Vitamin D Signaling Regulates Proliferation, Differentiation, and Myotube Size in C2C12 Skeletal Muscle Cells

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Vitamin D deficiency is linked to a range of muscle disorders including myalgia, muscle weakness, and falls. Humans with severe vitamin D deficiency and mice with transgenic vitamin D receptor (VDR) ablation have muscle fiber atrophy. However, molecular mechanisms by which vitamin D influences muscle function and fiber size remain unclear. A central question is whether VDR is expressed in skeletal muscle and is able to regulate transcription at this site. To address this, we examined key molecular and morphologic changes in C2C12 cells treated with 25-hydroxyvitamin D (25OHD) and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). As well as stimulating VDR expression, 25(OH)D and $1,25(OH)_2D$ dose-dependently increased expression of the classic vitamin D target cytochrome P450, family 24, subfamily A, polypeptide 1 (CYP24A1), demonstrating the presence of an autoregulatory vitamin D-endocrine system in these cells. Luciferase reporter studies demonstrated that cytochrome P450, family 27, subfamily B, polypeptide 1 (CYP27B1) was functional in these cells. Both 25OHD and $1,25(OH)_2D$ altered C2C12 proliferation and differentiation. These effects were related to the increased expression of genes involved in G_0/G_1 arrest (retinoblastoma protein [*Rb*], 1.3-fold; *ATM*, 1.5-fold, both $P < .05$), downregulation of mRNAs involved in G_1/S transition, including *myc* and cyclin-D1 (0.7- and 0.8-fold, both $P < .05$) and reduced phosphorylation of Rb protein (0.3-fold, $P < .005$). After serum depletion, $1,25(OH)_2D$ (100nM) suppressed myotube formation with decreased mRNAs for key myogenic regulatory factors (myogenin, 0.5-fold; *myf5*, 0.4-fold, $P < .005$) but led to a 1.8-fold increase in cross-sectional size of individual myotubes associated with markedly decreased myostatin expression (0.2-fold, $P < .005$). These data show that vitamin D signaling alters gene expression in C2C12 cells, with effects on proliferation, differentiation, and myotube size. **(*Endocrinology* 155: 347–357, 2014)**

In addition to established effects in bone and mineral homeostasis, vitamin D deficiency is linked to a range of muscle disorders (1). These include muscle weakness, myalgia, and drug-related myopathy. Type II (fast-twitch) muscle fibers atrophy in elderly individuals with vitamin D deficiency, exacerbating their tendency to fall (2).

Evidence suggests that vitamin D plays a role in muscle development (1). Children with vitamin D-deficient rickets develop profound muscle weakness and hypotonia that improves after sun exposure or vitamin D supplementation (3, 4). In animal studies, mice lacking the vitamin D receptor (VDR) have smaller muscle fibers at 3 weeks of age,

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Abbreviations: 7AAD, 7-amino-actinomycin D; AB, Alamar Blue; BrdU, bromodeoxyuridine; CYP24A1, cytochrome P450, family 24, subfamily A, polypeptide 1; CYP27B1, cytochrome P450, family 27, subfamily B, polypeptide 1; DBP, Vitamin D-binding protein; FITC, fluorescein isothiocyanate; MRF, myogenic regulatory factor; 25OHD, 25-hydroxyvitamin D; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; PI, propidium iodide; Rb, retinoblastoma protein; VDR, vitamin D receptor.

persistent expression of developmental muscle genes, and impaired motor coordination throughout adulthood (5–7).

However, the precise mechanisms by which vitamin D signaling influences muscle development and function are unclear. Importantly, the issue of whether the VDR is expressed in skeletal muscle is contentious (1, 8, 9).

The aim of this study was to assess the effects of vitamin D in an in vitro model of skeletal muscle. C2C12 muscle cells, a widely investigated model of myogenesis (10, 11), were used. To determine whether vitamin D could have direct effects on these cells, expression and functionality of key components of the vitamin D-endocrine system, specifically cytochrome P450, family 27, subfamily B, polypeptide 1 (CYP27B1), VDR, and cytochrome P450, family 24, subfamily A, polypeptide 1 (CYP24A1), was measured. We then assessed effects of 25-hydroxyvitamin D (25OHD) and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) on C2C12 muscle cell proliferation and differentiation.

Table 1. Primer Sequences

Gene	Primer
Akirin-1	
Forward	ccacctttacccttcgacaa
Reverse	taggcaagaaggcttcagga
ATM	
Forward	gagtgagacgggctgttacc
Reverse	catgctgcctccttcttttc
Cyclin D1	
Forward	agtgcgtgcagaaggagatt
Reverse	cacaacttctcggcagtcaa
Cyclophilin	
Forward	tggaccaaacacaaacggttcc
Reverse	acattgcgagcagatggggtag
CYP24A1	
Forward	cccttctgcaagaaaactgc
Reverse	ctcttgagggctctgattgg
CYP27B1	
Forward	ggctgcatctctacctgacc
Reverse	cagctggaagtggtagctca
Desmin	
Forward	gtgaagatggccttggatgt
Reverse	gtagcctcgctgacaacctc
p19	
Forward	tccattgaagaagggagtgg
Reverse	accgtttagatggctgttgc
p27	
Forward	cagaatcataagcccctgga
Reverse	tctgacgagtcaggcatttg
Myc	
Forward	gcccagtgaggatatctgga
Reverse	atcgcagatgaagctctggt
Myf5	
Forward	aggaaaagaagccctgaagc
Reverse	gcaaaaagaacaggcagagg
Myogenin	
Forward	ccttgctcagctccctca
Reverse	tgggagttgcattcactgg
Myostatin	
Forward	ctgtaaccttcccaggacca
Reverse	tcttttgggtgcgataatcc
Rb	
Forward	aacccagcagtgcgttatct
Reverse	ggtgttcgaggtgaaccatt
TBP	
Forward	tatcactcctgccacaccag
Reverse	atgatgactgcagcaaatcg
VDR	
Forward	ttggaaccctggtctcattc
Reverse	ccctctattggtgcttccag

Materials and Methods

Cell culture

C2C12 cells were propagated in DMEM-F12 with 10% heat-inactivated fetal calf serum at 37°C and 5% CO_2. On reaching 70% confluence, cells were distributed into appropriate plates for each assay and cultured in media with 10% fetal calf serum. At 24 hours after seeding (day 1), cells were treated with 100nM $1,25(OH)_2D$, 100nM 25OHD, or vehicle (ethanol). The medium was changed daily. From day 3 onward, serum was decreased from 10% to 2% and changed to horse serum to initiate cell cycle exit and myogenic differentiation (ie, serum depletion) (12, 13). Serum depletion has been previously used to examine effects of different treatments on myogenic differentiation (14–16). The 100nM dose of $1,25(OH)_2D$ is usual for studies examining its effect on cell lines and primary cells (17–20). In some assays specified below, additional doses of $1,25(OH)_2D$ and 25OHD were used to assess dose responses.

Cell proliferation

Cell proliferation was measured by 3 methods on day 3 before serum starvation.

1) For cell counting, cells cultured in 6-well plates were dislodged by trypsin and counted using a hemocytometer.
2) For Alamar Blue (AB) staining (Invitrogen), cells cultured in 96-well plates were stained with AB (10% vol/vol). AB is reduced in proliferating cells from resazurin to resorufin, the latter being a fluorescent compound. Measurement of AB fluorescence is therefore a marker of cell proliferation and viability (21). In our study, fluorescence excitation at 544 nm and emission at 590 to 610 nm was measured. AB fluorescence was determined at baseline and after 25OHD or $1,25(OH)_2D$ treatments as an index of cellular proliferation over this period.
3) For bromodeoxyuridine (BrdU) incorporation, using a fluorescein isothiocyanate (FITC) BrdU Flow Kit (BD Pharmingen), BrdU (100 mmol/mL) was added on day 2 for 24 hours. Staining for BrdU and 7-amino-actinomycin D (7AAD) was performed as described (22). Flow cytometric data was acquired using a FACS Canto (BD Biosciences), and fluorochromes/filters were BrdU FITC B530/30 and 7AAD R660/20. FlowJo software (Tree Star) was then used to analyze these data.

Apoptosis and necrosis

Annexin V and propidium iodide (PI) are established markers for apoptosis and cell necrosis (23). Annexin V binds to phos-

Table 2. Antibody Information

Protein Target	Name of Antibody	Manufacturer, Catalog No.	Species and Clonality	Dilution
VDR	VDR (D6)	Santa Cruz Biotechnology, sc13133	Mouse monoclonal	1:1000
Total Rb	RB (G3-245)	BD Pharmingen, 554136	Mouse monoclonal	1:1000
Phospo-Rb	Phospho-Rb (Ser807/811)	Cell Signaling, 9308	Rabbit polyclonal	1:1000
c-myc	c-*myc* (c19)	Santa Cruz Biotechnology, sc788	Rabbit polyclonal	1:1000
β-Actin	β-Actin (AC-74)	Sigma Aldrich, A2228	Mouse monoclonal	1:20 000

phatidylserine that becomes exposed on the cell surface during apoptosis, and PI is an intercalating agent that gains entry into the cell via membrane defects during necrosis (23). On day 3, cells cultured in 6-well plates were trypsinized and then resuspended in solution containing 10mM HEPES, 140mM NaCl, 2.5mM $CaCl_2$, and FITC-labeled annexin V antibody (1:50 dilution; BD Pharmingen). After 30 minutes incubation, cells were treated with PI (1:50 dilution). Flow cytometric data was acquired using a FACS Canto (BD Biosciences), and fluorochromes/filters were annexin V FITC B530/30 and PI B655lpor. FlowJo software (Tree Star) was then used to analyze these data.

Vitamin D luciferase reporter studies

Plasmids for GAL-4-VDR, UASTK-Luciferase, and pcDNA were generated by R.J.C.-B. (Kolling Institute). Plasmids were transformed in chemically competent Top-10′ *E. coli* (Invitrogen) and extracted using the Plasmid Mini Kit (QIAGEN) according to the manufacturer's protocols. C2C12 cells were split into 24-well culture plates and transfected at a high density before adhesion as previously described (24). Lipofectamine 2000 (Gibco) was used to transfect 800 ng of GAL4-VDR and 800 ng of UASTK-luciferase reporter into 21 wells per plate. Cells were also transfected with 800 ng β-galactosidase reporter to correct for transfection efficiency. The remaining 3 wells were transfected with pcDNA empty vector as negative control. Twenty-four hours after transfection, growth medium was replaced with serum-free medium, and cells were treated with 25OHD (1nM–100nM), $1,25(OH)_2D$ (1nM–100nM), or ethanol control (0.1% of media solution). Twenty-four hours later, luciferase activity was detected using the Steady-Glo luciferase assay system (Promega) and luminometry on a microplate scintillation counter (Packard). In this system, luciferase activity results from $1,25(OH)_2D$ binding to GAL-4-VDR and subsequent activation of the UASTK-luciferase gene via its GAL4 promoter. Detection of luciferase activity after treatment with 25OHD therefore shows conversion to $1,25(OH)_2D$. Luciferase readings were corrected for β-galactosidase activity as a transfection control. This was detected using the Galacto-Star System (Applied Biosystems).

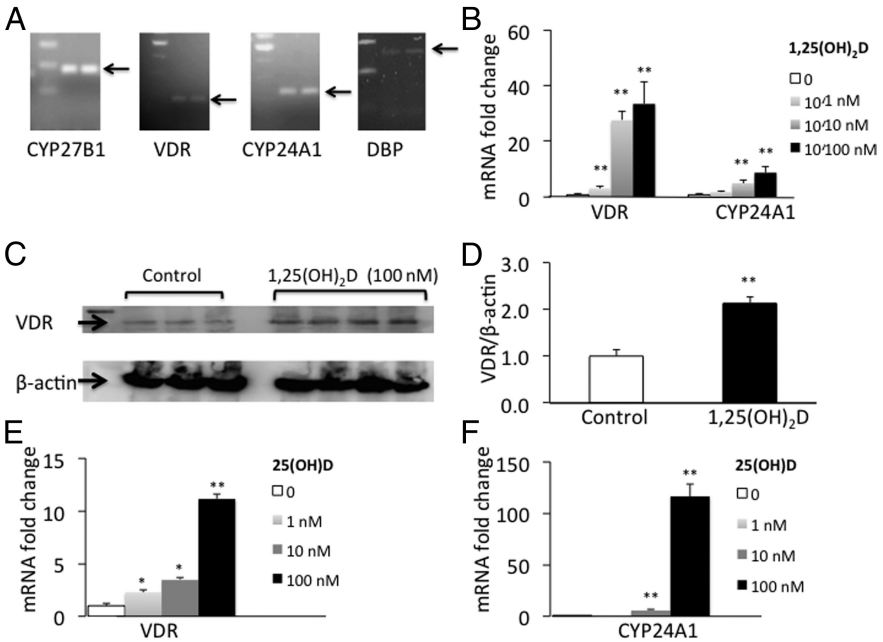

Figure 1. Components of the vitamin D-endocrine system in C2C12 muscle cells. A, C2C12 cells express CYP27B1, VDR, CYP24A1, and DBP mRNA as seen on semiquantitative PCR. Duplicates for each are shown. B, On RT-PCR, expression of VDR and CYP24A1 mRNA is stimulated in a dose-dependent fashion by 48 hours treatment with $1,25(OH)_2D$ (data are mean ± SEM, n = 3 per group). C and D, Western blots (C) and densitometric quantitation (D) show that VDR expression (normalized for β-actin) increased 2.2-fold in response to 72 hours treatment with $1,25(OH)_2D$ ($P < .005$, n = 6–8 per group). E and F, On RT-PCR, expression of VDR and CYP24A1 mRNA is stimulated in a dose-dependent fashion by 24 hours treatment with 25(OH)D (data are mean ± SEM, n = 3 per group). *, $P < .05$; **, $P < .005$.

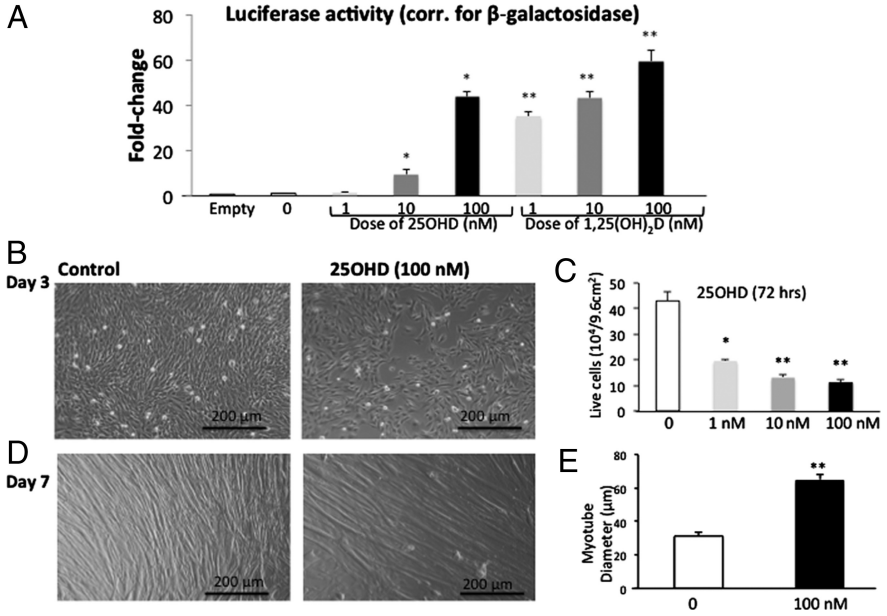

Figure 2. Effects of 25OHD in C2C12 muscle cells. A, 25OHD induced a dose-dependent increase in luciferase activity in C2C12 cells transfected with GAL4-VDR (switch) and UASTK-luciferase reporter ($P < .005$, n = 3–4 per group). Similar changes were observed in response to $1,25(OH)_2D$ ($P < .005$, n = 3–4 per group). These findings imply the presence of functional CYP27B1 in C2C12 cells converting 25OHD to $1,25(OH)_2D$. Negligible levels of luciferase activity were detected in cells transfected with empty vector pcDNA. Cells were also transfected with β-gal reporter; luciferase activity was corrected (corr.) for β-galactosidase activity as transfection control. B, As further evidence of functional CYP27B1, 25OHD induced an antiproliferative effect in C2C12 myoblasts. C, This effect was first noted after 72 hours of treatment and was dose-dependent on cell counting using a hematocytometer ($P < .05$, n = 3 per group in 6-well plates). D and E, After prolonged treatment with 25OHD throughout differentiation, there were fewer myotubes (D) and these were larger in size compared with control-cultured cells (E) ($P < .005$). Fibers were counted, and diameter was assessed on ImageJ (diameter measurement, 20 fibers randomly selected per field, 2 fields per sample). *, $P < .05$; **, $P < .005$.

Real-time PCR

RNA was isolated using the RNeasy Mini-kit (QIAGEN), and equal amounts were reverse transcribed using Superscript III first strand kit (Invitrogen) as previously described (25, 26). Real-time quantitative PCR was performed in 384-well plates. The protocol included melting for 10 minutes at 95°C and 40 cycles of 2-step PCR including melting for 15 seconds at 95°C and annealing for 1 minute at 60°C. Primers were designed using Primer 3 and BLAST (National Library of Medicine) and obtained from Invitrogen. Primer sequences are listed in Table 1. Every plate included housekeeping genes (TATA-box binding protein [*TBP*] and/or cyclophilin) for every sample. Semiquantitative PCR was also performed by separation of PCR products via agarose gel electrophoresis. Images were taken using a Gel Doc (Bio-Rad).

Western blot

Cell lysates (60 μg protein) were separated by SDS-PAGE as previously reported (27). A 10% gel was used, proteins were transferred to PVDF membrane, and the membrane was blocked with 5% skim milk powder in PBS plus 0.1% Tween 20. Primary antibody was applied overnight at 4°C. Washed membranes were incubated for 1 hour at room temperature with 1:1000 of horseradish peroxidase-conjugated secondary antibody in blocking buffer. After washing, immune-reactive bands were visualized using enhanced chemiluminescence (Santa Cruz Biotechnology) in a Bio-Rad chemiluminescence detection system. Bands were quantified using ImageJ (National Institutes of Health).

A list of primary antibodies, the dilutions used, and manufacturers' details have been included in Table 2. The VDR-D6 antibody was chosen for its previously reported specificity (28) and our own validation experiments that confirm the absence of signal in VDR-null tissues. The cell cycle antibodies targeting retinoblastoma protein (Rb), phospho-Rb, and c-myc have been widely used in cancer studies and validated by Western blot in small interfering RNA knockdown cell models (29, 30). We used protein lysates of MCF-7 human breast cancer cells as positive controls when assessing cell cycle protein expression.

Statistical analysis

Statistics were calculated in Excel or SPSS version 20. Unless otherwise specified, Student's unpaired *t* test with unequal variance was used to compare 2 groups. ANOVA with post hoc testing and Bonferroni correction was used where multiple comparisons were made. For all figures, data are presented as mean ± SEM. *P* values <.05 were considered significant.

Results

C2C12 cells express components of the vitamin D-endocrine system

There is ongoing debate about whether the effects of vitamin D on muscle are direct or indirect via effects in other tissues. To determine whether direct effects were possible, components of the vitamin D-endocrine system were measured in the absence or presence of $1,25(OH)_2D$ or 25(OH)D.

At a transcript level, C2C12 myotubes express *VDR*, *CYP27B1*, *CYP24A1*, and vitamin D-binding protein (*DBP*) (Figure 1A). Expression of VDR increased in a dose-dependent manner after 48 hours treatment with $1,25(OH)_2D$ ($P < .005$, Figure 1B). Translation of VDR mRNA into protein was confirmed using the VDR-D6 antibody (Santa Cruz). VDR protein increased >2-fold by 72 hours ($P < .005$, Figure 1, C and D), and, consistent with functional vitamin D signaling, the classic VDR target gene *CYP24A1* was markedly upregulated as well ($P < .005$, Figure 1B).

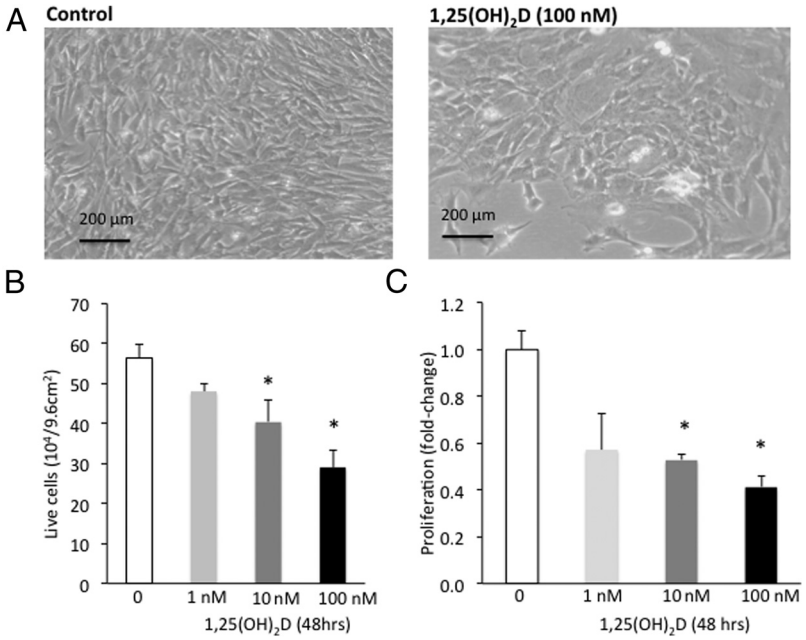

Figure 3. Antiproliferative effect of $1,25(OH)_2D$ on C2C12 myoblasts. A, There were notably fewer myoblasts in culture after 48 hours of treatment with $1,25(OH)_2D$. B, This effect was dose dependent and significant at 10nM and 100nM on cell counting using a hematocytometer ($P < .05$, n = 3 per group in 6-well plates). C) AB fluorescence assay also indicated a dose-dependent antiproliferative effect of $1,25(OH)_2D$ ($P < .05$, n = 4 per group). *, $P < .05$; **, $P < .005$.

C2C12 cells express functional CYP27B1

Apart from demonstrating the expression of CYP27B1 mRNA, we sought to determine whether this enzyme was functional in C2C12 cells. After treatment with 25OHD, there were dose-dependent increases in VDR and CYP24A1 mRNAs, strongly implying the local metabolism of 25(OH)D into $1,25(OH)_2D$ (Figure 1, E and F). Further evidence of functional CYP27B1 protein was sought. Luciferase reporter studies were performed in C2C12 cells that were transfected with GAL4-VDR (switch) and UASTK-luciferase reporter. After 24 hours treatment with 25OHD, there was a dose-dependent increase in luciferase activity ($P < .05$, Figure 2A), indicating the intracellular conversion of 25OHD to $1,25(OH)_2D$ by functional CYP27B1 and the subsequent activation of luciferase expression via $1,25(OH)_2D$-bound GAL4-VDR. In general, luciferase activity in response to 25OHD was comparable to that seen with the same concentrations of $1,25(OH)_2D$ (Figure 2A).Luciferase reporter studies have been previously used to demonstrate functional CYP27B1 in other cell types (31).

25OHD and $1,25(OH)_2D$ exert antiproliferative effects in C2C12 myoblasts

After 72 hours treatment with 25OHD, there were visibly fewer myoblasts in culture compared with control-cultured samples (Figure 2B). This antiproliferative effect was seen at 48 hours in cells treated with $1,25(OH)_2D$ (Figure 3A), was dose-dependent with both 25OHD and $1,25(OH)_2D$ (Figures 2C and 3B), and was confirmed with AB fluorescence (Figure 3C).

Decreased cell counts can be due to decreased proliferation, increased cell death, or both. Proliferation was assessed by flow cytometric analysis of BrdU incorporation and 7AAD staining. Myoblasts treated with 100 nM $1,25(OH)_2D$ had a higher percentage of cells in quiescent phases of the cell cycle (G_0/G_1) and a lower proportion of cells in the active (S and M) phases (Figure 4A). This suggested that the antiproliferative effect of $1,25(OH)_2D$ was associated with cell cycle arrest.

$1,25(OH)_2D$ does not induce apoptosis or necrosis in C2C12 myoblasts

In addition to the antiproliferative effect described above, the reduced cell number after $1,25(OH)_2D$ treatment could be due to increased cell death. To exclude this possibility, flow cytometry analysis was performed. Treatment with $1,25(OH)_2D$ had no effect on the proportion of apoptotic or necrotic cells on annexin V and PI staining (Figure 4B). In addition, there was no difference in the proportion of cells in the pre-G_0/G_1 phases on BrdU incorporation and 7AAD staining ($P = .54$, Figure 4A). As this phase contains both dying and apoptotic cells, this further confirms the lack of toxic effect of $1,25(OH)_2D$ in this study.

$1,25(OH)_2D$ alters expression of cell cycle markers

To assess mechanisms by which $1,25(OH)_2D$ exerted its antiproliferative effect, mRNA levels of genes controlling the cell cycle were measured. Treatment of C2C12 myoblasts with $1,25(OH)_2D$ (range 1nM–100nM) for 48 hours dose-dependently increased *ATM* and *Rb* and reduced *c-myc* and cyclin D1 mRNA levels (Figure 4D). These genes control the G_1-S restriction point, and the described changes are consistent with cell cycle arrest as seen in Figure 3A (32). VDR expression increased at the 48-hour time point (Figure 4D). Other cell cycle markers, including *p19* and *p27*, were unchanged by $1,25(OH)_2D$ treatment.

Treatment with $1,25(OH)_2D$ (48 hours, 100nM) did not alter levels of total Rb protein (Figure 4C). This could be related to increased turnover of Rb or perhaps

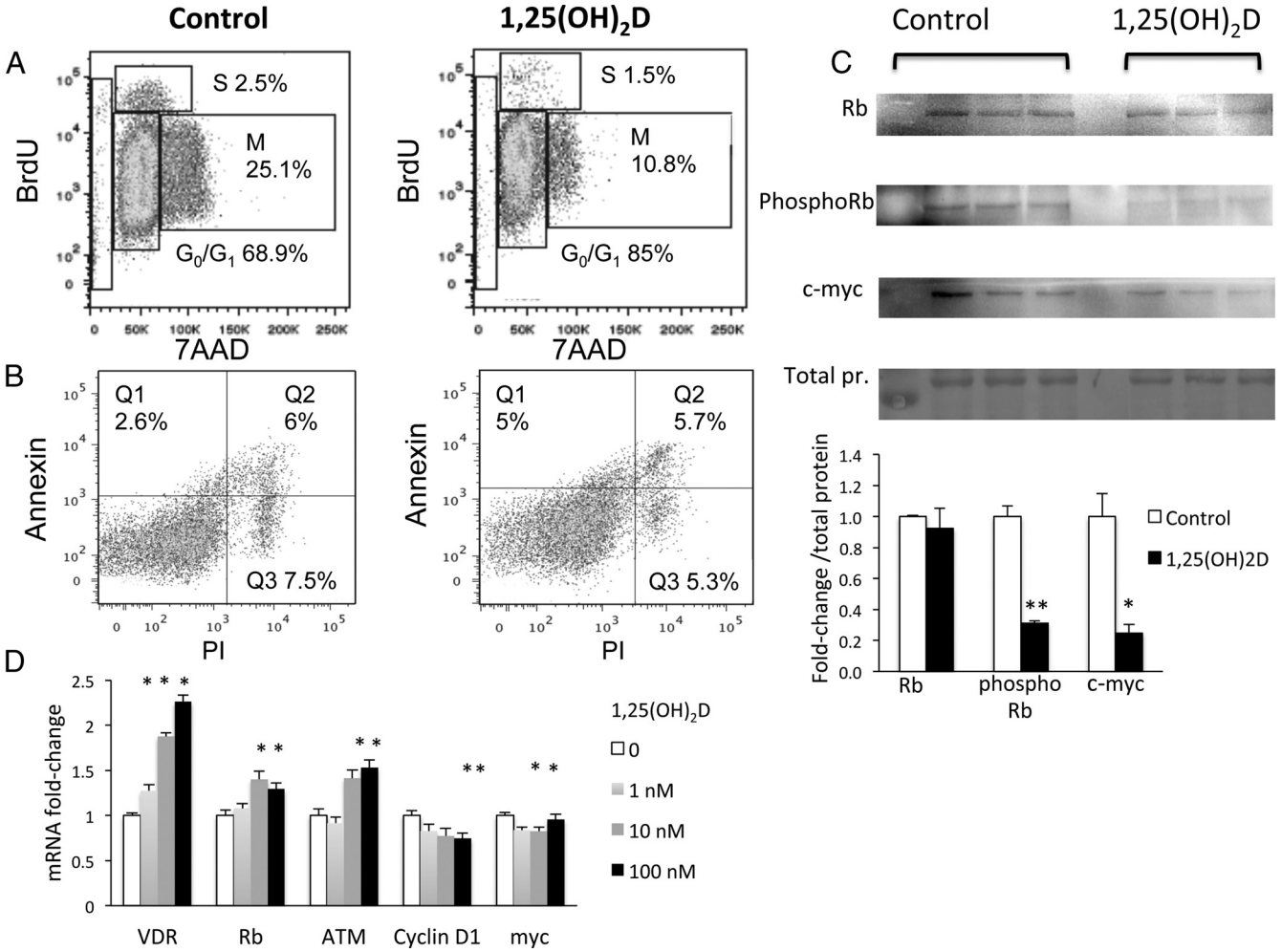

Figure 4. Cell cycle effects of 1,25(OH)$_2$D on C2C12 myoblasts. A, Representative fluorescence-activated cell sorting (FACS) plot of BrdU and 7AAD staining. 1,25(OH)$_2$D treatment resulted in significantly more C2C12 cells in the quiescent phases of the cell cycle (ie, G_0/G_1 phases, 80.1% ± 1.5% vs 68.9% ± 1.3%, mean ± SEM from n = 4 per group, $P < .05$) and significantly fewer in the active phases of the cell cycle (ie, M and S phases, 16.7% ± 1.3% vs 25.9% ± 2.1%, $P < .05$). B, Representative FACS plot of annexin V and PI staining. In 4 experiments, 1,25(OH)$_2$D treatment had no effect in the proportion of cells that stained positively for annexin V (ie, Q1, 2.3% ± 0.9% vs 2.0% ± 0.3%, $P = .7$), PI (ie, Q3, 4.3% ± 0.9% vs 13.8% ± 5.5%, $P = .2$), or both (ie, Q2, 5.0% ± 1.3% vs 4.8% ± 1.0%, $P = .9$). C, At a protein level, total Rb expression was not significantly altered despite the mRNA findings. However, Rb phosphorylation and c-myc protein levels were significantly reduced by 1,25(OH)$_2$D treatment ($P < .05$, corrected for total protein [pr,] on Coomassie blue stain). D, On RT-PCR, 1,25(OH)$_2$D treatment resulted in altered mRNA expression of cell cycle genes in C2C12 cells. Specifically, *Rb* and *ATM* mRNA expression increased in a dose-dependent fashion whereas cyclin D1 and *myc* expression decreased ($P < .05$, n = 3 per group). *, $P < .05$; **, $P < .005$.

to delay between mRNA and protein synthesis. Interestingly, despite similar total protein, there was reduced phosphorylation of Rb protein (Figure 4C). Dephosphorylated Rb binds to critical regulatory proteins including E2F transcription factors and induces cell cycle arrest (32, 33).

Treatment with 1,25(OH)$_2$D reduced c-myc (Figure 4C), a transcription factor that controls cell proliferation. C-myc activity is frequently elevated in cancer (34). In epithelium (35), the c-myc pathway represents a mechanism by which 1,25(OH)$_2$D and VDR influence proliferation, and decreased c-myc is likely to contribute to the decrease in proliferation.

25OHD and 1,25(OH)$_2$D decrease myotube formation

On day 3, myogenic differentiation was induced by changing 10% fetal calf serum to 2% horse serum. The rationale for this was to examine effects of 1,25(OH)$_2$D on myogenesis independent of its effects on proliferation, by inducing cell cycle arrest in general. Continued treatment with 1,25(OH)$_2$D delayed myotube formation and reduced the number of myotubes by 38% from days 3 to 10 ($P < .05$, Figure 5, A and C). This coincided with downregulation of myogenic regulatory factors (MRFs) *myf5*, myogenin (Figure 5B), and desmin (data not shown). These genes play central, overlapping roles in myogenesis, influencing cell cycle arrest and myotube formation. Con-

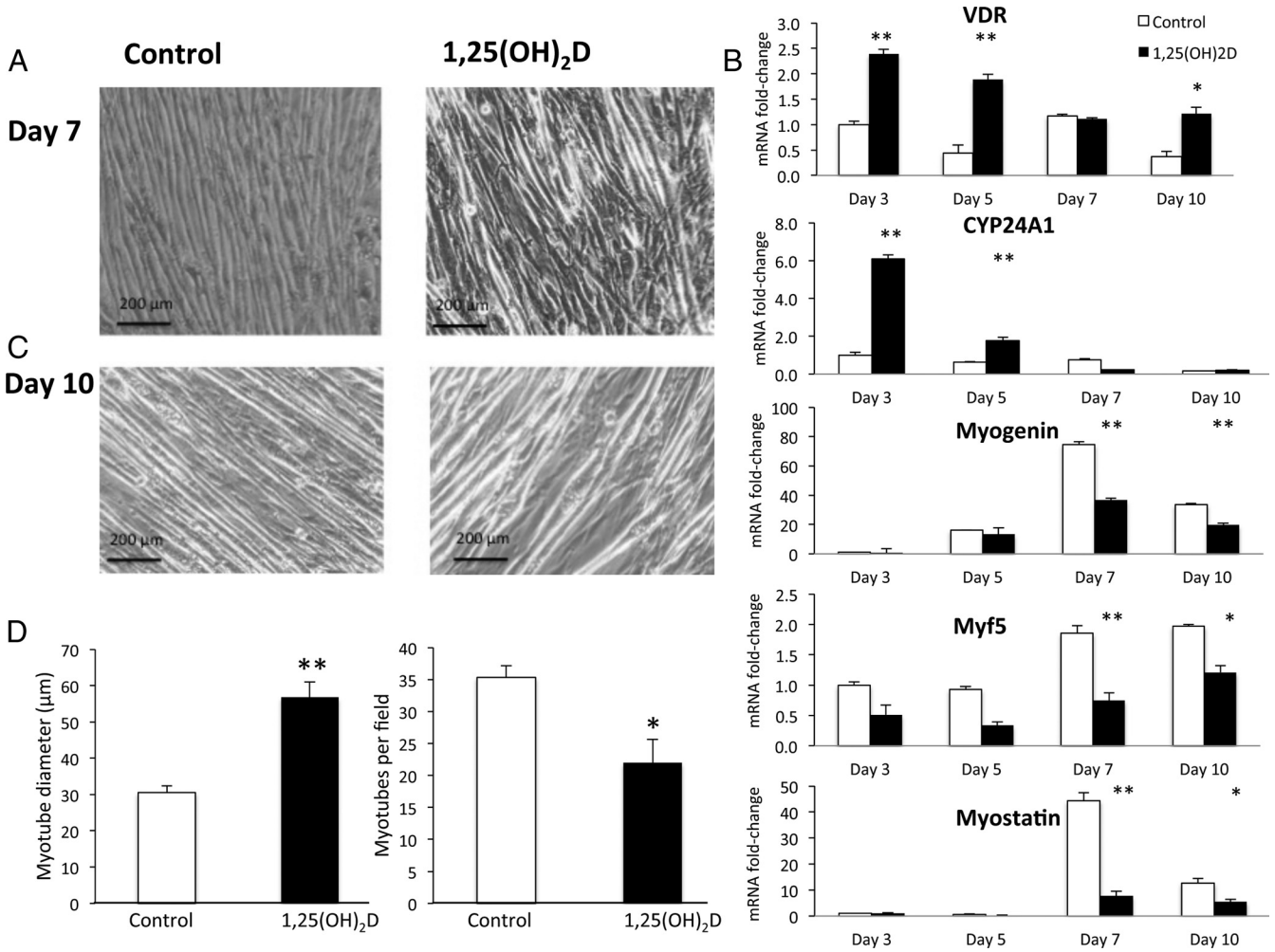

Figure 5. Effects of $1,25(OH)_2D$ on C2C12 myotube formation and size. A, At day 7, there were notably fewer myotubes in C2C12 cells treated with $1,25(OH)_2D$. B, On RT-PCR, myogenin, myf5, and myostatin mRNA levels were significantly reduced by $1,25(OH)_2D$ treatment throughout differentiation induced by serum starvation. VDR mRNA levels increased, indicating that changes in MRF expression were due to $1,25(OH)_2D$-VDR genomic activity. Increases in the expression of CYP24A1 confirms the presence of a vitamin D autoregulatory system in these cells, activated by $1,25(OH)_2D$-mediated VDR stimulation. The reference point for fold change is the day 3 control, error bars are ±SEM, and n = 3 per group. C, On day 10, C2C12 myotubes treated with $1,25(OH)_2D$ were notably larger in diameter. D, On image analysis, $1,25(OH)_2D$ treatment led to a significant reduction in myotube number (22 ± 3.6 vs 35 ± 1.9 myotubes per field, $P < .05$) and increases in myotube diameter (56.8 ± 4.3 vs 30.5 ± 1.8 μm, $P < .005$). Fibers were counted, and diameter was assessed on ImageJ (diameter measurement, 8 fibers randomly selected per field, 3 fields per sample). *, $P < .05$; **, $P < .005$.

sistent with this, $1,25(OH)_2D$ suppressed fusion of C2C12 myocytes to form myotubes. Fewer myotubes were also seen in response to 25OHD, indicating persistent CYP27B1 function and conversion to $1,25(OH)_2D$ throughout C2C12 cell differentiation (Figure 2D).

25OHD and $1,25(OH)_2D$ increase C2C12 myotube diameter, and $1,25(OH)_2D$ downregulates myostatin

Despite delayed proliferation and lower absolute number of myotubes, by day 10, cells treated with $1,25(OH)_2D$ were markedly larger (Figure 5, C and D, 1.8-fold increase in cross-sectional area, $P < .005$). A similar increase in myotube size was also seen in response to 25OHD (Figure 2, D and E, 2-fold increase in cross-sectional area, $P < .005$). Myostatin, a negative regulator of muscle mass, was downregulated 10-fold on day 7 in response to $1,25(OH)_2D$ (Figure 5B, $P < .005$), providing a potential mechanism for the notable difference in myotube size.

Discussion

The presence of a functional vitamin D system in C2C12 cells (Figures 1 and 2) implies that vitamin D signaling may play a direct role in muscle regulation. We found 3 distinct effects of treating C2C12 cells with 25OHD and $1,25(OH)_2D$: 1) inhibition of proliferation (Figures 2 and 3), 2) inhibition of myotube formation during serum starvation (Figures 2 and 5), and 3) increased size of individual

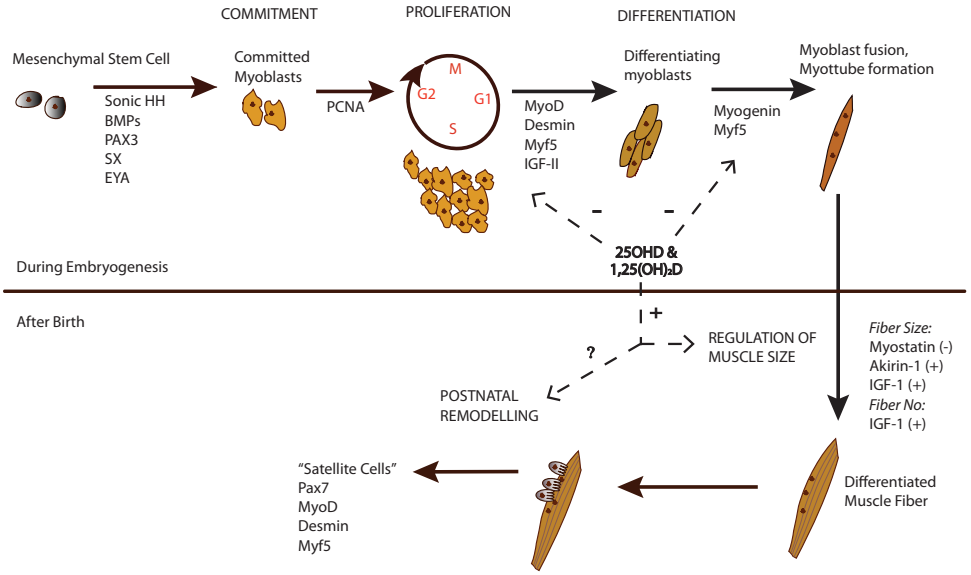

Figure 6. Simplified model of mammalian myogenesis and proposed effects of 25OHD and 1,25(OH)$_2$D. Genes regulating myoblast commitment at the mesenchymal stem cell level, myoblast proliferation and cell cycle withdrawal, myocyte fusion, myofiber size determination, and satellite cell activity are summarized. Potential effects of 25OHD and 1,25(OH)$_2$D on myogenesis based on data from C2C12 cells have also been shown (dashed lines). These include an antiproliferative effect (−), inhibition of myotube formation (−), and increased fiber size (+). The question that arises is whether vitamin D signaling may play a role in muscle regeneration at the level of the satellite cell. Abbreviations: BMP, bone morphogenetic protein; EYA, eyes absent transcription factor; Pax3, paired box protein 3; Pax7, paired box protein 7; PCNA, proliferating cell nuclear antigen; SHH, sonic hedgehog.

myotubes (Figures 2 and 5). VDR stimulation was effective at various time points (Figures 4 and 5), again suggesting a direct role in the transcriptional regulation of muscle development. Transcription factors that are known to regulate key steps in mammalian myogenesis have been summarized in Figure 6, including those involved in myoblast commitment, proliferation, differentiation, and the determination of muscle fiber size. Potential effects of 1,25(OH)$_2$D and 25OHD on myogenesis based on this work have also been depicted.

The antiproliferative effects of 1,25(OH)$_2$D in muscle cells were first described in 1985 (36). They are consistent with antiproliferative effects of 1,25(OH)$_2$D in a number of other cells and tissues including skin (35), cancer cells (37), and immune cells (38). We report a novel antiproliferative effect of the prehormone 25OHD in C2C12 cells associated with the presence of functional CYP27B1 as demonstrated by luciferase reporter studies. We also report, for the first time, underlying mechanisms for the antiproliferative effect of vitamin D signaling in C2C12 cells. By direct regulation of cell cycle gene expression (*ATM*, *myc*, *Rb*, and cyclin D1) and posttranslational Rb hypophosphorylation, 1,25(OH)$_2$D promotes cell cycle arrest and quiescence in C2C12 cells as displayed in BrdU/7AAD flow cytometry analysis (Figure 4). The antiproliferative effect of 1,25(OH)$_2$D was not related to an increase in cell death, making toxicity unlikely (Figure 4). Consistent with this, treatment with 1,25(OH)$_2$D markedly upregulated CYP24A1, which degrades 1,25(OH)$_2$D (Figures 1 and 5).

Another novel finding is the suppression of C2C12 myotube formation after 25OHD and 1,25(OH)$_2$D treatment and serum deprivation. These findings stand in contrast to the recent study by Garcia and colleagues (18). Without serum starvation, prolonged treatment of C2C12 cells with 1,25(OH)$_2$D resulted in a stimulatory effect on myotube formation via increased expression of MRFs in this earlier study (18). This discrepancy is interesting, because serum deprivation and prolonged confluent culture represent 2 distinct models of myogenesis. The former relies on withdrawal of mitogenic stimuli to induce cell cycle arrest and myogenin expression (12), and the latter relies on expression of endogenous IGFs (12, 39). Due to the protracted nature of C2C12 myogenesis in high-serum conditions, it is possible that Garcia et al (18) reporting a stimulatory effect of 1,25(OH)$_2$D on myogenesis corresponds with earlier cell cycle arrest, as seen before serum deprivation (ie, day 3) in our study.

In broader terms, the inhibitory effects of vitamin D signaling on myocyte proliferation and myotube formation may indicate the promotion of cell quiescence and protection from senescence. In a recent study, 1,25(OH)$_2$D (100nM) inhibited proliferation and delayed replicative senescence of human mesenchymal stem cells on the basis of ß-galactosidase staining and p16 expression, without

affecting their clonogenic capacity (40). Another study found direct links between the antiproliferative effect of VDR and proteins involved in cell survival, namely FoxO and Sirt1 (41). This may be particularly important in muscle, in which age-related dysfunction of stem cells is directly related to downregulation of their quiescent, self-renewing capacity (42).

A separate effect in this study, also seen with the high-serum method (18), was an anabolic effect of 25OHD and $1,25(OH)_2D$ on myotube size. We saw a pronounced downregulation of myostatin, a negative regulator of muscle mass, and upregulation of an upstream transcription factor, follistatin, has also been described (18). Effects of $1,25(OH)_2D$ and VDR on the TGF-β family, of which myostatin is a member, have also been demonstrated in mesenchymal stem cells (43), skin (44), and liver (45), suggesting widespread links between these pathways. $1,25(OH)_2D$ may also have effects on fiber size via insulin signaling pathways, relating specifically to insulin receptor substrate-1 and Akt phosphorylation (46).

There are limitations to this study. Although C2C12 cells express proteins necessary for muscle contraction and display the morphology of individual fiber units, there are striking differences between these cells and adult muscle, particularly in their degree of maturation (47) and mode of glucose transport (48). Therefore, effects in C2C12 cells do not always translate to adult muscle. Second, treatment of cells with pharmacological doses of 1,25(OH)D in vitro may not necessarily correspond with physiologic responses in vivo. Nevertheless, this in vitro model may provide insight into the role of the VDR in skeletal muscle development and transcriptional events mediated by its activation.

In vivo studies also support a role for vitamin D signaling in the development of skeletal muscle. Mice with deletion of VDR displayed muscle fibers that were smaller and more variable in size than wild-type mice (5). This was associated with higher expression of *myf5*, myogenin, and *E2A* in quadriceps muscle of the knockouts. Myostatin was not reported in this study (5). European sea bass that received dietary vitamin D after hatching demonstrated dose-dependent increases in white muscle fiber size and differences in the expression of MRFs (49). In humans, children born to vitamin D-deficient mothers displayed significantly smaller arm-muscle area (50), and another study reported a positive correlation between $1,25(OH)_2D$ levels and skeletal muscle mass on dual-energy x-ray absorptiometry scan in adults (51).

There are, however, no in vivo studies examining effects of vitamin D on muscle fiber number, an important question given its in vitro effects on proliferation and myotube formation. Overexpression of c-myc in VDR-knockout mice is associated with the local development of colonic epithelial hyperplasia and dysregulated epidermal differentiation, leading to alopecia (35, 52). We have also found that $1,25(OH)_2D$ directly regulates c-myc expression in muscle cells, suggesting in vivo relevance in this tissue. Other questions remain such as potential roles for vitamin D in muscle regeneration, a process that closely mimics muscle differentiation (53). Such a role has recently been suggested by the upregulation of VDR in a mouse model of muscle injury (54) and the modulation of key angiogenic factors vascular endothelial growth factor and fibroblast growth factor-1 in C2C12 cells treated with $1,25(OH)_2D$ (55).

In summary, this work reports the presence of functional CYP27B1 in C2C12 cells on the basis of luciferase reporter studies and novel effects of 25OHD in C2C12 cell proliferation and differentiation. This work also elucidates novel effects of vitamin D signaling in C2C12 cell cycle regulation, with effects in the expression and post-translational modification of genes controlling G_0/G_1 arrest and G_1/S transition. This offers mechanistic insight into the established antiproliferative effect of $1,25(OH)_2D$ in these cells. Independent of this effect, $1,25(OH)_2D$ inhibited myogenesis by the suppression of MRFs and had an anabolic effect on myotube formation, previously unreported findings in C2C12 cells after serum deprivation. Taken together, these findings raise the possibility of a direct effect of vitamin D on muscle. Further studies are needed to examine developmental effects of vitamin D in skeletal muscle and elucidate relevant signaling pathways at this site.

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