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In vitro effects of biologically active vitamin D on myogenesis: A Systematic Review

Kathryn H. Alliband[1], Sofia V. Kozhevnikova[1], Tim Parr[1], Preeti H. Jethwa[1*], John M. Brameld[1]

[1]School of Biosciences, Faculty of Science, University of Nottingham, United Kingdom

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KHA and JMB contributed to the conception and interpretation of the data and reviewing of the drafts. KHA contributed to writing the original draft, acquisition, and analysis of the data, SVK con-tributed to the data acquisition, TP and PHJ contributed to the revising and contributing intellectual content writing. JMB had final approval of the version to be published.

Keywords

Vitamin D, 25-dihydroxyvitamin D3, 25-hydroxyvitamin D2, myogenesis, Myogenin, differentiation, MyoD, Systematic review

Abstract

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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 pa-pers 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.

Contribution to the field

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 have systeamtically search the literature and identified that vitamin D, particularly the active form, has a direct effect by inhibiting proliferation and promoting differentiation of muscle cell in vitro, however whether this leads to the strengthening the muscle requires further studies.

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In vitro effects of biologically active vitamin D on myogenesis: A Systematic Review

Kathryn H. Alliband[1], Sofia V. Kozhevnikova[1], Tim Parr[1], Preeti H. Jethwa*[1] and John M. Brameld[1]

[1]School of Biosciences, Division of Food Nutrition and Dietetics, University of Nottingham Sutton Bonington Campus, Loughborough, LE12 5RD.

Correspondence

*Dr Preeti Jethwa

Preeti.Jethwa@nottingham.ac.uk.

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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)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 pa-pers 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.

1. 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 [1]. Muscle biopsies from VD deficient individuals show muscle wasting (mostly type II fibre atrophy), large interfibrillar spaces and fat infiltration within the muscle [2]. In general, deficiency occurs when levels of 25(OH)D3 (inactive vitamin D) fall below 25nmol/L, however this cut-off point can vary within the literature [3]. 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 [4].

Around 80-90% of VD is obtained via UV-B induced synthesis in the skin in humans, whilst 10-20% comes from dietary intake [3]. 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 hydroxylation by 25-hydroxylase to form 25(OH)D3 or calcidiol [5]. 25(OH)D3 is the major circulating form of VD and is measured as a marker of VD status [6]. 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 [5] (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 [2].

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 [7,8]. 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 [9]. 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 [10]. 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 [6].

Within the literature, VDRs have been described in different cell locations, one as a nuclear hormone receptor and the other as a membrane receptor [2]. 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 [3].

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 [11]. 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 [3]. 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 [1].

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. Materials and Methods

This review was constructed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [12].

2.1. 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.

2.2. 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)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.

2.3. 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. There were no secondary measured outcomes.

2.4. 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 to moles for consistency.

2.5. 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 VD used meant that direct comparisons in the form of a meta-analysis was not possible.

2.6. 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.

3. Results

3.1. 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. 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). A detailed list of excluded studies with reasoning for exclusion can be found in Supplementary Table 2.

135 *3.2. Quality Assessment*

136 All 12 papers received a score of 'low risk' when assessed against the quality assessment criteria
137 previously outlined in Supplementary Table 1. For three of the studies [13-15] no information could
138 be found regarding replicates and/or repeats therefore it was assumed that this was adequate when
139 giving a low overall bias score (Table 1).

140 *3.3. Study Characteristics*

141 All studies included within this review used the biologically active form of VD (1,25(OH)2D3) apart
142 from Saito et al, 2017 [16] where an analogue of the active form of VD called Eldecalcitol was used.
143 Four different cell types were used across the studies (C2C12, primary human myoblasts, primary
144 mouse satellite cells and primary chick myoblasts) and active VD concentration ranged from 10-5M
145 to 10-13M (Table 2).

146 *3.4. Effects on Proliferation*

147 From the relevant articles, eight [4,13-15,17-20] studied the effects of 1,25(OH)2D3 on proliferation
148 and all reported an inhibitory effect. Of these, four [13,15,18,19] quantified DNA content as a marker
149 of proliferation (Figure 3). Interestingly, one study [13] reported an initial short stimulatory effect of
150 1,25(OH)2D3 treatment on DNA synthesis on day 1 (1.5-fold increase) however, this was followed
151 by an inhibitory effect on day 4 (0.7-fold). The remaining three studies [15,18,19] all revealed a
152 decrease in DNA content of different magnitude (0.5 to 0.95-fold) (Figure 3).

153 The other four studies measured proliferation in various ways (Table 3). One study showed an
154 increase in p21 and p27 mRNA [17] whilst three studies revealed a decrease in cyclin mRNAs
155 [14,15,19]. Decreases in proliferation was also shown by an increase in number of cells in the
156 quiescent phase [14,17,18], decreased levels of proliferating cell nuclear antigen (PCNA) at the
157 protein level [4] and decreases in DNA synthesis as previously reported. It is important to note that

158 only two out of eight of these studies checked for differences in apoptosis between treated and control
159 cells [14,15].

160 *3.5. Effects on differentiation*

161 Differentiation of muscle cells was determined in all but one [19] of the final twelve studies. Markers
162 of differentiation included expression of mRNA or protein for myoD (early differentiation),
163 myogenin (early-mid stage), myosin/myosin heavy chain isoforms (late stage) or the measurement of
164 creatine kinase activity (mid-stage). However, it should be noted that the mRNA expression of
165 myogenin and myosin heavy chain isoforms have been shown to change during the time course of
166 differentiation in C2C12 cells [21] indicating that the time point at which these markers are measured
167 is important.

168 *3.6. Effects of vitamin D on early-stage myogenic differentiation*

169 Five studies measured myoD expression [4,15,16,20,22]. Three of these studies measured expression
170 on day 4 [4,16,20] whilst one measured expression on day 1 [15] and another on day 7 [22] (Figure
171 4). mRNA expression was measured in all cases except for one [22] where protein expression was
172 measured. Four out of five studies [4,16,20,22] reported an increase in expression of myoD which
173 ranged from 1.8-fold to 3-fold. However, one study [15] reported a decrease in expression of 0.5-fold
174 on day 1. These changes in myoD expression were in response to 10-7M 1,25(OH)2D3 for all cases
175 apart from one [16] which used 10-7M Eldecalcitol, an analogue of the active form of VD.

176 *3.7. Effects of vitamin D on early/mid-stage myogenic differentiation*

177 Myogenin expression in response to 1,25(OH)2D3 was investigated by nine of the twelve studies
178 included within this review [4,14,15,17,18,20,22-24]. The time points at which myogenin expression
179 was measured varied from day 1 to day 7. For eight of the nine studies which measured myogenin,
180 the concentration of 1,25(OH)2D3 used was 10-7M but one study [23] used 10-8M 1,25(OH)2D3. In
181 most cases mRNA expression was measured but in two studies [22,23] 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 [14,15,17,20,24] and four studies reporting an increase in expression [4,18,22,23] (Figure 5).

Three studies measured creatine kinase activity as a marker of differentiation [13,23,24]. Two of these studies reported their results as a time course [13,23] whilst one reported results for day 4 only [24]. A variety of concentrations of 1,25(OH)2D3 were used across the studies (Table 4). One study [24] 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 [13,23] whilst the other study found that creatine kinase activity decreased across all 1,25(OH)2D3 concentrations on day 4 [24].

3.8. 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 [15-18,20,23]. 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 [13], one study found and increase in MyHC protein [23], three studies reported an increase in expression of at least one MyHC isoform [16,17,20,23] and two studies reported a decrease in MyHC isoforms (MyHC neo, MyHC IIa and MyHCII subtype unspecified) [15,18].

Five studies measured the effects of 1,25(OH)2D3 on myotube size [4,14,18,20,23] (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 [23] whilst 10-7M 1,25(OH)2D3 was used in the other four [4,14,18,20]. All five studies

206 concluded that treatment with 1,25(OH)2D3 resulted in an increase in myotube size which ranged
207 from 1.1-fold to 2-fold.

208 **4. Discussion**

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

213 *4.1. Treatment with vitamin D inhibits proliferation.*

214 Of the eight studies which investigated the effects of active VD on muscle cell proliferation
215 [4,13-15,17-20], all of them found an inhibitory effect. One study observed a stimulatory effect on
216 day 1, however this was followed by inhibition on day 4 [13]. It is worth noting that only two studies
217 [14,15] checked for differences in apoptosis between treated and untreated groups therefore it cannot
218 be ruled out that the decrease in DNA observed in some of the other studies was not due to apoptosis.

219 Importantly, Okuno et al, 2012 [17] showed that active VD caused an increase in cell cycle arrest at
220 G0/G1 which occurred in parallel with increased expression of p21 and p27. Both p21 and p27 are
221 members of the Cip/Kip family and are able to bind to cyclin dependent kinases and inhibit their role
222 in cell cycle progression [25]. In order for a cell to proliferate, expression of p21 must decrease to a
223 level where it no longer forms a complex with p53 [26]. Additionally, cells must also reduce/eliminate
224 p27 to progress through proliferation, which is achieved via translocation of p27 to the cytoplasm
225 where it is degraded [25]. Both Okuno et al, 2012 [17] and Olsson et al, 2016 [15] reported increased
226 expression of both p21 and p27 suggesting that active VD treatment leads to increased transcription
227 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 [15,19]. 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 [27]. The D cyclins activate CDK4/6 enabling entry into S phase and down-regulation of cyclin D3 specifically inhibits G1 to S transition [28]. 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.

4.2. 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 8. Following withdrawal from the cell cycle, as described previously, myoblast fusion occurs to form multinucleated myotubes [4]. MyoD is a transcription factor involved in the early stages of differentiation [14]. 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 [29]. However, we previously observed no change in myoD mRNA over the time course of differentiation in C2C12 cells [21].

Four of the five studies which measured myoD reported an increase in expression following treatment with active VD [4,16,20,22], although the increase in expression was not significant for one study

[20]. Interestingly, the only study which found a decrease in myoD expression was also the only study which used primary human cells [15]. 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) [15] whereas the remaining four studies all reported increased myoD expression on either day 4 [4,16,20] or day 7 [22]. 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 [30]. One of the studies which found a 1.8-fold increase in myoD expression [22] 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 [31]. 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.

4.3. 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 MyHC isoforms) expressed by myotubes and muscle fibres [32]. Myogenin expression is therefore used as a marker of early to mid-stage differentiation and normally follows an increase in myoD expression [33]. 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 [33]. 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 [23].

However, the nature of this effect is controversial. As seen in Figure 5, five studies reported a decrease in myogenin expression [14,15,17,20,24] whilst four reported an increase [4,18,22,23]. 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 more cell-cell contacts, which leads to increased IGF expression and an increase in myogenin [14]. Garcia et al, 2011 [4], 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 [14] 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 [21,34] 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 [35] 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 [21] and primary chick myoblasts [13]. Two studies [13,23] 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 [24] 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.

4.4. 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 [23] and together they form a significant proportion of the proteins present in differentiated muscle [36]. Five studies reported an increase in myosin or MyHC isoforms following active VD treatment [13,16,17,20,23] whilst two studies reported a decrease in expression [15,18]. We previously showed that the MyHC isoforms are expressed in two distinct patterns during differentiation in C2C12 cells [21]. The first pattern is an increase then decrease, peaking around day 2-4 of mRNA for MyHC embryonic (MyHC emb), fetal (MyHC neo) and slow type 1 (MyHC I) isoforms [21]. The fast type II isoforms were all expressed much later in differentiation, being induced at days 2-4 in the order IIa > IIx > IIb [21]. Hence, an increase in differentiation would always results 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 [17] 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 [15] 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 [23] 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 [37]. 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 [16] so active VD may impact on muscle fibre type indirectly via induction of local IGFI expression. Supporting this, Braga et al, 2017 [22] 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 [38].

4.5. Vitamin D increases myotube size

Of the five studies which measured myotube size, all five reported an increase in myotube size [4,14,18,20,23], which suggests a stimulatory effect of active VD on differentiation. In addition, Garcia et al, 2011 [4] found an increase in expression of Follistatin (Fst). Fst is an antagonist of Myostatin (Mstn) a known negative regulator of muscle mass [31], 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 [14]

found myotube size was increased 1.8-fold on day 10 following a 10-fold decrease in Myostatin expression on day 7.

5. 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 [24] used concentrations of active VD within the physiological serum range (around 10-10M) [39] 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 [40]. 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 (10-7M).

Due to the presence of 1α-hydroxylase within skeletal muscle [40] 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 [4,9], but this review suggests that VD deficiency

during embryonic and fetal 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 [24], more research is needed to determine the impacts of supplementation during pregnancy/lactation or in the young offspring on muscle cell differentiation.

Author Contributions:

KHA and JMB contributed to the conception and interpretation of the data and reviewing of the drafts. KHA contributed to writing the original draft, acquisition, and analysis of the data, SVK contributed to the data acquisition, TP and PHJ contributed to the revising and contributing intellectual content writing. JMB had final approval of the version to be published.

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Conflicts of Interest: The authors of this review report that there were no conflicts of interest.

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Captions

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.

Figure 2. Selection and exclusion of studies in accordance with PRISMA guidelines (Moher *et al.*, 2015).

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.

Figure 5: Effect of $1,25(OH)_2D_3$ of Myogenin expression. The active form of vitamin D ($1,25(OH)_2D_3$) 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

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.

Figure 7: Effect 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.

Figure 8: Process of myogenic differentiation from myoblasts to multinucleated muscle fibers 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.

Table 1. Summary of quality assessment 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

383 **Table 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,	Myogenin, creatine kinase

			10^{-9}M, 10^{-11}M and 10^{-13}M	
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

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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, 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 ($p<0.05$)

Table 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	p<0.01
		+55% on day 2	P<0.01
		+30% on day 3	p<0.05
		+ 15% on day 6	p<0.05
Gili et al, 2016 (**C2C12**)	10^{-7}M $1,25(OH)_2D_3$	1.7-fold on day 1	Individual p values not given. ANOVA interaction p<0.05
		1.8-fold on day 2	
		1.3-fold on day 4	
Ryan et al, 2013 (**C2C12**)	$1,25(OH)_2D_3$ for all:	Day 4 for all:	Individual p values not given. ANOVA interaction p<0.001
	10^{-13}M	Same as control	
	10^{-11}M	6% decrease	
	10^{-9}M	12.5% decrease	
	10^{-7}M	25% decrease	
	10^{-5}M	62.5% decrease	

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

Note: 1,25(OH)2D3, 1,25 dihydroxyvitamin D; MyHC, myosin heavy chain,

Figure 1.JPEG

Figure 1

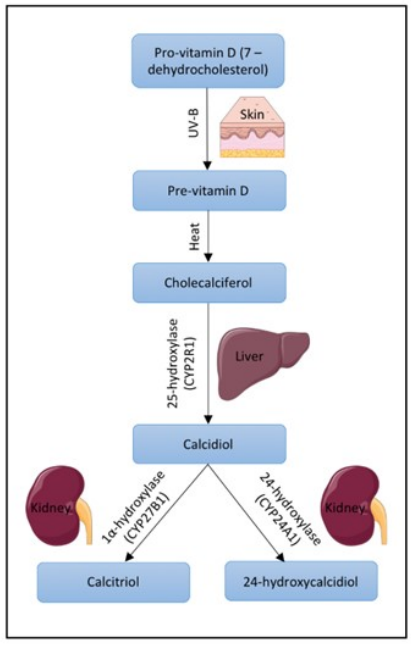

Figure 2.JPEG

Figure 2

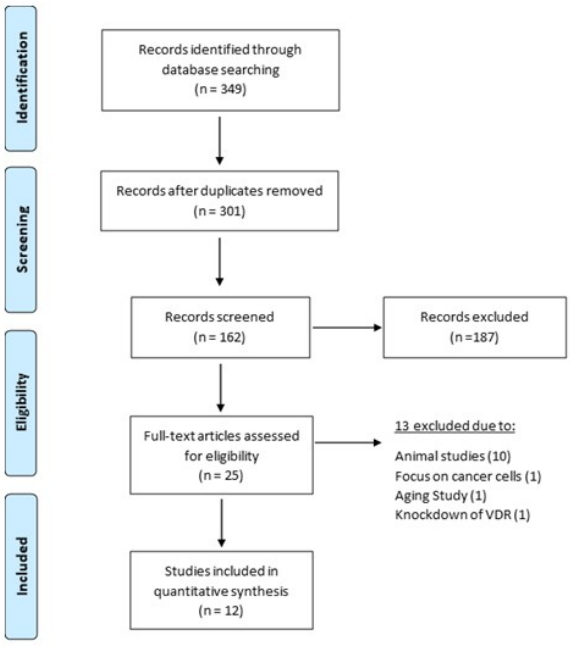

Figure 3.JPEG

Figure 3

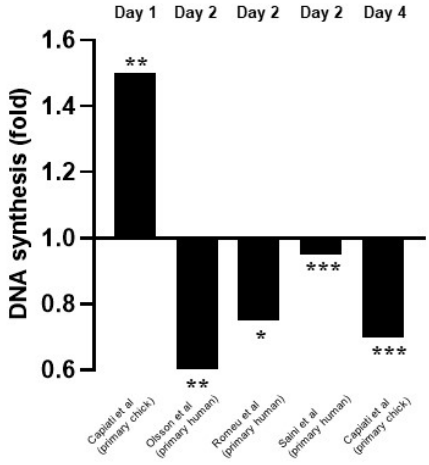

Figure 4.JPEG

Figure 4

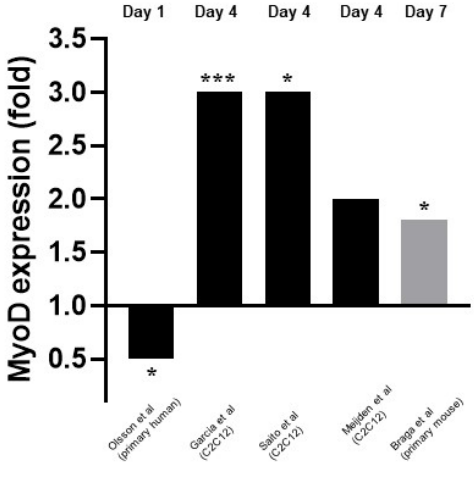

Figure 5.JPEG

Figure 5

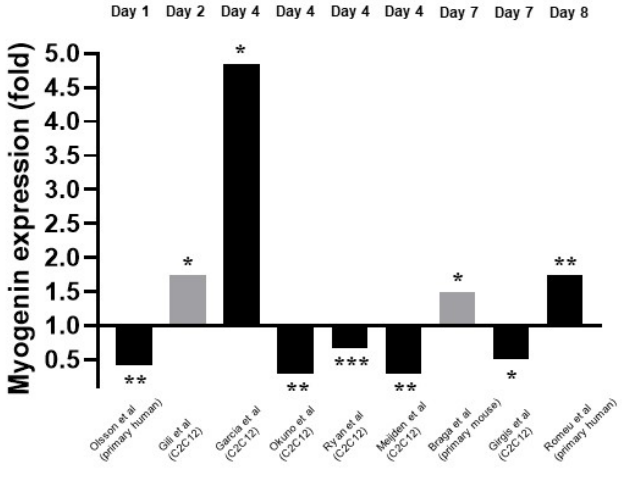

Figure 6.JPEG

Figure 6

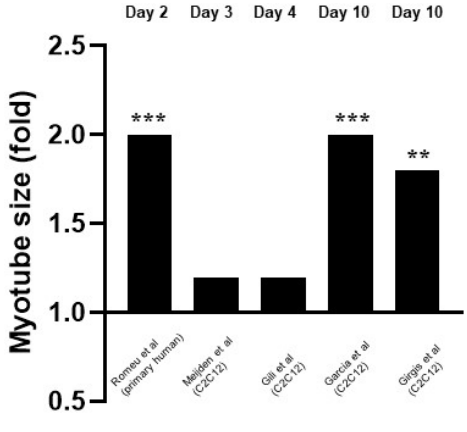

Figure 7.JPEG

Figure 7

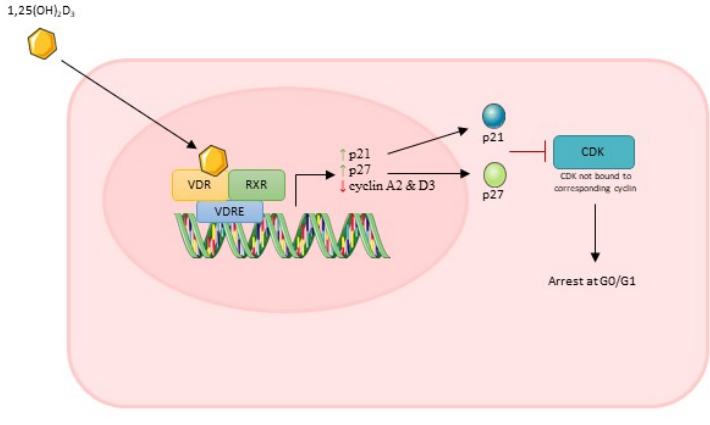

Figure 8.JPEG

Figure 8