Endocrine CONNECTIONS

Vitamin D induces myogenic differentiation in skeletal muscle derived stem cells

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Abstract

Skeletal muscle wasting is a serious disorder associated with health conditions such as aging, chronic kidney disease and AIDS. Vitamin D is most widely recognized for its regulation of calcium and phosphate homeostasis in relation to bone development and maintenance. Recently, vitamin D supplementation has been shown to improve muscle performance and reduce the risk of falls in vitamin D deficient older adults. However, little is known of the underlying molecular mechanism(s) or the role it plays in myogenic differentiation. We examined the effect of 1,25-D_3 on myogenic cell differentiation in skeletal muscle derived stem cells. Primary cultures of skeletal muscle satellite cells were isolated from the tibialis anterior, soleus and gastrocnemius muscles of 8-week-old C57/BL6 male mice and then treated with 1,25-D_3. The efficiency of satellite cells isolation determined by PAX7+ cells was 81%, and they expressed VDR. Incubation of satellite cells with 1,25-D_3 induces increased expression of: (i) MYOD, (ii) MYOG, (iii) MYC2, (iv) skeletal muscle fast troponin I and T, (v) MYH1, (vi) IGF1 and 2, (vii) FGF1 and 2, (viii) BMP4, (ix) MMP9 and (x) FST. It also promotes myotube formation and decreases the expression of MSTN. In conclusion, 1,25-D_3 promoted a robust myogenic effect on satellite cells responsible for the regeneration of muscle after injury or muscle waste. This study provides a mechanistic justification for vitamin D supplementation in conditions characterized by loss of muscle mass and also in vitamin D deficient older adults with reduced muscle mass and strength, and increased risk of falls.

Key Words
- VDR
- PAX7
- MYC
- FGF
- IGF
- MSTN
- FST

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Introduction

Vitamin D is universally recognized for its classical effects on calcium regulation and phosphate homeostasis, in relation to bone development and maintenance (1, 2). However, vitamin D deficiency has also been linked to the pathogenesis of several acute and chronic diseases such as musculoskeletal disorders, especially associated with reduced muscle mass and impaired physical performance in frail and elderly individuals (3, 4). While vitamin D deficiency has been associated with a reduction in upper and lower limb muscle strength and physical performance (5), a positive association between vitamin D supplementation on upper and lower body muscle strength has been described in healthy individuals (6).

At a cellular level there is also evidence of VDR expression and direct effects of vitamin D on human skeletal muscle precursor cells (7), which provides a

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rationale for a direct role of vitamin D in muscle function. Furthermore, mice lacking VDR show an abnormal skeletal muscle phenotype with smaller, variable muscle fibers and persistence of immature muscle gene expression during adult life, suggesting a role of vitamin D in muscle development (8, 9). However, more research needs to be done in order to decipher the underlying mechanism or the role vitamin D plays in association with myogenic differentiation. Vitamin D, a fat-soluble secosteroid prohormone, is obtained from sun exposure or from dietary sources. During exposure to sunlight 7-dehydrocholesterol in the skin is converted to previtamin D3, which is immediately converted by a heat-dependent process to vitamin D3. Vitamin D2 and vitamin D3 from dietary sources are incorporated into chylomicrons and transported by the lymphatic system into the venous circulation. Vitamin D in the circulation is bound to the vitamin D-binding protein, which transports it to the liver, where the enzyme, D-25 hydroxylase, converts vitamin D to 25-hydroxyvitamin D3. 25-HydroxyvitaminD_3 is biologically inactive and is converted primarily in the kidney by the 25-hydroxyvitamin D-1-hydroxylase to its biologically active form 1,25-D3 or calcitriol (10). Our group and others have previously delineated the role of vitamin D in myogenic cell differentiation in C2C12 cells (11, 12, 13, 14). Mouse C2C12 skeletal muscle myoblast cells are an '*in vitro*' cell line, which is widely used to study genes that regulate muscle growth and differentiation (15, 16).

Satellite cells, also known as 'skeletal muscle stem cells', have a remarkable ability to regenerate after muscle injury and are responsible for compensation of muscle turnover caused by daily wear and tear. In this study, we expand our previous results and conclusions (11, 12) by now utilizing a more clinically relevant cell model, which is the primary culture of skeletal muscle derived satellite cells. In conclusion, the aim of the present study was to test the hypothesis that 1,25-D_3 promotes myogenic cell differentiation by targeting skeletal muscle stem cells, and to determine the associated molecular mechanism(s). This was done by investigating the expression of key pro- and anti-cell differentiation lineage markers and select growth factors modulated by 1,25-D_3 in primary cultures of skeletal muscle satellite cells.

Materials and methods

Experimental animals

Male 8-week-old C57/BL6 mice (B.W.: 22.0 ± 0.3 g) from Jackson Laboratories were used for this study, which was approved by the Institutional Animal Care and Use Committee (IACUC) at Charles R. Drew University of Medicine and Science. Mice were housed for one week prior to experimental procedures to permit acclimatization. Animals were killed by CO_2 inhalation. After confirming the death of the animals, they were soaked in alcohol 70% for 5 min. The skin and fascia were then removed and the hind limb muscles tibialis, soleus and gastrocnemius were removed under aseptic conditions. Later we proceeded with the satellite cells isolation.

Cell culture and satellite cell isolation

Primary satellite cell cultures were isolated as previously described (17, 18). Briefly, once tendons, vessels and fat were removed, each muscle was cut into small fragments and enzymatically digested at 37°C in 0.2% collagenase solution for 1 h. Myofibers were further purified from interstitial cells and tendons by a series of trituration, sedimentation and washings. Myofiber fragments were passed through a 40-μm cell strainer, resuspended in growth media (DMEM medium containing 20% FBS, 10% horse serum and 1% chick embryo extract) and plated in culture dishes. Cells were allowed to adhere for 2 h to remove large debris, macrophages and fibroblasts that adhere to the plastic. The primary non-attached myoblasts were transferred onto collagen-coated T25 flask and left undisturbed for 3 days allowing the cells to attach. Upon reaching 70% confluency, the satellite cells were detached and replated according to different treatments. Cells were incubated with or without 100 nM 1,25-D_3 (Sigma-Aldrich) dissolved in less than 0.1% ethanol as vehicle in growth media for 1–12 days. The 100 nM supra-physiological concentration of 1,25-D_3 employed in the experimental designed was the optimal concentration established based on our prior dose–response studies (21, 22) and it is in alignment with a commonly used dose applied in the majority of publications related to 1,25-D_3 effects on different cell lines or in primary cell cultures (19–24). Because of the 10-h half-life of 1,25-D_3, the cell culture media, incubated with or without 1,25-D3 (100 nM), was replaced daily (11, 12).

Detection of PAX7, VDR and MYC by immunocytochemistry

Satellite cells incubated in growth media for 1 day in collagen-coated chamber slides were washed thrice with PBS (1×) and fixed by immersion in 2% *p*-formaldehyde.

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Cells were first quenched with 3% H_2O_2 and then blocked with normal goat or horse serum, and incubated with: (1) a rabbit polyclonal antibody against PAX7 at a dilution of 1:200 (Abcam), (2) a rabbit polyclonal antibody against VDR at a dilution of 1:50 (Santa Cruz Biotechnology) or (3) a mouse monoclonal antibody against MYC type II (1:400) from Abcam. The detection was followed by a 1:200 dilution of either anti-rabbit or anti-horse biotinylated secondary antibody from Calbiochem, followed by the ABC complex (1:100) (Vectastain Elite ABC System, Vector Laboratories) and 3,3-diaminobenzidine (DAB) (Sigma), without counterstaining. In negative controls, we either omitted the first antibody or replaced it with a rabbit IgG isotype antibody (11).

Double labeling immunofluorescence detection of PAX7 and VDR

The double localization of PAX7 and VDR was carried out on 2% *p*-formaldehyde-fixed satellite cells primary cultures plated on collagen-coated chamber slides. For VDR, cells were blocked with normal goat serum, and incubated with a rat monoclonal antibody against VDR at a dilution of 1:50 (Abcam), followed by a 1:200 dilution of anti-rat biotinylated secondary antibody (Vector Labs, Burlingame, CA, USA). The subsequent reaction was carried out by incubating the cells in a 20 mg/mL solution of streptavidin-FITC (Vector Labs), followed by 10% normal goat serum and then a 1:250 dilution of anti-PAX7 monoclonal antibodies (Abcam). Fluorescence labeling was performed with secondary antibody 'Texas Red' (13 mg/mL; Vector Labs). After several washes, the slides were detached and counterstained/mounted in 'vectashield' mounting medium with DAPI (Vector Labs). Slides were examined under a Leica DMLB fluorescence microscope equipped with the appropriate filters. The VDR/PAX7 co-localization images were obtained by merging the red and green filtered pictures, as well as the blue filter for the VDR/PAX7/DAPI merge. Fields were photographed with a Leica DFC310 FX digital camera and Leica acquisition software. Negative controls were done by either omitting the first antibody or using a rabbit non-specific IgG (25).

PCR array analysis of skeletal muscle growth and differentiation factors

RT^2 profiler PCR pathway focused arrays (SABiosciences, Qiagen) were applied in triplicate in order to detect changes in gene expression of skeletal muscle growth and differentiation factors. Total cellular RNA from satellite cells that were treated with or without 1,25-D_3 (100 nM) for 7 days, was isolated with TRIzol Reagent (Invitrogen). They were then subjected to reverse transcription, and the resulting cDNA was analyzed by the Mouse Skeletal Muscle: Myogenesis & Myopathy (PAMM-099Z) PCR Arrays (SABiosciences, Qiagen). The Mouse Skeletal Muscle: Myogenesis & Myopathy RT^2 Profiler™ PCR Array contains genes related to skeletal muscle differentiation, function and disease-related processes. Real-time PCRs were performed as follows: melting for 10 min at 95°C, 40 cycles of two-step PCR including melting for 15 s at 95°C and annealing for 1 min at 60°C. The raw data was analyzed using the C_t method following the manufacturer's instructions (SABiosciences Corp., Qiagen) (26).

Real-time quantitative PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen) and equal amounts (1 µg) of RNA were reverse transcribed using High Capacity RNA-to-cDNA PCR kit (Applied Biosystems). Mouse gene PCR primer sets (RT^2) for VDR, IGF1, IGF2, FGF1, FGF2, MSTN and FST were obtained from SABiosciences. The Power SYBR green PCR Master Mix (Applied Biosystems) was used with Step-One-Plus real-time PCR System (Applied Biosystems). The protocol included melting for 15 min at 95°C, 40 cycles of three-step PCR including melting for 15 s at 95°C, annealing for 30 s at 58°C, elongation for 30 s at 72°C with an additional detection step of 15 s at 81°C, followed by a melting curve from 55 to 95°C at the rate of 0.5°C per 10 s. The samples of 25°ng cDNA were analyzed in quadruplicate in parallel with GAPDH controls; standard curves (threshold cycle vs log pg cDNA) were generated by log dilutions of standard cDNA (reverse transcribed mRNA from satellite cells in GM) from 0.1 pg to 100 ng. Experimental mRNA starting quantities were then calculated from the standard curves and averaged using SABioscience software as described previously (26). The ratios of marker experimental gene (e.g., VDR, IGF1 and 2, FGF1 and 2, MSTN, and FST mRNA) to that of GAPDH mRNA were computed and normalized with control (untreated) samples as 100%.

Western blotting and densitometry analyses

The cell lysates (50 µg protein) were subjected to Western blotting analyses by 4–15% Tris–HCl PAGE (Bio-Rad) in a running buffer (Tris/glycine/SDS). The proteins were

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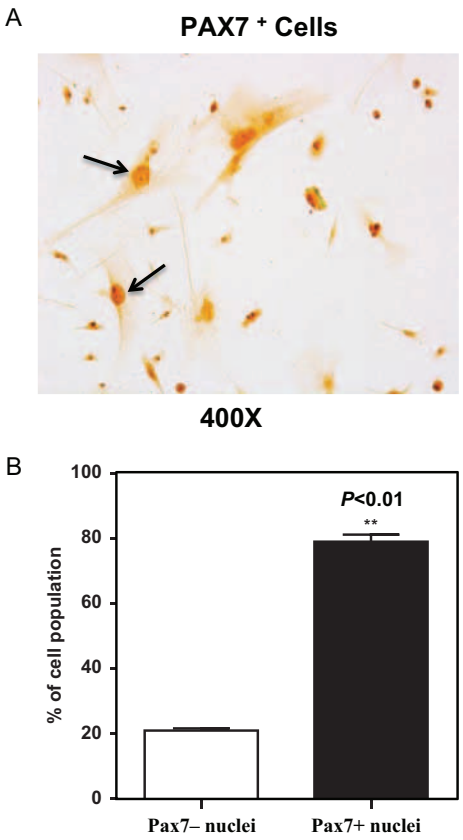

Figure 1
Efficiency in the isolation of primary cultures of satellite cells from skeletal muscle. Once the satellite cells were isolated and prior to starting the culture expansion, the efficiency of the applied technique was tested by the expression of PAX7 by immunocytochemistry (A). Magnification 400×. The counting of positive PAX7 nuclei with the Image-Pro software renders an 81% efficiency ($P<0.01$) in the isolation of satellite cells by the applied methodology (B).

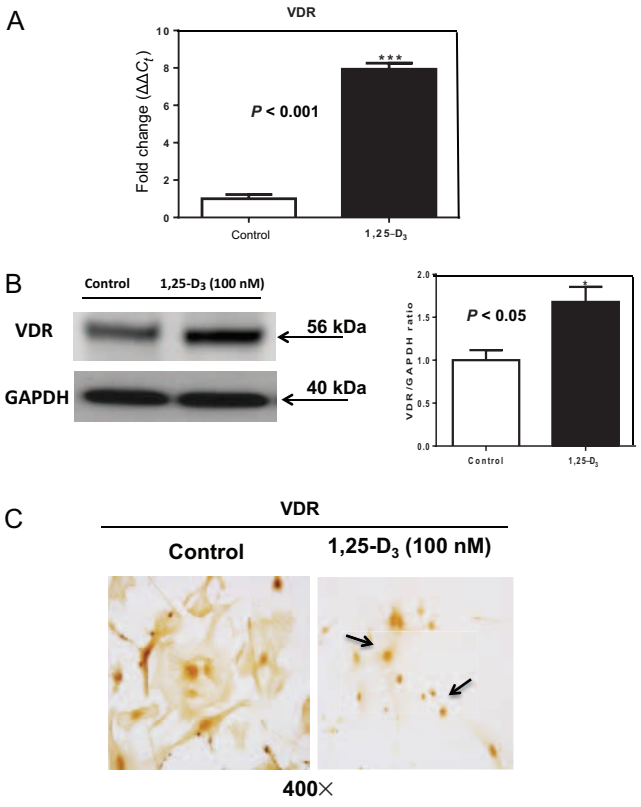

Figure 2
Expression, nuclear translocation and upregulation of steady-state mRNA and protein levels of VDR upon incubation of satellite cells with 1,25-D_3. Cultures of satellite cells were continuously incubated with or without 1,25-D_3 (100 nM) for 7 days on four-well removable chamber slides and then were subjected to real-time PCR (A); Western blotting with the corresponding densitometric analysis (B) and immunocytochemistry using a rabbit polyclonal antibody for VDR, magnification 400× (C). Mean ± S.E.M. corresponds to experiments done in triplicate, $P<0.001$ (A) and $P<0.05$ (B). Samples and controls were normalized to the GAPDH housekeeping mRNA/protein.

transferred onto PVDF membranes in a transfer buffer (Tris/glycine/methanol) using transblot semi-dry apparatus (Bio-Rad). The nonspecific binding was blocked by immersing the membranes into 5% nonfat dried milk and 0.1% (v/v) Tween 20 in PBS for 3 h at RT. After several washes with the washing buffer (PBS Tween 0.1%), the membranes were incubated with the primary antibodies for 3 h at RT or overnight at 4°C. Primary rabbit polyclonal antibodies from Abcam were used for: VDR (1:1000), IGF1 (1:500), IGF2 (1:500), FGF1 (1:500), FGF2 (1:500) and FST (1:500). The MYC type II (1:1000) was a monoclonal antibody also from Abcam. The MYOD (1:500) and the MYOG (1:500) were both polyclonal rabbit antibodies from Santa Cruz Biotechnology. The rabbit polyclonal MSTN antibody (1:500) and the mouse monoclonal GAPDH antibody (1:5000) were both from Millipore. After several washes with buffer, the membranes were incubated for 3 h at RT with 1/2000 dilution (anti-mouse) or 1:2000 dilution (anti-rabbit) of secondary antibody linked with HRP (Cell Signaling Technology). After several washes, the immunoreactive bands were visualized using the WesternSure PREMIUM chemiluminescent detection system (Li-COR Biotechnology, Lincoln, NE, USA). The scanning of the bands was done with the C-DiGit Blot Scanner (Li-COR Biotechnology) and the images were captured with the Image Studio Software, version 5.2 (Li-COR Biotechnology).

Statistical analysis

All data are presented as mean ± S.E.M., and between-group differences were analyzed using ANOVA. If the overall ANOVA revealed significant differences, then pair-wise comparisons between groups were performed

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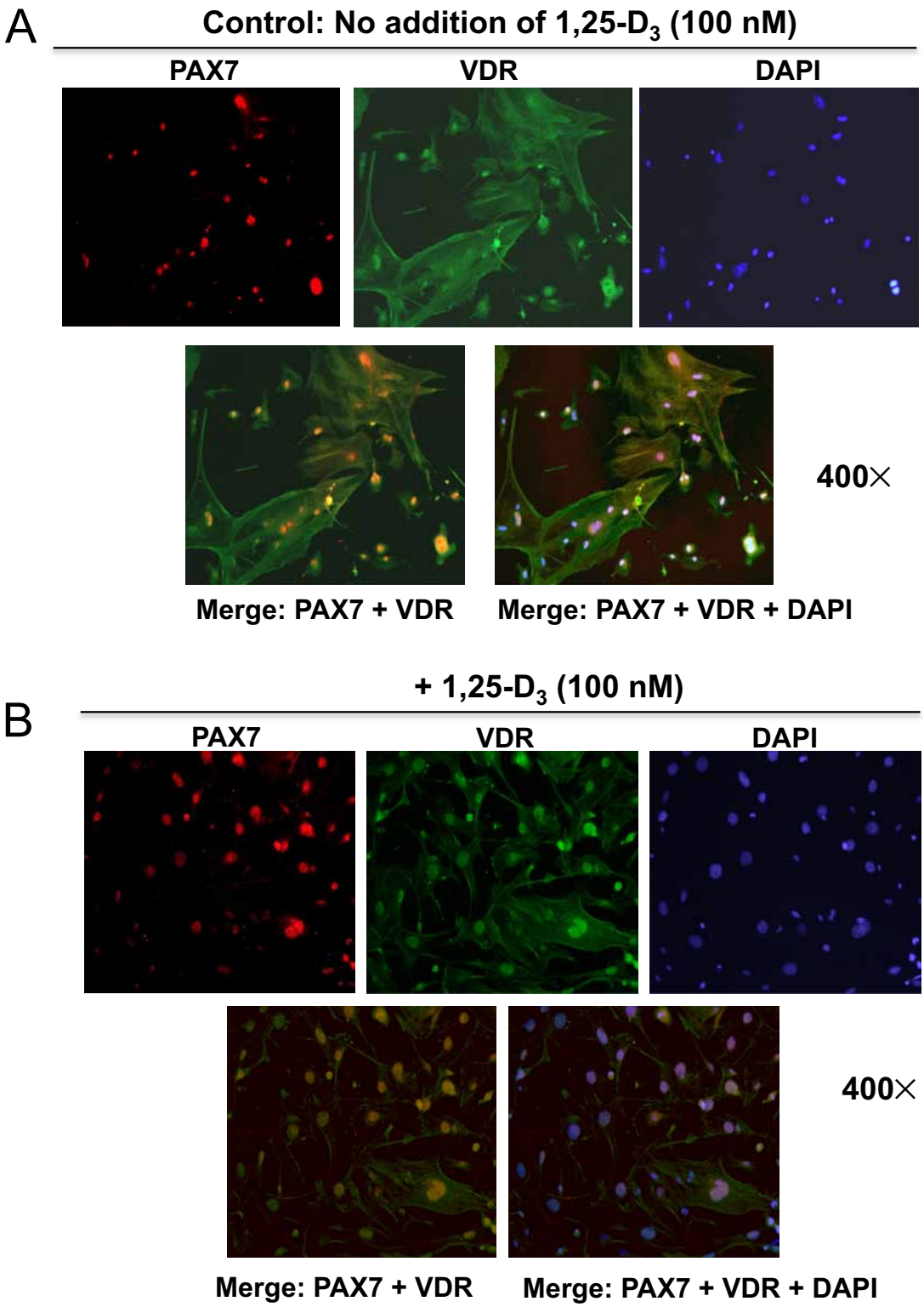

Figure 3
Co-expression of PAX7 and VDR in primary cultures of satellite cells. The experiment was carried out on primary cultures of satellite cells plated on collagen-coated chamber slides that were continuously incubated with or without 1,25-D_3 for 7 days, (A) no addition of 1,25-D_3 and (B) with 1,25-D_3. After the incubation period, cells were subjected to double immunofluorescence for VDR (FITC, green) and PAX7 (Texas red). The counterstaining was done with DAPI (blue). The 'yellow' VDR/PAX7 merge images were obtained by fusing the red and green filtered pictures, and the 'purple' picture were obtained by merging the VDR/PAX7/DAPI pictures. Magnification 400×.

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Table 1 Differential steady-state mRNA levels of pro- and anti-myogenic growth factors and myogenic markers between 1,25-D_3 treated and untreated satellite cells.

Ref. Seq	Symbol	Description	Fold Δ
NM_007554	Bmp4	Bone morphogenic protein 4	+2.7
NM_008006	Fgf2	Fibroblast growth factor 2	+2.52
NM_010512	Igf1	Insulin-like growth factor 1	+1.62
NM_010514	Igf2	Insulin-like growth factor 2	+6.01
NM_010834	MSTN	Myostatin	−2.6
NM_013599	MMP9	Matrix metallopeptidase 9	+3.2
NM_030679	Myh1	Myosin, heavy polypeptide 1, skeletal muscle adult	+3.99
NM_009405	Tnni2	Troponin I, skeletal, fast 2	+1.93
NM_011620	Tnnt3	Troponin T3, skeletal, fast	+2.2

Total RNA from cells treated as in Fig. 2 for 7 days was subjected to RT real-time PCR by the Skeletal Muscle: Myogenesis & Myopathy PCR Array, and the ratios between the 1,25-D_3-treated and 1,25-D_3-untreated cells corrected by GAPDH were calculated for assays performed in triplicate. All experiments were done in triplicates.

by Tukey's multiple comparison test. All comparisons were two-tailed, and $P<0.05$ were considered statistically significant. The *in vitro* experiments were repeated thrice, and data from representative experiments are shown. Specifically, the RT^2 Profiler PCR Arrays were done in triplicates and in some cases further confirmed by qRT-PCR done in triplicates.

Results

Efficiency in the isolation of primary cultures of satellite cells from skeletal muscle

To determine the efficiency of the satellite cell isolation from skeletal muscle by the methodology described by Danoviz and Yablonka-Reuveni, the primary cultures were tested for the expression of PAX7 by immunocytochemistry (Fig. 1A). PAX7 is a unique marker for satellite cells, which is expressed in the nuclei of myogenic progenitor cells during development and thought to be exclusively expressed in satellite cells of mature muscle (27, 28). The counting of positive PAX7 nuclei with the Image-Pro software renders an 81% of efficiency ($P<0.01$) in the isolation of satellite cells by the applied methodology (Fig. 1B).

Increase expression and nuclear translocation of VDR in satellite cells upon incubation with 1,25-D_3

To determine whether satellite cells expressed VDR at a basal level and whether its expression and nuclear translocation is induced upon incubating the cells with 1,25-D_3, real-time PCR, Western blots and immunocytochemistry were carried out after the cells were continuously incubated or not with

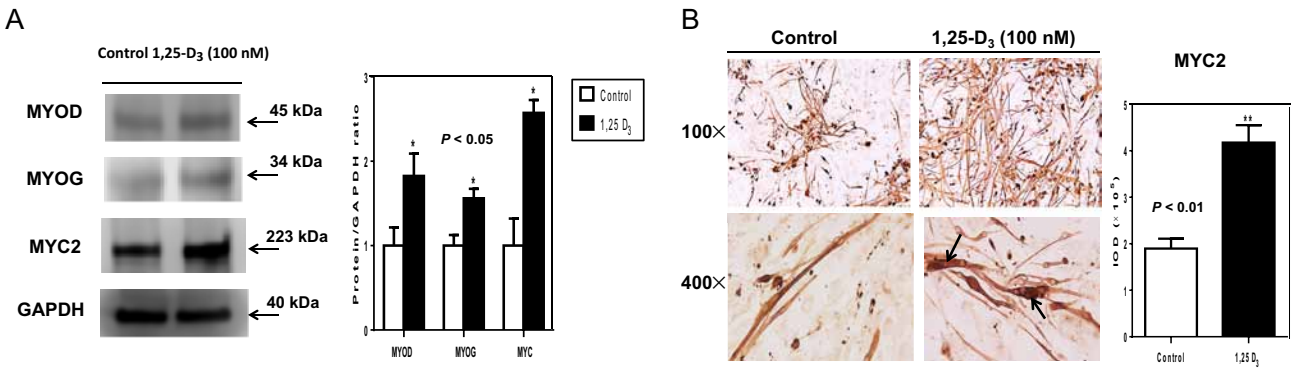

Figure 4
1,25-D_3 promotes myogenic differentiation in primary cultures of satellite cells. Primary cultures of satellite cells were treated as in Figs 2 and 3. Representative Western blots with the corresponding densitometric analysis (*$P<0.05$) are shown for MYOD, MYOG and MYC type II. Samples and controls were normalized with GAPDH housekeeping gene (A). Panel (B) shows representative immunocytochemistry pictures at 100× and 400× magnification of satellite expressing MYC type II with the corresponding image analysis expressing percentage IOD (area × intensity) for experiments done in triplicate (**$P<0.01$). Arrows indicate polynucleated myotubes.

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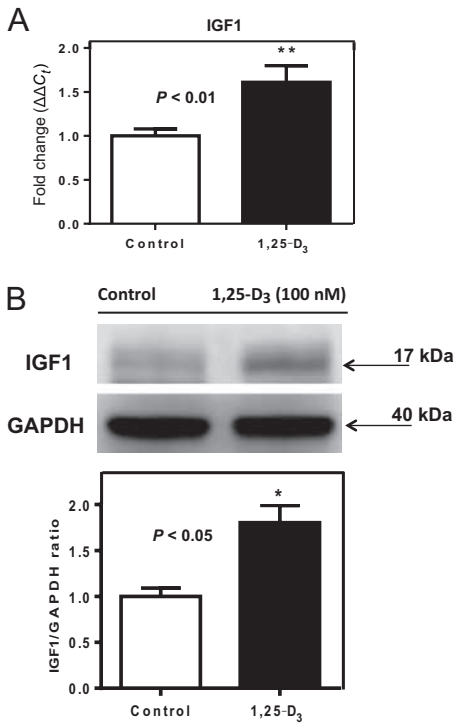

Figure 5
Upregulation of IGF1 steady-state mRNA and protein levels upon incubation of satellite cells with 1,25-D_3. Primary cultures of satellite cells were incubated as in Fig. 2 for 7 days. Total RNA and whole-protein extracts were isolated for qRT-PCR and Western blottings, respectively. (A) Mean ± S.E.M. corresponds to experiments done in triplicate for IGF1; $^{**}P < 0.01$. (B) Western blottings with the corresponding densitometric analysis for IGF1; $^{*}P < 0.05$. In both cases, real-time PCR and Western blottings, samples and controls were normalized with GAPDH housekeeping gene.

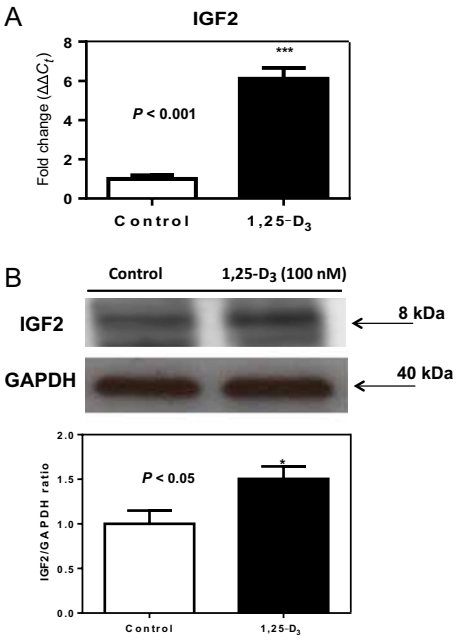

Figure 6
Steady-state mRNA and protein levels upregulation of IGF2 upon incubation of satellite cells with 1,25-D_3. Primary cultures of satellite cells were incubated as in Fig. 2 for 7 days. Total RNA and whole-protein extracts were isolated for qRT-PCR and Western blottings, respectively. (A) Mean ± S.E.M. corresponds to experiments done in triplicate for IGF2; $^{***}P < 0.001$. (B) Western blottings with the corresponding densitometric analysis for IGF2; $^{*}P < 0.05$. In both cases, real-time PCR and Western blottings, samples and controls were normalized with GAPDH housekeeping gene.

1,25-D_3 (100 nM) for 7 days. The dose of 1,25-D_3 used for these studies was derived from studies published by us and others on the effect of different concentrations of 1,25-D_3 at 3 and 4 days on the proliferation of C3H 10$T_{1/2}$ multipotent cells. In those studies, comparison of 1,25-D_3 at 10 and 50 nM to controls had no effect, while there was a dose-dependent significant decrease in cell proliferation with increasing doses that peaked at 1,25-D_3 (100 nM) (22). The increased expression of VDR upon incubation with 1,25-D_3 was demonstrated by real-time PCR (quantitative RT-PCR (qRT-PCR)) (Fig. 2A). VDR mRNA expression was increased by 8-fold at 7 days, compared with the controls (no 1,25-D_3 addition). The increased expression of VDR after 1,25-D incubation was further confirmed by Western blot analyses with the corresponding image analysis using whole-cell culture homogenates under the same conditions as above. Densitometric analysis of the bands revealed an increased VDR expression upon incubation with 1,25-D_3 for 7 days by 1.7-fold (Fig. 2B). Immunocytochemistry studies showed mostly a basal cytoplasmic localization of VDR in the control (no 1,25-D_3 incubation) compared with a predominant nuclear localization upon continuous incubation of the cells with 1,25-D_3 for 7 days (Fig. 2C).

Co-expression of PAX7 and VDR in primary cultures of satellite cells

In order to demonstrate that the isolated satellite cells are PAX7 positive and in addition express VDR, a double immunofluorescence experiment was carried out. Figure 3 shows first (panel A) that satellite cells not incubated with 1,25-D_3 express PAX7 (red) in the nuclei and express VDR (green) mostly located in the cytoplasm. Upon continuously incubating the satellite cells with 1,25-D_3 for 7 days (panel B), they still expressed PAX7 (red) in the nuclei, but this time the same cells expressed the VDR (green) mostly in the nuclei, denoting a typical translocation of the VDR receptors to the nuclei. DAPI was utilized to show the nuclear localization. The yellow merge picture denotes the co-expression between PAX7 (red) and VDR (green)

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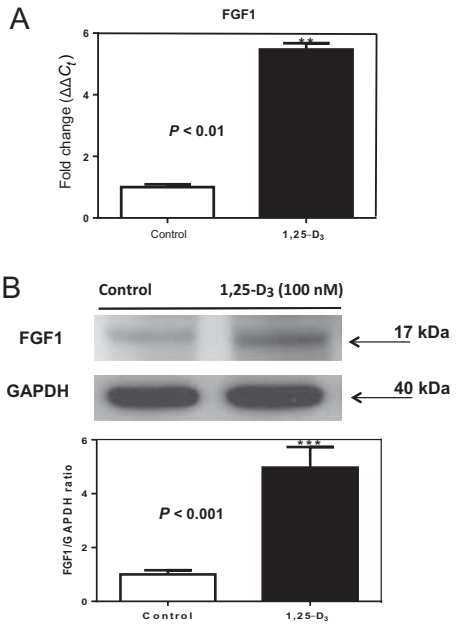

Figure 7
Steady-state mRNA and protein levels upregulation of FGF1 upon incubation of satellite cells with 1,25-D_3. Primary cultures of satellite cells were incubated as in Fig. 2 for 7 days. Total RNA and whole-protein extracts were isolated for qRT-PCR and Western blottings, respectively. (A) Mean ± S.E.M. corresponds to experiments done in triplicate for FGF1; $^{**}P<0.01$. (B) Western blottings with the corresponding densitometric analysis for FGF1; $^{***}P<0.001$. In both cases, real-time PCR and Western blottings, samples and controls were normalized with GAPDH housekeeping gene.

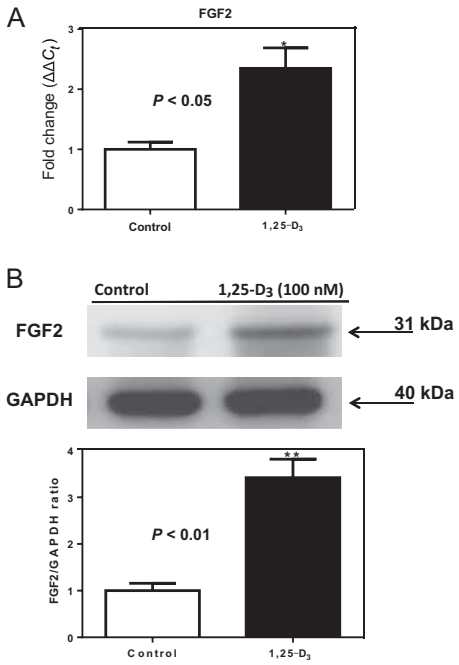

Figure 8
Steady-state mRNA and protein levels upregulation of FGF2 upon incubation of satellite cells with 1,25-D_3. Primary cultures of satellite cells were incubated as in Fig. 2 for 7 days. Total RNA and whole-protein extracts were isolated for qRT-PCR and Western blottings, respectively. (A) Mean ± S.E.M. corresponds to experiments done in triplicate for FGF2; $^{*}P<0.05$. (B) Western blottings with the corresponding densitometric analysis for FGF2; $^{**}P<0.01$. In both cases, real-time PCR and Western blottings, samples and controls were normalized with GAPDH housekeeping gene.

and the purple cells demonstrate the co-localization among PAX7, VDR and DAPI.

1,25-D_3 enhances myogenic differentiation in satellite cells by promoting the expression of myogenic markers, myotube formation and by modulating myogenic growth factors

The effect of 1,25-D_3 on specific myogenic markers of cell differentiation and growth factors were evaluated at the steady-state mRNA level by RT2 Profiler PCR Array (Skeletal Muscle: Myogenesis & Myopathy Pathways). Table 1 shows the differential steady-state mRNA levels between 1,25-D_3 treated (7 days) and untreated cells for determinations done in triplicate after 7 days of continuous incubation with 1,25-D_3. The PCR array analysis showed an upregulation in the expression of skeletal muscle myogenic markers such as MYH1 (+3.99), Tnni2 (+1.93) and Tnni3 (+2.2). Changes in the expression of pro- and anti-myogenic factors were also observed: upregulation in the expression of IGF1 (+1.62), IGF2 (+6.01) and FGF2 (+2.52), and most importantly a marked downregulation of MSTN (–2.6). Continuous incubation of satellite cells with 1,25-D_3 also increased the expression of BMP4 (+2.7), a positive marker of myotube formation/maturation (29). In addition, MMP9 was upregulated (+3.2); MMP9 is a marker of myogenic cell migration and engraftment (30) that is also known to coordinate and affect wound repair.

The pro-myogenic effect of vitamin D on satellite cells was further demonstrated by the increased expression of myogenic markers such as MYOD, MYOG and MYC type II by Western blots with the corresponding densitometric analysis upon incubation of satellite cells with 1,25-D_3 (Fig. 4A). In addition we were able to demonstrate that 1,25-D_3 induced myotube formation showing polynucleated myotube formation and increased expression of MYC type II by immunocytochemistry with the corresponding image analysis expressing percentage IOD (area × intensity) for experiments done in triplicate ($^{**}P<0.01$) (Fig. 4B).

The increased expression of IGF1, IGF2, FGF1 and FGF2 after 1,25-D_3 incubation was further confirmed

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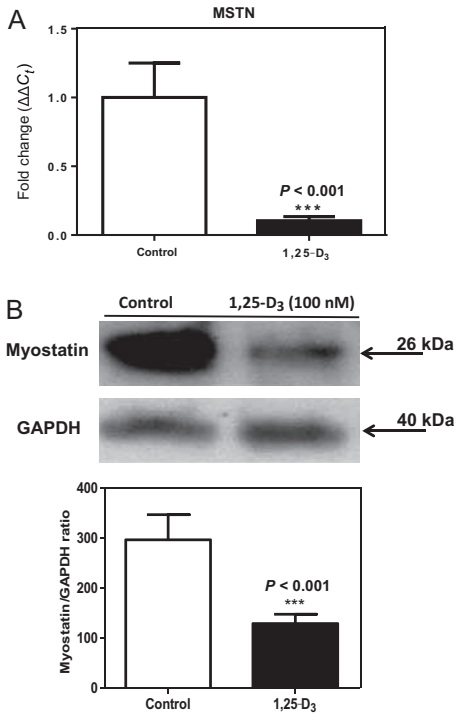

Figure 9
1,25-D_3 downregulates the expression of MSTN in satellite cells. Cultures of satellite cells were treated as in Fig. 2 for 7 days. Total RNA and whole-cell protein extracts were isolated for qRT-PCR and Western blottings, respectively. (A) Mean ± S.E.M. corresponds to experiments done in triplicate for MSTN; ***$P<0.001$. (B) MSTN Western blottings with the corresponding densitometric analysis; ***$P<0.01$. In both cases, qRT-PCR (A) and Western blottings (B), samples and controls were normalized with GAPDH housekeeping gene.

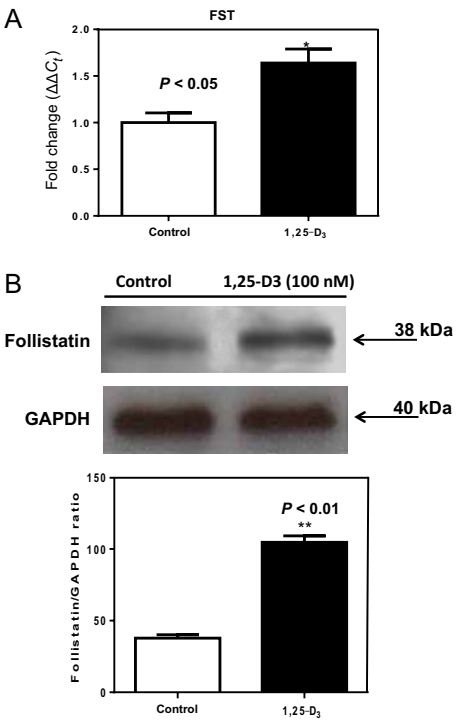

Figure 10
Steady-state mRNA and protein upregulation levels of FST expression upon incubation of satellite cells with 1,25-D_3. Cultures of satellite cells were incubated as in Fig. 2 for 7 days. Total RNA and whole-protein extracts were isolated for qRT-PCR and Western blottings. (A) Mean ± S.E.M. corresponds to experiments done in triplicate; *$P<0.05$. (B) FST Western blottings with the corresponding densitometric analysis; **$P<0.01$. In both cases, qRT-PCR (A) and Western blottings (B), samples and controls were normalized with GAPDH housekeeping gene.

at 7 days by quantitative real-time PCR, and at the protein level by Western blots with the corresponding densitometric analysis. Figure 5A shows the +1.5-fold increase in the expression of IGF1 by real-time PCR compared with the control ($P<0.01$), and Fig. 5B shows a similar pattern by Western blot compared with the control ($P<0.05$). Figure 6A shows a +4.0-fold increase of IGF2 mRNA expression by real-time PCR upon incubating the cells with 1,25-D_3 compared to the control with a similar pattern of expression at the protein level by Western blot (Fig. 6B). The increased expression of FGF1 (+3.9-fold) upon 1,25-D_3 incubation was also confirmed at the steady-state mRNA level by real-time PCR (Fig. 7A) and at the protein level by Western blots with the corresponding densitometric analysis (Fig. 7B). Similarly, an increased expression of FGF2 was confirmed by real-time PCR (+2.3-fold) (Fig. 8A) and by Western blot analysis (Fig. 8B).

Because the PCR arrays showed a decreased expression of MSTN upon incubating the cells with 1,25-D_3, and because MSTN is a known negative regulator of skeletal muscle mass, we further confirmed the PCR arrays by quantitative real-time PCR. The real-time PCR for MSTN performed at 7 days of continuous incubation with 1,25-D_3 showed a remarkable decrease in MSTN expression by 5-fold, compared with the control ($P<0.001$) (Fig. 9A). The remarkable decreased expression of MSTN at the protein level was shown by Western blotting, with the respective densitometric analysis (Fig. 9B).

1,25-D_3 increases FST expression in primary cultures of skeletal muscle satellite cells

To determine whether FST, which inhibits MSTN activity *in vitro* and *in vivo*, is involved in the mechanism by which 1,25-D_3 promotes muscle growth, we investigated FST expression in primary cultures of skeletal muscle derived satellite cells, with and without 1,25-D_3 incubation. Real-time qPCR revealed that after 7 days of continuous incubation with 1,25-D_3, FST expression was increased by 1.7-fold compared with controls ($P<0.05$) (Fig. 10A).

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Because MSTN expression was substantially downregulated at day 7 by 1,25-D_3, we determined the expression of FST at the protein level by Western blotting. Figure 10B shows that the expression of FST was significantly upregulated ($P<0.01$) compared with controls, suggesting that 1,25-D_3 promotes myogenic differentiation by inhibiting MSTN activity, possibly through an increase in FST expression.

Discussion

The data presented in this manuscript demonstrate that VDR is expressed in skeletal muscle satellite cells that are responsible for restoring muscle mass after injury. Furthermore, the addition of 1,25-D_3 to satellite cell primary cultures enhances myogenic differentiation through an increased expression and nuclear translocation of VDR; triggering an increase in the expression of myogenic markers, myotube formation, and a modulation of pro- and antimyogenic factors. The increase in VDR expression, to some extent, is expected, because it is known that 1,25-D_3 auto-regulates the expression of the VDR gene through intronic and upstream enhancers (32). In this manuscript, we also demonstrated that the 1,25-D_3 effect on skeletal muscle satellite cells involves: an increased expression of known pro-myogenic skeletal muscle markers such as (1) MYOD, (2) MYOG, (3) MYC type II, (4) muscle troponin I and troponin T, (5) MYH1 (myosin heavy chain I); and growth factors such as (6) IGF1 and 2, (7) FGF1 and 2, (8) BMP4 and (9) MMP9. Furthermore, exposure of satellite cells to 1,25-D_3 increases myotube formation. It also upregulates the expression of FST (a MSTN inhibitor) (33); even more, we showed that 1,25-D_3 downregulates MSTN expression, the most important negative regulator of muscle mass (34). Myogenesis of skeletal muscle cells is a highly ordered and sequential process: it starts with a period of proliferation followed by a differentiation process that generates myoblasts from mesodermal stem cells. Satellite cells are maintained in a quiescent state and upon requirement are activated to proliferate and fuse with other cells to form or repair myofibers. In addition, they are able to self-renew and replenish the stem pool. In this study, we were able to demonstrate that satellite cells express the VDR receptor, making them the main target for vitamin D. Satellite cells were characterized by the expression of PAX7, which is considered the main defining factor for this cell type (27, 35). The increase in expression of well-known myogenic markers such as MYOD, MYOG, MYC type II, MYH1 and muscle troponin I and T, upon incubation of satellite cells with 1,25-D_3, is a clear indication of the pro-myogenic effect of vitamin D in this primary culture cell system. We also demonstrated that the addition of 1,25-D_3 induced gene expressions of pathways involved in myogenic differentiation, such as IGF, FGF and TGF-β-related signaling pathways.

1,25-D_3 increases the expression of IGF1 and IGF2, which indicates that IGFs play an important role in pushing muscle differentiation in the proposed system. It has been shown that the IGF signaling pathway plays a key role in the regulation of skeletal muscle growth and differentiation. Both IGF1 and 2 are locally expressed in muscle cells contributing to the regulation of skeletal muscle growth, differentiation, and also maintaining adult muscle tissue homeostasis (36). Moreover, IGFs plays a critical role in adult muscle survival, regeneration and hypertrophy (37, 38, 39). Even more, it has been recently described that IGF1 suppresses MSTN signaling pathway during myogenic differentiation, adding one more mechanistic indication to the inhibition of MSTN by vitamin D (40). At the same time, it has been shown that IGF2 inhibition leads to a reduced expression of MYOD target genes, which suggests that IGF2 is also essential for amplifying and maintaining MYOD efficacy (41). Regarding the increase expression of FGF1 and FGF-2 by 1,25-D3, it has been previously described that both proteins are present in proliferating skeletal muscle cells, but are undetectable after terminal differentiation (42). The same publication reports that transient transfection of cells with FGF1 or FGF2 expression constructs exerted a global effect on myoblast DNA synthesis indicating that production of FGF1 and FGF2 by skeletal muscle cells may act as a paracrine and autocrine regulator of skeletal muscle development *in vivo*.

We also demonstrated the increased expression of Bmp4 upon incubation of satellite cells with 1,25-D_3, consistent with the concept that Bmp4 expressed in myoblasts has a positive role in myotube formation/maturation through MYOG expression (29).

Concerning the increased expression of MMP9 induced by vitamin D incubation, it has been shown that overexpression of MMP family members, especially MMP9, improves myogenic cell migration and engraftment (30). As it was mentioned earlier in the text, it also coordinates wound repair since deficient MMP9 mice were unable to remove the fibrinogen matrix during wound healing (31).

Regarding the TGF-β signaling pathway involvement in myogenic differentiation promoted by vitamin D, our study in primary cultures of satellite cells confirms our previous results obtained on the C2C12 myoblast

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cell line (11): that 1,25-D_3 administration to skeletal muscle cells reduced the expression of MSTN, the most relevant negative regulator of muscle mass known to date (34). Even more, we were also able to confirm in this highly relevant cell system that FST, which is a MSTN-binding protein that can inhibit MSTN activity *in vitro* and promote muscle growth *in vivo* (33) increases upon incubation with 1,25-D_3. It has been shown that FST antagonizes MSTN by a direct protein interaction, preventing MSTN from executing its inhibitory effect on muscle development (43).

These sets of results reinforce the pro-myogenic effect of 1,25-D_3 on skeletal muscle differentiation via regulation of different pathways and the decreased MSTN expression at the steady-state mRNA and protein level, by possibly inhibiting MSTN activity through an increase in FST and IGF1 expression.

In summary, we showed that satellite cells expressed VDR and that addition of 1,25-D_3 to satellite cells induces increased expression of: (i) MYOD, (ii) MYOG, (iii) MYC type II, (iv) muscle troponin I and T, (v) MYH1, (vi) IGF1 and 2, (vii) FGF1 and 2, (viii) BMP4, (ix) MMP9 and (x) FST. It also induces a decrease expression of MSTN. In conclusion, vitamin D exerts a clear pro-myogenic effect on satellite cells in charge of muscle reconstitution after muscle injury or muscle waste. We believe that this study provides a mechanistic justification for vitamin D replenishment in muscle waste conditions such as AIDS, cancer and renal failure, characterized by loss of muscle mass, and also in vitamin D deficient elderly adults who have an age-related loss of muscle mass and strength, and an increased rate of falls. The study even opens the door for an emerging potential role of therapies directed to trigger select vitamin D regulated muscle pathways in the treatment of adverse muscle conditions.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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