can be found at https://smart.servier.com.

can be triggered via two mechanisms *in vitro*: 1. Serum starvation, which leads to a decrease in mitogenic stimuli, withdrawal from the cell cycle and increase in myogenin expression. 2. Prolonged confluence leading to a high cell density and more cell-cell contacts, which leads to increased IGF expression and an increase in myogenin (Girgis et al., 2014). Garcia et al. (2011), who used

the latter method, found that myogenin expression was increased at day 4 following active VD treatment of C2C12 cells. On the other hand, Girgis et al. (2014) used the serum deprivation method and reported a decrease in myogenin expression in C2C12 cells at day 7. It is important to note that we previously showed (Brown et al., 2012; Brearley et al., 2019) using multiple time points, that myogenin mRNA initially increases upon induction of differentiation (via serum starvation) of C2C12 cells, reaching a peak around day 2-3, then decreases again. Therefore, induction of differentiation would be associated with an increase in myogenin mRNA at early time points (days 0–3), but increased differentiation could also be associated with a more rapid decline in expression at later time points. It is also worth noting that C2C12 cells differentiate more rapidly than primary human myoblasts (Cheng et al., 2014) so are likely to have an earlier peak in myogenin expression. Hence, the discrepancies in the observed effects of active VD on myogenin expression could be due to the cell type used, the timepoints of measurement or a combination of the two. It is important that future studies should include measurements at several time points in order to make clear interpretations. It is also plausible that the two different methods of inducing differentiation may have different time frames, such that the rates of increase and decrease in expression as well as the peak of myogenin may be different. Certainly, there were large differences between studies in time points at which myogenin expression was measured, which likely contributed to the conflicting results.

Creatine kinase (CK) is a mid-stage marker of differentiation reported to peak around day 4 to 6 in both C2C12 (Brown et al., 2012) and primary chick myoblasts (Capiati et al., 1999). Two studies (Capiati et al., 1999; Gili et al., 2016) reported an increase in CK activity following treatment with active VD both of which found expression to peak on day 2, earlier than the expected window of 4–6 days. The remaining study which looked at CK activity (Ryan et al., 2013) reported a decrease in activity in C2C12 cells on day 4 across all active VD concentrations studied. Once again this might relate to differences in the timing relative to the expected peak in expression, with an early increase and a later decrease in expression potentially indicating an increase in the rate of differentiation.

Overall, the data is conflicting for both markers of early to mid-stage differentiation (myogenin and creatine kinase), but this may be due to the varying time points that each marker was measured, the variation in cell type, the differing concentrations of active VD used or a combination of all three.

Vitamin D Stimulates Expression of Late-Stage Markers of Differentiation

Myosin and the myosin heavy chain (MyHC) isoforms are muscle specific proteins that are often used as markers of mature, differentiated muscle cells (Gili et al., 2016) and together they form a significant proportion of the proteins present in differentiated muscle (Zammit, 2017). Five studies reported an increase in myosin or MyHC isoforms following active VD treatment (Capiati et al., 1999; Okuno et al., 2012; Gili et al., 2016; van der Meijden et al., 2016; Saito et al., 2017) whilst two studies reported a decrease in expression (Olsson et al., 2016; Romeu Montenegro et al., 2019). We previously showed that the MyHC isoforms are expressed in two distinct patterns during differentiation in C2C12 cells (Brown et al., 2012). The first pattern is an increase then decrease, peaking around day 2–4 of mRNA for MyHC embryonic (MyHC emb), foetal (MyHC neo), and slow type 1 (MyHC I) isoforms (Brown et al., 2012). The fast type II isoforms were all expressed much later in differentiation, being induced at days 2–4 in the order IIa > IIx > IIb (Brown et al., 2012). Hence, an increase in differentiation would always results in an increase in expression of the fast (type II) isoforms, but effects on the embryonic, foetal, and slow (type I) isoforms would be time dependent, with an increase in expression at early time points, but a decrease in expression at later time points.

Okuno et al. (2012) found that MyHC type IIa expression in C2C12 cells was increased by active VD at day 8 and they suggest that this indicated an anabolic effect in muscle. On the other hand, Olsson et al. (2016) found that both MyHC neonatal and MyHC IIa expression were reduced in C2C12 cells at day. 1. Considering MyHC is a marker of late stage differentiation (Gili et al., 2016) it is unclear why this study chose to measure MyHC expression during the earlier stages of differentiation, possibly missing the timepoint where MyHC expression may have increased.

The majority of *in vitro* evidence suggests that active VD stimulates the expression of MyHC isoforms, suggesting that active VD stimulates differentiation. Additionally, one study showed that injection of active VD increased expression of MyHC type IIa *in vivo* (Korn et al., 2013). However, this could be due to effects on muscle fibre type rather than muscle cell differentiation. It is known that IGFI can alter MyHC isoform expression (Saito et al., 2017) so active VD may impact on muscle fibre type indirectly via induction of local IGFI expression. Supporting this, Braga et al. (2017) reported an increase in expression of both IGFI and IGFII following active VD treatment in primary mouse cells *in vitro*. However, some argue that non- genomic actions of active VD, such as increases in intracellular Calcium concentrations, may be responsible for its effects on MyHC mRNA expression (de Boland and Boland, 1987).

Vitamin D Increases Myotube Size

Of the five studies which measured myotube size, all five reported an increase in myotube size (Garcia et al., 2011; Girgis et al., 2014; Gili et al., 2016; van der Meijden et al., 2016; Romeu Montenegro et al., 2019), which suggests a stimulatory effect of active VD on differentiation. In addition, Garcia et al. (2011) found an increase in expression of Follistatin (Fst). Fst is an antagonist of Myostatin (Mstn) a known negative regulator of muscle mass (Retamales et al., 2015), including both muscle cell proliferation and differentiation. Therefore, active VD might increase myotube size directly and/or indirectly via increasing IFG1 or Fst expression, the latter then inhibits Mstn (an inhibitor of differentiation) but both result in increased differentiation. Supporting this, Girgis et al. (2014) found myotube size was increased 1.8-fold on day 10 following a 10-fold decrease in Myostatin expression on day 7.

CONCLUSIONS

There is reasonably strong evidence to suggest that active VD inhibits proliferation of myoblasts, and stimulates differentiation and increases myotube size, although the effects on each stage of differentiation are not entirely consistent. These inconsistencies may relate to the use of different cell types and measurements at variable time points which makes interpretation more difficult. However, understanding the normal time course of expression during differentiation allows for some consistency across studies, but it clearly indicates that future studies should involve multiple time points. Also, only one study (Ryan et al., 2013) used concentrations of active VD within the physiological serum range (around 10–10 M) (Hou et al., 2018) so future studies should also consider using concentrations of active VD which are more physiologically relevant. However, it is worth noting that muscle cells do express 1α-hydroxylase and therefore can locally convert inactive VD to the active form (Mori et al., 2020). As it is not possible to measure these transient, local fluctuations in active VD, it cannot be ruled out that it may be possible for intracellular physiological concentrations to reach levels used within some of the studies in this review (10–7 M). Due to the presence of 1α-hydroxylase within skeletal muscle (Mori et al., 2020) future studies should also investigate the effects of inactive VD on muscle cells to see whether this results in similar effects to the active form.

It does appear that active VD has effects on skeletal muscle, particularly muscle cell proliferation and differentiation, indicating potential effects during embryonic development; when these processes mainly take place. VD deficiency has been shown to increase the risk of poor muscle strength and therefore falls, particularly in the elderly population (Garcia et al., 2011; Ceglia and Harris, 2013), but this review suggests that VD deficiency during embryonic and foetal development (i.e., during pregnancy) may also impact upon muscle development and function. Whilst VD supplementation in deficient individuals appears effective in increasing muscle strength and therefore decreasing fall risk in the elderly (Ryan et al., 2013), more research is needed to determine the impacts of supplementation during pregnancy/lactation or in the young offspring on muscle cell differentiation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

KA and JB contributed to the conception and interpretation of the data and reviewing of the draft manuscript. KA contributed to writing the original draft manuscript, acquisition, and analysis of the data. SK contributed to the data acquisition. TP and PJ contributed to the revising and contributing intellectual content writing. JB had final approval of the version to be published. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.736708/full#supplementary-material

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Appendix C Manuscript 2

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Active vitamin D increases myogenic differentiation in C2C12 cells via a vitamin D response element on the myogenin promoter

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Background: Skeletal muscle development during embryogenesis depends on proliferation of myoblasts followed by differentiation into myotubes/multinucleated myofibers. Vitamin D (VD) has been shown to affect these processes, but there is conflicting evidence within the current literature on the exact nature of these effects due to a lack of time course data. With 20%–40% of pregnant women worldwide being VD deficient, it is crucial that a clearer understanding of the impact of VD on myogenesis is gained.

Methods: A detailed 8-day differentiation time course was used where C2C12 cells were differentiated in control media (2% horse serum) or with different concentrations of active VD, 1,25 $(OH)_2D_3$ (10^{-13} M, 10^{-11} M, 10^{-9} M or 10^{-7} M), and measurements were taken at 6 time points. DNA, creatine kinase and protein assays were carried out as well as quantitative PCR to determine expression of Myf5, MyoD, myogenin, MHC I, and MHC neonatal, MHC embryonic, MHC IIa, MHC IIx, and MHC IIb mRNAs. Transfections were carried out using one vector containing the myogenin promoter and another containing the same promoter with a 3 base mutation within a putative vitamin D response element (VDRE) to determine effects of 1,25 $(OH)_2D_3$ on myogenin transcription. Finally, a ChIP assay was performed to determine whether the VD receptor (VDR) binds to the putative VDRE.

Results: 1,25$(OH)_2D_3$ caused an inhibition of proliferation and an increase in differentiation in C2C12 cells. Myf5, myogenin, MHC I, and MHC neonatal, MHC embryonic, MHC IIa, MHC IIx, and MHC IIb expression were all increased by 1,25$(OH)_2D_3$. Myotube size was also increased by VD. When the putative VDRE on the myogenin promoter was mutated, the increase in expression by VD was lost. ChIP analysis revealed that the VDR does bind to the putative VDRE on the myogenin promoter.

Conclusion: Active VD directly increases myogenin transcription via a functional VDRE on the myogenin promoter, resulting in increased myogenic differentiation,

increased expression of both the early and late MHC isoforms, and also increased myotube size. These results highlight the importance of VD status during pregnancy for normal myogenesis to occur, but further *in vivo* work is needed.

KEYWORDS

vitamin D, vitamin D response element, vitamin D receptor, myogenin, differentiation, myogenesis

Introduction

It is well documented that vitamin D (VD) deficiency, defined in the UK by serum levels of inactive VD [25(OH)D_3] falling below 25 nmol/L (Griffin et al., 2021), is associated with muscle weakness, and that this is reversible with dietary supplementation (Braga et al., 2017), indicating a link between serum 25(OH)D_3 status and muscle function. There is also evidence of vitamin D receptor (VDR) expression in human muscle cells, as well as effects of VD treatment, suggesting that the effects on muscle may be direct and mediated through the VDR (Olsson et al., 2016). Furthermore, VDR knockout mice have smaller muscle fibers than wild type mice at 3 weeks of age, which becomes even more prominent by 8 weeks (Endo et al., 2003). Collectively, these findings suggest that VD and the VDR play an important role in the regulation of skeletal muscle function and development.

The biologically active form of VD, 1,25$(OH)_2D_3$ (also called calcitriol), has the highest affinity for the VDR with a k_D of 0.1 nM (Carlberg, 2019). The VDR belongs to the zinc finger class of transcription factors (Molnár, 2014), which can be activated by low (nanomolar) concentrations of 1,25$(OH)_2D_3$, and interacts in a sequence specific manner with genomic DNA (Carlberg and Campbell, 2013). Upon binding of 1,25$(OH)_2D_3$ to the VDR, a conformational change occurs allowing the VDR to bind to other proteins to form a dimer (Thompson et al., 1998). Hence, the VDR either forms a homodimer with another VDR, or a heterodimer by binding with a retinoic acid receptor (RAR) or a retinoid X receptor (RXR), with the latter being the predominant heterodimer formed (Carlberg and Molnár, 2015). Once formed, the homo- or heterodimer binds to a VD response element (VDRE) on a target gene, which are typically comprised of two conserved hexameric repeats separated by a 3-nucleotide spacer (Tsoumpra et al., 2020). In order for successful activation of transcription, the activated VDR needs to cause efficient dissociation of co-repressors as well as efficient association of co-activators (Carlberg and Molnár, 2015). Primarily, p160 coactivators and steroid receptor activators 1, 2, and 3 are recruited, which have histone acetylase activity resulting in the unwinding of DNA from the histones and allowing access for the transcriptional machinery (Christakos et al., 2016). In total, over 900 genes have been found to be regulated by VD (Wang et al., 2005).

Within the genes that have been shown to be regulated by VD, some genes relating to myogenesis have been identified (Girgis et al., 2014). Our recent systematic review (Alliband et al., 2021), evaluating the effects of active VD on muscle differentiation and expression of myogenic genes, showed a consistent anti-proliferative effect of active VD on muscle cells in culture, as well as an increase in myotube size. However, the effects on differentiation and the mRNA expression of myogenic regulatory factors (MRFs) and myosin heavy chain (MHC) isoforms were inconsistent. The main issue across the studies appeared to be a lack of time course data, with most only measuring gene expression at one or two time points, with large inconsistencies as to which day of differentiation this was done on. There was also variability in the concentrations of active VD used.

Hence, the aim of this study was to determine the effects of 1,25$(OH)_2D_3$ on muscle cell proliferation and differentiation, using the C2C12 muscle cell line, with the effects on differentiation being via a detailed time course for mRNA expression of the MRFs and MHC isoforms (Figure 1). This builds on our previous work which mapped the time course of mRNA expression of these genes during normal C2C12 differentiation (Brown et al., 2012). We used four different concentrations of 1,25$(OH)_2D_3$ which ranged from below physiological to above physiological concentrations (physiological range for 1,25$(OH)_2D_3$ = 4.3 × 10^{-11} M to 1.9 × 10^{-10} M) (Pagana et al., 2019) to determine whether any observed effects were dose dependent, and further investigated the mechanism for the observed effects of 1,25$(OH)_2D_3$ on myogenic differentiation.

Materials and methods

Cell culture

The C2C12 mouse myoblast cell line was obtained from the European Collection of Authenticated Cell Cultures (ECACC). C2C12 myoblasts were grown in growth medium consisting of Dulbecco's modified eagle's medium (DMEM; Sigma Aldrich, Poole, UK) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen, Paisley, UK) and 1% (v/v) 100 × penicillin streptomycin (P/S; Invitrogen, Paisley, UK) and incubated at 37°C and 5% CO_2. Cells were treated with different concentrations of 1,25$(OH)_2D_3$ [either 10^{-13} M, 10^{-11} M, 10^{-9} M or 10^{-7} M in 0.1% ethanol (v/v)]. Two controls were used, one treated with 0.1% (v/v) ethanol and the second with normal differentiation media to determine whether the vehicle was causing any of the effects.

For the proliferation experiment, cells were plated onto 96-well plates (at 5,000 cells/well) prior to 1,25$(OH)_2D_3$ treatments being added 24 h later. Plates were stopped at t = 0 (24 h after plating, before addition of treatments) and at 24 h and 48 h post 1,25$(OH)_2D_3$ treatment by removing media and washing with phosphate buffered saline (PBS). 100 µL of dH_2O was then added to each well and plates were frozen at −20°C prior to freeze-thawing and DNA assays.

For differentiation studies, cells were plated onto 6-well plates (1.5 × 10^5 cells/well). On day 0 (approximately 70% confluence) cells underwent serum starvation to encourage differentiation by switching to differentiation media consisting of DMEM

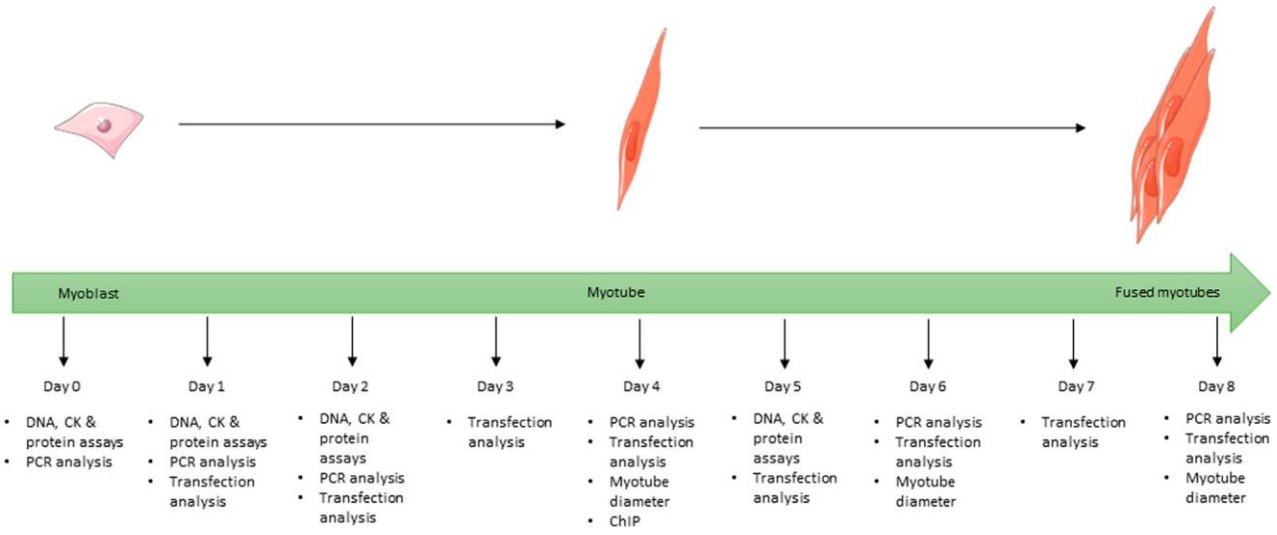

FIGURE 1

Schematic showing the full 8-day time course used for the differentiation of C2C12 cells during the study and at which time point each experiment was undertaken for all cells grown in differentiation media. Images used within this figure were obtained from smart servier medical art and can be found at https://smart.servier.com

supplemented with 2% (v/v) horse serum (HS; Invitrogen, Paisley, UK) and 1% (v/v) 100 X P/S. VD treatments were also added at this point. Cells were cultured for up to 8 days and media was replaced every 48 h. Cells for protein and creatine kinase assays were harvested on days 0, 1, 2, and 5 (Figure 1) by scraping into 500 µL of tri-sodium citrate (0.05M, pH 6.8, 4˚C) and placed on ice, then stored at −20˚C. Cells for gene expression analysis were harvested on days 0, 1, 2, 4, 6, and 8 (Figure 1) by scraping into 200 µL of ice cold RNase free PBS and stored at −80˚C prior to RNA extraction (see below).

DNA, protein and creatine kinase assays

To lyse the cells prior to DNA assays, plates underwent two freeze/thaw cycles. DNA contents were determined using the Hoechst fluorescent dye method as previously described (Rago et al., 1990; Hurley et al., 2006). In brief, 100 µL of working dye solution was added to 100 µL cell lysates on the 96-well plates. A standard curve of known concentrations of calf thymus DNA was run alongside the samples. Fluorescence was measured using a fluorescent plate reader (Fluostar Optima, BMG Labtech) at excitation of 350 nm and emission 460 nm.

For protein and creatine kinase assays, cells in tri-sodium citrate buffer were thawed on ice and then sonicated (Soniprep150, MSE, UK) using 10 µm amplitude for roughly 30 s, or until the sample was clear. Cell lysates were used immediately after sonication for the creatine kinase assay and were stored at −20˚C again prior to protein and DNA assays. Creatine kinase activity (U/l) was determined as previously described (Hurley et al., 2006; Brown et al., 2012) using a CK kinetic assay kit (Thermo Electron Corporation, Fisher Scientific, Loughborough, UK). Samples were run on a 96-well plate and absorbance at 340 nm was read every 30 s for a total of 5 min at 37˚C to determine enzyme activity (BioRad 680XR Microplate reader). Protein contents were determined using the Lowry method as previously described (Hurley et al., 2006; Brown et al., 2012). Samples were run on a 96-well plate alongside a standard curve of known concentrations of bovine serum albumin and absorbance was measured at 655 nm (BioRad 680XR Microplate reader).

A DNA assay was also carried out (as above) on these samples to normalise protein and CK activity against.

Gene expression analysis via quantitative RT-qPCR

An RNA isolation kit (ROCHE high pure isolation kit) was used according to the manufacturer's protocol to extract total RNA from cells previously scraped into 200 µL RNase free PBS, after thawing on ice. Total RNA concentrations were determined using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, United States). All samples were diluted to 50 ng/µL and stored at −80˚C. First strand cDNA synthesis was carried out using a Thermo Scientific RevertAid RT kit (Thermo Scientific, Vilnius, Lithuania) according to manufacturer's protocol using 500 ng total RNA per sample. First strand cDNA samples were then diluted 5- fold using RNase free water and stored at −20˚C.

A LightCycler® 480 (Roche, Burgess Hill, UK) was used to carry out RT-qPCR analysis. Each well on a 384 well PCR plate contained the following: 5 µL first strand cDNA, 7.5 µL SYBR Green I Master mix (Roche, Burgess Hill, UK), 0.45 µL forward primer (10 µM), 0.45 µL reverse primer (10 µM), 1.6 µL RNase free H_2O. The PCR amplification protocol was as follows: pre-incubation at 95˚C for 5 min followed by 45 amplification cycles (denaturation of strands at 95˚C for 10 s, annealing at 60˚C for 15 s, elongation at 72˚C for 15 s) (Brown et al., 2012). All reactions were carried out in triplicate. Initially, qPCR analysis was run on every cDNA sample using primers for a potential reference gene (TATA binding protein) to check that

the crossing point (CP) for each sample was within 2 standard deviations of the mean of all samples. Once this was confirmed, a cDNA pool was made by combining 10 µL aliquots from every sample. This pool was then used in serial dilutions to create a standard curve to determine the PCR efficiencies for all primer pairs/ genes and subsequently the transcript concentrations of the samples. Melt curve analysis was used to ensure only one peak was present (indicating a single amplicon product) for all primers in all samples. Supplementary Data S1 includes a list of genes measured and their corresponding forward and reverse primers. All PCR primers were designed to cross exon-exon boundaries and working primers were diluted from stock primers.

Oligreen quantification of total cDNA

As in our previous study (Brown et al., 2012), we were unable to find a reference gene that did not significantly change across the time course studied (data not shown). Therefore, an Oligreen ssDNA Assay kit (Invitrogen, Eugene, Oregon) was used to quantify cDNA content for each sample on a 384 well PCR plate according to manufacturer's protocol. Fluorescence was quantified using the LightCycler® 480 (Roche, Burgess Hill, UK) with increasing temperature at a continuous ramp rate of 0.11°C/s until it reached 95°C. Fluorescence measurements at 80°C were plotted against a cDNA standard curve (as described above) to determine relative cDNA quantity in each sample and this was subsequently used for normalisation of all genes.

Generation of myogenin promoter plasmids

The mouse myogenin promoter sequence was obtained from the NCBI database (accession M95800). The typical VDRE sequence obtained from (Tsoumpra et al., 2020) was aligned to the myogenin promoter sequence using Clustal Omega, which identified a potential VDRE (12/15 bases, 80% match) on the myogenin promoter at −1,260 to −1,246 from the transcription start site (TSS). A vector containing the mouse myogenin promoter sequence, including the potential VDRE, linked to a Turbo-green fluorescent protein (GFP) reporter (MyoG-VDRE-GFP) was purchased from VectorBuilder. Another vector with a mutated VDRE (MyoG-mutVDRE-GFP) was also purchased (VectorBuilder) which was identical in sequence apart from 3 bases in positions 2, 3, and 4 of the VDRE sequence (−1,261 to −1,264) which were changed from GAA to AGG. Full vector components and sequences are shown in Supplementary Data S2. Plasmids were prepared using a Promega PureYield Plasmid Miniprep kit (Madison, Wisconsin, USA) according to manufacturer's protocol. Plasmid concentrations were measured using a nanodrop® ND-1000 (Thermo Scientific, Wilmington, United States) and were stored at −20°C.

Myogenin promoter transfection studies

Transfection of C2C12 cells with the different vectors was carried out at 60%–70% confluency on day −1 (i.e. 24 h before switching to differentiation media). C2C12 cells were cultured in 24- well plates, at 30,000 cells/well, and were transfected 24 h after plating (whilst in the log phase of growth) using Lipofectamine 2000 (Invitrogen, California, United States) according to manufacturer's protocol. Each well contained 500 ng vector (400 ng MyoG-GFP vector plus 100 ng pDsRed-Express-NI vector with a CMV promoter followed by red fluorescence protein (RFP) (Brown et al., 2014). This was to check for transfection efficiency after 24 h due to myogenin not being expressed until mid-stage differentiation and so the MyoG-GFP cells did not appear green until day 2. 2.5 µL lipofectamine in Opti-MEM medium (Gibco, Paisley, UK) was used per well. 30 min prior to cell imaging, media was replaced with Fluorobrite DMEM (Fisher Scientific, Loughborough, UK) with 2 drops per ml of NucBlue Hoechst nuclear stain (Fisher Scientific, Loughborough, UK) and incubated at 37°C, 5% CO_2. Cell images were taken on a fluorescent microscope (Evos FL) and fluorescence was measured every 24 h for 8 days with a fluorescent plate reader (Fluostar Optima, BMG Labtech) using the matrix scan setting. This is where 30 × 30 measurements are taken over a 1.5 cm^2 area for each well, and the average for each well calculated. GFP was measured at an excitation of 485 nm and emission of 520 nm and NucBlue was measured at an excitation of 355 nm and emission of 460 nm. All GFP data was normalised to NucBlue to account for any differences in cell number.

Images of GFP transfected myotubes were analysed for myotube diameter using ImageJ as described previously (Brown et al., 2018; Brearley et al., 2021). Briefly, four images at ×10 magnification were used for each treatment at each time point and five myotubes were analysed per image, a total of 20 myotubes per treatment at each time point. Myotube diameter was measured at 25%, 50%, and 75% of total myotube length to provide an average diameter per myotube. Length in number of pixels was converted to µM by measuring the length of a 400 µM scale bar in pixels.

Chromatin immunoprecipitation (ChIP) assay

Cells were plated on 6 well plates at 1.5 × 10^5 cells/well and cultured in growth medium for 48 h until 70% confluent. Media was then removed and replaced with differentiation media supplemented with either 0.1% (v/v) EtOH, 10^{-11} M or 10^{-7} M $1,25(OH)_2D_3$ for 4 days, with media changed every 48 h 4 days after switching to differentiation media, cells were cross linked using 1% (v/) formaldehyde. ChIP assay was carried out using a Pierce Agarose ChIP kit (Pierce, Thermo Scientific, Wilmington, United States). According to manufacturer's protocol. After digestion, genomic DNA fragment size was checked by running on a 1% (w/v) agarose gel to ensure fragments were between 200–1000 bp. Anti- vitamin D receptor antibody (ab3508 Abcam, UK) was used to isolate genomic DNA fragments containing a VDRE. The myogenin promoter VDRE primers (Supplementary Data S1) used for PCR were designed using Primer Express. Each PCR reaction contained the following: 5 µL genomic DNA, 7.5 µL SYBR Green I Master mix (Roche, Burgess Hill, UK), 0.45 µL forward primer (10 µM), 0.45 µL reverse primer (10 µM), 1.6 µL RNase free H_2O and PCR conditions were the same as described above. PCR products were run on a 2.5% (w/v) Metaphor Agarose gel in 1 × TAE buffer at 100 V to confirm fragment size.

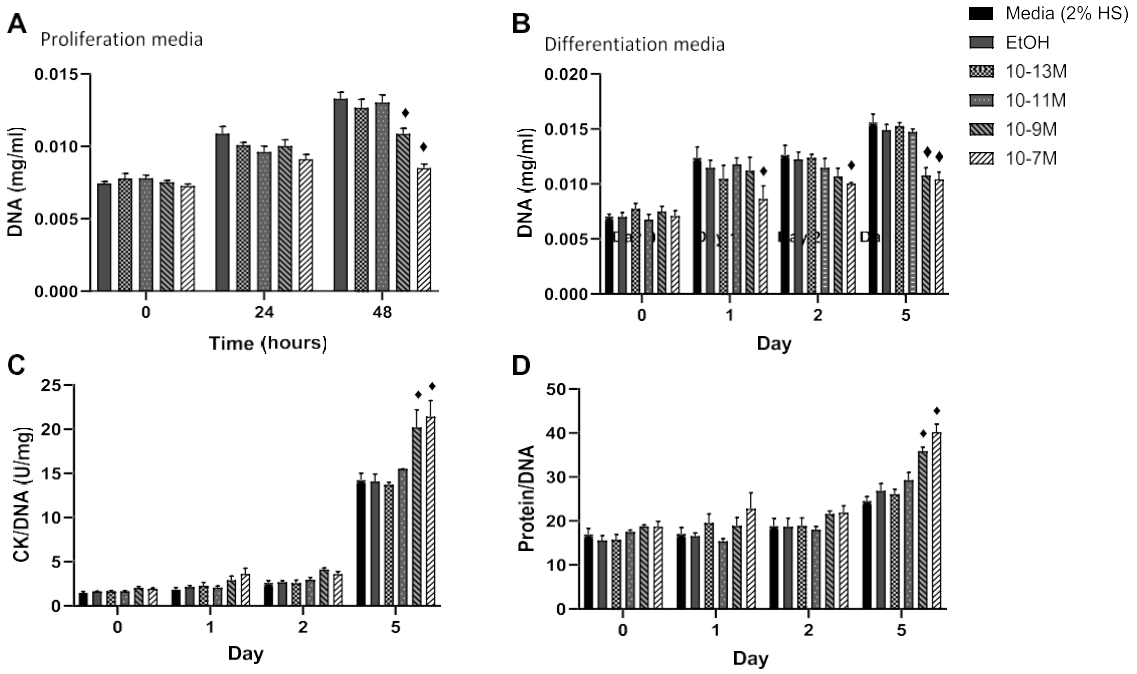

FIGURE 2

(A) Effects of 1,25 $(OH)_2D_3$ treatment in C2C12 cells on DNA, creatine kinase and protein. (A) DNA (mg/mL) of C2C12 cells grown in proliferation media for 48 h with varying concentrations of 1,25 $(OH)_2D_3$ in 0.1% (v/v) ethanol. (B–D) C2C12 cells grown in differentiation media for 5 days with varying concentrations of 1,25$(OH)_2D_3$ in 0.1% (v/v) ethanol. (B) DNA (mg/mL) (C) Creatine Kinase activity normalised to DNA (U/L) (D) Protein content normalised to DNA. ♦ indicates where a bar is significantly different ($p < 0.05$) to both media and ethanol controls [media (2% HS) and EtOH] within the same time point. Error bars show standard deviation. 4 biological replicates (wells) were used throughout and all assays were carried out in triplicate. Statistical analysis was carried out via a 2-way ANOVA with a *post hoc* Bonferroni test where time*treatment interaction was significant ($p < 0.05$).

Statistical analysis

Four biological replicates (i.e., wells) were used throughout. Assays and PCR analyses were carried out in triplicate for each sample. Statistical analyses were carried out using GenStat 22nd edition. Most data were analysed by 2-way ANOVA (day x treatment) with the exception of the second promoter transfection experiment where two different vectors were used so a 3-way ANOVA (day x treatment x vector) was carried out. *Post hoc* Bonferroni tests were then used to identify groups that were significantly different. Results were deemed significant if $p < 0.05$.

Results

Vitamin D decreases proliferation and increases differentiation of C2C12 cells

In order for myoblasts to undergo myogenic differentiation they must first exit from the cell cycle so that they can switch from a proliferative to a differentiative state (Kitzmann and Fernandez, 2001). DNA content of C2C12 cells was measured as a marker of cell proliferation with a decrease in DNA content indicating a decrease in cell number. A significant day × treatment interaction was observed for DNA content ($p = 0.001$) for cells in proliferation media, Figure 2A. The two highest concentrations of 1,25$(OH)_2D_3$ resulted in significantly lower levels of DNA at 48 h compared to controls ($p < 0.05$) but were similar to DNA levels at the time
0 point. For cells in differentiation media a significant day × treatment interaction was also observed for DNA content ($p < 0.001$), Figure 2B. By day 1 (24 h post switching to differentiation media) cells treated with 10^{-7} M 1,25$(OH)_2D_3$ had significantly lower DNA content than controls ($p < 0.05$) indicating decreased proliferation. By day 5, both 10^{-7} M and 10^{-9} M 1,25$(OH)_2D_3$ had significantly lower DNA content than day 5 controls ($p < 0.05$). Within differentiating muscle cells in culture, creatine kinase (CK) begins to be expressed after proliferating myoblasts exit from the cell cycle and begin to express differentiation specific factors (Tai et al., 2011). Therefore, CK enzyme activity was measured as a marker of mid stage differentiation (Figure 2C). There was a significant day × treatment interaction ($p < 0.001$) for CK activity. Activity did not increase between day 0 and 2 for all treatment groups, but CK enzyme activity for both 10^{-9} M and 10^{-7} M 1,25$(OH)_2D_3$ was significantly higher than controls by day
5 ($p < 0.05$).
A significant day × treatment interaction was also seen for the protein/DNA ratio in response to 1,25$(OH)_2D_3$ treatment ($p < 0.01$, Figure 2D). Again, there were no differences until day 5, when both 10^{-7} M and 10^{-9} M 1.25$(OH)_2D_3$ resulted in significantly higher protein contents than controls ($p < 0.05$). The increases in both CK enzyme activity and protein contents, relative to DNA, indicate a significant increase in differentiation with both 10^{-7} M and 10^{-9} M 1,25$(OH)_2D_3$ compared to the controls.

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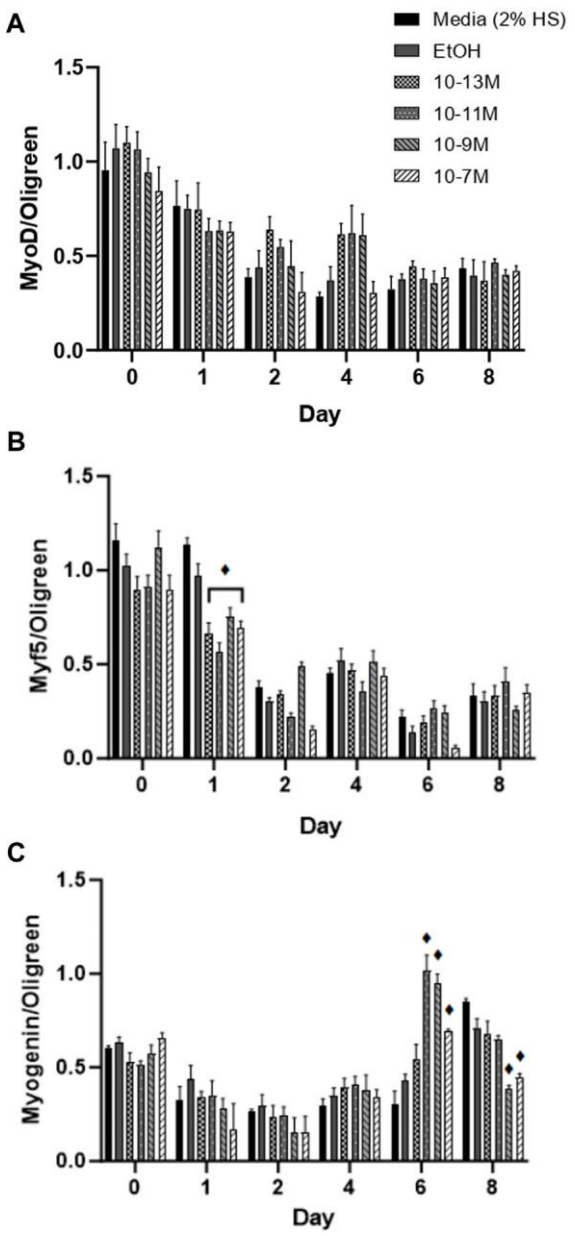

FIGURE 3

Effects of 1,25 $(OH)_2D_3$ on C2C12 cells over 8 days on myogenic regulatory factor gene expression. (A) MyoD (B) Myf5 and (C) Myogenin mRNA expression normalised to Oligreen during C2C12 differentiation over an 8-day time course with varying concentrations of 1,25$(OH)_2D_3$ dissolved in 0.1% (v/v) ethanol. ◆ symbol indicates where a bar is significantly different ($p < 0.05$) to both media and ethanol controls [media (2% HS) and EtOH] within the same time point. Error bars show standard deviation. 4 biological replicates (wells) were used throughout and all PCR experiments were carried out in triplicate. Statistical analysis was carried out via a 2-way ANOVA with a *post hoc* Bonferroni test where time*treatment interaction was significant ($p < 0.05$).

Vitamin D influences mRNA expression of two myogenic regulatory factors

Myf5 and MyoD are myogenic regulatory factors (MRFs) responsible for cell commitment to the myogenic lineage, exit from the cell cycle and the initiation of terminal differentiation (Montenegro et al., 2019) and therefore are markers of early stage differentiation. For MyoD mRNA expression (Figure 3A), there was no significant day × treatment interaction ($p = 0.49$) and no significant effect of treatment ($p = 0.12$). As expected, there was a significant effect of time ($p < 0.001$) with mRNA expression decreasing from day 0 to day 1 ($p < 0.05$) and from day 1 to day 2 ($p < 0.05$). For Myf5 mRNA expression (Figure 3B) there was a significant day × treatment interaction ($p < 0.001$). As expected, Myf5 mRNA expression decreased over time for all treatment groups, but all 1,25$(OH)_2D_3$ treatment groups significantly decreased on day 1 compared to day 0 ($p < 0.05$) whereas controls did not decrease until day 2 ($p < 0.05$).

Myogenin is an MRF which is expressed following MyoD and Myf5 expression, and is necessary for the terminal differentiation of myoblasts to myotubes (Montenegro et al., 2019), so is often used as the main marker of myogenic differentiation. There was a significant day × treatment interaction ($p < 0.001$) for myogenin mRNA expression (Figure 3C). There were no significant differences in expression from day 0 to day 4, but there was an increase in expression for the 3 highest doses of 1,25$(OH)_2D_3$ on day 6 compared to day 4, although only 10^{-11} M and 10^{-9} M 1,25$(OH)_2D_3$ were significant ($p < 0.05$). Myogenin mRNA expression did not significantly increase until day 8 for controls ($p < 0.05$), at which point, expression for 10^{-9} M and 10^{-7} M 1,25$(OH)_2D_3$ was significantly lower than controls ($p < 0.05$). Hence, there was an earlier peak for myogenin expression with 1,25$(OH)_2D_3$ treatment indicating that the differentiation process had been induced sooner.

Vitamin D influences mRNA expression of myosin heavy chain (MHC) isoforms

(Brown et al., 2014) showed that expression of the myosin heavy chain (MHC) isoforms can be categorised into early or late during C2C12 muscle cell differentiation. The MHC type I, neonatal and embryonic isoforms are expressed earlier during differentiation. For all 3 of these isoforms, mRNA expression remained low from day 0 to day 4, peaked on day 6, then fell on day 8 (Figures 4A–C). Importantly, on day 6, mRNA expression of both MHCI and MHC embryonic was significantly higher for the 3 highest concentrations of 1,25$(OH)_2D_3$ compared to controls ($p < 0.05$), whereas only 10^{-9} M 1,25$(OH)_2D_3$ was significantly higher than controls for MHC neonatal mRNA on day 6 ($p < 0.05$). Expression of all three early MHC isoforms was positively correlated with myogenin mRNA expression (Supplementary Data S3), with expression peaking on day 6. The strongest correlation was observed between myogenin and MHCI with an R^2 value of 0.59 ($p < 0.05$), followed by MHC neonatal ($R^2 = 0.55$, $p < 0.05$) then MHC embryonic ($R^2 = 0.49$, $p < 0.05$).

The late MHC isoforms are expressed after the early isoforms in the order MHC IIa, IIx, IIb towards the latter stages of C2C12 differentiation (Brown et al., 2012), hence an increase in mRNA expression was only seen on days 6 and 8 (Figures 4D–F). MHC IIa mRNA expression was significantly increased by 10^{-7} M 1,25$(OH)_2D_3$ ($p < 0.05$), whilst MHC IIx expression was significantly increased by both 10^{-9} M and 10^{-7} M 1,25$(OH)_2D_3$ ($p < 0.05$), and

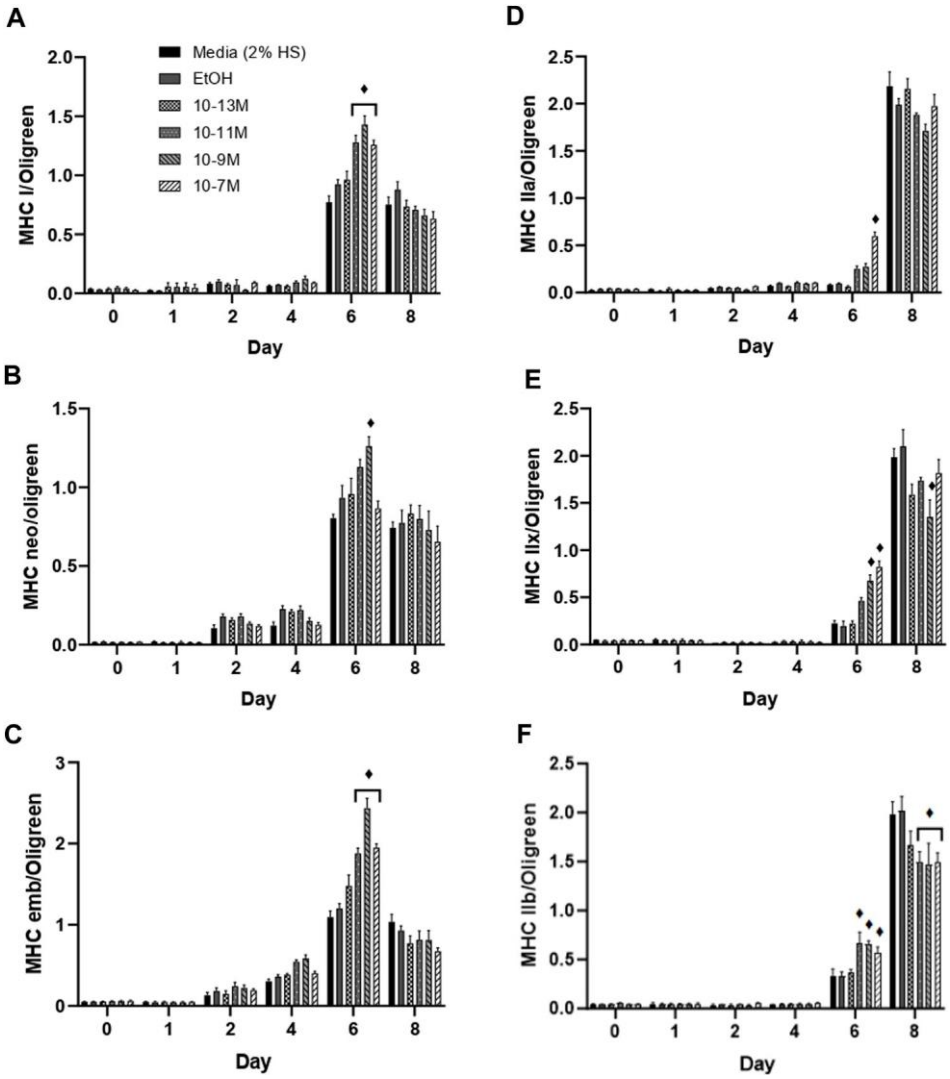

FIGURE 4

Effects of 1,25$(OH)_2D_3$ on C2C12 cells over 8 days on myosin heavy chain gene expression. (A) MHC I (B) MHC neonatal (C) MHC embryonic (D) MHC IIa (E) MHC IIx (F) MHC IIb mRNA expression normalised to Oligreen during C2C12 differentiation over an 8-day time course with varying concentrations of 1,25 $(OH)_2D_3$ dissolved in 0.1% (v/v) ethanol. ◆ symbol indicates where a bar is significantly different ($p < 0.05$) to both media and ethanol controls [media (2% HS) and EtOH] within the same time point. Error bars show standard deviation. 4 biological replicates (wells) were used throughout and all PCR experiments were carried out in triplicate. Statistical analysis was carried out via a 2-way ANOVA with a *post hoc* Bonferroni test where time*treatment interaction was significant ($p < 0.05$).

MHC IIb expression was significantly increased by 10^{-11} M, 10^{-9} M and 10^{-7} M 1,25$(OH)_2D_3$ ($p < 0.05$) on day 6 compared to controls. Importantly, expression of all 3 late MHC isoforms did not change from day 0 to day 6 in the control cells, and only significantly increased on day 8 ($p < 0.05$) indicating an earlier induction of differentiation with 1,25$(OH)_2D_3$ treatment.

Vitamin D increases myogenin expression via a functional VDRE on the myogenin promoter

Since myogenin is a transcription factor known to induce expression of many of the genes associated with myogenic differentiation, including the MHC isoforms, we hypothesised that 1,25$(OH)_2D_3$ effects on differentiation might be via increases in mRNA myogenin expression, which subsequently increases the expression of other genes. This was backed up by the observed positive correlations between myogenin and MHC early isoform mRNA expression. We therefore investigated whether the myogenin promoter contained a putative VDRE, which was identified at −1,260 to −1,246 base pairs from the transcriptional start site, and then tested its responsiveness to 1,25$(OH)_2D_3$ by performing transfection studies using a vector containing the myogenin promoter sequence (with the putative VDRE) upstream of a GFP reporter sequence (MyoG-VDRE-GFP, Supplementary Data S2). Over the 8-day time course, there was a significant day × treatment interaction for myogenin promoter activity ($p < 0.001$, Figure 5A). On day 4, GFP fluorescence was significantly higher with 10^{-7} M and 10^{-11} M 1,25$(OH)_2D_3$ compared to controls ($p < 0.05$). By day 5, all 3 1,25$(OH)_2D_3$ treatment groups were significantly higher than

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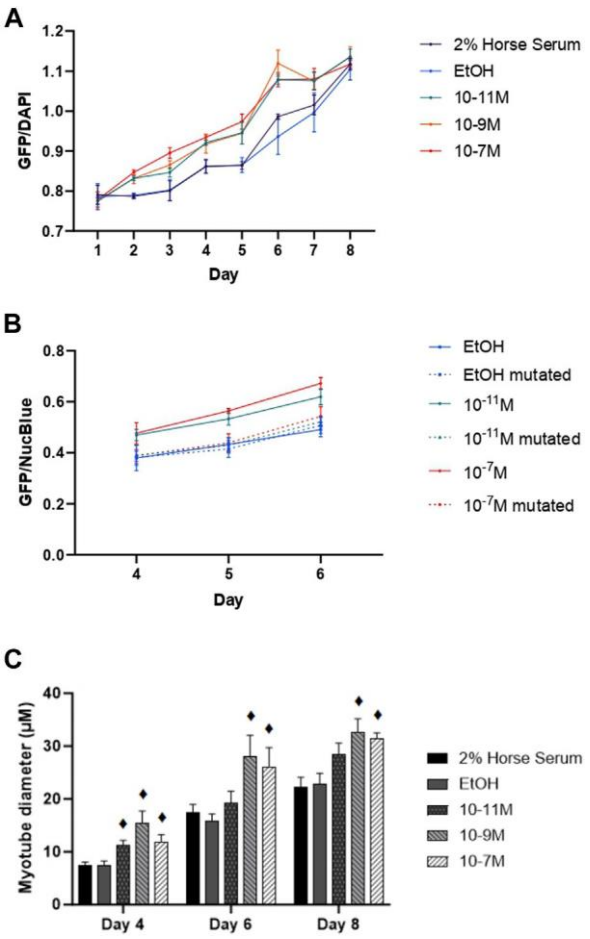

FIGURE 5

Effects of 1,25 $(OH)_2D_3$ on C2C12 cells transfected with wild-type or mutated mouse myogenin GFP vectors. (A) GFP fluorescence (normalised to NucBlue) using a vector containing the wild type myogenin promoter sequence 5′ to a turbo GFP sequence during C2C12 differentiation over an 8 day time course with varying concentrations of 1,25$(OH)_2D_3$ dissolved in 0.1% (v/v) ethanol. (B) GFP fluorescence (normalised to NucBlue) using both the wild type myogenin promoter sequence as well as the myogenin promoter sequence with a 3-base mutation in positions 2, 3, and 4 on the VDRE sequence using a 0.1% (v/v) ethanol control and two 1,25$(OH)_2D_3$ treatment concentrations (10^{-7} M and 10^{-11} M in 0.1% (v/v) ethanol) based on results from A on days 4, 5, and 6. (C) Myotube diameter (μM) of C2C12 cells, transfected with the wild type myogenin promoter vector 5′ to a turbo GFP sequence, grown in differentiation media with varying concentrations of 1,25$(OH)_2D_3$ dissolved in 0.1% (v/v) ethanol on days 4, 6, and 8. ◆ symbol indicates where a bar is significantly different ($p < 0.05$) to both media and ethanol controls [media (2% HS) and EtOH] within the same time point. Error bars show standard deviation and 4 biological replicates (wells) were used throughout. Statistical analysis was carried out via a 2-way ANOVA for A and C and a 3-way ANOVA for B, with a *post hoc* Bonferroni test where time*treatment interaction was significant ($p < 0.05$). There was a significant time × treatment interaction for A with GFP fluorescence for 10^{-7} M, 10^{-9} M and 10^{-11} M 1,25$(OH)_2D_3$ being 15%, 20% and 15% higher than the EtOH control on day 6 ($p < 0.05$). In B this effect of 1,25$(OH)_2D_3$ on fluorescence was lost for cells transfected with the mutated vector.

controls ($p < 0.05$). The largest effects were seen on day 6, with GFP fluorescence for 10^{-7} M, 10^{-9} M, and 10^{-11} M 1,25$(OH)_2D_3$ being 15%, 20%, and 15% higher than the EtOH control ($p < 0.05$).

Transfections were then repeated using the myogenin promoter sequence with a 3-base mutation within the putative VDRE at −1,261 to −1,264 (MyoG-mutVDRE-GFP), with the MyoG- VDRE-GFP run alongside for comparison. Due to consistent results at the 3 timepoints used (days 4, 5, and 6), the day × treatment × vector interaction was not significant ($p = 0.769$, Figure 5B) but there was a significant treatment × vector interaction ($p < 0.001$). As previously observed, in cells treated with the normal VDRE construct (MyoG- VDRE-GFP), both 10^{-7} M and 10^{-11} M 1,25$(OH)_2D_3$ caused a significant increase in fluorescence compared to controls on days 4, 5, and 6 ($p < 0.05$). However, there was no effect of 1,25$(OH)_2D_3$ treatment in cells transfected with that mutated VDRE construct (MyoG-mutVDRE- GFP), indicating that responsiveness to 1,25$(OH)_2D_3$ was lost when the putative myogenin VDRE site was mutated.

Myotube diameter was measured in fluorescent myotubes and was also affected by 1,25$(OH)_2D_3$ treatment (Figure 5C). The time × treatment interaction was not significant ($p = 0.8$), but there was a significant effect of treatment ($p < 0.001$), with 10^{-7} M, 10^{-9} M and 10^{-11} M 1,25$(OH)_2D_3$ resulting in larger myotube diameters compared to controls on day 4 ($p < 0.05$), and 10^{-7} M and 10^{-9} M 1,25$(OH)_2D_3$ resulting in larger myotube diameters compared to controls on days 6 and 8 ($p < 0.05$). Cell images can be seen in Supplementary Data S4.

To further confirm that the identified VDRE sequence was functional, and that the VDR binds to this region, a ChIP assay was performed. Following the pull-down of genomic DNA fragments using a VDR-specific antibody, 3 different primer pairs were used for PCR to amplify slightly different regions of DNA around the VDRE. Amplicon lengths for primer pairs 1, 2, and 3 were 138 bp, 62 bp, and 82 bp respectively (Supplementary Data S1). Amplification curves are shown in Figures 6A a single PCR amplicon was produced for all 3 primer pairs and melt curve analysis also showed a single peak, again indicating a single product. The PCR products were run on a 2.5% metaphor agarose gel to confirm that they were the correct length (Figure 6B; Supplementary Data S5). For primer pairs 1 and 2 (which both spanned the VDRE), the Cp values for 1,25$(OH)_2D_3$ treated cells were significantly lower than the control ($p < 0.05$, Figure 6C) indicating an increase in binding of the VDR to the VDRE with 1,25$(OH)_2D_3$ treatment. However, Cp values for primer pair 3 were not significantly different (Figure 6C), likely because they bind ~97 bp upstream of the VDRE (Figure 6D) and therefore some DNA fragments containing the VDRE were not detected by this primer pair.

When the newly identified mouse myogenin VDRE sequence was aligned to the human myogenin genomic sequence (Accession AF050501.1) using Clustal Omega, it revealed an 11/15 (73%) base pair match (Figure 6E). This could indicate that this VDRE sequence on the myogenin promoter is conserved across species. However, alignment with cow, pig and sheep myogenin sequences was attempted but not possible due to the available sequences not containing enough of the 3' promoter.

Discussion

For the first time, this detailed C2C12 differentiation time course study has shown a clear stimulatory effect of active VD [1,25$(OH)_2D_3$] on myogenic differentiation which appears to be via a functional VDRE on the myogenin gene promoter.

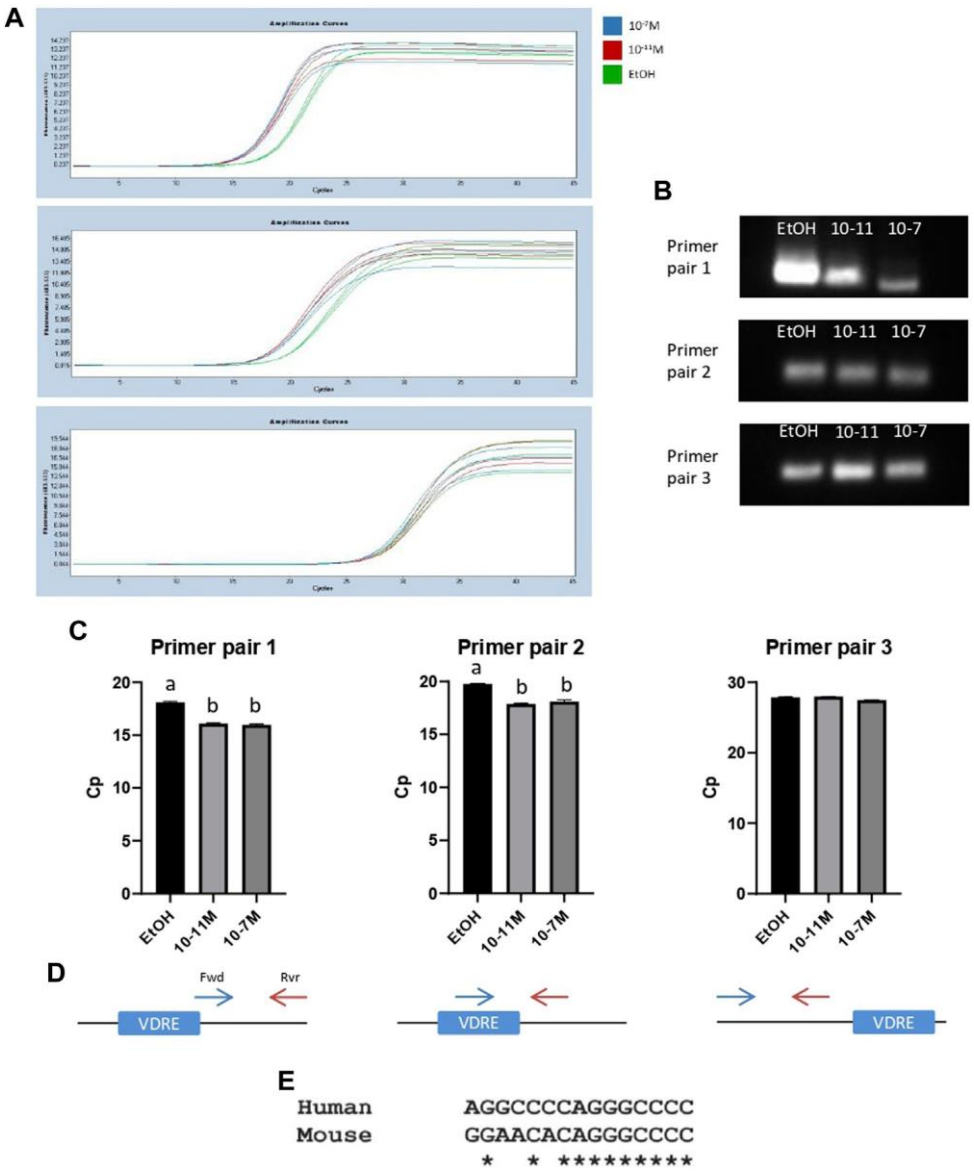

FIGURE 6
PCR products from ChIP assays for C2C12 cells treated with $1,25(OH)_2D_3$. (A) PCR amplification curves for ethanol (EtOH), 10^{-11} M and 10^{-7} M $1,25(OH)_2D_3$ generated by LightCycler 480 software following a ChIP assay using an anti-VDR antibody and primers to amplify a region of DNA around the VDRE sequence (−1,260 to −1,246). (B) PCR products from (A) run on a 2.5% (w/v) metaphor agarose gel to confirm fragment size and that a single PCR product was produced. Positive and negative controls were also run (for full gel image including ladder see Supplementary Data S5). (C) Average Cp values for ethanol, 10^{-11} M and 10^{-7} M $1,25(OH)_2D_3$. Error bars show standard deviation and samples were run in triplicate. A is significantly different to b ($p < 0.05$) determined by one way ANOVA and *post hoc* Bonferroni. (D) Diagram to show forward (Fwd) and reverse (Rvr) primer positions in relation to the VDRE. Primer pair 1: Fwd primer starts on the last base pair of the VDRE sequence (+13 from VDRE start site), Rvr primer position is +128 from start of VDRE. Primer pair 2: Fwd primer position is +1 from start of VDRE, Rvr primer position is +41 from start of VDRE. Primer pair 3: Fwd primer position is −179 from start of VDRE, Rvr primer position is −124 from start of VDRE. (E) Sequence alignment using Clustal Omega between the human myogenin promoter sequence (Accession AF050501.1) and the newly identified mouse myogenin VDRE sequence.

Firstly, we showed that $1,25(OH)_2D_3$ inhibits cell proliferation, inducing a switch towards a differentiative state. At 48 h, DNA content for the highest two $1,25(OH)_2D_3$ concentrations were similar to day 0 indicating that the reduced level of DNA compared to controls was due to an inhibition of proliferation rather than an increase in apoptosis. This agrees with previous work in C2C12 cells showing a 75% decrease in proliferating cell nuclear antigen (PCNA), a marker of proliferation, after 7 days in $1,25(OH)_2D_3$ treated cells (Garcia et al., 2011), and also agrees with work in chick myoblasts where proliferation was significantly decreased by $1,25(OH)_2D_3$ on days 4 and 6 compared to controls (Capiati et al., 1999). It has been reported that myoblasts treated with active VD have a higher percentage of cells in the G_0/G_1 (quiescent) phase and a lower percentage of cells in the S/M (active) phase compared to controls (Girgis et al., 2014) suggesting that this anti- proliferative effect of VD is associated with cell cycle arrest. Our results show that creatine kinase (CK) activity, which is expressed after myoblasts exit from the cell cycle and begin to differentiate (Tai et al., 2011), is significantly higher for $1,25(OH)_2D_3$ treated cells than

controls. Again, this agrees with (Capiati et al., 1999) who showed that CK activity was increased in 1,25(OH)$_2$D$_3$ treated chick myoblasts on days 2, 4 and, 6 compared to controls, which suggests that active VD inhibits proliferation and stimulates cells to differentiate.

The MRFs are basic helix-loop-helix transcription factors that recognise and bind to E box sequences (CANNTG) on target genes (Adhikari et al., 2021). They possess potent signaling effects, such that inducing expression of any of the MRFs on a variety of tissue culture cells results in conversion to muscle-type cells (Hasty et al., 1993). Myf5 is the first MRF to be expressed, followed closely by MyoD and then myogenin (Zammit, 2017). The myogenin promoter contains E box sequences both within the proximal promoter sequence (−130 to +18 bp) and in enhancer regions −4.5 kb and −6.5 kb upstream of the coding sequence, which become bound by MyoD/E-protein heterodimers to activate transcription during differentiation (Faralli and Dilworth, 2012). Our results show that Myf5 mRNA expression is significantly lower in 1,25(OH)$_2$D$_3$ treated cells than controls on day 1, possibly indicating a faster down regulation of early differentiation markers, which may suggest that cells are differentiating more quickly. These results are in agreement with previous work which found a decrease in Myf5 gene expression just 6 h post 1,25(OH)$_2$D$_3$ injection in chick embryos (Chen et al., 2021). In addition, work in C2C12 cells showed that both inactive [25(OH)D$_3$] and active VD treatment reduced Myf5 mRNA expression (Girgis et al., 2014). MyoD mRNA expression was not found to be affected by 1,25(OH)$_2$D$_3$ in this study. The current literature reports inconsistent effects of active VD on MyoD mRNA expression *in vitro* with some finding increased expression (Garcia et al., 2011; Saito et al., 2017), one study finding decreased expression (Olsson et al., 2016) and another finding no significant effect (van der Meijden et al., 2016). It is worth noting that there is large variability between these studies on the day that MyoD expression was measured, often at only a single time point, which ranged from day 1 to day 7. Despite this study finding no change in MyoD mRNA expression, it is worth noting that MyoD increases transcription of p21 which inhibits various cyclin dependent kinases (CDKs), thus leading to cell cycle arrest, allowing myoblasts to enter a differentiative state (Christ and Brand-Saberi, 2002). Previous studies have shown an increase in p21 mRNA expression in response to vitamin D (Okuno et al., 2012; Olsson et al., 2016), therefore it cannot be ruled out that vitamin D could also be acting via cell cycle regulatory factors to stimulate cells to enter the differentiation phase more quickly. As far as we are aware, this study has used the most detailed time course to measure the effects of 1,25(OH)$_2$D$_3$ on MRF expression, measuring at 6 different time points across differentiation. Our study shows that 1,25(OH)$_2$D$_3$ causes an earlier increase in myogenin mRNA expression compared to controls. There are also inconsistencies within the literature on the effect of active VD on myogenin expression *in vitro* with some studies finding a decrease in mRNA expression (Okuno et al., 2012; Ryan et al., 2013; Girgis et al., 2014; Olsson et al., 2016; van der Meijden et al., 2016) and others finding an increase in mRNA expression (Garcia et al., 2011; Gili et al., 2016; Braga et al., 2017; Montenegro et al., 2019), and protein expression (2, 36). Again, most of these studies measure expression at one or two time points, rather than using time courses, with variation on day of measurement. *In vivo*, dietary VD [25(OH)D$_3$] has been shown to increase myogenin expression in piglets from mothers fed a high VD diet, with higher levels of myogenin mRNA observed in the longissimus dorsi and psoas major muscles compared to controls (Zhou et al., 2016). Myogenin is an essential component of the regulatory pathway that leads to skeletal muscle formation during embryogenesis. In myogenin knockout mice, pups survive fetal development but are born immobile and die shortly after birth with a lack of differentiated skeletal muscle, despite normal levels of MyoD (Hasty et al., 1993). In contrast, knockout of either MyoD or Myf5 result in normal muscle formation (Asfour et al., 2018), but knockout of both Myf5 and MyoD results in complete lack of skeletal muscle (Rudnicki et al., 1993), with mice being born alive but immobile, and dying soon after birth. A previous study has shown that myogenin mRNA expression is completely blocked when the VDR is knocked down *in vitro* (Gili et al., 2016) suggesting that VD is essential for myogenin expression. The VDRE sequence that we have found is, to our knowledge, the first time that a functional VDRE has been identified on the myogenin promoter. Our results show that when this VDRE sequence is mutated, the increase in myogenin mRNA expression previously seen in 1,25(OH)$_2$D$_3$ treated cells becomes abolished, suggesting that this sequence is essential for the upregulation of myogenin gene transcription by 1,25(OH)$_2$D$_3$. In addition to this, ChIP analysis showed that the VDR does in fact bind to the sequence identified suggesting that the effects of 1,25(OH)$_2$D$_3$ on myogenin mRNA expression are direct and VDR mediated.

A recent study has shown that depleting myogenin levels via shRNA in C2C12 cells resulted in a significant reduction in myosin heavy chain protein levels (Adhikari et al., 2021). In addition, over expression of rat myogenin during mouse fetal development results in increased levels of oxidative enzymes within skeletal muscle (Hughes et al., 1999). Both of these suggest that myogenin plays a role in regulating expression of the slow twitch, or early, MHC isoforms which are oxidative in nature. In agreement with this, our results show a positive correlation between myogenin and early MHC isoform mRNA expression. Therefore, the increase in mRNA expression of the early MHC isoforms observed in response to 1,25(OH)$_2$D$_3$ is likely to be due, at least in part, to the increase in myogenin expression.

Our previous work has found that during C2C12 differentiation the MHC fast isoforms are expressed in the order IIa, IIx, and IIb (Brown et al., 2012). Our results show an earlier increase in expression of all three of the fast isoforms with 1,25(OH)$_2$D$_3$ compared to controls, suggesting that treatment causes cells to move through differentiation more quickly. In agreement, other work has found that eldecalcitol (a VD analogue) increased mRNA expression of all three fast isoforms in C2C12 cells (Saito et al., 2017). In addition to this, one study found that 1,25(OH)$_2$D$_3$ caused an increase both MHC IIa mRNA and protein expression in already differentiated C2C12 cells (Okuno et al., 2012) suggesting that the effects of VD on MHC expression may not be isolated to embryogenesis, but may also have effects in differentiated muscle. Finally, we show that 1,25(OH)$_2$D$_3$ causes a significant increase in myotube diameter. In our recent systematic review (Alliband et al., 2021) there was strong agreement across the literature with all 5 *in vitro* studies that measured myotube size finding a significant increase with 1,25(OH)$_2$D$_3$. This is reflected *in vivo* since rat pups from VD deficient mothers had smaller muscle fibers and larger interfibrillar spaces within the muscle (Max et al., 2014). Cluster gene analysis showed that VD sufficient pups showed an upregulation of genes involved in cell differentiation and a down regulation of genes involved in protein

catabolism compared to VD deficient pups (Max et al., 2014). In addition, piglets born to mothers fed a high VD diet show a larger muscle fiber cross sectional area in the psoas major and longissimus dorsi muscles (Zhou et al., 2016). Hence, there appear to be a clear effects of VD on muscle fiber size both *in vitro* and *in vivo*.

Overall, the results presented show that active VD directly increases myogenin transcription via a functional VDRE on the myogenin promoter, resulting in increased myogenic differentiation, increased mRNA expression of both the early and late MHC isoforms, as well as increased myotube size. However, we cannot rule out that $1,25(OH)_2D_3$ may also be exerting direct effects on expression of the MHC isoforms. Future work could investigate the effects of VD at the protein level to check that the effects on gene expression found in this study correlate with effects at the protein level. Future work also should investigate the impact of VD deficiency during embryogenesis *in vivo,* in relation to muscle fibre development, MRF and MHC expression in the muscle of the offspring, as well as the long term impact on postnatal muscle growth and body composition. Finally, an 11/15 base pair match was found between the mouse myogenin VDRE sequence and a potential VDRE sequence on the human myogenin genomic sequence. Therefore, further work to investigate the impact of $1,25(OH)_2D_3$ on human myogenesis is needed. Currently, it is estimated that between 20%-40% of pregnant women worldwide are vitamin D insufficient or deficient (Urrutia-Pereira and Solé, 2015), therefore it is crucial that a clearer understanding of the impact of maternal VD deficiency on offspring development and health is gained.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

KA: Writing–original draft, Writing–review and editing. TP: Supervision, Writing–review and editing. PJ: Supervision, Writing–review and editing. JB: Supervision, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at:
https://www.frontiersin.org/articles/10.3389/fphys.2023.1322677/ full#supplementary-material

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Appendix D PIP Summary

In 2022 I took part in a professional internship for PhD students (PIP) for 3 months (April, May and June). I worked as a public health nutritionist at Andra Health under Rose Smith, who is a registered nutritionist and director of Andra Health. Below is a summary of the work that I completed and the skills that were gained as a result.

Week 1 (4th – 10th April)

Tested positive for COVID on Friday 1st April so was off ill on Monday 4th and Tuesday 5th April. Felt better by Wednesday 6th so worked from home from Wednesday to Friday (could not go into the clinic because was still testing positive and had some symptoms).

Wrote a blog post for the website/social media channels on the new legislation by the government to include calories on menus. I really enjoyed this, and it was something I felt very passionate about, I wrote mainly from an eating disorder perspective and the effect that this will have on people struggling with their relationship with food.

In addition to this I put together all of the information for an E book on the gut microbiome and an E book on the gut brain axis for the new IBS packages that Andra Health are launching soon. It was extremely interesting to research these areas and I felt that I was able to critically evaluate the literature well, then write about it in a way that the general public can understand.

Finally, I have been appointed as the line manager for the current undergraduate nutrition intern. I had a meeting with him via zoom and set him a task list of everything he needs to do over the next week. I probably found this part of the week the most challenging because I have never managed somebody before and felt a little out of my comfort zone, however the meeting went well, and I felt that I was friendly and approachable towards the intern.

Weeks 2, 3 and 4 (11th April – 1st May)

During this time I was helping to get everything ready and finalised for the launch of the gut health packages at Andra Health. This involved proof reading / fact checking resources, helping to go over the appointment protocols, doing critical analysis of research to make sure that the protocols contained the most up to date 'gold standard' information, and helping to create gut friendly recipes which were healthy, balanced meals.

During week 2 and 3, the undergraduate nutrition intern was still on placement and so my role was to continue to manage him. I feel that I grew in confidence with these management skills, which was helped by the fact that both the undergraduate intern and I got on so well. I set him tasks such as writing blog posts and social media content and then checked over his work once it was completed before sending it off to the manager at Andra Health. We had weekly meetings so I was able to give feedback to the intern on what he did well and how he could improve the work.

Weeks 5, 6, 7, 8 and 9 (2nd May – 5th June)

The gut health packages were launched, and Andra Health wanted to host a webinar about it and asked whether I would like to create and present it using all of the information I had learnt so far on gut health. I created a presentation on gut health, IBS, probiotics and prebiotics and I then presented this as a webinar to around 15 people (a mixture of existing and prospective clients) on 16th May.

During this second half of my placement, I also started to shadow appointments. This involved sitting in on nutrition appointments with clients and making notes. After each appointment, I would then have a meeting with Rose (registered nutritionist and director) where we would go over the notes I had made, the notes she had made, and how to approach treatment for that client.

After each appointment the client is sent an appointment report containing all of the information from their appointment, as well as an action plan of what they need to do going forwards. Towards the end of the placement, I was given the opportunity to write some of the appointment reports, which were then sent to Rose to check over before being sent to the client.

I also had the opportunity to do a couple of food diary analyses for clients. This was where the client had kept a food diary for a week and my role was then to input all of that information into a database. After this, I was able to make graphs showing energy intake, carbohydrate, fat and protein intake, essential amino acid intake and vitamin and mineral intake. I then made tables showing their breakdown of fat intake (saturated, unsaturated, polyunsaturated, cholesterol), fruit/veg portions per day, sugar intake, fibre intake etc. I was able to sit in on the appointments for these clients where Rose went over their food diary analysis with them. I was then able to write the appointment reports up for these clients with all of the graphs, tables etc.

In summary, I have thoroughly enjoyed my placement at Andra Health and have gained an insight into private practice which I previously had no experience with. I have gained management experience, which was a completely new role for me, and have been able to build confidence in this area. I also feel that I have improved my writing skills and am able to take scientific information and present it in a way that is easy to understand by members of the public with a non-science background (leaflets, resources, social media content, blog posts etc).
Rose has kindly offered that I can continue to shadow appointments in return for writing some blog posts / social media content etc which I will definitely take the opportunity to do.