Calcif Tissue Int (2015) 97:602–610
DOI 10.1007/s00223-015-0054-x

ORIGINAL RESEARCH

Vitamin D Receptor Ablation and Vitamin D Deficiency Result in Reduced Grip Strength, Altered Muscle Fibers, and Increased Myostatin in Mice

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Received: 9 January 2015 / Accepted: 17 August 2015 / Published online: 4 September 2015

Abstract Vitamin D deficiency is associated with muscle weakness, pain, and atrophy. Serum vitamin D predicts muscle strength and age-related muscle changes. However, precise mechanisms by which vitamin D affects skeletal muscle are unclear. To address this question, this study characterizes the muscle phenotype and gene expression of mice with deletion of vitamin D receptor (VDRKO) or diet-induced vitamin D deficiency. VDRKO and vitamin D-deficient mice had significantly weaker grip strength than their controls. Weakness progressed with age and duration of vitamin D deficiency, respectively. Histological assessment showed that VDRKO mice had muscle fibers that were significantly smaller in size and displayed hypernuclearity. Real-time PCR also indicated muscle developmental changes in VDRKO mice with dysregulation of myogenic regulatory factors (MRFs) and increased myostatin in quadriceps muscle (>2-fold). Vitamin D-deficient mice also showed increases in myostatin and the atrophy marker E3-ubiqutin ligase MuRF1. As a potential explanation for grip strength weakness, both groups of mice had down-regulation of genes encoding calcium-handling and sarco-endoplasmic reticulum calcium transport ATPase (*Serca*) channels. This is the first report of reduced strength, morphological, and gene expression changes in VDRKO and vitamin D-deficient mice where confounding by calcium, magnesium, and phosphate have been excluded by direct testing. Although suggested in earlier in vitro work, this study is the first to report an in vivo association between vitamin D, myostatin, and the regulation of muscle mass. These findings support a direct role for vitamin D in muscle function and corroborate earlier work on the presence of VDR in this tissue.

Keywords Vitamin D · Vitamin D receptor · Skeletal muscle · Strength · Development · Aging

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Introduction

In addition to established effects in bone and mineral homeostasis, increasing evidence suggests that vitamin D exerts effects in skeletal muscle [1]. Muscle weakness and pain are prominent features of vitamin D deficiency that respond to replacement [1]. Serum 25(OH)-vitamin D (25OHD) levels correlate with muscle strength in healthy individuals and predict the risk of functional decline and sarcopenia in older populations [2, 3]. A cross-sectional

observational study also reported an association between serum levels of 25OHD and myostatin, a negative regulator of muscle mass, in older men [4].

The biologically active form of vitamin D, $1,25(OH)_2D$, relies on the photochemical conversion of 7-dehydrocholesterol to vitamin D in skin by ultraviolet type-B radiation and subsequent hydroxylation steps. Active vitamin D then binds to its cognate receptor: the vitamin D receptor (VDR). VDR is a member of the nuclear receptor superfamily that regulates expression of many genes including those involved in calcium/phosphate homeostasis and cellular proliferation and differentiation [1]. The $1,25(OH)_2D$-VDR complex also exerts rapid, non-genomic effects on intracellular signaling, and calcium flux [1].

Precise mechanisms to explain vitamin D's effects in muscle are unclear. Biochemical abnormalities associated with vitamin D deficiency, particularly altered serum phosphate, and calcium levels, independently lead to muscle disease [5]. Whether skeletal muscle expresses VDR and may therefore be a direct target of $1,25(OH)_2D$ has been controversial [6–10]. We recently reported that VDR *is* expressed in murine skeletal muscle and modulates the uptake of vitamin D in this tissue [11]. In addition, a study published in this journal recently demonstrated VDR in human skeletal muscle and its activation following vitamin D supplementation [12].

Upon entry into muscle cells, vitamin D alters calcium flux by altering protein kinase C and rapid inositol triphosphate (IP3)-dependent calcium shifts from the sarcoplasmic reticulum to the cytosol, thereby potentially influencing muscle contraction and relaxation [13, 14]. Vitamin D treatment of cultured muscle cells leads to doubling in the size of differentiated myotubes [15] and opposing effects are seen following VDR knockdown by siRNA [16]. These in vitro effects rely on interaction between vitamin D signals and myostatin, a member of the TGF-β superfamily that negatively regulates muscle mass [15, 17]. However, in vivo studies examining direct effects of VDR in muscle function, fiber size, and TGF-β are lacking. A pilot study of 21 older women recently reported increases in muscle fiber size following vitamin D supplementation [10], raising the intriguing possibility that VDR-related mechanisms regulate muscle mass.

In this study, we examine the effect of vitamin D deficiency and deletion of VDR on grip strength, muscle bulk, fiber size, and gene expression. In the absence of altered mineral levels following adequate dietary supplementation, these mice displayed a distinct muscle phenotype with morphological differences, reduced strength, and altered gene expression. This provides further evidence in support of a functional role of vitamin D signaling in muscle, corroborating the presence of VDR in this tissue.

Materials and Methods

Animals and Ethics

Whole-body VDR knockout (VDRKO) mice were maintained on a γ-irradiated "rescue chow" (SF08-002, Specialty Feeds, Glen Forest, NSW) containing 2 % calcium, 1.2 % phosphorus, 0.2 g/g lactose, and 1 IU vitamin D/g from weaning. WT littermates were used as controls for the VDRKO mice. Rescue chow is essential to normalize the blood mineral ion levels of VDRKO mice [18]. In a second group of C57BL6 mice, regular chow was given from 0 to 3 weeks of age following which mice received either a vitamin D-deplete diet or a vitamin D-control diet. The vitamin D-deplete diet contains no vitamin D but increased calcium (2 %), magnesium (0.2 %) and phosphorus (1.2 %) to prevent abnormal mineral levels in association with vitamin D deficiency (SF085-003, Specialty Feeds, Glen Forest, NSW). The vitamin D-control diet contains vitamin D (cholecalciferol) 2.2 IU/g, 1 % calcium, 0.2 % magnesium, and 0.7 % phosphorus (SF085-034, Specialty Feeds, Glen Forest, NSW). Serum levels of vitamin D and related minerals were tested at various time-points following the commencement of vitamin D-free and control diets. In each group of mice (VDRKO vs WT littermates, Vitamin D deficiency vs replete), grip strength, muscle mass, histology, and gene expression were examined. Study time-points differed between these models. Muscle parameters were examined at 6 months of age in vitamin D-deficient mice to assess effects of prolonged deficiency, as an earlier study in rats had shown modest phenotypic changes following just 4 months of vitamin D deficiency [19]. Due to their reduced life span and increased morbidity with aging, muscles from VDRKO mice were culled at an earlier time point (i.e., 3 months of age) [18]. Sequential changes in grip strength were examined at earlier time points in both models prior to sacrifice. Use of animals was approved by the Animals Ethics Committee of the Garvan Institute of Medical Research (AEC 12/26). All animals were sacrificed by general anesthesia followed by cervical dislocation.

Serum Parameters

Serum calcium, phosphate, and magnesium levels were measured using Roche reagents on a Roche Modular analyser. Serum 25OHD levels were measured by Diasorin Liaison assay (Royal North Shore Hospital, Sydney, Australia).

Grip Strength Measurement

A grip strength meter (Columbus Instruments, OH, USA) was used to test mouse forearm grip strength as recorded in

Newtons (N). Mice were held by the base of the tail and allowed to grip the trapeze with their front paws and then pulled with their body parallel to the floor. Each mouse was trialed 15 times in sets of 3 with a short rest in between sets. The highest and lowest readings were excluded for each mouse and the remaining readings were averaged and corrected for individual body weight.

Muscle Histology

Quadriceps muscles were isolated from VDRKO mice and their WT littermates, vitamin D-deficient mice and their controls. Upon isolation, muscles were rapidly frozen in isopentane (2-methylbutane) cooled with liquid nitrogen. Frozen 8 μm muscle sections were cut using a Cryostat (Leica). Sections were incubated with Mayer's hematoxylin solution for 4 min, washed in deionized water, and then incubated with eosin solution for 4 min. Sections were then washed in deionized water, dehydrated in ethanol, and then mounted. Using ImageJ (National Institutes of Health), diameters of muscle fibers and nuclei per fiber were measured in $\sim$ 150 fibers per mouse (fibers selected randomly). Fiber quantification was performed blinded for genotype and diet.

Real-Time PCR (RT-PCR)

Total RNA was isolated from whole quadriceps muscle using TRIZOL Reagent (Sigma-Aldrich, Australia), as previously described [20]. cDNA was synthesized from 1 μg of total RNA using the Maxima First Strand cDNA synthesis kit (Thermo Scientific). RT-PCR was performed in 10 ul reactions using the SYBR Green master mix (Promega) within 384-well plates (ABI Prism 7900HT Sequence Detection System). The amplification protocol included 10 min at 95 °C and 40 cycles of two-step PCR including melting for 15 s at 95 °C and annealing for 1 min at 60 °C. Triplicates were used for each sample. Primers were designed using Primer 3 and BLAST (National Library of Medicine) and obtained from Invitrogen. Every plate included house-keeping genes (TATA-box binding protein (*Tbp*), cyclophilin) for every sample. For each experiment, a house-keeping gene that did not differ significantly between groups was used to normalize CT (cycle threshold) values. CT is the number of PCR cycles at which fluorescence above background crosses a set threshold. Relative expression levels were calculated by comparing the logarithm of the difference of total cycle number and CT for specific groups (i.e., ΔΔCT). Primer sequences are presented in Table 1.

Statistical Analyses

Data are presented as mean ± SEM. Statistical tests are Student's paired *t* tests conducted at a significance level of 5 % unless otherwise stated. Statistical tests and graphs were performed using Excel and/or Prism (GraphPad Prism Version 6 for Windows; GraphPad Software, San Diego, CA, USA).

Table 1 Primer sequences

Gene	Primer
Calbindin D-28 K	F: gccagccaatagagttgctc
	R: ttcctcgcaggacttcagtt
Cyclophylin	F: tggaccaaacacaaacggttcc
	R: acattgcgagcagatgggtag
MAFbx	F: ctctgctgtgagtgccacat
	R: caatgagcctgggtaccact
MuRF1	F: tggaaacgctatggagaacc
	R: aacgacctccagacatggac
Myf5	F: aggaaaagaagccctgaagc
	R: gcaaaaagaacaggcagagg
MyoD	F: agtgaatgaggccttcgaga
	R: gcatctgagtcgccactgta
Myogenin	F: ccttgctcagctccctca
	R: tgggagttgcattcactgg
Myostatin	F: ctgtaaccttcccaggacca
	R: tcttttgggtgcgataatcc
Serca 1	F: ctgtccatgtccctccactt
	R: gggtggttatccctccagat
Serca 2A	F: gatcctctacgtggaacctttg
	R: ggtagatgtgttgctaacaacg
Serca 2B	F: gatcctctacgtggaacctttg
	R: ccacagggagcaggaagat
Serca 3	F: gcattttcttatcctcctggtg
	R: tctgctcccaggaacctttg
TBP	F: tatcactcctgccacaccag
	R: atgatgactgcagcaaatcg
TGF-B1	F: ttgcttcagctccacagaga
	R: tggttgtagagggcaaggac

Results

Serum Values

Serum calcium, magnesium, and phosphate levels were measured in a subgroup of mice (Fig. 1a, b). Due to adequate dietary supplementation, VDRKO and vitamin D-deficient mice did not display differences in mineral levels compared to their respective controls. Vitamin D deficiency was initially noted at 1 month following the commencement of the vitamin D-free diet and by 3 months, these mice had no detectable 25OHD (Fig. 1c).

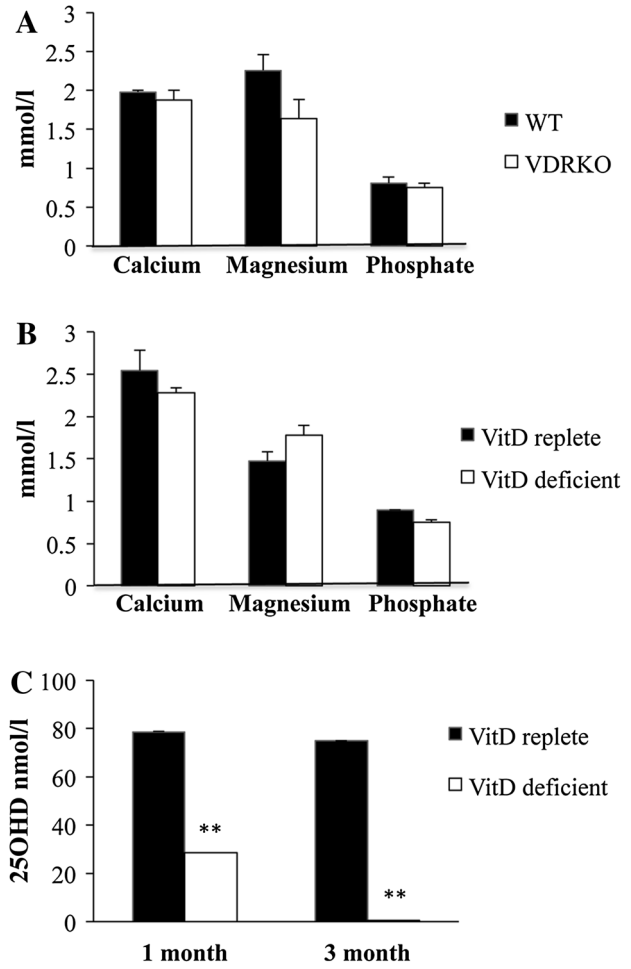

Fig. 1 Serum mineral and vitamin D levels. **a** Due to rescue diet, serum calcium, magnesium, and phosphate levels were not significantly different between WT and VDRKO mice at 2 months age. **b** After being on vitamin D-free and replete diets for 3 months, serum calcium, magnesium, and phosphate levels were not significantly different between these mice, owing to increased supplementation of these minerals. **c** Serum 25(OH)Vitamin D levels dropped significantly after 1 month of the diet and was undetectable at 3 months ($n = 4$ mice per group, *error bars* SEM, $^{**}p < 0.005$)

Grip Strength

VDRKO were significantly weaker than WT controls with 43 % reduction in grip strength at 2 months of age (Fig. 2a). At 3 months of age, the difference was greater at 48 % reduction in grip strength ($p < 0.005$, Fig. 2a). There were also differences in vitamin D-deficient versus replete mice. At 2 months of age, vitamin D-deficient mice were 15 % weaker than controls ($p < 0.05$, Fig. 2b). At 3 months, vitamin D-deficient mice were 25 % weaker than controls ($p < 0.05$, Fig. 2b).

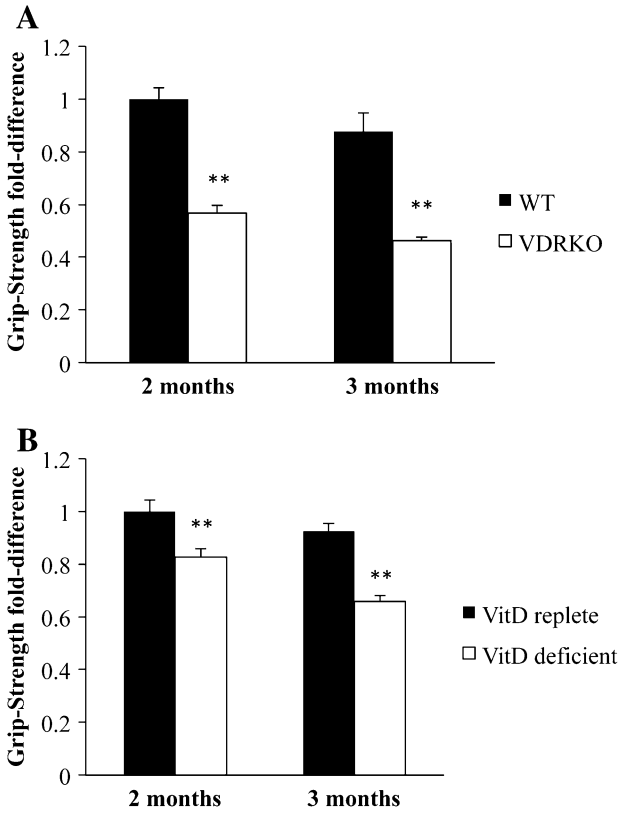

Fig. 2 Grip strength analysis. **a** At 2 and 3 months age, VDRKO mice displayed significantly reduced grip strength corrected for body weight (reported as fold-difference compared to grip strength of WT mice at 2 months). Age-related decline over this period was also more prominent in VDRKO mice compared to WT littermates (18 vs. 12 % decline in grip strength, respectively). **b** At 2 months, vitamin D-deficient mice were significantly weaker than replete mice and this difference increased by 3 months ($n = 6$–12 mice per group, *error bars* SEM, $^{*}p < 0.05$, $^{**}p < 0.005$)

Muscle Mass and Histology

VDRKO mice had significantly lighter quadriceps muscles even after adjustment for total body mass (Fig. 3c). They displayed smaller quadriceps muscle fibers (Fig. 3a–d) and a higher nuclei to fiber ratio compared to WT littermates (Fig. 4). These changes imply defects in the regulation of muscle fiber size and myocyte proliferation in the absence of VDR, respectively. Similar changes were seen in tibialis anterior muscles (data not shown), suggesting diffuse muscle defects in VDRKO mice. There were no histologic changes to indicate necrosis in muscle from VDRKO mice, nor was there any increase in the proportion of central nuclei in fibers from VDRKO mice (i.e., an indication of fiber remodeling/regeneration). By contrast, quadriceps muscle mass proportionate to total body mass did not differ in mice

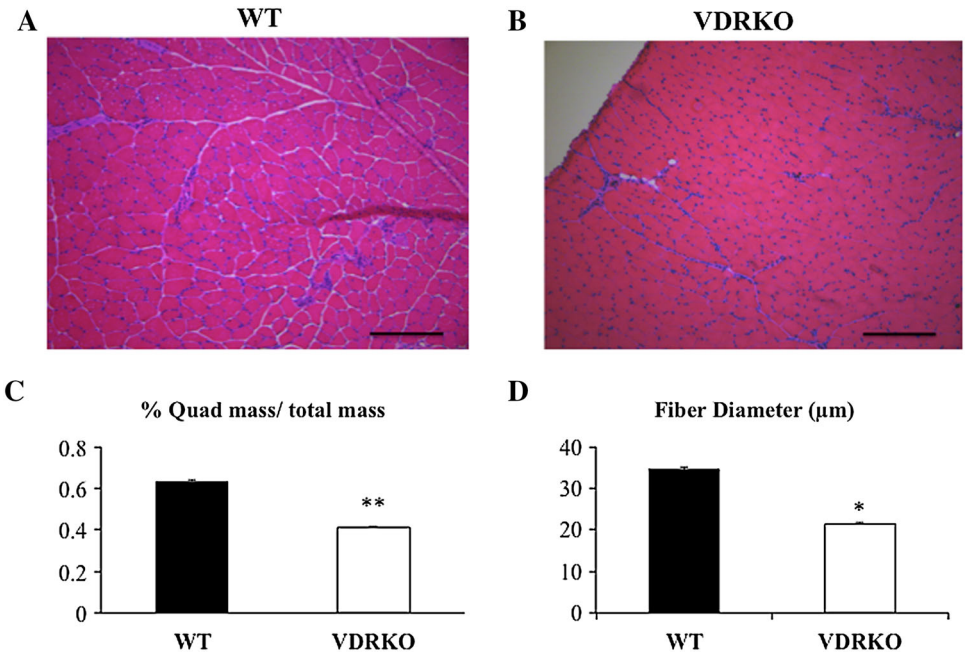

Fig. 3 Muscle Histology in VDRKO mice. **a**, **b** On H&E stain, VDRKO mice showed smaller muscle fibers than WT counterparts (quadriceps, 3-month-old male mice, $n = 5$ per group). **c** After correction for body mass, muscles remained significantly lighter in VDRKO mice. **d** On ImageJ quantification, VDRKO muscles displayed $\sim$ 30 % reduction in fiber diameter ($n = 5$ mice per group, 150 fibers analyzed per mouse, *scale bars* 200 µm, *error bars* SEM, $^{*}p < 0.05$, $^{**}p < 0.005$)

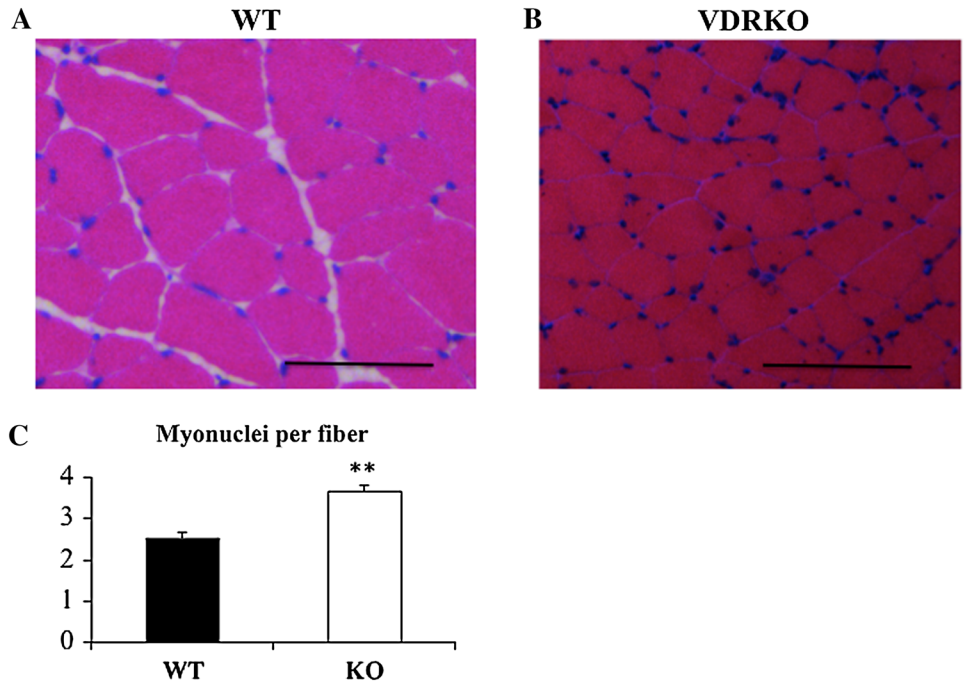

Fig. 4 Muscle histology in VDRKO mice. **a–c** Muscle fibers from VDRKO mice displayed a significant increase in nuclear number compared to WT mice. ($n = 5$ mice per group, 150 fibers analyzed per mouse, *scale bars* 50 µm, *error bars* SEM, $^{*}p < 0.05$, $^{**}p < 0.005$)

with vitamin D deficiency (Fig. 5c). Muscle fiber size did not differ between vitamin D-deficient and replete mice (Fig. 5). These parameters were not adjusted for physical activity, which was not measured in this cohort of mice.

Gene Expression

Differential expression of several groups of mRNAs was examined in the quadriceps muscles of VDRKO, vitamin D-deficient mice and their controls (Fig. 6). Myogenic regulatory factors *MyoD* and *Myf5* were significantly upregulated in VDRKO mice (Fig. 6a, $p < 0.05$), consistent with impaired muscle development and maturity. Expression of *Myostatin* mRNA was greater than twofold in VDRKO mice compared to WT littermates (Fig. 6a, $p < 0.005$), explaining the substantial reduction in fiber size and muscle mass seen in these mice. To a lesser extent, vitamin D-deficient mice also displayed a significant

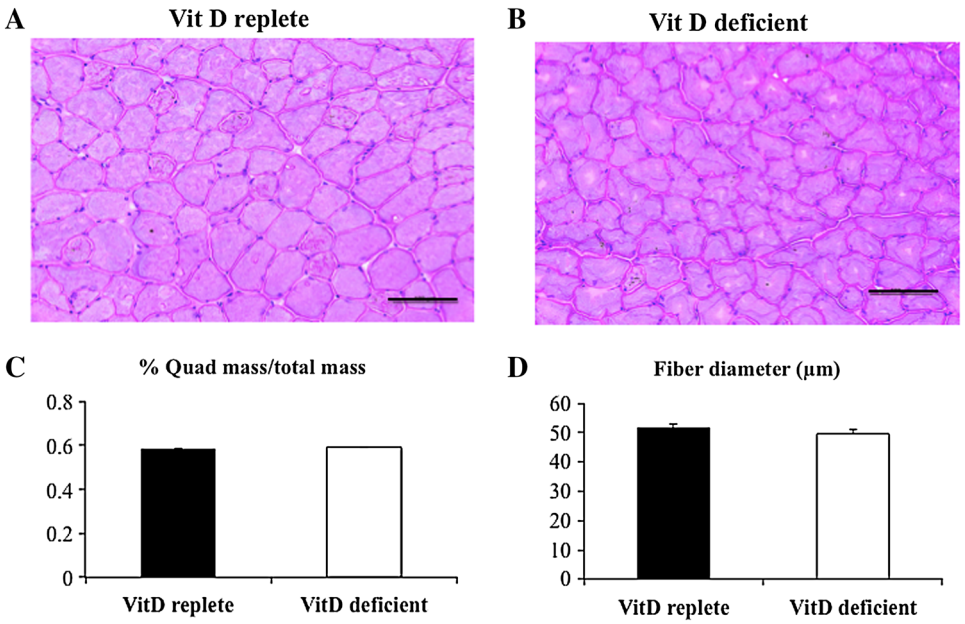

Fig. 5 Muscle histology in vitamin D-deficient mice. **a**, **b** On H/E stain, there was no significant difference in muscle fiber size in vitamin D-deficient versus replete mice. **c** Quadriceps muscle mass, normalized to total body mass, did not differ between vitamin D-deficient and replete mice. **d** On Image-J analysis, muscle fiber size was not significantly different (quadriceps, 6-month-old mice, $n = 5$ mice per group, 150 fibers analyzed per mouse, *scale bars* 100 μm)

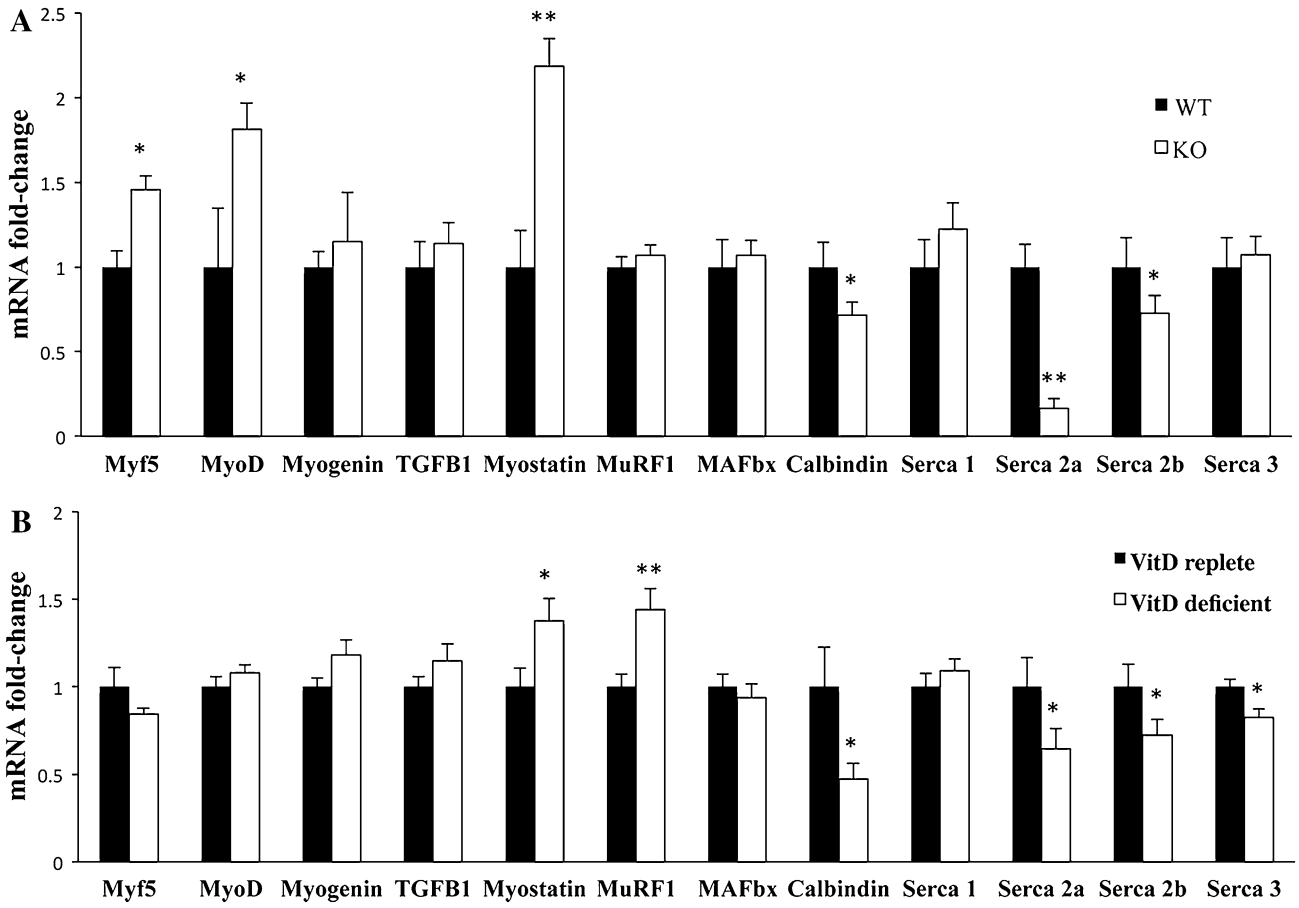

Fig. 6 Gene expression in muscle from VDRKO and vitamin D-deficient mice. **a** VDRKO mice showed significant upregulation of mRNAs for myogenic regulatory factors, >2-fold increase in *Myostatin* mRNA and significant reduction in *Calbindin-28 K*, and genes encoding *Serca2a* and *2b* mRNAs. **b** Vitamin D-deficient mice showed upregulation of the atrophy marker *MuRF1*, modest increase in *Myostatin* mRNA and down-regulation of *Calbindin-28 K*, *Serca2a*, *2b* and *3* mRNAs. ($n = 6$ mice per group, *error bars* SEM, $*p < 0.05$, $**p < 0.005$)

increase in *Myostatin* mRNA (Fig. 6b, $p < 0.05$). This was associated with increase in the E3-ubiqutin ligase *MuRF1* (Fig. 6b, $p < 0.005$), suggesting upregulation of proteolysis and atrophy pathways in the muscles of these mice. Both vitamin D-deficient and VDRKO mice showed significant down-regulation of calcium-handling genes, specifically genes encoding for Calbindin and sarcoendoplasmic reticulum calcium transport ATPase (*Serca*) channels (Fig. 6, $p < 0.05$). This supports a critical role for vitamin D in intramuscular calcium handling via genomic mechanisms in skeletal muscle.

Discussion

This is the first study to examine muscle strength, histology, and gene expression in VDRKO and vitamin D-deficient mice whilst correcting serum calcium, phosphate and magnesium levels. By assessing function, morphology, and mRNAs in these mouse models, we addressed the important question: *how* does vitamin D signaling alter muscle strength and morphology?

Previous studies reported functional differences in VDRKO mice such as impaired swimming, motor coordination, and reduced stride length compared to WT littermates [21–23]. Other studies reported impaired muscle contraction and recovery in vitamin D-deficient rats and chicks compared to their vitamin D-replete counterparts [24–26]. With the exception of one study [27], these studies did not correct for associated biochemical abnormalities and could therefore not conclude *direct effects* of vitamin D. These studies also did not associate functional differences in vitamin D-deficient or VDRKO mice with specific morphologic changes in their skeletal muscle.

On grip strength testing, vitamin D-deficient and VDRKO mice were significantly weaker than their controls and this difference increased with duration of vitamin D deficiency and age. Interestingly, differences in grip strength were greater in VDRKO versus WT than vitamin D-deficient versus replete mice, perhaps explained by complete ablation of vitamin D signaling in the former. We hypothesized that muscle weakness in these mice may be related to defects in Ca^{2+} flux and intracellular handling during excitation–contraction coupling. Indeed, rapid non-genomic effects of vitamin D on calcium handling in cultured muscle cells have been reported by the Boland group for over 30 years [13, 28, 29]. In this study, we report that vitamin D also exerts *genomic effects* in the calcium-handling apparatus within skeletal muscle. Although this has been previously reported in tissues with classic vitamin D responses, namely intestine and kidney [30], this is the first time such an effect has been shown in skeletal muscle. Muscle from VDRKO and vitamin D-deficient mice showed reduced mRNAs for the calcium-binding gene, Calbindin-28 K, and SERCAs, potentially resulting in defective Ca^{2+} flux from sarcoplasmic reticulum (SR) into cytosol following excitation, impaired muscle contraction and reduced strength. An association between SERCA expression and muscle strength in obese mice has been reported [31] and the same may apply to vitamin D models used in this study. To shed further light on the matter, in vivo contractile studies are needed to examine effects of vitamin D in muscle force, fatigue and recovery, all of which are intrinsically related to calcium signals. In vitro calcium flux studies may also inform on functional differences of reduced expression of calcium-handling genes in these models. It is also possible that non-genomic mechanisms related vitamin D availability, and unrelated to VDR activity, may be responsible for changes seen in the deficiency model.

Muscles from VDRKO mice were significantly lighter, even after correction for lower body weight, than WT mice. On histology, VDRKO mice displayed smaller and hypernuclear muscle fibers. A role for VDR in the regulation of muscle fiber size is supported by anabolic effects of $1,25(OH)_2D$ in C2C12 myotubes and opposing growth-inhibitory effects following VDR knockdown in these cells [16]. Similarly, muscle hyper-nuclearity in the absence of VDR concurs with anti-proliferative effects of VDR in cultured muscle cells [8, 15]. In the notable study by Endo and colleagues, smaller muscle fibers were seen in mice with a different model of VDR deficiency (exon 2 deletion) and this progressed in the absence of a high-calcium/phosphorus rescue diet [32]. Our study is the first to demonstrate an in vivo role for VDR in muscle fiber size that is independent of phosphate or calcium levels. Increased expression of myogenic regulatory factors (MRFs) *Myf5* and *MyoD* was also seen in adult VDRKO mice, supporting defects in myogenesis and post-natal muscle maturation in these mice. Most strikingly, *Myostatin* mRNA was greater than twofold higher in muscle from VDRKO mice, offering a potential explanation for the reduced muscle mass and fiber size seen in these mice.

Mice with vitamin D deficiency showed increased expression in the atrophy-related gene MuRF1. This ubiquitin ligase triggers muscle protein degradation and is considered to play a role in pathological muscle wasting [33]. However, muscle fiber size and MAFBx, another ubiquitin ligase, were not significantly altered by dietary vitamin D deficiency. Interestingly, a modest increase in *Myostatin* mRNA was also seen, favoring the activation of atrophy-related mechanisms in vitamin D-deficient mice despite the absence of morphologic changes. Possible explanations for this discrepancy are that a longer duration of vitamin D deficiency (>6 months) may be necessary for atrophy-related mechanisms to lead to overt atrophy.

Alternatively, normal mineral levels in these mice may have muted the overt development of muscle wasting in vitamin D deficiency. In support of this, the use of a high-calcium diet in Sprague–Dawley rats with vitamin D deficiency led to partial reversal in increased ubiquitin proteosomal activity in muscle [19]. However, this study showed no change in the expression of myostatin and this may be related to the shorter duration of vitamin D deficiency in this study (i.e., 4 months). It is therefore possible that vitamin D deficiency and associated calcium defects exert overlapping, interconnected effects in muscle atrophy via activation of the ubiquitin-proteosome and myostatin.

Importantly, both models showed significant increases in myostatin expression. Myostatin, a member of the TGF-β superfamily which negatively regulates muscle mass, was first discovered in 1997 [34]. A great deal of research has examined the immense potential of targeting myostatin or its receptor (ActRIIB) in the treatment of muscle wasting and sarcopenia [35, 36]. We have previously shown that treatment of cultured muscle cells with vitamin D results in pronounced inhibition of myostatin and subsequent doubling in myotube size [15]. The STRAMBO study also displayed a significant correlation between serum myostatin and 25OHD levels in older human subjects [4]. The current work advances the in vivo link between vitamin D signaling and myostatin in muscle and raises the pertinent question: may vitamin D present another pathway for myostatin inhibition and the reversal of muscle wasting?

Differences in the muscle phenotypes of VDRKO versus vitamin D-deficient mice reflect diverse effects of aberrant vitamin D signaling. Reduced muscle mass and smaller muscle fibers in mice with congenital ablation of VDR highlight the developmental role of vitamin D signaling. By contrast, changes seen in 6-month-old mice with vitamin D deficiency may relate to the muscle effects of aging, as supported by the activation of the atrophy-related gene MuRF1 in this model. Conversely, VDR deficiency may also play a role in muscle aging as suggested by down-regulation of VDR in muscles of older subjects [37]. Vitamin D may also exert effects in muscle development. This is supported by the association of Vitamin D deficiency during pregnancy with reduced muscle fiber size in newborn offspring [38, 39]. These observations suggest that vitamin D signaling plays concerted roles in skeletal muscle at opposing ends of the age spectrum. However, comparison between the mouse models used in this study is limited by distinct differences in their genotype, diet, and alteration in vitamin D biology (i.e., genetic ablation of VDR versus acquired deficiency of 25OHD via altered diet).

Although calcium and phosphorus levels were normalized in mice with abnormal vitamin D signaling, other variables such as FGF23 and parathyroid hormone (PTH) could not be adjusted in these models. Nevertheless, this study indicates coordinated effects of vitamin D and VDR in muscle strength, morphology, and gene expression, independent of calcium and phosphorus levels. This study indicates that skeletal muscle may be a direct target of vitamin D, a point of heated contention, and lays the foundation for further enquiry such as the generation of a skeletal muscle-specific VDRKO model. The precise delineation of vitamin D's effects, namely in muscle development and aging, and its potential interactions with myostatin, will be the subject of future research.

Acknowledgments CMG received salary support from the NHMRC (National Health Medical and Research Council, Australia) as a post-doctoral early career fellow. JEG and RJC-B are also supported by NHMRC.

Compliance with Ethical Standards

Conflict of Interest Christian M. Girgis, Kuan Minn Cha, Peter J. Houweling, Renuka Rao, Nancy Mokbel, Mike Lin, Roderick J. Clifton-Bligh and Jenny E. Gunton have no conflicts of interest or disclosures to report.

Human and Animal Rights and Informed Consent All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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