25-hydroxyvitamin D upregulates L6 muscle cell differentiation induced by mononuclear cells via the Notch signaling pathway

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Research

Keywords: Myoblast, PBMC, Desmin, Myogenin, VDR, CYP27B1, IL-6, IL-10

Posted Date: January 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-156796/v1

Abstract

During muscle regeneration, myoblasts engage in cross-talk with immune cells to achieve optimal proliferation and differentiation. In this process, cytokines secreted by immune cells are described to modulate the kinetic of muscle differentiation. Taking into account that immune and muscle cells are both targets of vitamin D, we investigated *in vitro* the impact of 25-hydroxyvitamin D (25(OH)D) on the transcriptional response of muscle cells in presence of mononuclear cells.

To address this objective, an *in vitro* model of co-culture using L6 myogenic cell line and peripheral blood mononuclear cell (PBMC) isolated from rat was used and compared to L6 cultured alone. Cells were treated with 25(OH)D (125 nM) during the 6 days of differentiation. Gene expression of 25(OH)D metabolism actors, muscle differentiation and metabolism markers, and of Notch signaling pathway effectors were studied in L6 cells by qPCR.

In mono-cultured L6 cells, a 25(OH)D treatment induced a 3-fold increase ($p < 0.05$) in *VDR* mRNA expression at 24 h while no change in mRNA expression of the muscle differentiation markers i.e. *Myog*, *Myh2* and *Des* was observed. In the presence of PBMCs, the mRNA expression of these markers was enhanced (27.5 times for *myogenin,* $p < 0.05$) resulting in an overexpression of the Notch pathway effectors (*Dll*: 6.8-fold and *Hes1*: x3.8-fold, $p < 0.05$). The 25(OH)D counteracted these effects of the PBMCs on L6 gene expression with the exception of the interleukin 6 transcript and protein.

In the present study, our *in vitro* approach demonstrates the importance of immune cells in stimulating muscle cell differentiation. Taken as a whole, the data show that 25(OH)D attenuates *in vitro* the Notch pathway-dependent effects of immune cells on muscle cell differentiation and energy metabolism.

Introduction

Adult skeletal muscle has a remarkable ability to regenerate following trauma. Because adult myofibers are terminally differentiated, the regeneration of skeletal muscle is largely dependent on a small population of resident cells termed satellite cells. The contribution of immune cells (i.e. macrophages and lymphocytes) in regulating satellite cell migration, proliferation and differentiation is a major process of muscle regeneration (1). Fundamentally, pro-inflammatory cytokines (notably Interleukin (IL)-6) produced by immune cells promote muscle cell proliferation, whereas anti-inflammatory cytokines (IL-10) activate muscle cell differentiation (2, 3).

Muscle regeneration is a tightly coordinated process composed of four consecutive interlinked phases: (i) necrosis, (ii) inflammation, (iii) activation and differentiation of satellite cells i.e., muscle stem cells, in myocytes and (iv) fusion of myocytes and maturation of newly formed myofibers (4). Alternatively, after activation and proliferation, satellite cells return to their quiescent state until the next regeneration process (5). The signaling pathways and the transcription factors orchestrating muscle regeneration have been studied extensively. In sum, myogenesis is controlled by the sequential action of lineage determination markers (i.e., Pax3/Pax7) that act together with Six and with Myocyte enhancer factor-2 (Mef2) proteins to regulate muscle gene expression. Pax7 and Pax3 are thought to be the principal regulators of muscle cell specification and tissue (6, 7). Satellite cells can be activated by numerous signals from the regenerative microenvironment, including those mediated by adhesion molecules or by growth factors as well as cytokines produced by neighboring cells such as resident immune cells (7).

Vitamin D seems a likely candidate to stimulate muscle recovery and performance, as muscle and immune cells are preferred targets of this nutrient (8). The infusion of vitamin D *in vivo* led to an increase in muscle regeneration in different experimental models (9). Moreover, it is known to shift the T-cell response from a T helper 1 (Th1) to a Th2-mediated one, which reduces inflammation and promotes an immunosuppressive state (10) by decreasing the production of type 1 cytokines (IL-6, Interferon-γ (IFN-γ)) and increasing the production of type 2 cytokines (IL-4, IL-10) (11).

A recent clinical trial has failed to support the effectiveness of vitamin D supplementation on physical performance and infection rates in older adults (12). In contrast, epidemiologic studies have shown that circulating 25(OH)D level and muscle strength/function are positively correlated suggesting that a target of vitamin D is the skeletal muscle (13). Indeed, skeletal L6 muscle cells have been demonstrated to express the 1 α-hydroxylase enzyme (CYP27B1) and therefore are able to metabolize 25(OH)D in 1,25 dihydroxyvitamin D ($1,25(OH)_2D$ or calcitriol) which interacts with VDR (14).

In vitro studies have established that $25(OH)_2D$ positively controls muscle anabolism and inhibits muscle cell proliferation, but stimulates myogenesis (15).

Furthermore, PBMCs including monocytes, T and B cells, express VDR and CYP27B1 enzyme and most likely contribute to the majority of the $1,25(OH)_2D$ formed locally in the tissues (16, 17). $1,25(OH)_2D$ plays numerous roles through both genomic and non-genomic pathways (8, 18). The genomic effects of 1,25(OH)2D are mediated by an interaction with a cytoplasmic nuclear vitamin D receptor (VDR) from the superfamily of ligand-activated transcription factors. The 1,25(OH)2D-VDR forms an heterodimeric complex with the Retinoid-X-Receptor (RXR) and regulates the expression of target genes with a vitamin D response element (VDRE) in their promoter. The non-genomic effects, still poorly understood, are initiated by the binding of $1,25(OH)_2D$ to a distinct membrane receptor (mVDR) (19). This complex, after internalization, induces the entry of calcium *via* activation of calcium channels and thus activate the protein kinase C (PKC). Subsequently, this stimulates the activation of the Mitogen-Activated Protein Kinase (MAPK) and Extracellular-Regulated Protein Kinase (ERK) pathways (8, 20).

There is evidence of VDR expression and a direct effect of vitamin D on precursor (15, 21) and mature skeletal muscle cells (22), which provides a rationale for a role of vitamin D in muscle function. Our team have demonstrated that, in old rats, vitamin D deficiency down-regulates the Notch pathway, known to play a leading role in muscle regeneration (23). Furthermore, mice lacking VDRs show an abnormal skeletal muscle phenotype with smaller, variable muscle fibers and the persistence of immature muscle gene expression during adult life, suggesting a role of vitamin D in muscle development (22).

Taking into account these data, we planned to characterize the impact of 25(OH)D on the transcriptional response of muscle cells in presence of mononuclear cells. For this, we assessed the influence of 25(OH)D on L6 myogenic cell co-cultured with fresh mononuclear cells isolated from rat's blood by evaluating (i) the muscle differentiation and metabolism markers by transcriptomic analysis and (ii) cytokine production.

Materials And Methods

L6 myoblast monoculture

The rat skeletal muscle-derived cell line L6 myoblasts from the American Type Culture Collection were handled according to the guidelines of the European Center for the Validation of Alternative Methods Task force based on the Guidance on Good Cell Culture Practices (24). Cells were grown on plates (2.10^4 cells/mL) in a proliferation medium composed of DMEM (PAN Biotech, France) supplemented with 10% fetal bovine serum (Biowest, France), 4 mM glutamine (Sigma, France), 1% of penicillin-streptomycin (PAN Biotech, France) and 1X non-essential amino acids (Sigma, France) in an atmosphere of 5% CO_2 at 37°C. After 24 h of proliferation, the culture medium was replaced by a differentiation medium containing 2% horse serum (Dominic Dutscher, France) instead of the fetal bovine serum. Then, the L6 cells were cultured for six days in the presence or not (control) of 125 nM 25(OH)D (Dedrogyl®, Desma Pharma, France) with a change of medium after 72 h. The RNA cells were extracted at 24 h (D1), 72 h (D3) and 144 h (D6) of culture and stored at − 80°C until RT-PCR experiments.

PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were obtained from 3-mo young male rat's blood in accordance with the European Guidelines for Animal Experiments. The PBMCs were freshly isolated using Histopaque-1119® and Histopaque-1077® (Sigma, France) density gradients as previously described (25) and then suspended (1×10^6 cells/mL) in the differentiation medium.

Insert co-culture of L6 and PBMC

The PBMCs (4×10^5) were seeded in the upper part of 0.4 µm inserts (ThinCerts, Greiner Bio-One, France) and placed over the L6-containing wells (2.10^4 cells/mL) for three days in the presence or not (control) of 125 nM of 25(OH)D. Media collections and RNA cell extractions were performed at 24 h (D1) and 72 h (D3) of culture and stored at −80°C until analysis.

Quantitative RT-PCR analysis

Total RNA was extracted from the L6 mono-cultures (n = 6) and from the co-cultures (n = 6) with Tri-Reagent® (Sigma, France) according to the manufacturer's instructions. RNA concentrations were determined using a Nanodrop ND-8000 spectrophotometer (Thermo Scientific, France) and reverse transcription was performed according to the manufacturer's protocol. A quantitative RT-PCR array was carried out on a Rotor Gene Real-Time PCR system (Qiagen, France). For the mono-cultured L6 RNA, the primers used are listed in Table 1 and for the co-cultured RNA, PCR plates with 36 genes were used (rat RT^2 profiler™, Qiagen, Table 2). Data were analyzed using the comparative CT method, based on the formula $2^{-\Delta\Delta CT}$(26). Each transcript level was normalized to the *Hprt1* housekeeping gene and compared to the transcript expression in the D1 control sample without 25(OH)D treatment.

Immunofluorescence staining

At D1, D3 and D6, the mono-culture L6 samples (n = 3) were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton for VDR immunostaining. After nonspecific binding sites/epitopes blocking, the cells were incubated overnight at 4°C with rabbit anti-VDR polyclonal antibodies (Abcam®, France), then 1 h in the dark at room temperature with AlexaFluor 488 conjugated donkey anti-rabbit (Invitrogen, Paisley, UK). Concurrently, the cells were counterstained using DAPI (4',6-diamidino-2-phenylindole, 0.5 µg/mL). After three washes, cells fluorescence were analyzed by confocal laser-scanning microscopy (Leica Microscope, Heidelberg, Germany).

Cytokine quantification

Co-cultured L6-PBMC supernatants were collected at D1 and D3 for quantification of IL-6 and IL-10 concentrations using Milliplex kit (map rat cytokine, Millipore, France) according to the manufacturer's instructions. The fluorescence intensity was determined with Luminex System (Bio-Rad Laboratories, Germany).

Statistical analysis

Data analyses were performed using GraphPad Prism® 5.03 for Windows (GraphPad Software Inc., San Diego, CA, USA). The experimental design required a two-way ANOVA to discriminate between the time and the treatment effects followed by Newman-Keüls post-hoc test. The results are expressed as mean ± SEM. Differences could be considered statistically significant when the P value was less than 0.05. For transcriptome analysis, we considered a fold change lower than 0.5 or greater than 2 as significant.

Results

25(OH)D induces an overexpression of VDR mRNA in L6 cells without effect on muscle differentiation gene expression

We assessed the impact of vitamin D on the expression of *VDR* mRNA in L6 cells. At D1, *VDR* expression was 3-fold higher in the presence of 25(OH)D than in the control (1.0 *vs* 3.1 ± 0.5; $p < 0.001$; Fig. 1-A). A significant lower overexpression compared to the control was also observed at D3 (1.3-fold, $p < 0.05$) and D6 (2-fold, $p < 0.05$). VDR protein expression was determined by immunofluorescence staining (Fig. 1-B). The VDR protein expression was more pronounced at D1 and D3 in 25(OH)D conditions than in the control. Moreover, this protein localization was in the perinuclear space at D1 and in the cytoplasm at D3 according to the histomorphologic changes of the cell during the differentiation. We also studied the expression of the 1α-hydroxylase gene (*Cyp27b1)*, an enzyme involved in the transformation of 25(OH)D into 1,25(OH_2)D. The *Cyp27b1* transcript level was 2-fold lower at D1, in the presence of 25(OH)D, compared to the control (1.0 *vs* 0.5 ± 0.2; $p < 0.05$; Fig. 1-C). At D3 and D6, *Cyp27b1* mRNA expression was significantly increased independently of the 25(OH)D treatment.

About muscle differentiation biomarkers, mRNA expression of *Desmin (Des) and Myosin heavy chain 2 (Myh2)* was unchanged from D1 to D6 whatever the 25(OH)D treatment (Fig. <link rid="fig2">2</link>-A and 2-B). *Myogenin* (*Myog*) mRNA expression was increased at D3 in comparison with D1 both in control and treated cells (Fig. 2-C).

25(OH)D counteracts the effects of PBMCs on L6 gene expression except for IL-6 transcript

We determined the L6 cells gene expression co-cultured with PBMCs in presence or not (control) of 25(OH)D treatment for 3 days (125 nM) (Table 2). Firstly, we considered mRNA levels in D3 controls against D1 ones to characterize the effect of PBMCs alone on L6 cells gene expression. At D3, a significant overexpression of myogenesis marker mRNA was observed for: *Des* (3.3-fold); *Myog* (27.5-fold) and *Myh2* (3.2-fold). This upregulation was in accordance with Notch pathway *Delta-1 (Dll)* as well as *Hairy and enhancer of split 1* (*Hes1*) overexpression (D3 *vs* D1: 6.8-fold and 3.8-fold, respectively). The *Bone morphogenetic protein 4* (*Bmp4*) mRNA, a cell proliferation factor induced by the Notch pathway, was overexpressed 2.9-fold at D3. *Interleukin-6* (*Il-6*) mRNA expression was also increased significantly (9.1-fold). The mRNA expression of *F-box protein 32* (*Fbxo32*), one of the four subunits of the ubiquitin protein ligase complex, was upregulated at D3 in the presence of PBMCs (10.2-

fold). Two metabolic marker transcripts were overexpressed i.e. *Solute carrier family 2 member 4* (*Slc2a4* or *Glut 4*) (2.6-fold) and *ATPase sarco/endoplasmic reticulum Ca^{2+} transporting 1* (*Atp2a1*), a Ca^{2+}-ATPase pump gene (42.1-fold).

Secondly, we assessed the impact of 25(OH)D on L6 co-cultured with PBMCs considering mRNA levels in the 25(OH)D group against the control for each D1 and D3 (Table 2). At D1, any transcript was significantly changed with vitamin D treatment. At D3, 4 transcripts were downregulated: *Myog* (25(OH)D *vs* control: 12.5-fold *vs* 27.5; $p < 0.05$); *Hes1* (2.0 *vs* 3.8; $p < 0.05$); *Fbxo32*, (7.9 *vs* 10.2; $p < 0.05$) and *Atp2a1*, (20.5 *vs* 42.1; $p < 0.05$). Interestingly, *Il-6* mRNA expression was upregulated by 2-fold with the 25OHD treatment at D3 (25(OH)D *vs* control: 18.2-fold *vs* 9.1; $p < 0.05$).

No effect of 25(OH)D treatment on cytokine production from L6 and PBMCs co-culture

No difference in IL-6 and IL-10 levels was observed at D1 between 25(OH)D treated cells *vs* control (Table 3). Comparison of IL-6 and IL-10 concentrations at D3 *vs* D1 in the 25(OH)D treated cells showed an increase but not significant according to the high inter-individual variability (IL-6: 2124 ± 1072 *vs* 545 ± 474 pg/mL; IL-10: 21.6 ± 9.9 *vs* 72.6 ± 27.3 pg/mL; Table 3). Notably, the increase of the IL-6 level appeared more pronounced (12.2 ± 5.0) than the IL-10 one (5.8 ± 2.1). No significant increase in the IL-6/IL-10 ratio was observed between D3 and D1 (Table 3).

Discussion

This study aimed to evaluate, *in vitro*, how muscle cell differentiation is influenced by a 25(OH)D treatment in the presence of immune cells. The dose of 125 nM of 25(OH)D was chosen to mimic blood physiological condition (27, 28).

The 25(OH)D treatment on mono-cultured L6 cells induced an upregulation of *VDR* mRNA and protein expressions with a decreasing effect during differentiation kinetics (from D1 to D6). This suggests that 25(OH)D is converted into active $1,25(OH)_2D$ which auto-regulates the expression of the *VDR* gene through intronic and upstream enhancers as previously described (29, 30). The 25(OH)D to $1,25(OH)_2D$ conversion is performed by the CYP27B1 enzyme expressed in muscle cells. As expected, the mRNA of this enzyme was underexpressed at D1 25(OH)D treatment due to the repressive effect of VDR on gene transcription (31). After that, the *Cyp27b1* mRNA expression was upregulated simultaneously to the decrease of the *VDR* mRNA and protein expressions associated to the migration of VDR protein from the perinuclear space to the cytoplasm. In the light of these findings, the enhancement of the *Cyp27b1* expression seems to be a major key to the effects of 25(OH)D on muscle cell metabolism. Similarly, in a recent study, Sustova et al showed that 25(OH)D on C2C12 muscle cells induced an overexpression of *Cyp27b1* either directly or upon IL-6 stimulation (32).

Among the muscle differentiation markers determined, only *Myog* mRNA expression was overexpressed from 72 h without effects of 25(OH)D treatment. This is in agreement with the van der Meijden's study which reported in C2C12 mouse myoblasts that the expression of MyoD and ki67 were not significantly affected by both 25(OH)D or $1,25(OH)_2D3$(33)

Considering that, *in vivo*, the proliferation and the differentiation of muscle cells are facilitated by the surrounding immune cells (34), we used an *in vitro* model of L6 muscle cell and PBMCs insert co-culture in the presence of

125nM of 25(OH)D or not. We focused on a 24 h and a 72 h time lapse for differentiation when gene expression is most liable to be modulated (15, 35).

In the presence of PBMCs without 25(OH)D, eleven L6 muscle cell genes from myogenesis, Notch signaling, cell proliferation and metabolism clusters were upregulated over time. In fact, PBMCs induced a significant overexpression of *Des, Myog, Myh2 and Musk* mRNA in the L6 cells in conjunction with *Dll1* and *Hes1*, effectors of Notch signaling. Once the pathway is activated, the Notch receptor is cleaved and its intracellular domain acts as a transcription factor to induce *Hes1* gene expression. (23, 36). This highlight, for the first time to our knowledge, the importance of the cross-talk between immune and muscle cells to promote the expression of muscle differentiation genes without a direct cell-cell contact. An overexpression of both *Bmp4*, a key gene in the regulation of cell proliferation (37, 38), and *Il-6*, a myokine described as a muscular proliferative factor (39), was observed. This follows with Serrano's study showing that IL-6 promotes murine satellite cell proliferation *via* regulation of the cell-cycle-associated genes *cyclin D1* and *c-myc* (40). Finally, an overexpression of genes implicated in some energetic metabolism pathways was observed. This included proteolysis (*Fbxo32*) as described before (41), glucose transport (*Slc2a4*) as shown in Broydell's study (42) and calcium influx (*Atp2a1*) which plays an essential role in the regulation of intracellular Ca^{2+} level and in the skeletal muscle differentiation (43). Taken together, these observations confirm the hypothesis of the crosstalk between immune and muscle cells to promote metabolic changes and muscle differentiation.

The treatment with 25(OH)D reduced the L6 gene overexpression induced by PBMCs for 5 genes: *Myog*, *Myh2*, *Hes1*, *Fbxo32* and *Atp2a1*. Concerning *Myog*, this finding is coherent with the study undertaken by Endo which demonstrated an upregulation of myogenin mRNA expression in VDR null mice (22). For *Myh2*, the data are consistent with a previous study on L6 cell line showing a downregulation by a VDR knockdown, reversed after vitamin D treatment (44). The decreased level of *Hes1* mRNA appears to be consistent with the overexpression of the other Notch signaling factors such as *Dll1* and *Bmp4* (23). Hence, *Hes1* protein could contribute to the negative feedback regulation of Notch signaling (45). The underexpression of *Fbxo32* (or *Atrogin 1*) in C2C12 muscle cells was previously described in Chen's study (46) involving the FOXO1 signaling pathway as clarified recently by Dzik (47). Moreover, the *Il-6* mRNA overexpression in L6 cells induced by PBMCs is majored by 2-fold in the presence of 25(OH)D in link to the increase of IL-6 level in culture medium (12.2-fold). That can reinforce the promotion of L6 cells differentiation (48). The larger increase observed for IL-6 as opposed to IL-10 may be due to its double origin from both muscle and immune cells whereas IL-10 is exclusively produced by immune cells. Unexpectedly, vitamin D is described as having adverse effects on IL-6 production from these two cell types: i.e. inhibition on PBMCs (49, 50) and activation on L6 cells (48).

Conclusions

In the present study, our *in vitro* approach confirmed the importance of 25(OH)D and immune cells in stimulating muscle cell differentiation. Taken as a whole, the data highlight that 25-hydroxyvitamin D attenuates the Notch pathway-dependent effects induced by immune cells on muscle differentiation and cell energy metabolism. Further investigations on metabolic pathways are needed to better understand the effects of vitamin D in the immune and muscle cells crosstalk.

Abbreviations

25(OH)D, 25-hydroxyvitamin D; 1,25$(OH)_2$D, 1,25 dihydroxyvitamin D; Atp2a1, ATPase sarco/endoplasmic reticulum Ca^{2+} transporting 1; Bmp4, Bone morphogenetic protein 4; Dl1, Delta-1; Des, Desmin; Fbxo32, F-box protein 32; Hes1, Hairy and enhancer of split 1; IL, Interleukin; Myog, Myogenin; Myh2, Myosin heavy chain 2; PBMC, Peripheral blood mononuclear cell; RXR, Retinoid-X-Receptor; Slc2a4, Solute carrier family 2 member 4; Th, T helper cell; VDR, Vitamin D Receptor, VDRE, Vitamin D response element.

Declarations

Ethics approval

All animal procedures were approved by the institution's animal welfare committee (Comité d'Ethique en Matière d'Expérimentation Animale Auvergne: CEMEAA; Permit number 00872.02) and were conducted in accordance with the European's guidelines for the care and use of laboratory animals (2010-63UE). Animals were housed in the animal facility of the INRA Research for Human Nutrition (Agreement N°: C6334514). Rats were purchased from JANVIER (Le Genest St Isle, France). At the end of the experiment, the rats were sacrificed by decapitation after isoflurane anaesthesia and all efforts were made to minimize animal suffering.

Consent for publication

All authors support the submission to this journal.

Availability of data and materials

All data generated or analyzed during this study are included in this manuscript or are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

Funding

This work was supported by the University Clermont Auvergne and by the SFNEP Bernard Beaufrère prize, 2015.

Author's Contributions

The authors' responsibilities were as follows- CDF, NGM, MPV, SW: conceived and designed the experiments; CDF, SR: performed the experiments; CDF, VP, JS, NGM, MPV: analyzed the data and performed the statistical analysis; MPV, SW: contributed reagents/material/analysis tools; CDF, NGM, MPV: wrote the paper; MPV, SW, NGM: sourced the funding; and all authors: read and approved the manuscript.

Acknowledgements

We are grateful to Daniel Taillandier (UMR1019-INRAE/Clermont Auvergne University, Clermont-Ferrand, France) for supplying the L6 cell line, to Vincent Sapin (Université Clermont Auvergne, CNRS, INSERM, GReD, Clermont-Ferrand, France) for his assistance with Luminex® assay and to Caroline Vachias (Université Clermont Auvergne, CNRS, INSERM, GReD, Clermont-Ferrand, France) for her technical assistance with confocal microscopy.

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Tables

'able 1. Primer sequences for the quantitative analysis of gene expression in mono-cultured L6.

Gene name	RefSeq	Forward sequence	Reverse sequence
VDR	NM_017058.1	5’-TATTCTCCAAGGCCCACACT-3’	5’-CGGATGGTTCCATCATGTCT-3’
Cyp27b1	NM_053763.1	5’-CCGACCCTGCACTTGTAGA-3’	5’-TACAGCGCTCTGGACAATGA-3’
Desmin	NM_022531.1	5’-TCAAGGGCACCAACGACT-3’	5’-GGTCTGGATCGGAAGGTTGAT-3’
Myogenin	NM_017115.2	5’-GCAGTGCCATCCAGTACATTGAGC-3’	5’-GGAAGGTGACAGACATATCCTCCAC-3’
Myh2	NM_001135157	5’-TGATGCAGGAGAAAAATGACC-3’	5’-TTCCTCAGCATCAGCCAAG-3’
Hprt1	Hs.422707	5’-AGTTGAGAGATCATCTCCAC-3’	5’-TTGCTGACCTGCTGGATTAC-3’

Table 2. Gene expression levels in co-cultured L6 cells were analyzed by a RT profiler custom PCR array.

Functional gene grouping	RefSeq	Official name	Gene	Fold change D1		Fold change D3		ANOVA	
				Control	25(OH)D	Control	25(OH)D	Time	Treatment
Myogenesis	NM_022531	Desmin	Des	1.0 [a]	1.1 ± 0.3 [a]	3.3 ± 0.4 [b]	3.8 ± 0.5 [b]	0.0001	ns
	NM_017115	Myogenin	Myog	1.0 [a]	0.6 ± 0.1 [b]	27.5 ± 1.2 [c]	12.5 ± 4.3 [d]	0.0001	0.0043
	NM_001135157	Myosin heavy chain 2	Myh2	1.0 [a]	1.3 ± 0.6 [a]	3.2 ± 0.9 [b]	1.5 ± 0.1 [a]	0.0023	0.048
	NM_031061	Muscle associated receptor tyrosine kinase	Musk	1.0 [a]	0.8 ± 0.1 [a]	2.2 ± 0.5 [b]	1.8 ± 0.3 [b]	0.0077	ns
	NM_001106783	Myogenic factor 5	Myf5	1.0	1.0 ± 0.1	1.2 ± 0.3	1.1 ± 0.3	ns	ns
	NM_013172	Myogenic factor 6	Myf6	1.0	1.0 ± 0.2	1.2 ± 0.3	1.2 ± 0.2	ns	ns
	NM_001135158	Myosin heavy chain 1	Myh1	1.0	0.9 ± 0.0	1.7 ± 0.3	1.3 ± 0.2	ns	ns
	NM_019212	Actin alpha 1	Acta1	1.0	0.9 ± 0.1	0.7 ± 0.2	0.7 ± 0.3	ns	ns
	NM_053357	Catenin beta 1	Ctnnb1	1.0	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	ns	ns
Notch signalling	NM_032063	Delta-like 1	Dll1	1.0 [a]	1.0 ± 0.2 [a]	6.8 ± 0.1 [b]	6.7 ± 0.2 [b]	0.0001	ns
	NM_001105721	Notch 1	Notch1	1.0	1.2 ± 0.3	1.4 ± 0.2	1.3 ± 0.3	ns	ns
	NM_024360	Hairy and enhancer of split 1	Hes1	1.0 [a]	1.1 ± 0.3 [a]	3.8 ± 0.4 [b]	2.0 ± 0.3 [c]	0.0002	0.0108
Cell proliferation	NM_012827	Bone morphogenetic protein 4	Bmp4	1.0 [a]	1.2 ± 0.2 [a]	2.9 ± 0.4 [b]	2.4 ± 0.5 [b]	0.0013	ns
	NM_019305	Fibroblast growth factor 2	Fgf2	1.0	1.3 ± 0.3	1.8 ± 0.1	1.8 ± 0.3	ns	ns
	NM_012589	Interleukin 6	Il-6	1.0 [a]	1.7 ± 0.1 [b]	9.1 ± 1.6 [c]	18.2 ± 1.9 [d]	0.0001	0.0149
Apoptosis	NM_016993	BCL2, apoptosis regulator	Bcl2	1.0	1.1 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	ns	ns
	NM_012922	Caspase 3	Casp3	1.0	1.1 ± 0.1	1.8 ± 0.1	1.7 ± 0.2	ns	ns
Cell signaling	NM_053842	Mitogen activated protein kinase 1	Mapk1	1.0	1.1 ± 0.0	1.3 ± 0.1	1.3 ± 0.2	ns	ns
	NM_031020	Mitogen activated protein kinase 14	Mapk14	1.0	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.3	ns	ns
	NM_033230	AKT serine/threonine kinase 1	Akt1	1.0	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	ns	ns
	NM_019142	Protein kinase AMP-activated catalytic subunit alpha 1	Prkaa1	1.0	1.0 ± 0.1	1.5 ± 0.2	1.5 ± 0.2	ns	ns
	NM_057132	Ras homolog gene family member A	Rhoa	1.0	0.9 ± 0.0	1.1 ± 0.0	1.0 ± 0.1	ns	ns
Metabolism	NM_019906	Mechanistic target of rapamycin	Mtor	1.0	0.9 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	ns	ns
	NM_053857	Eukaryotic translation initiation factor 4E binding protein 1	Eif4ebp1	1.0	1.1 ± 0.1	1.3 ± 0.3	1.5 ± 0.4	ns	ns
	NM_031985	Ribosomal protein S6 kinase B1	Rps6kb1	1.0	0.9 ± 0.0	1.3 ± 0.1	1.2 ± 0.2	ns	ns
	NM_133521	F-box protein 32	Fbxo32	1.0 [a]	0.8 ±	10.2 ±	7.9 ±	0.0001	0.0500

				0.3 [a]	0.6 [b]	0.9 [c]		
NM_130755	Citrate synthase	Cs	1.0	0.8 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	ns	ns
NM_012751	Solute carrier family 2 member 4 (GLUT4)	Slc2a4	1.0 [a]	0.9 ± 0.2 [a]	2.6 ± 0.2 [b]	2.6 ± 0.3 [b]	0.0087	ns
NM_012735	Hexokinase 2	Hk2	1.0	1.2 ± 0.2	1.4 ± 0.0	1.6 ± 0.3	ns	ns
NM_032080	Glycogen synthase kinase 3 beta	Gsk3b	1.0	1.1 ± 0.1	1.3 ± 0.2	1.4 ± 0.2	ns	ns
NM_058213	ATPase sarco/endoplasmic reticulum Ca^{2+} transporting 1	Atp2a1	1.0 [a]	1.2 ± 0.2 [a]	42.1 ± 5.4 [b]	20.5 ± 3.0 [c]	0.0001	0.0032
NM_001110139	ATPase sarco/endoplasmic reticulum Ca^{2+} transporting 2	Atp2a2	1.0	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	ns	ns

Each transcript level was normalized to the *Hprt1* housekeeping gene and compared to the transcript expression in the day 1 control sample without the 25(OH)D treatment by the $2^{-\Delta\Delta CT}$ method. Data are means of fold change ± SEM (n = 6); D1: day 1; D3: day 3; Statistical analysis was performed using a two-way ANOVA to discriminate between the time and the treatment effects ($p < 0.05$). When the ANOVA indicated significant interactions, the Newman-Keüls post-hoc test was used. Superscript letters (a, b, c, d) indicate significant differences ($p < 0.05$); ns: not significant.

Table 3. Cytokine assay.

	D1 Cont	D1 25(OH)D	D3 25(OH)D
IL-6 (pg/mL)	438 ± 357	545 ± 474	2124 ± 1072
IL-10 (pg/mL)	21,8 ± 8,8	21,6 ± 9,9	72,6 ± 27,3
IL-6 / IL-10 ratio	14,4 ± 6,5	13,8 ± 7,3	26,0 ± 13,8

Data are means ± SEM (n = 6); D1: day 1; D3: day 3.

Figures

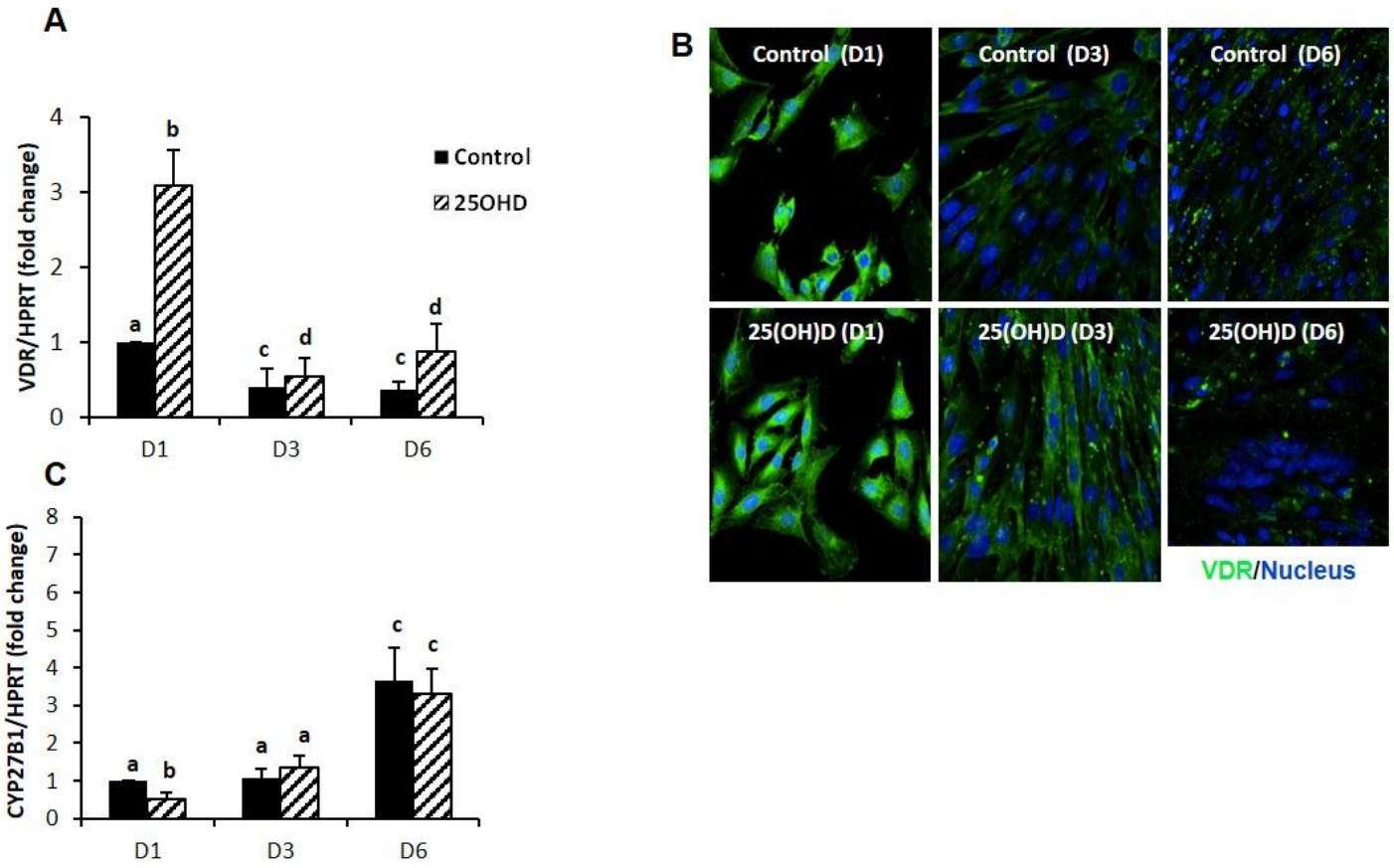

Figure 1: Domingues-Faria et *al.*

Figure 1

Effect of the 25(OH)D treatment on VDR and Cyb27b1 expression in mono-cultured L6. After 1-day (D1), 3 days (D3) and 6 days (D6) of treatment with or without 25(OH)D in the differentiation medium, VDR mRNA expression (A) and Cyp27b1 mRNA expression (B) in mono-cultured L6 was quantified by the comparative 2−ΔΔCT method. The transcript level was normalized to the Hprt1 housekeeping gene and compared to the transcript expression in the day 1 control sample without the 25(OH)D treatment (n = 6 per group). The data presented are mean ± SEM. *P < 0.05 vs D1 control group, indicating the difference has statistical significance. ***P < 0.001 vs D1 control group, indicating the difference has statistical significance. #P < 0.01 vs 25(OH)D group, indicating the difference has statistical significance. (C) After 1 day (D1), 3 days (D3) and 6 days (D6) of treatment with or without 25(OH)D in the differentiation medium, mono-cultured L6 were labelled by indirect immunofluorescence staining for VDR (green) and with DAPI as a nuclear counterstain (blue) (n = 3).

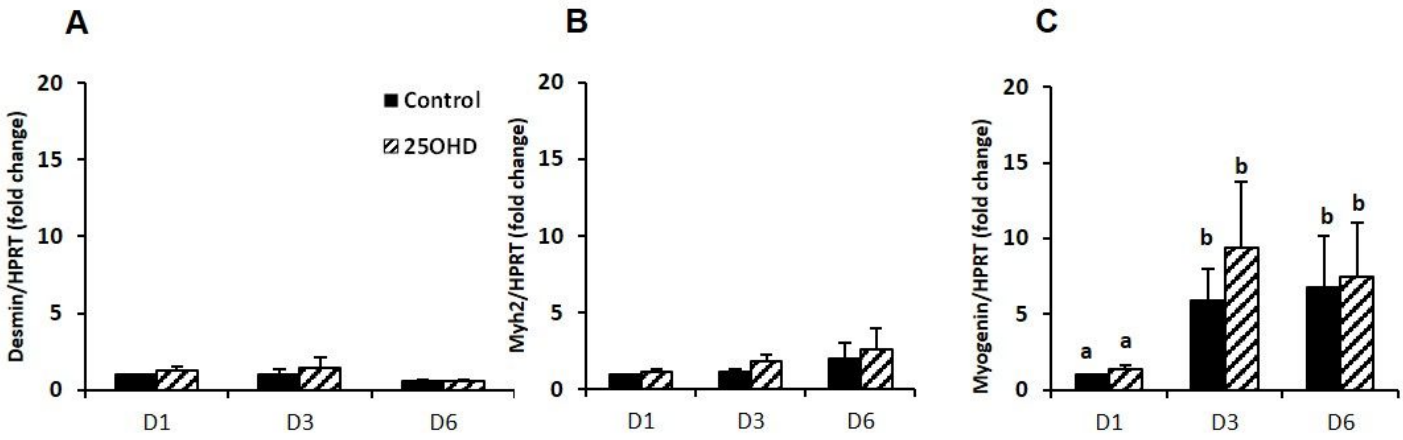

Figure 2: Domingues-Faria et *al.*

Figure 2

Effect of the 25(OH)D treatment on the mRNA expression of muscle differentiation markers in mono-cultured L6. After 1 day (D1), 3 days (D3) and 6 days (D6) of treatment with or without 25(OH)D in the differentiation medium, the mRNA expression of desmin (A), Myh2 (B) and myogenin (C) in mono-cultured L6 was quantified by the comparative 2−ΔΔCT method. Each transcript level was normalized to the Hprt1 housekeeping gene and compared to the transcript expression in the day 1 control sample without the 25(OH)D treatment (n = 6 per group). The data presented are mean ± SEM. *P < 0.05 vs D1 control group, indicating the difference has statistical significance. #P < 0.01 vs 25(OH)D group, indicating the difference has statistical significance.