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Effects of 1,25(OH)$_2$D$_3$ and 25(OH)D$_3$ on C2C12 Myoblast Proliferation, Differentiation, and Myotube Hypertrophy

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An adequate vitamin D status is essential to optimize muscle strength. However, whether vitamin D directly reduces muscle fiber atrophy or stimulates muscle fiber hypertrophy remains subject of debate. A mechanism that may affect the role of vitamin D in the regulation of muscle fiber size is the local conversion of 25(OH)D to 1,25(OH)$_2$D by 1α-hydroxylase. Therefore, we investigated in a murine C2C12 myoblast culture whether both 1,25(OH)$_2$D$_3$ and 25(OH)D$_3$ affect myoblast proliferation, differentiation, and myotube size and whether these cells are able to metabolize 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$. We show that myoblasts not only responded to 1,25(OH)$_2$D$_3$, but also to the precursor 25(OH)D$_3$ by increasing their VDR mRNA expression and reducing their proliferation. In differentiating myoblasts and myotubes 1,25(OH)$_2$D$_3$ as well as 25(OH)D$_3$ stimulated VDR mRNA expression and in myotubes 1,25(OH)$_2$D$_3$ also stimulated MHC mRNA expression. However, this occurred without notable effects on myotube size. Moreover, no effects on the Akt/mTOR signaling pathway as well as MyoD and myogenin mRNA levels were observed. Interestingly, both myoblasts and myotubes expressed CYP27B1 and CYP24 mRNA which are required for vitamin D$_3$ metabolism. Although 1α-hydroxylase activity could not be shown in myotubes, after treatment with 1,25(OH)$_2$D$_3$ or 25(OH)D$_3$ myotubes showed strongly elevated CYP24 mRNA levels compared to untreated cells. Moreover, myotubes were able to convert 25(OH)D$_3$ to 24R,25(OH)$_2$D$_3$ which may play a role in myoblast proliferation and differentiation. These data suggest that skeletal muscle is not only a direct target for vitamin D$_3$ metabolites, but is also able to metabolize 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$.

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Aging is associated with a loss of muscle mass, bone mass, and strength, which may result in reduced mobility and an increased risk for falls and fractures (Cederholm et al., 2013; Rizzoli et al., 2014). An adequate vitamin D status is essential to reduce the risk for falls and fractures and to optimize bone mineral density and muscle strength (Morgan, 2008; Lips and van Schoor, 2011; Bischoff-Ferrari, 2012). Vitamin D stimulates calcium absorption from the intestine and maintains serum calcium levels which is required for normal bone mineralization and muscle function (Lips, 2006). Regarding bone metabolism, vitamin D reduces osteoblast proliferation, stimulates osteoblast differentiation, and induces RANKL expression in osteoblasts which is involved in stimulation of osteoclast formation and bone resorption (Lips, 2006; Anderson and Atkins, 2008; van der Meijden et al., 2014). However, whether vitamin D directly reduces muscle fiber atrophy or stimulates muscle fiber hypertrophy remains subject of debate.

Several in vivo studies suggest a role for vitamin D in the regulation of muscle mass and function. Observational studies demonstrate that vitamin D deficiency in elderly people is associated with reduced muscle mass (Tieland et al., 2013) and strength (Bischoff et al., 1999; Zamboni et al., 2002), lower physical performance (Wicherts et al., 2007; Tieland et al., 2013), and an increased risk of falling (Snijder et al., 2006). Furthermore, a meta-analysis of 17 randomized controlled trials showed that vitamin D supplementation in subjects with a baseline serum 25-hydroxyvitamin D (25(OH)D) lower than 25 nmol/L did have a positive effect on hip muscle strength (Stockton et al., 2011). In animal models, reduced muscle function was reported in vitamin D deficient rats (Rodman and Baker, 1978; Pleasure et al., 1979) and chickens

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(Bischoff-Ferrari, 2012) compared to control animals. The studies described above suggest that vitamin D can affect muscle mass and function, however it is not clear whether vitamin D plays a direct or indirect role.

In vitro studies on myoblasts and myotubes show that the active metabolite 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) is able to directly affect myogenesis (Garcia et al., 2011; Buitrago et al., 2012; Girgis et al., 2014a). Myogenesis, a process that is essential for muscle regeneration, growth and hypertrophy, includes satellite cell activation, myoblast proliferation, differentiation, and myotube formation (Zanou and Gailly, 2013). Regarding myoblast proliferation in vitro, most studies show inhibitory effects of $1,25(OH)_2D$ (Simpson et al., 1985; Garcia et al., 2011; Okuno et al., 2012; Srikuea et al., 2012; Girgis et al., 2014a) likely due to a cell cycle arrest at the G1 to S transition (Girgis et al., 2014a). However, $1,25(OH)_2D$ effects on proliferation have also been reported to be absent (Stio et al., 2002) or stimulatory (Drittanti et al., 1989a; Capiati et al., 1999; Buitrago et al., 2012). Furthermore, whether $1,25(OH)_2D$ affects myoblast differentiation and hypertrophy of differentiated myotubes is not well known. Recently, it has been shown that when myoblasts were cultured in growth medium and subsequently in differentiation medium which were supplemented with $1,25(OH)_2D$ from the start of the culture resulted in less myotubes (Girgis et al., 2014a), but myotubes were larger in diameter than those that were differentiated in medium without supplemented $1,25(OH)_2D$ (Garcia et al., 2011; Girgis et al., 2014a). Since in these experiments the number of myoblasts was not standardized due to the anti-proliferating effects of $1,25(OH)_2D$, the larger myotube size could not be ascribed to a direct effect of $1,25(OH)_2D$ per se. As, yet it is still unknown what the effects are of $1,25(OH)_2D$ on myotube formation and size in cultures starting with the same number of cells.

A mechanism that may affect the role of vitamin D in the regulation of muscle fiber size and contractile function in vivo is the local conversion of 25(OH)D to $1,25(OH)_2D$. The metabolite $1,25(OH)_2D$ is primarily synthesized in the kidney from the precursor 25-hydroxyvitamin D (25(OH)D) (Lips, 2006). In addition, $1,25(OH)_2D$ synthesis has been demonstrated in several other cell types, such as in osteoblasts (Howard et al., 1981; van Driel et al., 2006a; Atkins et al., 2007; van der Meijden et al., 2014), prostate cells (Schwartz et al., 1998), and monocytes (Bacchetta et al., 2013). In osteoblasts, the function of locally synthesized $1,25(OH)_2D$ is supposed to be regulation of cell proliferation and differentiation (van Driel et al., 2006a; Atkins et al., 2007; Bikle, 2009). Recent studies have shown that rat muscle as well as C2C12 myoblasts and myotubes also express CYP27B1, which encodes the enzyme 1α-hydroxylase (Testerink et al., 2011b; Srikuea et al., 2012; Girgis et al., 2014a). Moreover, CYP27B1 activity has indirectly been demonstrated in muscle cells by performing luciferase reporter studies (Girgis et al., 2014a) and CYP27B1 silencing experiments (Srikuea et al., 2012). However, to the best of our knowledge, whether C2C12 cells do convert 25(OH)D to $1,25(OH)_2D$ has not been investigated yet. In addition to its possible conversion into $1,25(OH)_2D$, 25(OH)D may also be converted to 24R,25-dihydroxyvitamin D ($24R,25(OH)_2D$). This conversion is catalyzed by the 24-hydroxylase enzyme, encoded by the CYP24 gene (St-Arnaud, 2010). In osteoblasts in vitro, $24R,25(OH)_2D$ synthesis has been shown (Turner et al., 1980; Howard et al., 1981; van der Meijden et al., 2014) and this metabolite may stimulate cell differentiation through binding to the VDR (van Driel et al., 2006a; Curtis et al., 2014; van der Meijden et al., 2014). In myoblasts and myotubes, CYP24 expression has also been shown (Girgis et al., 2014a) and CYP24 activity may, therefore, affect skeletal muscle tissue as well. However, whether skeletal muscle cells are capable of synthesizing $24R,25(OH)_2D$ is still unknown.

The aim of this study was to investigate in a murine C2C12 myoblast culture model whether both $1,25(OH)_2D_3$ and $25(OH)D_3$ affect myoblast proliferation, differentiation, and myotube size, and which regulatory mechanisms, including myogenic regulatory factors and signaling pathways, are involved. We hypothesized that $1,25(OH)_2D_3$ affects the expression of genes in the regulation of vitamin D_3 signaling in myoblasts and myotubes, inhibits myoblast proliferation and stimulates myoblast differentiation and myotube hypertrophy. Moreover, we hypothesized that actions of $25(OH)D_3$ occur via its conversion to $1,25(OH)_2D_3$. Therefore, we investigated in C2C12 myoblast and myotube cultures whether supplementation of $1,25(OH)_2D_3$ or $25(OH)D_3$ to culture medium alters mRNA levels of genes involved in vitamin D metabolism and/or signaling pathways for protein synthesis. We further tested whether myotubes were able to synthesize $1,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ from supplemented $25(OH)D_3$.

Materials and Methods

Cell culture

Mouse C2C12 myoblast cell line was obtained from ATCC (American Type Culture Collection; Manassas, VA). Myoblasts were cultured in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM-31885, low glucose, phenol red; Gibco, Life Technologies, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (FBS; Gibco), 10 μg/ml penicillin (Sigma–Aldrich, St. Louis, MO), 10 μg/ml streptomycin (Sigma–Aldrich), 50 μg/ml fungizone (Gibco) and incubated at 37°C in humidified air with 5% CO_2. Passages between 4 and 10 were used for experiments and all culture media, including those of treated and control groups, contained 0.1% ethanol (vehicle).

C2C12 myoblast proliferation

C2C12 cells were plated out in 96-well plates or 6-well plates at a density of 500 cells per cm^2. After 24 h cells were cultured in medium with 1000 nmol/L $25(OH)D_3$ (Sigma–Aldrich), 100 nmol/L $1,25(OH)_2D_3$ (Sigma–Aldrich) or without supplements (i.e., control). Medium was replaced every day by growth medium containing $25(OH)D_3$, $1,25(OH)_2D_3$, or control. At day 1 and 4, the proliferation of C2C12 myoblasts in the 96-well plate was measured using XTT Cell Proliferation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Briefly, cells were incubated with XTT solution at 37°C, whereby the viable cells formed an orange formazan dye by cleaving the yellow tetrazolium salt XTT. After 2 h the orange formazan solution was quantified by a photospectrometer (Berthold Technologies, Bad Wildbad, Germany) at 450 nm. Cells from the 6-well plate were lysed and stored at −80°C until total RNA isolation.

C2C12 myoblast differentiation

C2C12 cells were plated out in 6-well plates and were grown until 90% confluence. To induce myotube formation, growth medium was changed to differentiation medium consisting of DMEM supplemented with 2% horse serum (Gibco), 10 μg/ml penicillin (Sigma–Aldrich), 10 μg/ml streptomycin (Sigma–Aldrich) and 50 μg/ml fungizone (Gibco) At day 1 and 3 of differentiation, cells were lysed and stored at −80°C until total RNA isolation or western blotting. Myotube thickness was measured at day 3 of the differentiation by obtaining images using a Leica inverted microscope type DM-IL. Subsequently, myotube thickness was determined from four images per well using ImageJ (v.1.41o, National Institute of Health, USA; http://rsbweb.nih.gov/ij/). Myotubes (>90 per experiment) were measured at three locations along their lengths (25%, 50%, and 75% of the length).

RNA isolation and RT-qPCR

Total RNA of C2C12 cells was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For removing residual DNA amounts an additional on-column DNase treatment was accomplished. Total RNA concentration was measured by the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE).

RNA was reverse transcribed from 500 ng total RNA in a 20 μl reaction mixture using the High Capacity RNA-to-DNA Master Mix (Applied Biosystems, Foster City, CA). Reverse transcription was performed using the following thermal cycler conditions: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. The PCR reaction of total 20 μl contained 5 μl cDNA, 200 nmol/L reverse and forward primer (Table 1) and SYBR Green Master Mix (Applied Biosystems). cDNA was diluted 1:10. qPCR was performed in duplicate on a StepOne real-time PCR System (Applied Biosystems): 20 sec at 95°C, 40 cycles consisting of 3 sec at 95°C, and 30 sec at 60°C. Several housekeeping genes were tested (18S, hypoxanthine phosphoribosyltransferase and glyceraldehyde 3-phosphate dehydrogenase) of which assessment of 18S rRNA expression was shown to be the more reproducible. Therefore, 18S rRNA was used as reference and the relative gene expression was calculated by the $2^{-\Delta Ct}$ method.

Western blot

C2C12 cells for western blot were scraped in cold radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Rockford, IL) containing protease and phosphatase inhibitors (Thermo Fisher Scientific). Protein concentrations were measured by the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific). Samples (one per experiment) were denaturated in SDS–PAGE sample buffer for 5 min at 90°C, loaded onto a SDS PAGE gel and transferred to a nitrocellulose membrane (GE Healthcare, Little Chalfont, UK). After transfer, the membrane was blocked overnight at 4°C with 2% ECL Advance Blocking Agent (GE Healthcare) in TBS with 0.01% Tween 20 (Sigma–Aldrich). Subsequently, the membrane was washed and incubated for 1 h at room temperature with primary antibody against p-Akt (Ser473; 1:4000), total Akt (1:4000), p-S6 (Ser235/236; 1:1000), total S6 (1:1000), and β-tubulin (1:2000) (all Cell Signaling Technology). After washing, the membrane was incubated for 1 h at room temperature with horseradish peroxidase-conjugated polyclonal goat anti-rabbit secondary antibody (1:4000; DakoCytomation, Glostrup, Denmark) and the membrane was analyzed with the enhanced chemiluminescence method (ECL Advance; GE Healthcare). Western blots were quantified using ImageJ. Total Akt, p-Akt, total S6, and p-S6 were normalized to β-tubulin.

TABLE 1. Primer Sequence

Target Gene	Primer Sequence (5′- 3′)
CYP27B1	Forward: CATCATGGGCAGAGCACCGT Reverse: TCACCATCCGCCGTTAGCAA
Vitamin D receptor (VDR)	Forward: TCCTGCTCGATGCCCACCACA Reverse: TGCACGAATTGGAGGCCGGAA
CYP24	Forward: AACAGCACGACACACTGGCAGA Reverse: CTCGGCGAGCCCAGATGCAG
MyoD	Forward: CATCCAGCCCGCGCTCCAAC Reverse: GGGCCGCTGTAATCCATCATGCC
Myogenin	Forward: CCAGCCCATGGTGCCCAGTGA Reverse: CCAGTGCATTGCCCCACTCCG
PGC1α	Forward: ACACAACCGCAGTCGCAACA Reverse: GGGAACCCTTGGGGTCATTTGG
Ki67	Forward: GGTGGGCACCTAAGACCTGAA Reverse: TCCTAGGACTAGGAGCTGGAG
MHC-I (MYH7)	Forward: AGATCCGAAAGCAACTGGAG Reverse: CTGCCTTGATCTGGTTGAAC
MHC-IIA (MYH2)	Forward: GCAGAGACCGAGAAGGAG Reverse: CTTTCAAGAGGGACACCATC
MHC-IIX (MYH1)	Forward: GCGACAGACACCTCCTTCAAG Reverse: TCCAGCCAGCCAGCGATG
MHC-IIB (MYH4)	Forward: CAACTGAGTGAAGTGAAGACC Reverse: AGCTGAGAAACCATAGCGTC
MHC embryonic (MYH8)	Forward: ACTGAGGAAGACCGCAAGAA Reverse: CAGGTTGGCATTGGATTGTTC
18S rRNA	Forward: GTAACCCGTTGAACCCCATT Reverse: CCATCCAATCGGTAGTAGCG

Measurement of medium concentrations of $1,25(OH)_2D_3$, $25(OH)D_3$, and $24R,25(OH)_2D_3$

C2C12 myoblasts were seeded into 6-well plates and were grown until 90% confluence. Cells were induced to form myotubes by changing growth medium to differentiation medium. After 3 days of differentiation, cells were cultured in medium supplemented with 0, 400, 1000, or 2000 nmol/L $25(OH)D_3$ for 24 h. Medium was collected and stored at −20°C until measurement of vitamin D_3 metabolites. As positive control, primary human osteoblasts were cultured in medium supplemented with 0, 400, and 1000 nmol/L $25(OH)D_3$ in 6-well plates with a cell density of 500.000 cells per well for 24 h, as described previously (van der Meijden et al., 2014). Medium was collected and stored at −20°C until measurement of vitamin D_3 metabolites.

The metabolite $1,25(OH)_2D_3$ was measured in non-conditioned and conditioned medium using a radioimmunoassay (Immunodiagnostic Systems, Boldon, UK). Cross reactivity with $25(OH)D_3$ and $24R,25(OH)_2D_3$ was 0.01% and <0.01% respectively. The intra-assay variation was 8% at a level of 25 pmol/L and 9% at a level of 70 pmol/L. The inter-assay variation was 11% at a concentration of 25 and 70 pmol/L.

The metabolites $25(OH)D_3$ and $24R,25(OH)_2D_3$ were analyzed in non-conditioned and conditioned medium using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Briefly, samples were incubated with deuterated internal vitamin D standards (d6-$25(OH)D_3$ and d6-$24R,25(OH)_2D_3$) and protein-precipitated using acetonitrile. Supernatant was, after PTAD derivatization, purified using a Symbiosis online solid phase extraction (SPE) system (Spark Holland, Emmen, The Netherlands), followed by detection with a Quattro Premier XE tandem mass spectrometer (Waters Corp., Milford, MA). Intra-assay variation of $25(OH)D_3$ was 9.6%, 6.0%, and 8.5% at a level of 58, 191, and 516 nmol/L, respectively. Intra-assay variation of $24R,25(OH)_2D_3$ was 5.4% and 9.1% at a level of 46 and 150 nmol/L, respectively.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed using SPSS version 20.0 software (SPSS Inc., Chicago, IL). Differences between groups were assessed using a one-way ANOVA followed by Bonferroni's post hoc test to examine the effects of treatment on myotube diameter and myotube number. A two-way ANOVA followed by Bonferroni's post hoc test was used to examine the effects of time and vitamin D_3 treatment on absorbance values, mRNA expression levels and protein expression levels. A three-way ANOVA was used to examine whether time and vitamin D_3 treatment affect expression levels of myosin heavy chain isoforms. A P-value < 0.05 was considered to be significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Results

Both $25(OH)D_3$ and $1,25(OH)_2D_3$ attenuated C2C12 myoblast proliferation

Figure 1 shows effects of 1 and 4 days of supplementation of $25(OH)D_3$ and $1,25(OH)_2D_3$ on estimates of viable cell numbers determined by the absorbance of the colored formazan product which is directly proportional to the number of viable cells. Two-way ANOVA revealed a significant effect of

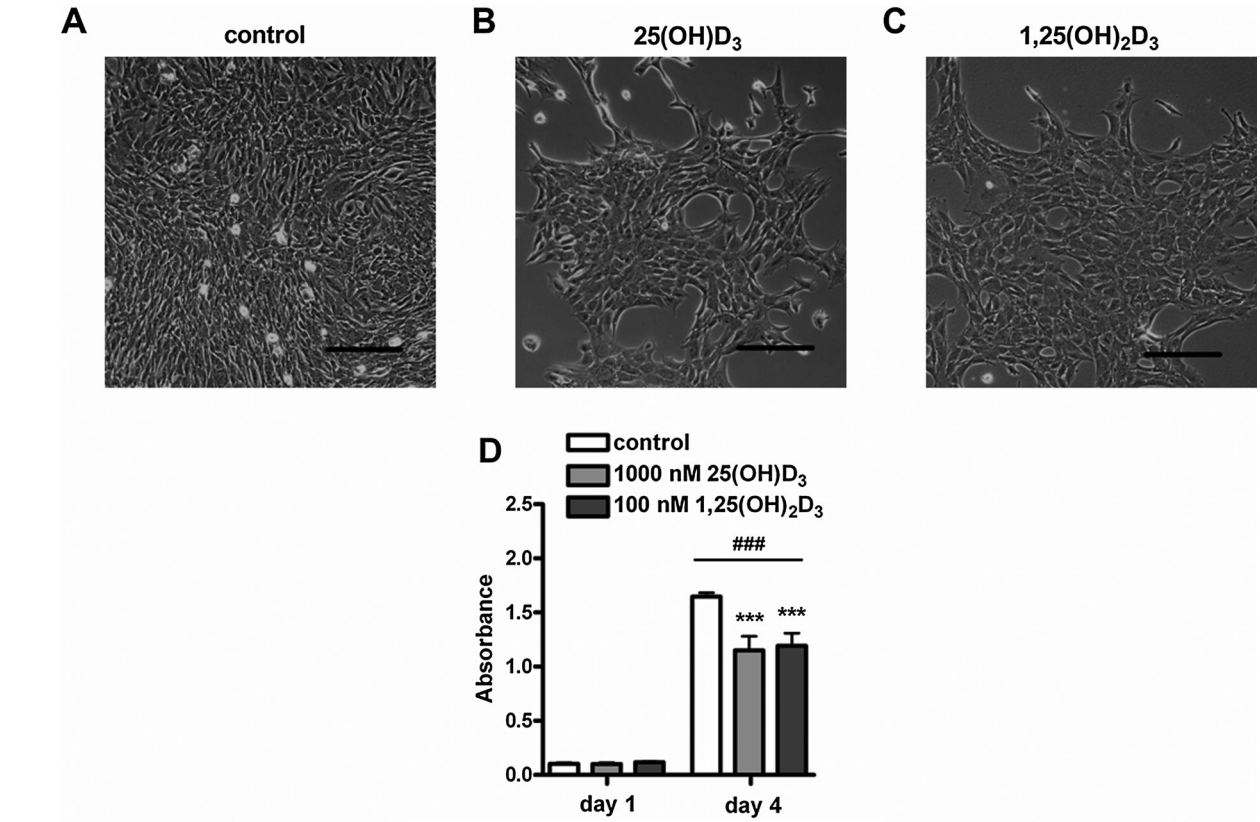

Fig. 1. Both 25(OH)D3 and 1,25(OH)2D3 attenuated C2C12 myoblast proliferation. Micrographs of C2C12 myoblasts cultured for 4 days in growth medium (A), in growth medium supplemented with 1000 nmol/L 25(OH)D3 (B), or 100 nmol/L 1,25(OH)2D3 (C). Myoblast proliferation was quantified at day 1 and 4 (D). Scale bar indicates 100 μm. Data were analyzed using a two-way ANOVA followed by Bonferroni's post hoc comparisons test. Values are mean ± SEM (n = 20). $^{***}P < 0.001$; $^{\#\#\#}P < 0.001$ ($^{\#}$ between time period, * between vitamin D3 concentrations).

time as well as an interaction between time and vitamin D_3 treatment ($P < 0.001$). As expected, for all treatment groups the number of viable cells was significantly increased at day 4 compared to day 1 ($P < 0.001$; Fig. 1D). At day 4, both 1000 nmol/L 25(OH)D_3 and 100 nmol/L 1,25(OH)$_2D_3$ significantly reduced the number of viable cells compared to control culture ($P < 0.001$). In the presence of 1000 nmol/L 25(OH)D_3 and 100 nmol/L 1,25(OH)$_2D_3$ the proportion of viable cells was shown to be 30.1% and 27.6% lower than in the control condition, respectively.

Both 25(OH)D_3 and 1,25(OH)$_2D_3$ increased CYP24 and VDR mRNA levels and reduced myogenin mRNA levels in proliferating myoblasts

Since myoblast proliferation was reduced by vitamin D_3 metabolites, we next investigated whether myoblasts expressed mRNA of proteins involved in vitamin D_3 metabolism and whether expression levels were modulated by vitamin D_3 metabolites. Myoblasts did express CYP27B1, CYP24, and VDR. At day 4, CYP27B1 mRNA levels were lower compared to those at day 1 ($P < 0.05$; Fig. 2A), but significant effects of 25(OH)D_3 or 1,25(OH)$_2D_3$ were not observed. CYP24 mRNA levels were higher at day 4 compared to day 1 ($P < 0.01$; Fig. 2B). At day 1 and day 4, CYP24 and VDR mRNA levels were both significantly increased by 25(OH)D_3 ($P < 0.01$) as well as by 1,25(OH)$_2D_3$ ($P < 0.01$ and $P < 0.05$, respectively; Fig. 2B and C). To investigate mechanisms underlying the anti-proliferative effects of both vitamin D_3 metabolites, we determined mRNA levels of ki67 and MyoD. Ki67 mRNA was significantly reduced at day 4 compared to day 1 ($P < 0.001$; Fig. 2D), but 25(OH)D_3 and 1,25(OH)$_2D_3$ supplementation did not change these mRNA levels. MyoD mRNA levels were also not affected by 25(OH)D_3 or 1,25(OH)$_2D_3$ supplementation (Fig. 2E). For myogenin, two-way ANOVA showed a significant interaction between the effects of time and vitamin D_3 treatment ($P < 0.05$; Fig. 2F). Post hoc analysis revealed that 25(OH)D_3 and 1,25(OH)$_2D_3$ reduced myogenin mRNA levels at day 4 of proliferation ($P < 0.01$). In addition, myogenin mRNA levels in control cultures were significantly increased at day 4 compared to day 1 ($P < 0.001$), but mRNA levels at day 4 were still 44 times lower than myogenin mRNA levels in myotubes (Fig. 4E).

Effects of 25(OH)D_3 and 1,25(OH)$_2D_3$ on myotube diameter

Confluent (90%) cultures of myoblasts differentiated into myotubes during 3 days of culture in differentiation medium. To examine whether 25(OH)D_3 and 1,25(OH)$_2D_3$ stimulated myotube hypertrophy, we measured myotube diameter at day 3 of the differentiation. Figure 3 shows micrographs of control myotubes, and 25(OH)D_3 or 1,25(OH)$_2D_3$ treated cells taken at day 3 of treatment (Fig. 3A–C). The diameter of myotubes exposed to 25(OH)D_3 was slightly increased compared to control myotubes (19%; $P < 0.05$; Fig. 3D), but an

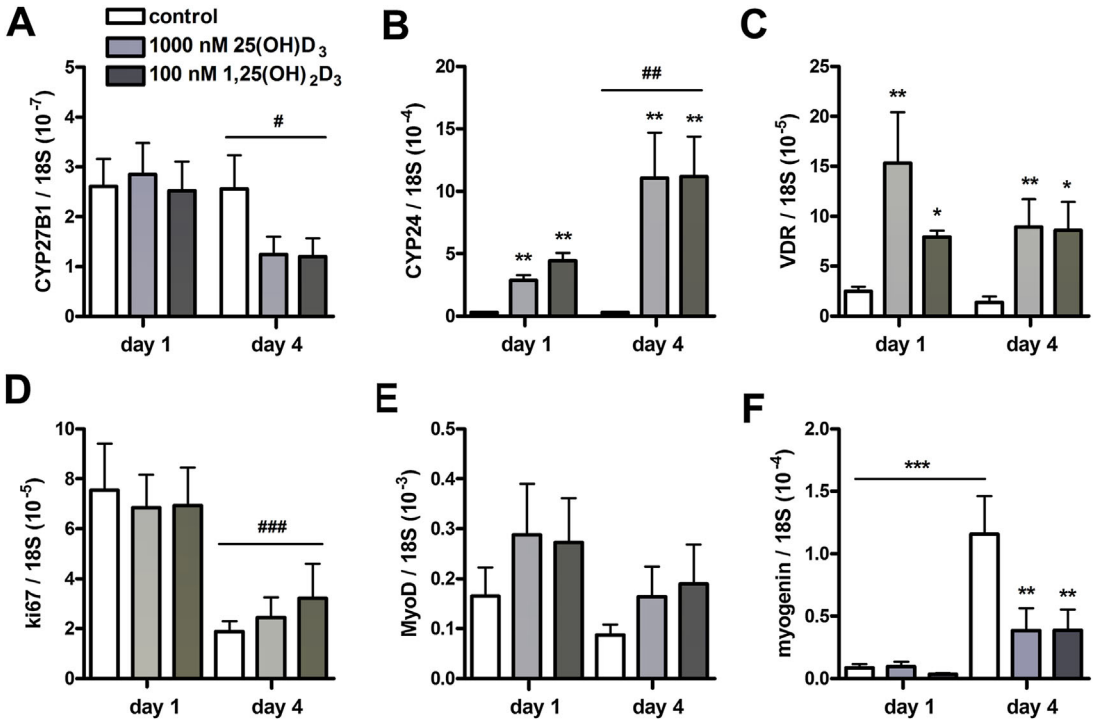

Fig. 2. Both 25(OH)D3 and 1,25(OH)2D3 increased CYP24 and VDR mRNA levels and reduced myogenin mRNA levels in myoblasts. Myoblasts were cultured for 4 days in growth medium supplemented with 1000 nmol/L 25(OH)D3, 100 nmol/L 1,25(OH)2D3, or without any supplements. After 1 and 4 days, mRNA levels of CYP27B1 (A), CYP24 (B), VDR (C), ki67 (D), MyoD (E), and myogenin (F) were determined. Data were analyzed using a two-way ANOVA followed by Bonferroni's post hoc comparisons test. Values are mean ± SEM (n = 8). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ ($^{\#}$ between time period, * between vitamin D3 concentrations).

effect of $1,25(OH)_2D_3$ on myotube diameter was not observed. The number of myotubes per mm^2 was not altered by both vitamin D_3 metabolites (Fig. 3E).

To investigate whether the lack of hypertrophy was associated with the high dose of the vitamin D_3 metabolites, we tested whether low concentrations of vitamin D_3 metabolites would induce myotube hypertrophy. For this purpose myoblasts were differentiated into myotubes in the presence of 100 nmol/L $25(OH)D_3$ and 1 nmol/L $1,25(OH)_2D_3$. However, these low concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ did not affect myotube diameter or the number of myotubes per mm^2 (Fig. S1A and B, Supplementary Data).

Both $25(OH)D_3$ and $1,25(OH)_2D_3$ increased CYP24 and VDR mRNA levels in differentiating myotubes

Figure 4 shows mRNA levels of CYP27B1, CYP24, VDR, MyoD, myogenin, and PGC1α during myotube formation. CYP27B1 mRNA levels after 3 days of culture in differentiation medium were increased compared to those after 1 day ($P < 0.001$; Fig. 4A). CYP24 mRNA levels were substantially increased by both $25(OH)D_3$ and $1,25(OH)_2D_3$ ($P < 0.001$; Fig. 4B). For mRNA levels of VDR, two-way ANOVA showed significant interaction effects between time and vitamin D_3 treatment ($P < 0.05$; Fig. 4C). Post hoc analyses showed that after 3 days of differentiation both $25(OH)D_3$ and $1,25(OH)_2D_3$ increased VDR mRNA levels ($P < 0.05$ and $P < 0.001$, respectively). In addition, within $1,25(OH)_2D_3$ treated myotubes, VDR mRNA levels at day 3 were increased compared to those measured at day 1 ($P < 0.001$). MyoD mRNA levels were not affected by time or vitamin D_3 treatment (Fig. 4D). After 3 days of culture in differentiation medium, myogenin and PGC1α mRNA levels were significantly higher than those after 1 day of culture in differentiation medium ($P < 0.001$ and $P < 0.01$, respectively; Fig. 4E and F), but effects of vitamin D_3 treatment could not be shown.

We also verified whether low concentrations of $25(OH)D_3$ (100 nmol/L) and $1,25(OH)_2D_3$ (1 nmol/L) were able to affect mRNA levels of CYP27B1, CYP24, VDR, MyoD, and myogenin during myotube formation (Fig. S2A–E, Supplementary Data). Low concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ did not affect CYP27B1 mRNA (Fig. S2A). CYP24 and VDR mRNA levels were markedly increased by 1 nmol/L $1,25(OH)_2D_3$ ($P < 0.001$; Fig. S2B and C), but 100 nmol/L $25(OH)D_3$ did not induce CYP24 or VDR mRNA. MyoD and myogenin mRNA levels were not affected by low concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ (Fig. S2D and E).

Effects of $25(OH)D_3$ and $1,25(OH)_2D_3$ on mRNA levels of myosin heavy chain (MHC)

Using a three-way ANOVA, we tested whether vitamin D_3 metabolites altered myotube phenotype by changing MHC isoform expression. After 3 days of culture in differentiation medium, mRNA levels of MHC-I, MHC-IIA, MHC-IIX, MHC-IIB, and MHC embryonic were increased compared to those at day 1 ($P < 0.001$; Fig. 5A–E). Since MHC expression levels are a hallmark of differentiation, effects of vitamin D_3 metabolites may have been obscured due to variations in MHC expression levels between experiments as the degree of differentiation may differ from experiment to experiment. Therefore, data were also normalized (treatment/control ratio). Three-way

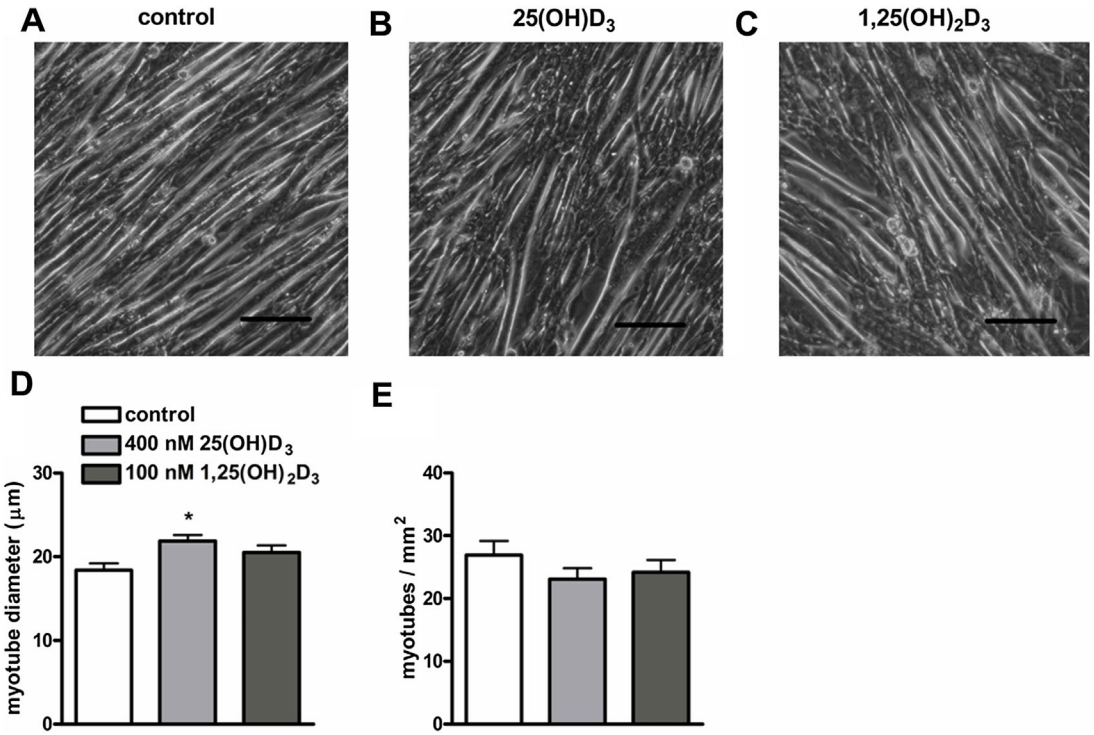

Fig. 3. Effects of 25(OH)D3 and 1,25(OH)2D3 on myotube diameter. Micrographs of C2C12 myoblasts cultured for 3 days in differentiation medium (A), in differentiation medium supplemented with 400 nmol/L 25(OH)D3 (B), or 100 nmol/L 1,25(OH)2D3 (C). After 3 days of culture, myotube diameter (μm) (D) and myotubes/mm^2 (E) were determined. Scale bar indicates 100 μm. Data were analyzed using a one-way ANOVA followed by Bonferroni's post hoc test. Values are mean ± SEM (n = 8). $^*P < 0.05$.

ANOVA on normalized data showed a significant interaction between time and vitamin D_3 treatment ($P < 0.001$). A main effect of vitamin D_3 treatment on MHC mRNA levels was significant at day 3, but not at day 1. Post hoc analysis revealed that for all conditions MHC mRNA levels were higher at day 3 than those at day 1 ($P < 0.001$) and revealed also that at day 3, $1,25(OH)_2D_3$ significantly increased mRNA levels of MHC compared to control ($P < 0.01$).

We also verified whether low concentrations of $25(OH)D_3$ (100 nmol/L) and $1,25(OH)_2D_3$ (1 nmol/L) were able to affect mRNA levels of different types of MHC during myotube formation (Fig. S3A–E, Supplementary Data). We found that MHC mRNA levels were higher at day 3 than those at day 1 ($P < 0.05$), but low concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ did not affect mRNA levels of any of the MHC isoforms.

Both $25(OH)D_3$ and $1,25(OH)_2D_3$ did not affect levels of p-Akt, total Akt, p-S6, and total S6 during myotube formation

To explain the positive effect of $25(OH)D_3$ on myotube size, we examined whether $25(OH)D_3$ and $1,25(OH)_2D_3$ activate components of the Akt/mTOR signaling pathway, including Akt and S6. Levels of p-Akt and p-S6 were not affected by time and treatment with vitamin D_3 metabolites (Fig. 6A and B). Total Akt protein levels after 3 days of culture were increased compared to those at day 1 ($P < 0.01$; Fig. 6C), but the two vitamin D_3 metabolites did not change this. Total S6 levels were not affected by time and vitamin D_3 treatment (Fig. 6D). Ratio of p-Akt/total Akt was lower at day 3 compared to day 1 ($P < 0.01$; Fig. 6E), whereas a higher ratio of p-S6/total S6 was found at day 3 compared to day 1 ($P < 0.05$; Fig. 6F). These results indicate that both vitamin D_3 metabolites did not enhance Akt/mTOR signaling.

Myotubes did not synthesize detectable $1,25(OH)_2D_3$ levels, but synthesized $24R,25(OH)_2D_3$ after exposure to $25(OH)D_3$

To test whether the effects of $25(OH)D_3$ occur via conversion to $1,25(OH)_2D_3$, we investigated whether C2C12 cells were able to synthesize $1,25(OH)_2D_3$ from $25(OH)D_3$. Because CYP27B1 mRNA levels in cells at day 3 of the differentiation (myotubes) were higher than those at day 1 (myoblasts), we chose to examine the conversion in myotubes. Myotubes were exposed to 0, 400, 1000, or 2000 nmol/L $25(OH)D_3$ and after 24 h $25(OH)D_3$ concentrations were strongly reduced to, respectively, 42%, 34%, and 36% of non-conditioned values (data not shown). However, after 24 h of culture the metabolite $1,25(OH)_2D_3$ could not be detected in medium (Table 2A). As positive control, osteoblasts were cultured in medium supplemented with 0, 400, or 1000 nmol/L $25(OH)D_3$. After 24 h, mean concentrations of, respectively, <10.0, 110.3, and 183.0 pmol/L $1,25(OH)_2D_3$ were measured in medium, whereas mean $1,25(OH)_2D_3$ concentrations in non-conditioned medium of, respectively, <10.0, 43.3, and 64.7 pmol/L were measured. These results indicate that primary human osteoblasts do convert $25(OH)D_3$ to $1,25(OH)_2D_3$, whereas C2C12 myotubes do not.

Because CYP24 mRNA levels were strongly induced after $25(OH)D_3$ treatment, we examined whether myotubes were

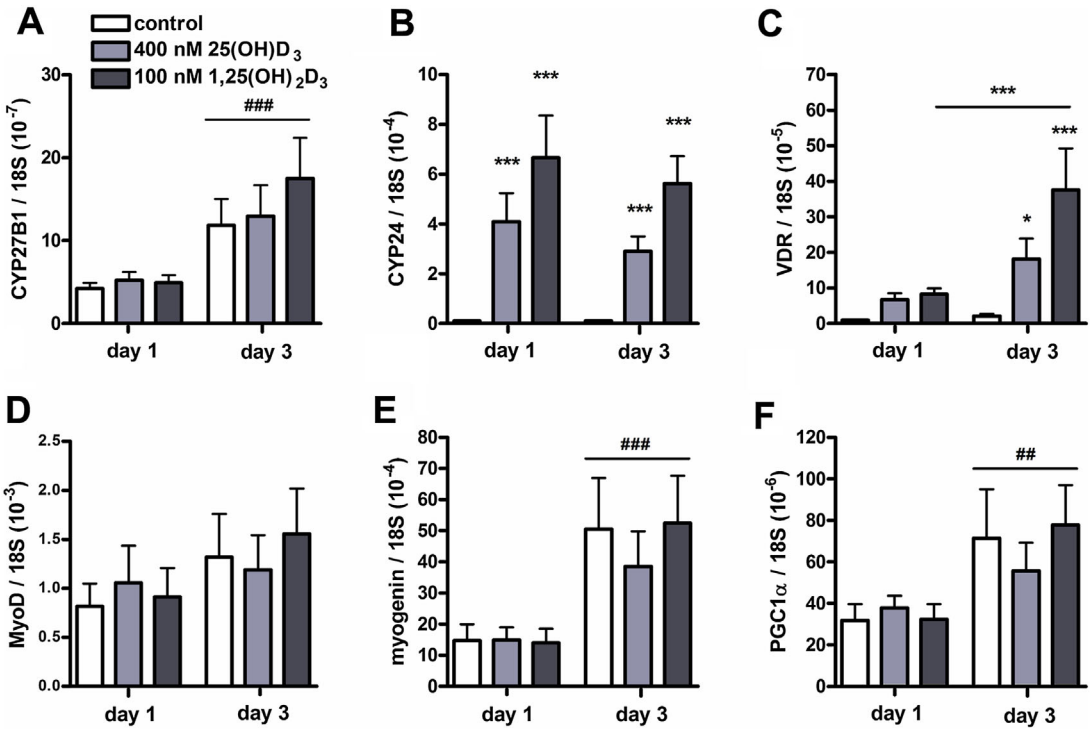

Fig. 4. Both 25(OH)D3 and 1,25(OH)2D3 increased CYP24 and VDR mRNA levels in differentiating C2C12 cells. C2C12 cells were cultured for 3 days in differentiation medium supplemented with 400 nmol/L 25(OH)D3, 100 nmol/L 1,25(OH)2D3, or without any supplements. After 1 and 3 days of culture, mRNA levels of CYP27B1 (A), CYP24 (B), VDR (C), MyoD (D), myogenin (E), and PGC1α (F) were determined. Data were analyzed using a two-way ANOVA followed by Bonferroni's post hoc comparisons test. Values are mean ± SEM (n = 6). $^{*}P < 0.05$, $^{***}P < 0.001$; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ ($^{\#}$ between time period, * between vitamin D3 concentrations).

able to synthesize 24R,25$(OH)_2D_3$ from 25$(OH)D_3$. Myotubes were exposed to 0, 400, 1000, or 2000 nmol/L 25$(OH)D_3$ and after 24 h of culture we, respectively, measured mean concentrations of <3, 6.8, 7.4, and 14.5 nmol/L 24R,25$(OH)_2D_3$ in medium (Table 2B). In non-conditioned medium, 24R,25$(OH)_2D_3$ concentrations were below their detection limit (<3 nmol/L). As positive control, osteoblasts were cultured in medium supplemented with 0, 400, or 1000 nmol/L 25$(OH)D_3$. After 24 h, mean concentrations of, respectively, <3, 70.2, and 105.4 nmol/L 24R,25$(OH)_2D_3$ were measured in medium, whereas 24R,25$(OH)_2D_3$ concentrations in non-conditioned medium were below their detection limit (<3 nmol/L). These results indicate that myotubes are able to convert 25$(OH)D_3$ to 24R,25$(OH)_2D_3$.

Discussion

The aim of this study was twofold: (i) to investigate the effects of 1,25$(OH)_2D_3$ and 25$(OH)D_3$ on proliferation and differentiation of myoblasts and myotube size; and (ii) to investigate 25$(OH)D_3$ metabolism within C2C12 muscle cells. With respect to our first aim, we demonstrated in myoblasts that both 25$(OH)D_3$ and 1,25$(OH)_2D_3$ increased VDR mRNA levels, reduced proliferation and decreased myogenin mRNA levels. During differentiation, both 25$(OH)D_3$ and 1,25$(OH)_2D_3$ increased VDR mRNA levels, but did not activate the Akt/mTOR pathway. Only 25$(OH)D_3$ slightly increased myotube size. Regarding our second aim, we hypothesized that effects of 25$(OH)D_3$ occur after its conversion to 1,25$(OH)_2D_3$, but despite the presence of CYP27B1 mRNA in myoblasts and myotubes we could not demonstrate 1,25$(OH)_2D_3$ synthesis in medium of myotubes after exposure to 25$(OH)D_3$. Interestingly, in myoblasts and myotubes CYP24 mRNA levels were increased in response to 25$(OH)D_3$ and accompanied by elevated 24R,25$(OH)_2D_3$ levels in medium. These results suggest that skeletal muscle cells not only respond to vitamin D_3 metabolites, but are also able to reduce vitamin D signaling by the activity of CYP24.

Proliferation

During myoblast proliferation, VDR mRNA expression was higher in myoblasts treated with 1,25$(OH)_2D_3$ than those without treatment. This observation is consistent with previous studies (Garcia et al., 2011; Srikuea et al., 2012; Girgis et al., 2014b) and suggests not only the presence of genomic transcriptional effects via the VDR, but also an increased responsiveness to 1,25$(OH)_2D_3$. Genomic effects of 1,25$(OH)_2D_3$ via the VDR were confirmed by strongly increased mRNA levels of CYP24, which is a target gene of the VDR. Treatment with 1,25$(OH)_2D_3$ also resulted in a reduction of myoblast number which is in line with several other studies (Simpson et al., 1985; Garcia et al., 2011; Okuno et al., 2012; Srikuea et al., 2012; Girgis et al., 2014a). This reduction of cell number in our study may in part be regulated by the genomic pathway of 1,25$(OH)_2D_3$, since it has been shown that expression of cell cycle genes is altered by 1,25$(OH)_2D_3$ (Drittanti et al., 1989b; Girgis et al., 2014a). In addition to genomic actions, non-genomic actions of 1,25$(OH)_2D_3$ such as stimulation of ERK1/2 (Ronda et al., 2007) and p38 MAPK (Buitrago et al., 2006) have been reported to modulate proliferation of myoblasts. In addition to

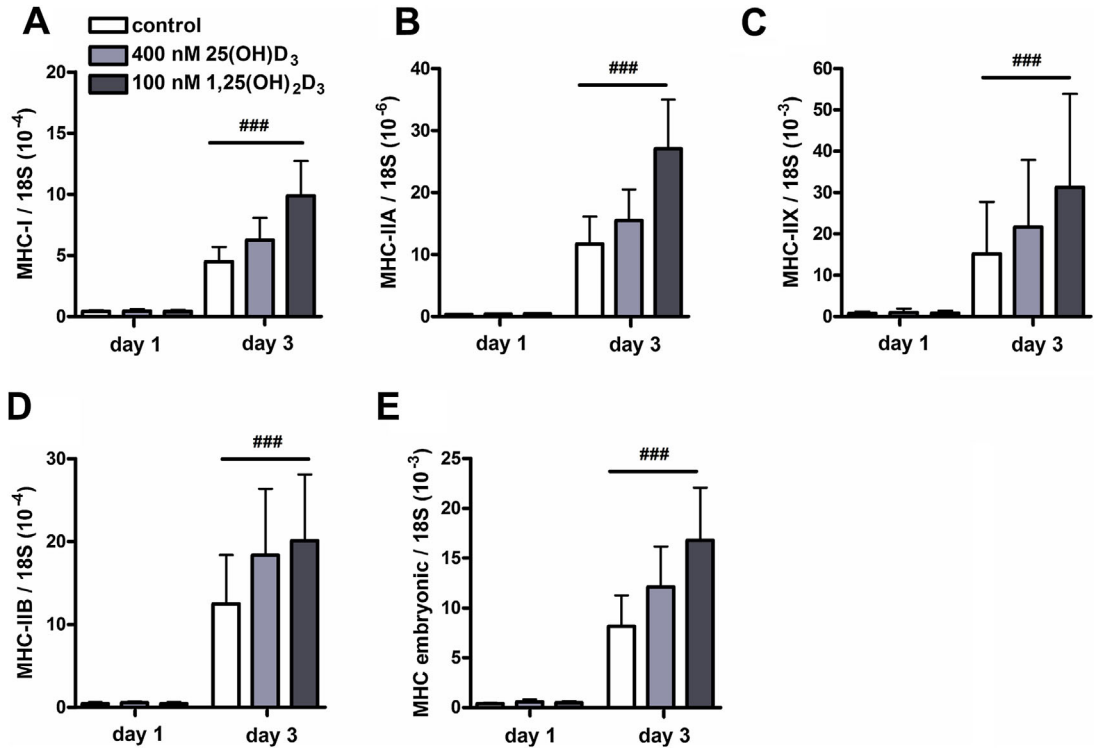

Fig. 5. Effects of 25(OH)D3 and 1,25(OH)2D3 on mRNA levels of myosin heavy chain. C2C12 cells were cultured for 3 days in differentiation medium supplemented with 400 nmol/L 25(OH)D3, 100 nmol/L 1,25(OH)2D3, or without any supplements. After 1 and 3 days of culture, mRNA levels of MHC-I (A), MHC-IIA (B), MHC-IIX (C), MHC-IIB (D), and MHC embryonic (E) were determined. Data were analyzed using a three-way ANOVA. Values are mean ± SEM (n = 6). $^{###}P < 0.001$ ($^{\#}$ between time period).

the inhibitory effects of $1,25(OH)_2D_3$, a few studies reported a stimulatory effect of $1,25(OH)_2D_3$ (Bellido et al., 1987; Buitrago et al., 2012). These stimulatory effects of $1,25(OH)_2D_3$ on myoblast proliferation were only demonstrated at early time-points (4–24 h), whereas inhibitory effects of $1,25(OH)_2D_3$ were mainly found at later time-points suggesting that the effect of $1,25(OH)_2D_3$ on proliferation is time-dependent. Serum concentration in medium is also important for the effects of $1,25(OH)_2D_3$ on proliferation, since it has been reported that $1,25(OH)_2D_3$ induces inhibitory effects in cultures with lower serum concentrations (5–10%), while higher serum concentrations (15–20%) result in stimulatory effects (Drittanti et al., 1989a). Both factors, serum and time, do probably affect the differentiation state of the cell which may determine the response of the cell to $1,25(OH)_2D_3$. Furthermore, we observed that myoblast number was not only lower after treatment with $1,25(OH)_2D_3$, but also after treatment with its precursor $25(OH)D_3$. This result confirms recent studies that found an anti-proliferative effect of $25(OH)D_3$ on myoblasts as well (Srikuea et al., 2012; Girgis et al., 2014a). It shows that muscle cells have the capacity to take up $25(OH)D_3$ (Abboud et al., 2013) and that $25(OH)D_3$ is directly or indirectly able to trigger mechanisms to reduce cell number or inhibit proliferation. Effects of $25(OH)D_3$ may occur via conversion to $1,25(OH)_2D_3$ since C2C12 myoblasts express CYP27B1, however as our results on myotubes show that myotubes do not convert $25(OH)D_3$ to $1,25(OH)_2D_3$ a direct effect may also be possible. Although $25(OH)D_3$ has a low affinity for the VDR (Lips, 2007), supra-physiological concentrations of $25(OH)D_3$ may activate the VDR leading to altered gene expression levels.

MyoD and ki67 were not significantly affected by both $25(OH)D_3$ or $1,25(OH)_2D_3$, but myogenin mRNA levels were lower after treatment with both metabolites compared to non-treated myoblasts which suggests that $25(OH)D_3$ and $1,25(OH)_2D_3$ inhibit the differentiation in growth medium. However, it is also possible that the higher mRNA levels of myogenin in control cultures were due to the almost confluent cell culture at the end of the proliferation experiment. An increased cell density will lead to more cell–cell contact which results in an earlier initiation of the differentiation (Mudera et al., 2010).

Differentiation and hypertrophy

As in myoblasts, differentiated myotubes also showed increased VDR mRNA levels by treatment with $25(OH)D_3$ or $1,25(OH)D_3$, suggesting the presence of genomic transcriptional effects via the VDR. However, vitamin D_3 signaling did not result in hypertrophic effects; we observed only a minor increase in myotube diameter (19% in 3 days) by $25(OH)D_3$. Other studies demonstrated increases in myotube diameter by 80–100% after $1,25(OH)_2D_3$ or $25(OH)D_3$ treatment (Garcia et al., 2011; Girgis et al., 2014a). Differences in experimental set up may clarify the conflicting results. Studies which observed an effect of $1,25(OH)_2D_3$ or $25(OH)D_3$ on myotube diameter, used a prolonged cell culture model in which proliferation was immediately followed by myotube formation. Due to anti-proliferative effects of $1,25(OH)_2D_3$ or $25(OH)D_3$, a lower number of cells was present at the start of the differentiation in myotubes in the cultures that had been treated with $1,25(OH)_2D_3$ or

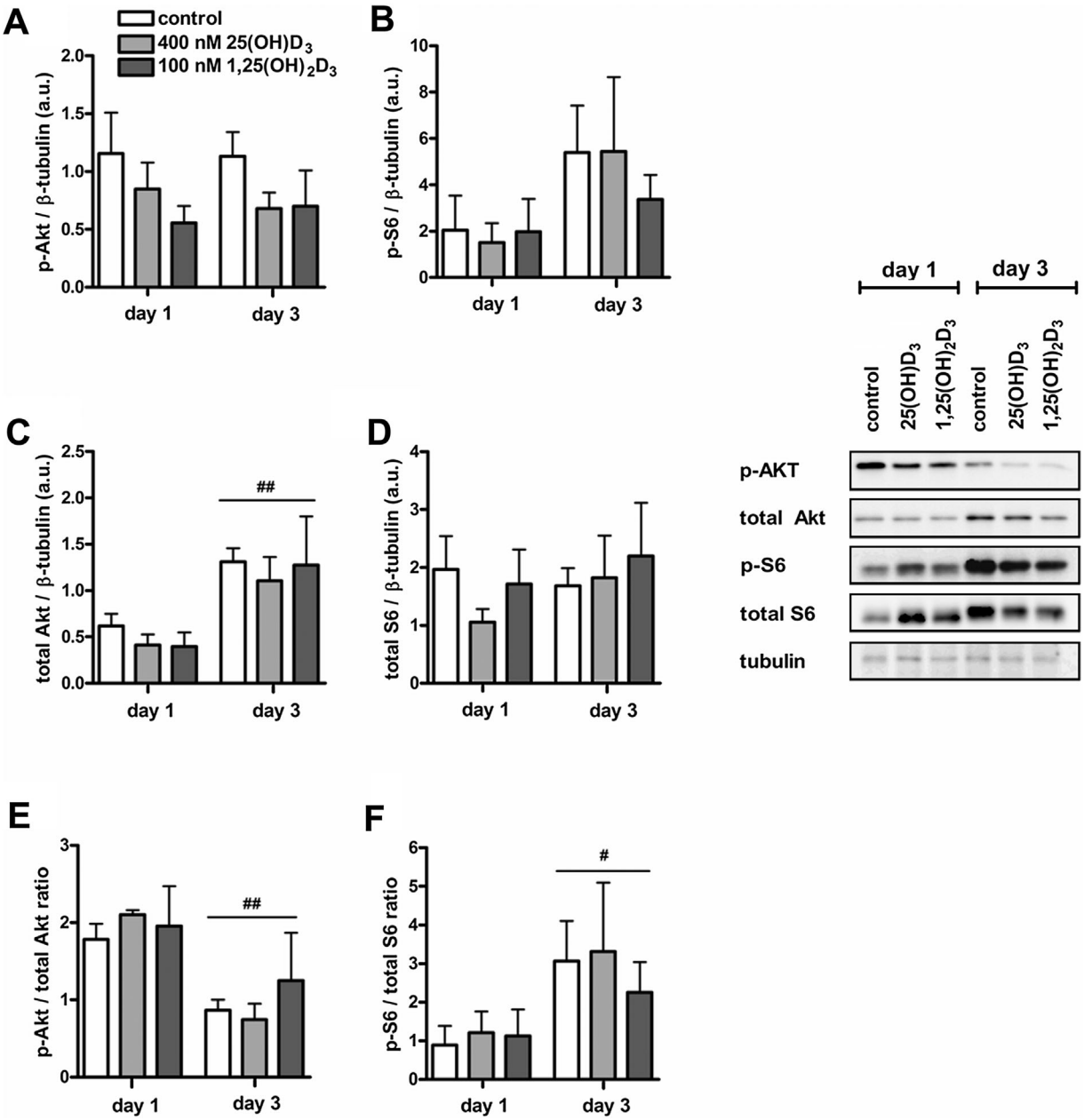

Fig. 6. Both 25(OH)D3 and 1,25(OH)2D3 did not affect levels of p-Akt, total Akt, p-S6, and total S6 in differentiating C2C12 cells. C2C12 cells were cultured for 3 days in differentiation medium supplemented with 400 nmol/L 25(OH)D3, 100 nmol/L 1,25(OH)2D3, or without any supplements. After 1 and 3 days of culture, levels of p-Akt (A), p-S6 (B), total Akt (C), total S6 (D) the ratio of p-Akt/total Akt (E), and p-S6/total S6 (F) were determined. Data were analyzed using a two-way ANOVA followed by Bonferroni's post hoc comparisons test. Values are mean ± SEM (n = 4). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ ($^{\#}$ between time period, * between vitamin D3 concentrations).

$25(OH)D_3$ compared to cell cultures without treatment. The lower number of myoblasts may have resulted in a lower number of myotubes, and thicker myotubes due to extra space in the culture well. This hypothesis is supported by data showing an optimal seeding density of human myoblasts in a 3D engineered collagen construct to obtain maximal force production of myotubes (Mudera et al., 2010). A high myoblast density may have a negative impact on myoblast force generating capacity and is associated with slow myosin expression (Mudera et al., 2010). In our study, myotube formation was investigated after starting with the same cell number and we did not observe any effect of $1,25(OH)_2D_3$ and only a minor effect of $25(OH)D_3$ on myotube number and size suggesting that both metabolites are not potent hypertrophic agents like for instance insulin-like growth factor-1 (IGF-1) (Stitt et al., 2004). To verify whether the lack of substantial hypertrophy was due to a lack of hypertrophic signaling, we investigated the activity of the Akt/mTOR signaling pathway, a key pathway involved in skeletal myotube hypertrophy (van Wessel et al., 2010) in response to $1,25(OH)_2D_3$ and $25(OH)D_3$. Akt mediates a wide range of cellular functions including cell proliferation, differentiation, gene transcription,

TABLE 2. Myotubes did not synthesize detectable $1,25(OH)_2D_3$ levels after exposure to $25(OH)D_3$

A	Myotubes		Osteoblasts positive control	
	$1,25(OH)_2D_3$ (pM) PRE	$1,25(OH)_2D_3$ (pM) POST	$1,25(OH)_2D_3$ (pM) PRE	$1,25(OH)_2D_3$ (pM) POST
0 nM $25(OH)D_3$	13.0	12.0 ± 1.0	<10.0	<10.0
400 nM $25(OH)D_3$	43.0	30.5 ± 0.5	43.3 ± 2.2	110.3 ± 13.5
1000 nM $25(OH)D_3$	76.0	52.5 ± 0.5	64.7 ± 4.8	183.0 ± 26.6
2000 nM $25(OH)D_3$	125.0	105.0 ± 3.0	–	–
B	Myotubes		Osteoblasts positive control	
	$24R,25(OH)_2D_3$ (nM) PRE	$24R,25(OH)_2D_3$ (nM) POST	$24R,25(OH)_2D_3$ (nM) PRE	$24R,25(OH)_2D_3$ (nM) POST
0 nM $25(OH)D_3$	<3.0	<3.0	<3.0	<3.0
400 nM $25(OH)D_3$	<3.0	6.8 ± 0.1	<3.0	70.2 ± 4.4
1000 nM $25(OH)D_3$	<3.0	7.4 ± 1.3	<3.0	105.4 ± 8.2
2000 nM $25(OH)D_3$	<3.0	14.5 ± 2.1	–	–

Myotubes and osteoblasts (positive control) were cultured in medium supplemented with increasing concentrations of 25(OH)D3. After 24 h, 1,25(OH)2D3 (A) and 24R,25(OH)2D3 (B) concentrations in non-conditioned (PRE) and conditioned (POST) culture medium were measured. Data are presented as mean ± SEM. Regarding the osteoblast culture, concentrations of 1,25(OH)2D3 and 24R,25(OH)2D3 in non-conditioned and conditioned medium have been published previously (van der Meijden et al., 2014).

and the rate of mRNA translation (Bodine et al., 2001; Glass, 2003; Gardner et al., 2012). Akt activation has been demonstrated by $1,25(OH)_2D_3$ during proliferation and differentiation of C2C12 myoblasts (Buitrago et al., 2012, 2013). However, in our study during differentiation no effect of $1,25(OH)_2D_3$ or $25(OH)D_3$ on the phosphorylation of Akt was observed. Moreover, downstream p-S6 was also not affected by both metabolites. Therefore, these results suggest that both $1,25(OH)_2D_3$ and $25(OH)D_3$ did not activate the Akt/mTOR signaling pathway in our model. These observations are in line with those of an in vivo rat study in which supra-physiological $1,25(OH)_2D_3$ levels did not result in muscle hypertrophy, but rather in muscle atrophy (Testerink et al., 2011a). This in vivo negative effect on muscle mass could be indirect, but in our cell culture model we investigated direct hypertrophic effects of $1,25(OH)_2D_3$ and $25(OH)D_3$ which were not present.

Expression of transcription factors that are essential for differentiation, including MyoD and myogenin, were also not affected by $25(OH)D_3$ or $1,25(OH)_2D_3$. However, at day 3 of differentiation MHC mRNA levels were increased by high concentrations of $1,25(OH)_2D_3$. Effects of $1,25(OH)_2D_3$ on MHC expression levels have been reported before, but those were not consistent (Okuno et al., 2012; Tanaka et al., 2014). In differentiating C2C12 myoblasts $1,25(OH)_2D_3$ decreased embryonic MHC, while in C2C12 differentiated myotubes $1,25(OH)_2D_3$ increased MHC-IIA mRNA expression (Okuno et al., 2012). In vivo injection of $1,25(OH)_2D$ in steers also showed an increased MHC-IIA expression (Korn et al., 2013). In contrast, in differentiating C2C12 myoblasts $1,25(OH)_2D_3$ supplementation has also been reported to increase mRNA levels of MHC-I, MHC-IIB, and MHC embryonic, without an effect on type IIA mRNA (Tanaka et al., 2014). Such differences may be due to differences in differentiation phases, medium composition or species differences (Okuno et al., 2012; Tanaka et al., 2014). In our study, $1,25(OH)_2D_3$ increased MHC mRNA levels in general, however an effect on myotube diameter was not observed. This suggests that the mRNA availability was sufficient and that the rate of mRNA translation was likely not affected yet. The mechanism by which $1,25(OH)_2D$ affects mRNA levels of MHC is not fully elucidated. Direct regulation of MHC mRNA levels by $1,25(OH)_2D$ is possible through binding to its receptor (Tanaka et al., 2014), but non-genomic actions of $1,25(OH)_2D$ such as the increase in intracellular calcium concentrations (de Boland and Boland, 1987) may also play indirectly a role in the regulation of MHC mRNA expression. Thus, based on our results and those reported in above mentioned studies, we conclude that $1,25(OH)_2D$ is able to increase mRNA levels of MHC isoforms, however effects seem to be determined by multiple factors. Effects of $25(OH)D_3$ on MHC expression were not observed.

Vitamin D_3 metabolism

CYP27B1 mRNA and protein expression have recently been shown in C2C12 myoblasts and C2C12 myotubes (Girgis et al., 2014a,b) as well as in primary murine myotubes (Girgis et al., 2014b) and regenerating murine muscle fibers in vivo (Srikuea et al., 2012). We confirmed the presence of CYP27B1 mRNA levels in both myoblasts and myotubes. Moreover, we also showed that myotubes have even higher levels of CYP27B1 mRNA compared to myoblasts, which suggests that myotubes were able to synthesize higher quantities of $1,25(OH)_2D_3$ than myoblasts. In addition, myotubes also have a higher uptake of $25(OH)D_3$ than myoblasts (Abboud et al., 2013). However, myotubes exposed to $25(OH)D_3$ did not synthesize detectable levels of $1,25(OH)_2D_3$. This is an unexpected finding as the presence of functional CYP27B1 has been reported in C2C12 myoblasts and primary mouse myotubes by performing luciferase reporter studies (Girgis et al., 2014a,b). Furthermore, it has been shown that CYP27B1 knockdown in C2C12 myoblasts abolishes the anti-proliferative effects of $25(OH)D_3$ (Garcia et al., 2011), which suggests that CYP27B1 is required for the actions of $25(OH)D_3$. The questions arises why in muscle cells in our study the presence of 1α-hydroxylase activity did not result in the synthesis of detectable $1,25(OH)_2D_3$ after $25(OH)D_3$ treatment. A possible explanation is that $1,25(OH)_2D_3$ was soon converted to $1,24R,25(OH)_3D_3$ by 24-hydroxylase, which is supported by the finding that CYP24 mRNA levels were strongly increased by $1,25(OH)_2D_3$. This explanation is supported by the observation that after 24 h $25(OH)D_3$ levels were strongly reduced to 34–42% of the non-conditioned concentrations, suggesting the presence of a very high vitamin D_3 metabolism in muscle cells. In osteoblasts, $25(OH)D_3$ levels are also reduced to 16–33% of non-conditioned concentrations (van der Meijden et al., 2014), but medium of these osteoblasts did show detectable levels of $1,25(OH)_2D_3$. Therefore, it is also possible that in C2C12 muscle cells 1α-hydroxylase activity was inhibited causing extremely low or absent $1,25(OH)_2D_3$ levels. In primary human osteoblast cultures, medium was supplemented with bovine serum albumin (BSA), but medium used in C2C12 cell cultures was supplemented with horse serum which may contain inhibiting factors such as transforming growth factor-β (TGF-β) (Turner et al., 2007) or growth factor independent-1 (GFI-1) (Dwivedi et al., 2005). Another explanation for the absent $1,25(OH)_2D_3$ levels in

C2C12 cells may be a loss of 1α-hydroxylase activity due to post-transcriptional abnormalities or deficient cofactors such as ferredoxin reductase or ferredoxin (Henry et al., 1992).

In addition to CYP27B1 expression, myoblasts and myotubes also expressed CYP24 mRNA. We show that $25(OH)D_3$ strongly increased CYP24 mRNA in myotubes and that myotubes were able to metabolize $25(OH)D_3$ to $24R,25(OH)_2D_3$. This result shows that muscle cells have a functional enzyme, that is, 24-hydroxylase, to regulate local $25(OH)D_3$ and and $1,25(OH)_2D_3$ concentrations. The 24-hydroxylase has been proposed to be responsible for the first step in degradation of $25(OH)D_3$ and $1,25(OH)_2D_3$, but several studies demonstrate that $24R,25(OH)_2D_3$ and $1,24R,25(OH)_3D_3$ may also play a role in bone tissue (Galus et al., 1980; Yamate et al., 1994; Erben et al., 1997; Seo et al., 1997; Yamamoto et al., 1998; van Driel et al., 2006b). The metabolites $24R,25(OH)_2D_3$ and $1,24R,25(OH)_3D_3$ stimulate osteoblast differentiation in vitro (van Driel et al., 2006b; van der Meijden et al., 2014). This raises the question whether the synthesized $24R,25(OH)_2D_3$ from $25(OH)D_3$ in our model is able to affect myoblast proliferation and differentiation. To the best of our knowledge, there is no literature available about $24R,25(OH)_2D_3$ actions on skeletal muscle cell proliferation and differentiation. Only in cardiac and vascular smooth muscle cells actions of $24R,25(OH)_2D_3$ have been reported, but these actions are all associated with calcium uptake by the cells and not with myogenesis. In vascular smooth muscle cells, $24R,25(OH)_2D_3$ is able to stimulate Ca^{2+}-ATPase and to reduce membrane L-type calcium channel activity as well as the intracellular calcium concentration (Shan et al., 1996). In cardiac myocytes, $24R,25(OH)_2D_3$ stimulates the calcium uptake by these cells, but less efficiently than $1,25(OH)_2D_3$ (Selles et al., 1994). Thus, it is possible that $24R,25(OH)_2D_3$ affects calcium uptake by skeletal muscle cells. Regarding the actions of $24R,25(OH)_2D_3$ in bone cells (van Driel et al., 2006b; van der Meijden et al., 2014), it is also possible that $24R,25(OH)_2D_3$ plays a role in skeletal muscle cell development or regeneration. Therefore, additional research is needed to investigate whether $24R,25(OH)_2D_3$ affects calcium uptake by skeletal muscle cells as well as skeletal muscle cell proliferation, differentiation and hypertrophy.

Limitations

Doses of $25(OH)D_3$ and $1,25(OH)_2D_3$ used in this study were relatively high compared to normal serum concentrations and care should be taken in the translation of these results to in vivo. However, differentiation experiments were also performed with low concentrations of $1,25(OH)_2D_3$ (1 nM) and $25(OH)D_3$ (100 nM) (see Figs. S1–S3). Low concentrations of $1,25(OH)_2D_3$ increased VDR and CYP24 mRNA levels in differentiating myoblasts similarly as the higher concentrations. Incubation of differentiating myoblasts with low concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ did not lead to altered MyoD or myogenin mRNA levels nor to myotube hypertrophy. Thus, low concentrations of $1,25(OH)_2D_3$ and $25(OH)D_3$ also did not have marked effects in our cell culture model with respect to differentiation and myotube size. Regarding the rate of proliferation, the anti-proliferative effects of $25(OH)D_3$ and $1,25(OH)_2D_3$ at lower concentrations have been reported previously in literature (1–100 nmol/L $25(OH)D_3$; 1–100 nmol/L $1,25(OH)_2D_3$) (Okuno et al., 2012; Girgis et al., 2014a). Note, however, that tissue concentrations of $1,25(OH)_2D_3$ can be higher than serum concentrations because of the local conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$. The use of relatively high doses of $25(OH)D_3$ and $1,25(OH)_2D_3$ did allow us to compare our results with those from other studies using C2C12 myoblasts and myotubes (Garcia et al., 2011; Srikuea et al., 2012; Girgis et al., 2014a).

The metabolite $1,25(OH)_2D_3$ was not detected in medium after treatment of C2C12 myotubes with $25(OH)D_3$ which was probably due to very fast vitamin D_3 metabolism in these cells. Synthesized $1,25(OH)_2D_3$ may be rapidly converted to $1,24R,25(OH)_3D_3$ and therefore further research is needed to examine whether there is detectable $1,25(OH)_2D_3$ synthesis on earlier time points.

Conclusion

This in vitro study shows that C2C12 myoblasts not only respond to $1,25(OH)_2D_3$, but also to the precursor $25(OH)D_3$ by reducing their proliferation and increasing their VDR expression. In differentiating myoblasts and myotubes, $1,25(OH)_2D_3$ as well as $25(OH)D_3$ stimulate VDR mRNA and in myotubes $1,25(OH)_2D_3$ also stimulates MHC mRNA expression. However, this occurs without notable effects on expression of myogenic regulatory factors and myotube size. Interestingly, C2C12 myoblasts and myotubes express CYP27B1 and CYP24 mRNA which are required for vitamin D_3 metabolism. Although CYP27B1 activity could not be shown in myotubes, after treatment with $1,25(OH)_2D_3$ or $25(OH)D_3$ C2C12 muscle cells showed strongly increased CYP24 mRNA levels and were able to synthesize $24R,25(OH)_2D_3$ from $25(OH)D_3$. Since $24R,25(OH)_2D_3$ stimulates osteoblast differentiation in vitro, this metabolite may play a role in myoblast differentiation as well. These data suggest that skeletal muscle is not only a direct target for vitamin D_3 metabolites, but is also able to metabolize $25(OH)D_3$ and $1,25(OH)_2D_3$.

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