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Chapter 9

Role of Vitamin D in Myogenesis

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Abstract

The secosteroid, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] plays a crucial role in regulating bone formation, remodeling and repair. Beyond its well-established role in skeletogenesis, gene-targeting studies support a physiological role for 1α,25$(OH)_2D_3$ in muscle development. There is evidence for expression of vitamin D receptor/vitamin D synthesizing enzyme/transport protein, local production of 1α,25$(OH)_2D_3$ and uptake of 25$(OH)D_3$, implying the existence of vitamin D-endocrine system in myogenic cells. Recently, much interest has been devoted to the effects of 1α,25$(OH)_2D_3$ on myogenesis. Simply stated, 1α,25$(OH)_2D_3$ has potent antiproliferative activity on myoblasts and inhibits myoblast differentiation. Intriguingly, recent studies suggest that 1α,25$(OH)_2D_3$ may stimulate protein synthesis in myotubes and have a role in self-renewal of muscle stem cells. 1α,25$(OH)_2D_3$ regulates myogenesis probably through its genomic or nongenomic actions. Understanding how vitamin D signaling contributes to muscle homeostasis may provide a valuable insight into an effective intervention strategy for muscle disorders. In this review, we summarize the current knowledge about a possible role of vitamin D in myogenesis.

Keywords: skeletal muscle, proliferation, differentiation, vitamin D, myogenesis

1. Introduction

The biologically active metabolite of vitamin D, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] functions by binding to vitamin D receptor (VDR) [1]. Thus, accurate identification of VDR in tissues is critical to understand the physiological and pathological significance of vitamin D [2]. The VDR has shown to be expressed in a wide variety of tissues, including bone, bronchus, intestine, kidney, mammary gland, pancreas, parathyroid, pituitary gland, prostate gland, spleen, testis and thymus [2]. However, in skeletal muscle, some controversies have existed [3–9]. Despite conflict, gene-targeting studies suggest a physiological role for 1α,25(OH)$_2$D$_3$ in muscle development. VDR null-mutant mice exhibit impaired muscle development [10, 11]. Intriguingly, there is evidence for expression of vitamin D receptor/vitamin D synthesizing enzyme/transport protein [6, 9, 12–16], local conversion of 25(OH)D$_3$ to 1α,25(OH)$_2$D$_3$ [6, 15] and uptake of 25(OH)D$_3$ [17], implying the existence of vitamin D-endocrine system in myogenic cells.

Recently, much interest has been devoted to the effects of 1α,25(OH)$_2$D$_3$ on myogenesis. It has been generally supposed that 1α,25(OH)$_2$D$_3$ exerts its effects through binding to VDR, inducing genomic or nongenomic actions. A genomic action occurs through nuclear transportation of 1α,25(OH)$_2$D$_3$-VDR complex [1]. VDR heterodimerizes with 9-cis-retinoic acid receptor (RXR), which modulates gene expression via binding to specific target gene promoter regions, known as vitamin D response elements (VDREs) [1]. The very large number of genes (estimated at approximately 3% of the mouse or human genome) is regulated, directly and/or indirectly, by vitamin D-endocrine system [18]. A nongenomic response to 1α,25(OH)$_2$D$_3$ is characterized by a rapid (the seconds to minutes range) activation of signaling cascades and an insensitivity to inhibitors of transcription and protein synthesis [19]. Although a consensus cannot be developed about how the nongenomic actions are initiated [1], several data suggest that nongenomic actions begin at the plasma membrane and occur through a putative plasma membrane-associated receptor [VDR(mem)] present in a caveolae [20] and a 1α,25(OH)$_2$D$_3$-membrane-associated rapid response steroid (1α,25D$_3$-MARRS) binding protein [21]. Although relative contribution of genomic and nongenomic action to myogenesis is currently unknown, 1α,25(OH)$_2$D$_3$ appears to have antiproliferative effect on myoblasts [9, 12–15] and inhibits myoblast differentiation [9, 16, 18]. Some hypotheses are currently proposed that 1α, 25(OH)$_2$D$_3$ may have anabolic effects on protein metabolism in myotubes [12, 15] and may be involved in self-renewal of muscle stem cells [9].

Better characterization of effects of vitamin D in myogenic cells will help to provide a valuable insight into an effective intervention strategy for muscle disorders such as sarcopenia, myopathy and neuromuscular diseases. Here, we will summarize the current evidence for a role of vitamin D in myogenesis.

2. VDR expression in skeletal muscle and myogenic cells

2.1. Animal studies

Although a number of studies have been published on the expression of VDR in skeletal muscle by immunohistochemistry, the specificity of various commercially available VDR antibodies used is called into question by Wang et al. [22]. They systematically characterize these antibodies in terms of their specificity and immunosensitivity using negative control samples from VDR knockout mice. For example, despite widely used for immunohistochemical studies, the rat monoclonal antibody (9A7, Affinity BioReagents) that recognizes an epitope between amino acid residues 89 and 105 of human VDR has been shown to bind the VDR but nonspecifically cross-react with other unidentified proteins from mice [22]. They identify the mouse monoclonal VDR antibody against the C-terminus of human VDR (D-6, Santa Cruz Biotechnology), which possesses high specificity, high sensitivity and versatility [22]. They show that VDR protein is not detected in muscle fibers from 6- to 7-week-old C57BL/6 mice [8]. In contrast, Girgis et al. [6] demonstrated that VDR protein is localized to nuclei of muscle fibers from 3-week-old C57BL/6 mice using the same antibody. These data suggest that VDR expression level may be dependent on the age of animal because its expression is progressively decreased with advancing age [6]. However, intriguingly, VDR has shown to be re-expressed in the regenerating muscle fibers from 12-week-old C57BL/6 mice [13]. Immunohistochemical analysis using a rabbit polyclonal antibody demonstrated that VDR is clearly detected in the central nuclei of newly formed regenerating muscle fibers but not in noninjured muscle fibers, suggesting that vitamin D signal via VDR may be involved in muscle regeneration irrespective of age. Besides immunohistochemical evidence, Srikuea et al. [13] provide strong evidence for the presence of VDR in mouse C2C12 cells, by combining PCR-based cloning and DNA sequencing. The full-length VDR mRNA transcript could be isolated from myoblasts and myotubes.

2.2. Human studies

Costa et al. [23] initially identify $1\alpha,25(OH)_2D_3$ binding protein in cloned human skeletal muscle cells derived from patients undergoing orthopedic surgery by a radiolabelled-ligand binding assay. The cloned human myogenic cells have a binding protein compatible with classical $1\alpha,25(OH)_2D_3$ receptors and functionally responded to $1\alpha,25(OH)_2D_3$ at physiological concentrations [23]. Bischoff et al. [3, 4] report the nuclear localization of VDR in muscle fibers from middle-aged and older female patients with osteoarthritis/osteoporosis or undergoing surgery using a rat monoclonal antibody (9A7). Ceglia et al. [5] investigate VDR expression in skeletal muscles from healthy postmenopausal women aged 65–85 years using a mouse monoclonal VDR antibody (H4537, R&D systems) whose accuracy is confirmed by immunoblot analysis using a mouse monoclonal VDR antibody (D-6). They report that VDR is detected in the nuclei of muscle fibers without relation to muscle fiber subtype. Ryan et al. [7] demonstrate that VDR is detectable in human muscle fibers by immunohistochemistry and immunoblot analysis using a polyclonal antibody against the human VDR. However, Olsson et al. [9] show that VDR is undetectable in muscle fibers from healthy individuals of age 20–27 years by immunoblot analysis using a mouse monoclonal VDR antibody (D-6). Overall, we cannot rule out the possibility of VDR expression in both animal and human muscle fibers at low levels. However, conflicting data should be carefully discussed because the detection of VDR expressed in muscle fibers may be dependent on the methodology for protein extraction and primary antibody. Indeed, Girgis et al. [6] note that the importance of specific conditions in the detection of VDR proteins expressed at low levels in skeletal muscle.

3. Vitamin D metabolism

3.1. Vitamin D metabolism in the liver and kidney

The process of 1α,25(OH)$_2$D$_3$ synthesis is highly regulated. Vitamin D, in the form of vitamin D$_3$, is nonenzymatically synthesized from 7-dehydrocholesterol in the skin through the action of ultraviolet irradiation [24]. Alternatively, vitamin D, in the form of either vitamin D$_2$ or vitamin D$_3$, can also be taken in the diet [24]. A biologically active form, 1α,25(OH)$_2$D$_3$, is synthesized from vitamin D$_3$ through two hydroxylation steps [24]. Vitamin D$_3$ is converted to 25-hydroxyvitamin D$_3$ [25(OH)D$_3$] in the liver by 25-hydroxylases (encoded by the gene *CYP27A1*) [24]. The generated 25(OH)D$_3$ is further hydroxylated to 1α,25(OH)$_2$D$_3$ by 25-hydroxyvitamin D$_3$ 1α-hydroxylase (encoded by the gene *CYP27B1*) in the kidney [24]. The synthesis of 1α,25(OH)$_2$D$_3$ from 25(OH)D$_3$ is stimulated by parathyroid hormone (PTH) and suppressed by calcium, inorganic phosphate and 1α,25(OH)$_2$D$_3$ itself [1]. As just described, it is generally recognized that vitamin D is metabolized sequentially in the liver and kidney. However, CYP27B1 seems to be more widely distributed in several extrarenal tissues than previously expected, such as skin, placenta, colon, pancreas, vasculature and brain [25–27]. Several lines of evidence suggest that myogenic cells have the ability to internalize circulating 25(OH)D$_3$ into the cell cytoplasm and subsequently locally convert it to 1α,25(OH)$_2$D$_3$. In the following section, we will briefly summarize our understanding of the uptake of 25(OH)D$_3$ and local synthesis of 1α,25(OH)$_2$D$_3$ occurred in myogenic cells.

3.2. Uptake of 25(OH)D$_3$ in myogenic cells

Vitamin D metabolites are lipophilic molecules with low aqueous solubility that must be transported in the circulation bound to plasma proteins [28]. The most important of these carrier proteins is the vitamin D-binding protein (DBP) [29]. Almost all circulating vitamin D metabolites are bound to DBP, to a lesser extent, to albumin and lipoproteins [28], providing a major impact on their pharmacokinetics. DBP-bound vitamin D metabolites restrict access to target cells [29] and, therefore, are less susceptible to hepatic metabolism and subsequent biliary excretion, leading to a longer circulating half-life [28]. 25(OH)D$_3$ does not simply diffuse into the cells. In the kidney proximal tubule, uptake of the 25(OH)D$_3$-DBP complex by epithelial cells occurs depending on receptor-mediated endocytosis via the multiligand megalin/cubilin tandem receptor [30]. Both receptors are expressed primarily in polarized epithelial cells [28]. Once internalized by epithelial cells, DBP is degraded in lysosomes, releasing 25(OH)D$_3$ for activation to 1α,25(OH)$_2$D$_3$ by CYP27B1 [30]. Intriguingly, it seems likely that muscle fibers endocytose 25(OH)D$_3$ through a similar mechanism. In support of this idea, megalin and cubilin are expressed in muscle fibers [17]. Uptake of ^{3}H-labeled 25(OH)D$_3$ into C2C12 myotubes was drastically reduced by megalin inhibitor [17]. These data suggest that 25(OH)D$_3$ may be internalized through megalin/cubilin-mediated endocytosis in myogenic cells.

3.3. Conversion of $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$ in myogenic cells

Several studies demonstrate that myogenic cells express key components of the vitamin D-endocrine system, including VDR, CYP27B1, CYP24A1 and DBP [6, 12–16]. It is supposed therefore that localized, muscle-specific, conversion of $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$ might drive many of the recognized effects of vitamin D. Srikuea et al. [13] report that C2C12 cells express the full-length CYP27B1 mRNA transcript and CYP27B1 protein is detected in the cytoplasm of myoblasts, exhibiting partially overlapping with the mitochondria to which CYP27B1 has been reported to be typically localized [31]. Girgis et al. [6, 15] confirm this possibility using a luciferase reporter assay system. In this system, luciferase activity results from $1\alpha,25(OH)_2D_3$ binding to GAL-4-VDR and subsequent activation of the UASTK-luciferase gene through its GAL4 promoter. The assay system show a dose-dependent increase in luciferase activity after 24 hours treatment with $25(OH)D_3$, indicating the intracellular conversion of $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$ by CYP27B1 and the subsequent activation of luciferase expression through $1\alpha,25(OH)_2D_3$-bound GAL4-VDR [6, 15]. Although the functional importance of CYP27B1 in myogenic cells has not been fully understood, these data provide evidence that this enzyme is biologically active and mediates to convert $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$.

4. Vitamin D signaling

4.1. Genomic pathway

Microarray analysis in squamous cell carcinoma treated with $1\alpha,25(OH)_2D_3$ identifies many genes including cell adhesion, cytoskeleton, extracellular matrix, growth factors/receptors, signal transduction, transcription factors, cell cycle and channels/transporters [32–34]. Profiling data provide insight into a much broader range of action for $1\alpha,25(OH)_2D_3$. With respect to several genes related to myogenesis, we will describe them in more detail. A consensus DR3-type VDRE has been identified in the dysferlin (DYSF) promoter [34], which plays a crucial role in membrane repair in skeletal muscle [35]. $1\alpha,25(OH)_2D_3$ increases dysferlin expression in human myotubes from carriers of one mutation in the DYSF gene probably through the binding of VDR to promoter of the DYSF gene [36]. FOXO1 has also the same element in the promoter [34]. Foxo1 is a member of the Foxo subfamily of forkhead/winged helix family of transcription factors, governs muscle growth, metabolism and myoblast differentiation. Foxo1 physically and functionally interacts with Notch by promoting corepressor clearance from DNA-binding protein, CSL [CBF1/RBPjk/Su(H)/Lag-1], leading to inhibition of myoblast differentiation through activation of Notch target genes [37]. Integrin β3 has a consensus sequence (−–756/−770) in the immediate 5′-flanking region [38]. The integrins are heterodimeric cell surface receptors, formed by the combination of 18 α-subunits and 8 β-subunits, mediate adhesion to the extracellular matrix [39]. Deficiency of integrin β3 in myoblast decreases Rac1-GTPase activity, downregulates myogenin expression, disrupts focal adhesion formation and interrupts actin organization, resulting in impaired myoblast migration and fusion [40]. Similarly, a β3-integrin-neutralizing antibody blocks myotube formation [40]. Consistent with in vitro studies, integrin β3 null-mutant mice reveals a defective muscle regeneration [40]. Id (inhibitor of differentiation) gene is also known as target of $1\alpha,25(OH)_2D_3$ [41]. $1\alpha,25(OH)_2D_3$ exerts its negative effect on Id1 gene transcription via the 57 bp upstream response sequence (−1146/−1090) [41]. Id proteins (Id1, Id2, Id3 and Id4) dimerize and neutralize the transcriptional activity of basic helix-loop-helix proteins [42]. Id inhibits MyoD activity either by forming transcriptionally inactive complexes of MyoD-Id or by forming heterodimers with E-proteins and effectively blocking the formation of active MyoD/E-protein complexes [43]. VDR null-mutant mice show no differences in expression levels of Id1 and Id2 in skeletal muscle [44]. Finally, expression of insulin-like growth factor binding proteins (IGFBPs) is known to be under the control of multiple VDREs [45]. The IGF system includes not only three ligands (insulin, IGF-I and IGF-II), three receptors [the insulin receptor (IR), the IGF-I receptor (IGF-IR) and the mannose-6-phosphate IGF-II receptor (M6P/IGF-IIR)] but also six IGFBPs. IGFs through its receptor signal can stimulate both myoblast proliferation and differentiation, which are two mutually exclusive biological events during myogenesis [46]. In the circulation, IGFBPs act as carrier proteins for the IGFs and regulate IGFs turnover, transport and half-life [47]. At the local level, they function as modulators of IGF activity [48]. Of six human IGFBP genes, IGFBP-1, -3 and -5 have been shown to be primary $1\alpha,25(OH)_2D_3$-target genes [45]. IGFBP-5 is the major IGFBP secreted by skeletal muscle and appears to mediate IGF-II expression via an autoregulatory loop mechanism [46]. In belief, the induction of IGFBP-5 occurs earlier than the induction of IGF-II in early stages of myogenesis. IGFBP-5 located on the cell surface binds to autocrine IGF-II and potentiates its interaction with IGF-IR, leading to the enhanced activation of the IGF-IR-PI3K-Akt signaling pathway. Consequently, IGF-II expression is further increased, promoting myoblast differentiation [49]. Besides the regulation of IGF bioavailability, IGFBP-5 directly regulates the transcriptional response of osteoblasts to $1\alpha,25(OH)_2D_3$. IGFBP-5 interacts with VDR and prevents VDR:RXRα heterodimerization, probably leading to impair vitamin D-stimulated transcription and cell cycle arrest [49].

Recently, Ryan et al. [7] report that 1947 mRNAs are differentially expressed in human myogenic cells following treatment of $1\alpha,25(OH)_2D_3$. The most significantly increased or repressed mRNA are CYP24A1 (>25,000-fold) or calpain 11 (0.1-fold), respectively. Messenger RNAs encode proteins involved in muscle relaxation (Parvalbumin), protein synthesis (Ig-like, fibronectin type III domain-containing 1), cytoskeletal dynamics [Rho Guanine Nucleotide Exchange Factor (GEF) 16], RNA and nucleotide binding (Tudor domain-containing protein 10), cellular energy metabolism (insulin-like growth factor 3), apoptosis (Fas apoptotic inhibitory molecule 2) and nucleosome function (Histone cluster 1, H3j) are significantly upregulated, whereas messenger RNAs encode proteins involved in cell migration (Podocan) and cellular proliferation (WAP four-disulfide core domain 1) are significantly downregulated [7]. Very little is currently known about the direct $1\alpha,25(OH)_2D_3$-target genes in myogenic cells, it is to be hoped that future research will clarify this issue.

4.2. Nongenomic pathway

After short-term treating (1–10 min) chick myoblasts with $1\alpha,25(OH)_2D_3$, translocation of VDR from the nucleus to the plasma membrane rapidly occurs [50]. Microtubule-depolymerizing agents block the translocation [50]. The translocation depends on intact caveolae that are specialized plasmalemmal microdomains [51]. Methyl-beta-cyclodextrin, which disrupts the caveolae structure, abolishes $1\alpha,25(OH)_2D_3$-dependent VDR translocation to the plasma membrane [52]. In addition, chemically-induced disruption of caveolae and small-interfering RNA (siRNA)-mediated silencing of caveolin-1 suppress $1\alpha,25(OH)_2D_3$-dependent activation of proto-oncogene, c-Src [52]. Caveolin-1 colocalizes with c-Src near the plasma membrane under basal conditions [52]. After treating with $1\alpha,25(OH)_2D_3$, the colocalization is disrupted and they are redistributed into cytoplasm and nucleus [52]. Therefore, it can be hypothesized that (1) interaction caveolin-1/c-Src inactivates the kinase under basal conditions and (2) when $1\alpha,25(OH)_2D_3$ stimulates VDR translocation to the plasma membrane, it dissociates the caveolin-1/c-Src complex, allowing c-Src activation [52]. The initial activation of c-Src by $1\alpha,25(OH)_2D_3$ is assumed to be a gateway to the nongenomic actions in myogenic cells [53, 54]. $1\alpha,25(OH)_2D_3$ can potentially activate multiple signaling pathways, including cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA), PKC, calmodulin/calmodulin-dependent kinase, protein kinase B (PKB/Akt) and multiple mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 and c-Jun NH_2-terminal 1 and 2 [53]. For example, short hairpin RNA (shRNA)-mediated silencing of VDR in C2C12 myoblasts reduces activation of c-Src, ERK1/2, p38, and Akt induced by $1\alpha,25(OH)_2D_3$ [55, 56], suggesting that $1\alpha,25(OH)_2D_3$ exerts nongenomic actions through VDR in myogenic cells. It should be noted, however, that their relative contribution to myogenesis remains to be established. For further details on nongenomic pathways in myogenic cells, excellent reviews are available [53, 54].

The rapid nongenomic effect observed in myogenic cells is likely to be mediated, at least in part, by VDR-independent mechanisms. Indeed, studies using myoblasts lacking VDR demonstrates that ERK1/2 and Akt phosphorylation by $1\alpha,25(OH)_2D_3$ is only partially suppressed [55, 56]. A putative membrane-associated receptor for $1\alpha,25(OH)_2D_3$, $1,25D_3$-MARRS has been purified from chick intestinal basal lateral membranes [21], cloned and sequenced [57]. A full-length cDNA of $1,25D_3$-MARRS is identical to that previously described as glucose-regulated thiol-disulfide oxidoreductase protein precursor, ERp57, as it has also been referred to as GRP58, ERp60/61, PDI-Q2 and PDIA3 [58]. Studies using neutralizing antibodies [59, 60], ribozyme technology [58] and *Cre/loxP* technology [61, 62] demonstrate that $1,25D_3$-MARRS mediated at least some nongenomic actions of $1\alpha,25(OH)_2D_3$. A rapid activation of PLC and PKC by $1\alpha,25(OH)_2D_3$, which is normally observed in chondrocytes derived from the growth zone, is not reduced in cells from VDR null-mutant mice [59]. However, these responses are blocked by rabbit polyclonal antibody against the N-terminal sequence of the $1,25D_3$-MARRS [59]. Although no experimental data are currently available, $1\alpha,25(OH)_2D_3$, possibly through $1,25D_3$-MARRS, may act directly on the muscle cell membrane.

5. Role of vitamin D signaling in muscle development

5.1. VDR null-mutant mice

Gene knockout mice provide an excellent possibility to investigate complex regulatory systems that cannot be modeled in cell culture systems in vitro. In two independent laboratories [63, 64], VDR null-mutant mice are generated by gene targeting. Yoshizawa et al. [63] disrupt exon 2 of the VDR gene, which encodes the first zinc finger motif in the DNA-binding domain essential for the biological functions of VDR, whereas Li et al. [64] ablate a VDR fragment spanning exons 3–5, which encode the second zinc finger motif in the DNA-binding domain. Both VDR null-mutant mice are phenotypically normal at birth and grow properly until weaning, but thereafter show various pathological conditions including hypocalcemia, hypophosphatemia, secondary hyperparathyroidism and osteomalacia, as a typical feature of human vitamin D-dependent rickets type II [63, 64]. This phenotype is progressively exacerbated until VDR null-mutant mice are fed a rescue diet containing the high-calcium, -phosphorus and -lactose [65].

5.2. Muscle morphology and gene expression in VDR null-mutant mice

At the age of 3 week, no significant differences are observed between VDR null-mutant mice [63] and wild-type mice in body weight or serum concentrations of calcium, phosphate, alkaline phosphatase and vitamin D metabolites [10]. At the cellular level, the skeletal muscle from VDR null-mutant mice has already begun to atrophy (approximately −20%, regardless of muscle fiber types) compared with the wild-type mice [10], implying that muscle atrophy observed in 3-week-old VDR null-mutant mice occurs independent of secondary systemic metabolic changes. By 8 weeks of age, the morphological changes further progress probably because of the absence of VDR or the systemic metabolic changes that had not been present at 3-week [10]. The morphological abnormalities are observed in VDR null-mutant mice fed a rescue diet [10], suggesting that the absence of VDR is likely to be a major cause rather than the secondary systemic metabolic changes. Finally, neither degenerative nor necrotic changes are observed in skeletal muscle from VDR null-mutant mice [10]. Similar results are obtained with biceps femoris, medial gastrocnemius, anterior tibial and soleus muscles [10]. Therefore, the skeletal muscle abnormalities in VDR null-mutant mice may occur diffusely spread throughout the body. At the gene level, Myf5, myogenin, E2A, embryonic and neonatal myosin heavy chain (MyHC) genes, which should have already been downregulated in wild-type mice, are still expressed in VDR null-mutant mice [10]. These data suggest two possibilities as to impaired muscle development. One possibility is that the absence of VDR affects myogenesis during embryonic stage. In favor of the first possibility, it is noteworthy that VDR is primarily expressed in chick skeletal muscle at embryonic stage rather than at adult stage and VDR-binding activity gradually decreased between embryonic and perinatal stages to levels that is equal to low activity observed in adult stage [66]. Consistent with VDR null-mutant mice, newborn rats from vitamin D-deficient mothers have smaller muscle fibers compared to newborn rats from vitamin D-adequate mothers [67]. When VDR expression is suppressed by siRNA in myoblasts, they failed to differentiate into myotubes [16].

Taken together, impaired muscle development may be a consequence of defective myogenesis during embryonic and fetal stages. On the other hand, if overall myoblast differentiation occurred normally as the authors noted [10], another possibility should be taken into account. Vitamin D-deficient rats show a decrease in type II muscle fiber size concomitantly with an increase in protein degradation and decrease in protein synthesis [68]. Vitamin D deficiency leads to upregulation of muscle-specific E3 ubiquitin ligases, muscle atrophy F-box (MAFbx)/Atrogin-1 and/or muscle ring-finger protein 1 (MuRF1) [11, 68] that are transcriptionally increased before and at the onset of muscle wasting [69] and subsequently activated ubiquitin-proteasome pathway [68]. MAFbx/Atrogin-1 targets MyoD for degradation in several models of skeletal muscle atrophy [70], whereas MuRF1 is involved in degradation of MyHC protein in dexamethasone-treated skeletal muscle [71]. More recently, some ubiquitin-proteasome-related genes are shown to be upregulated in skeletal muscle of newborn rat from vitamin D-deficient mothers compared with that of newborn rat from vitamin D-adequate mothers [67]. The newborn rats show smaller muscle fiber size than the control rats [67]. Therefore, impaired muscle development may result from an imbalance between protein synthesis and degradation. To more effectively address the issue, tissue-specific deletion of VDR at early or late stage of muscle development or conditional postnatal deletion may help to clarify the role of VDR.

6. Effects of vitamin D on myogenesis in vitro

6.1. Myoblast proliferation

Some studies show that $1\alpha,25(OH)_2D_3$ stimulates myoblast proliferation. Giuliani et al. [72] report that $1\alpha,25(OH)_2D_3$ (0.13 nM) increases cell density of chick myoblasts. Drittanti et al. [73] show the biphasic effects of $1\alpha,25(OH)_2D_3$ (0.1 nM) that exhibited a mitogenic effect during the stage of myoblast proliferation. In contrast, most studies suggest that $1\alpha,25(OH)_2D_3$ or $25(OH)D_3$ has antiproliferative effect on myogenic cells [9, 12–16]. $1\alpha,25(OH)_2D_3$ (1–100 nM) inhibits proliferation of C2C12 myoblasts in a dose-dependent manner [11–13] without inducing necrotic and apoptotic cell death [9, 15]. To elucidate the mechanism by which $1\alpha,25(OH)_2D_3$ exerts its antiproliferative effect, Okuno et al. [14] perform cell cycle analysis using flow cytometry. $1\alpha,25(OH)_2D_3$ arrests the cells in the G0/G1 phase concomitantly with induction of cyclin-dependent kinase (CDK) inhibitors, $p21^{WAF1/CIP1}$ that facilitates cell cycle withdrawal [74] and $p27^{Kip1}$ that inhibits a wide range of CDKs essential for cell cycle progression [75]. Girgis et al. [4] also report the increased expression of genes involved in G0/G1 arrest including Rb (retinoblastoma protein) and ATM (ataxia telangiectasia mutated) and decreased expression of genes involved in G1/S transition, such as c-myc and cyclin D1. Hypophosphorylated Rb protein is decreased [15] that is active form, blocks entry into S-phase by inhibiting the E2F transcriptional program [76, 77]. Overall, these data support the antiproliferative role of $1\alpha,25(OH)_2D_3$ in myogenic cells.

6.2. Myoblast differentiation

Some studies [78, 79] report that $1\alpha,25(OH)_2D_3$ (0.1 or 1 nM) has inhibitory effects on DNA synthesis in differentiating chick myoblasts, with an increase in MyHC expression, an increase in myofibrillar and microsomal protein synthesis and an elevation of creatine kinase activity. Garcia et al. [12] report that prolonged treatment of C2C12 myoblasts with $1\alpha,25(OH)_2D_3$ (100 nM) enhanced myoblast differentiation by inhibiting cell proliferation and modulating the expression of promyogenic and antimyogenic growth factors. $1\alpha,25(OH)_2D_3$ downregulates insulin-like growth factor-I (IGF-I) and myostatin expression and upregulates IGF-II and follistatin expression [12]. Follistatin antagonizes myostatin-mediated inhibition of myogenesis [80]. Intriguingly, inhibition of myostatin is characterized by increased expression of IGF-1 and IGF-II [81–86], which are known to be potent stimulus of myogenesis [45, 87]. Therefore, it can be hypothesized that $1\alpha,25(OH)_2D_3$ may contribute to myogenesis by inducing IGF-II expression through modulation of myostatin-follistatin system. It should be noted, however, that only small thin myotubes with few nuclei are observed until day 10 [12]. This may not recapitulate normal C2C12 myoblast differentiation as previously reported [88].

In general, C2C12 myoblasts normally proliferate and are mononucleated when kept subconfluently in high-mitogen medium (e.g., 10–20% fetal bovine serum). To initiate cell cycle exit and myogenic differentiation, by switching from high-mitogen medium to low-mitogen medium (e.g., 2% horse serum), they fuse and differentiate into postmitotic, elongated and multinucleated myotubes. Using this C2C12 myoblast differentiation system, Buitrago et al. [89] show that $1\alpha,25(OH)_2D_3$ (1 nM) enhanced the expression of MyHC and myogenin at 72 h after treatment. Okuno et al. [14] examine the effects of $1\alpha,25(OH)_2D_3$ (1–100 nM) on differentiating and differentiated stage of C2C12 myoblasts. In differentiating phase, $1\alpha,25(OH)_2D_3$ treatment downregulates the expression of neonatal MyHC and myogenin and inhibits myotube formation in a dose-dependent manner [14]. The expression of fast-type MyHC isoforms is increased when fully differentiated myotubes are treated with $1\alpha,25(OH)_2D_3$ [14, 16]. Girgis et al. [15] investigate the prolonged treatment of $1\alpha,25(OH)_2D_3$ (100 nM) on C2C12 myoblast differentiation. When myoblast is treated with $1\alpha,25(OH)_2D_3$ throughout proliferative, differentiating and differentiated stages, myotube formation is delayed by day 10 concomitantly with downregulation of Myf5 and myogenin [15]. However, myotubes treated with $1\alpha,25(OH)_2D_3$ exhibited larger cell size than nontreated myotubes [15]. These results suggest that $1\alpha,25(OH)_2D_3$ may biphasically act in the process of early and late myoblast differentiation. They show that $1\alpha,25(OH)_2D_3$-mediated hypertrophic effect on myotubes is accompanied with downregulation of myostatin [15]. Several studies have provided evidence that myostatin acts as a negative regulator of the Akt/mammalian target of rapamycin (mTOR) signaling pathway [90–93], which plays a key role in the regulation of protein synthesis [94]. For example, Trendelenburg et al. [92] show that myostatin reduces Akt/mTOR signaling complex 1 (TORC1)/p70 S6 kinase (p70S6K) signaling, inhibiting myoblast differentiation and reducing myotube size. Intriguingly, $1\alpha,25(OH)_2D_3$ sensitizes the Akt/mTOR signaling pathway to the stimulating effect of leucine and insulin, resulting in a further activation of protein synthesis in C2C12 myotubes [95]. Recently, however, Olsson et al. [9] report that myostatin expression remains unchanged in response to 1α,25$(OH)_2D_3$ in human muscle precursor cells. These conflicting results may be due to the variable methods of analysis and to the species differences. Therefore, further studies are required to clarify whether 1α,25$(OH)_2D_3$ affects myoblast differentiation by modulating myostatin/Akt/mTOR signaling. Olsson et al. [9] report that 1α,25$(OH)_2D_3$ inhibits myotubes formation with concomitant downregulation of myogenic regulatory factors, myocyte enhancer factor 2 (MEF2) transcription factors and muscle structural proteins including MyHCs, myosin light chains (MyLCs), troponin and titin. They suggest the possibility that 1α,25$(OH)_2D_3$ may play a role in the promotion of self-renewal and maintenance of the satellite stem cell pool, through the modulation of the FOXO and Notch signaling pathways [9]. The proposed concept regarding the direct effects of 1α,25$(OH)_2D_3$ on myogenesis is intriguing because FOXO3 promotes quiescence in muscle stem cells by activating Notch signaling [96], which is required to maintain quiescent state in myogenic stem cells [97, 98]. Notch signaling inhibits myoblast differentiation [99]. For example, Notch signaling activated Hes1, which inhibits MyoD expression [100] and the ability of MEF2C to cooperate with MyoD and myogenin to activate myogenesis [101]. Further studies will be required to fully elucidate the molecular mechanisms of 1α,25$(OH)_2D_3$ actions on myogenesis.

7. Conclusion

This review highlighted the role of 1α,25$(OH)_2D_3$ in myogenesis. Although VDR null-mutant mice exhibit impaired muscle development, the precise mechanisms remains to be elucidated. However, they provide insight into the physiological roles of vitamin D in muscle develop-

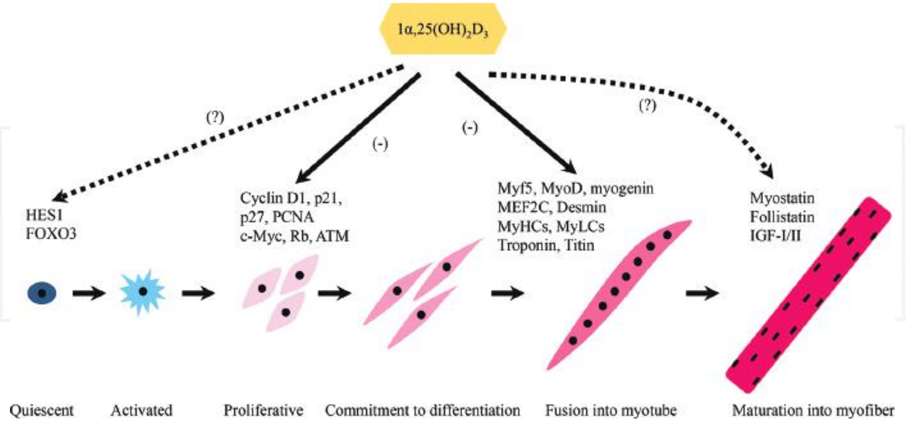

Figure 1. Proposed effects of 1α,25$(OH)_2D_3$ on myogenesis based upon in vitro data. Illustration of myogenesis was cited from Zammit et al. [102] with minor modifications. 1α,25$(OH)_2D_3$ modulates expression of key components of the vitamin D-endocrine system in myoblasts/myotubes. Potential effects of 1α,25$(OH)_2D_3$ are shown (dashed lines). 1α,25$(OH)_2D_3$ has antiproliferative effect and inhibits myoblast differentiation.

ment. Myogenic cells appear to retain vitamin D-endocrine system. $1\alpha,25(OH)_2D_3$ regulates myogenesis probably through genomic and nongenomic actions. **Figure 1** shows proposed effects of $1\alpha,25(OH)_2D_3$ on myogenesis. Simply stated, $1\alpha,25(OH)_2D_3$ has potent antiproliferative activity on myoblasts and inhibits myoblast differentiation. Further studies will be required to clarify whether $1\alpha,25(OH)_2D_3$ enhances protein synthesis in myotubes and promotes self-renewal of muscle stem cells. Although the effects of $1\alpha,25(OH)_2D_3$ on myogenesis have gradually come to be known, several questions remain unanswered and clouded. For example, what are the genes specifically induced by $1\alpha,25(OH)_2D_3$? How does multiple signaling pathways activated by $1\alpha,25(OH)_2D_3$ contribute to myogenesis? What are the molecular events underlying the cross-talk that occurs between genomic and nongenomic pathways and how, ultimately, are these pathways regulated? Given the beneficial role of $1\alpha,25(OH)_2D_3$ in myogenesis, answering these questions will be critical if we want to develop vitamin D therapy to treat muscle disorders.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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