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Mice with myocyte deletion of vitamin D receptor have sarcopenia and impaired muscle function

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Abstract

Background It has long been recognized that vitamin D deficiency is associated with muscle weakness and falls. Vitamin D receptor (VDR) is present at very low levels in normal muscle. Whether vitamin D plays a direct role in muscle function is unknown and is a subject of hot debate. Myocyte-specific deletion of VDR would provide a strategy to answer this question.

Methods Myocyte-specific vitamin D receptor (mVDR) null mice were generated by crossing human skeletal actin-Cre mice with floxed VDR mice. The effects of gene deletion on the muscle phenotype were studied in terms of body tissue composition, muscle tissue histology, and gene expression by real-time PCR.

Results Unlike whole-body VDR knockout mice, mVDR mice showed a normal body size. The mVDR showed a distinct muscle phenotype featuring reduced proportional lean mass (70% vs. 78% of lean mass), reduced voluntary wheel-running distance (22% decrease, $P = 0.009$), reduced average running speed, and reduced grip strength (7–16% reduction depending on age at testing). With their decreased voluntary exercise, and decreased lean mass, mVDR have increased proportional fat mass at 20% compared with 13%.

Surprisingly, their muscle fibres showed slightly increased diameter, as well as the presence of angular fibres and central nuclei suggesting ongoing remodelling. There were, however, no clear changes in fibre type and there was no increase in muscle fibrosis. VDR is a transcriptional regulator, and changes in the expression of candidate genes was examined in RNA extracted from skeletal muscle. Alterations were seen in myogenic gene expression, and there was decreased expression of cell cycle genes *cyclin D1*, *D2*, and *D3* and cyclin-dependent kinases *Cdk-2* and *Cdk-4*. Expression of calcium handling genes sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCA) *Serca2b* and *Serca3* was decreased and *Calbindin* mRNA was lower in mVDR muscle.

Conclusions This study demonstrates that vitamin D signalling is needed for myocyte function. Despite the low level of VDR protein normally found muscle, deleting myocyte VDR had important effects on muscle size and strength. Maintenance of normal vitamin D signalling is a useful strategy to prevent loss of muscle function and size.

Keywords Vitamin D; Vitamin D receptor; Muscle; Sarcopenia; Weakness

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Introduction

Vitamin D plays a critical role in the regulation of calcium homeostasis, and skeletal muscle requires calcium for normal development and physiology. Vitamin D deficiency and inactivating mutations in the vitamin D receptor (VDR) are associated with muscle weakness in humans and in mouse models.[1–3]

The role of vitamin D and VDR in muscle is the subject of many reviews.[2–5] Despite this, it remains unclear whether vitamin D has direct effects in muscle. Vitamin D regulates absorption of dietary calcium and phosphate, renal calcium handling, and bone calcium homeostasis. Normal serum calcium and phosphate are needed for normal muscle function, complicating study of the role of vitamin D in muscle function.

We have previously described a mouse model of vitamin D deficiency with normal calcium and phosphate in which muscle weakness is present.[1] In addition, mice with whole-body deletion of VDR have decreased muscle mass and are weak, even if fed rescue diet from weaning to maintain normal serum calcium and phosphate.[1] Unfortunately, the whole-body VDR-null mice have many potential confounding factors, including liver cirrhosis and poor bone health.[4,6] Despite this, these two models suggest that direct effects of vitamin D on muscle are plausible, as in both cases, calcium and phosphate did not differ from the controls.

The presence of VDR in muscle is controversial, particularly since the meticulous demonstration that many of the previously used antibodies for VDR demonstrate clear bands in the absence of VDR protein.[7] However, a number of recent studies have demonstrated the presence of VDR in muscle with well-validated antibodies for mouse[7,8] and human muscle.[9] However, in uninjured normal muscle, the expression of VDR is very low, making its direct biological importance unclear.

We have shown that treatment of a cultured muscle cell line with 25-hydroxyvitamin D or 1,25-dihydroxyvitamin D caused important changes in the myotubes including increased myofibre diameter and changes in gene expression.

In order to examine the direct role of vitamin D in myocytes, mice with inactivation of VDR in myocytes (mVDR) were generated and compared with floxed littermate controls. Analysis of mVDR mice was performed including phenotype analysis of body composition, muscle tissue histology, and expression of putative candidate genes downstream of VDR signalling.

Methods

Mice

Mice with floxed VDR genes were created as previously described and were kindly supplied by Professor Geert Carmeliet.[10] Floxed VDR mice were bred with mice expressing Cre-recombinase under control of the human skeletal actin (HSA) promoter. HSA-Cre is highly expressed in myocytes and is not expressed in cardiac myocytes.[11] The resulting mice have deletion of VDR in skeletal myocytes. Confirmation of homozygosity for VDR-flox was done every 4–6 generations. Mouse genotypes were classified based on presence or absence of Cre as mVDR and floxed control respectively as we have previously reported. Deletion efficacy was assessed by measuring expression of *Vdr* mRNA in whole muscles, as shown in *Figure* 1.

Mice were bred at Australian Bioresources (Mossvale, Australia), and studies were conducted in the Biological Services Facility at the Westmead Institute for Medical Research. Male mice were used for all studies. Mice were fed a standard chow diet and food and water were freely available. The rooms have a standard 12 h light and 12 h dark cycle and the temperature is maintained at 22°C. Studies were approved by the WSLHD Animal Ethics Committee.

DEXA

Dual X-ray absorptiometry was performed using a Lunar PIXImus small animal scanner (GE Lunar) to assess body composition as previously reported.[12] During the scans, animals were kept anaesthetized using inhaled isoflurane.

Grip strength

Grip was measured using a grip strength metre from Columbus Instruments, Ohio, USA, as we have previously reported.[1] Results are expressed as Newtons/gram of mouse weight. At the ages indicated in *Figure* 1, mice were lifted by the base of the tail and placed so that their front paws gripped the trapeze with their body horizontal. Each mouse was tested 15 times in sets of three with a short rest between each set.

Wheel running

Mice were placed in individual cages with running wheels (Promethion) that measured wheel use including distance travelled, speed, and proportion of time in the wheel. Mice were allowed to acclimatize to the cages for 24 h and then running distance was measured over the following 24 h. Running speed was measured in revolutions per second, and percentage of time running was measured each hour.

Tissue collection and histology

At study completion, mice were anaesthetized with a cocktail of ketamine and xylazine. Blood was collected by cardiac puncture and then the great vessels were severed while the

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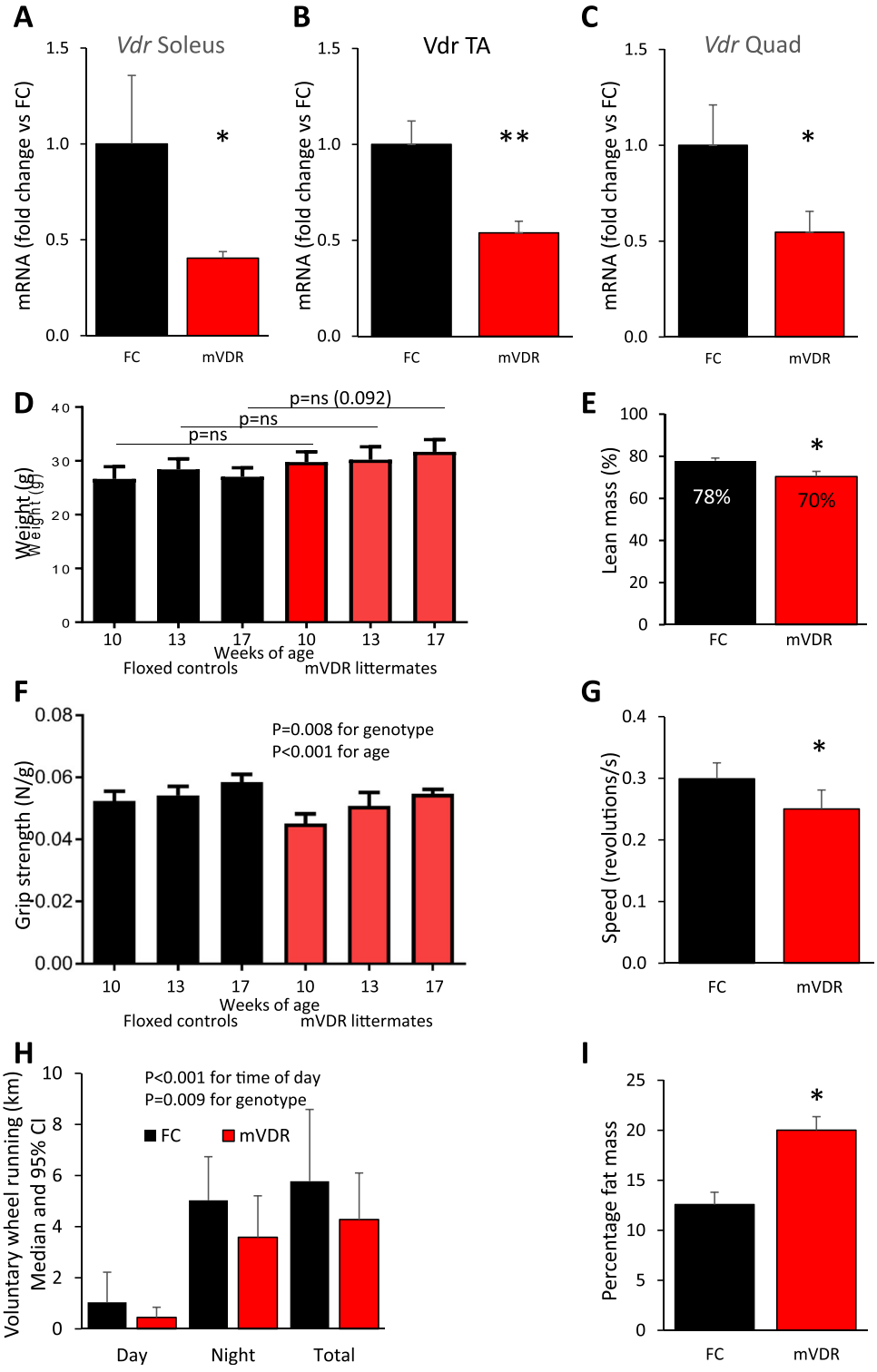
Figure 1 Phenotype of mVDR mice mRNA for *Vdr* in six mice per group at 13 weeks in (A) soleus muscle (B) tibialis anterior, and (C) quadriceps. (D) Mouse body weight (N = 6–16); (E) lean mass measured by DEXA at 13 weeks; N = 5–6; (F) grip strength (N = 6–16); (G) wheel-running speed; and (H) running distance at 13 weeks of age (N = 7–8, median, and 95% CI). (I) Fat mass in mice at 13 weeks of age, measured by DEXA (N = 5–6). FC, floxed control; mVDR, myocytes vitamin D receptor null. * = $P < 0.05$, ** = $P < 0.01$.



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mouse was still deeply anaesthetized. Muscles were dissected and weighed. Samples for RNA/western blotting were snap frozen in liquid nitrogen. Samples intended for histology were collected in OCT for frozen sections or in 10% paraformaldehyde for paraffin sections.

Haematoxylin and eosin (H&E), succinate dehydrogenase (SDH) and Sirius red stains were performed as previously reported[13,14] on paraffin sections. Fibre counting at the quadriceps midpoint used the whole muscle section, stained with H&E, and the whole quadriceps section was scanned for counting using a Nanozoomer.

Content of VDR protein in muscle was examined using paraffin-embedded quadriceps sections from 13-week-old mice. Antigen retrieval was carried out using pH 9 buffer from Dako. Blocking was with Phosphate buffered saline (PBS) and 2% Bovine serum albumin (BSA). Primary antibody was the well-validated D-6 antibody (Santa Cruz, sc-13133 at 1:50 dilution in PBS with 0.2% BSA).[7,8] Washes were carried out with the diluent (PBS with 0.2% BSA). Secondary antibody was Cy3 (Jackson ImmunoResearch, 711-166-152, used at 1:250 dilution). Pictures were captured with an Olympus VS120 slide scanner.

Apoptosis was assessed in muscle using cleaved caspase 3 staining as previously reported,[13,15] with the same Cy3 secondary antibody and images were acquired with the Olympus VS120 slide scanner.

Western blot

A total of 40 mg of snap-frozen quadriceps muscle was homogenized in ice-cold RIPA buffer [65 mM Tris·HCl, 150 mM NaCl, 5 mM EDTA, 1% NP-40 (v/v), 0.5% sodium deoxycholate (w/v), 0.1% SDS (w/v), and 10% glycerol (v/v), (pH 7.4) containing 1 mg/L aprotinin, 1 mg/L leupeptin, 10 mmol/L NaF, 1 mmol/L Na3VO4, and 1 mmol/L PMSF]. Cleared lysates (50 μg per lane) were electrophoresed in 12% polyacrylamide gels and transferred to PVDF membranes (Thermo Scientific, #88518). Total OXPHOS Rodent WB Antibody Cocktail (Abcam, ab110413) was used to measure components of the mitochondrial oxidative phosphorylation pathway. Membranes were washed and probed with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling), and proteins were visualized using Super Signal West Pico Chemiluminscent Substrate (Thermo) and a ChemiDoc Imaging System (Bio-Rad). Densitometry was performed using ImageJ software (NIH freeware).

Fibre typing

Fibre typing was performed as previously described.[16–18] Briefly, serial transverse 8 μm cryosections of mouse quadriceps muscle were cut onto slides and blocked with 2% BSA for 10 min, followed by further blocking with AffiniPure Fab fragment goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.) for 1 h at room temperature to prevent cross reactivity with endogenous mouse antibodies. Sections were then incubated with primary antibodies to either slow myosin type I (MAB1628; Chemicon, 1:300), myosin heavy chain IIA (SC71; DSHB, University of Iowa, neat), or myosin heavy chain IIB (BF-F3, DSHB, University of Iowa, USA, neat) overnight at 4°C. The next day, sections were washed three times with PBS, re-blocked with 2% BSA for 10 min, and incubated with either Alexa555 goat anti-mouse IgG (for MAB1628 and SC71, A21424, Molecular Probes, 1:300) or Alexa555 goat anti-mouse IgM (for BF-F3, A-21426, Molecular

Table 1 Sequences of real-time PCR primers used

Genes	Forward primer	Reverse primer
Cyclophilin	5′-TGGACCAAACACAAACGGTTCC-3′	5′-ACATTGCGAGCAGATGGGGTAG-3′
Calbindin	5′-GCCAGCCAATAGAGTTGCTC-3′	5′-TTCCTCGCAGGACTTCAGTT-3′
Cdk2	5′-AAATTCATGGATGCCTCTGC-3′	5′-ACAGGGACTCCAAAGGCTCT-3′
Cdk4	5′-ACTCTGAAGCCGACCAGTTG-3′	5′-CCAGACTCCTCCATCTCTGG-3′
CyclinD1	5′-AGTGCGTGCAGAAGGAGATT-3′	5′-CACAACTTCTCGGCAGTCAA-3′
CyclinD2	5′-TCGATGATTGCAACTGGAAG-3′	5′-ATGCTGCTCTTGACGGAACT-3′
CyclinD3	5′-CGCCCCTGACTATTGAGAAG-3′	5′-GTCTGGGCATGCTTTTTGAC-3′
Cyp24a1	5′-CCCTTCTGCAAGAAAACTGC-3′	5′-CTCTTGAGGGCTCTGATTGG-3′
Mafbx	5′-CTCTGCTGTGAGTGCCACAT-3′	5′-CAATGAGCCTGGGTACCACT-3′
Murf1	5′-TGGAAACGCTATGGAGAACC-3′	5′-AACGACCTCCAGACATGGAC-3′
Myf5	5′-AGGAAAAGAAGCCCTGAAGC-3′	5′-GCAAAAAGAACAGGCAGAGG-3′
MyoD	5′-AGTGAATGAGGCCTTCGAGA-3′	5′-GCATCTGAGTCGCCACTGTA-3′
Myogenin	5′-CCTTGCTCAGCTCCCTCA-3′	5′-TGGGAGTTGCATTCACTGG-3′
Myostatin	5′-CTGTAACCTTCCCAGGACCA-3′	5′-TCTTTTGGGTGCGATAATCC-3′
p19	5′-TCCATTGAAGAAGGGAGTGG-3′	5′-ACCGTTTAGATGGCTGTTGC-3′
p21	5′-GCCTTAGCCCTCACTCTGTG-3′	5′-AGGGCCCTACCGTCCTACTA-3′
p27	5′-CAGAATCATAAGCCCCTGGA-3′	5′-TCTGACGAGTCAGGCATTTG-3′
Serca2a	5′-GATCCTCTACGTGGAACCTTTG-3′	5′-GGTAGATGTGTTGCTAACAACG-3′
Serca2b	5′-GATCCTCTACGTGGAACCTTTG-3′	5′-CCACAGGGAGCAGGAAGAT-3′
Serca3	5′-GCATTTTCTTATCCTCCTGGTG-3′	5′-TCTGCTCCCAGGATTTACTTC-3′
TgfB1	5′-TTTGGAGCCTGGACACACAGTACA-3′	5′-TGTGTTGGTTGTAGAGGGCAAGGA-3′

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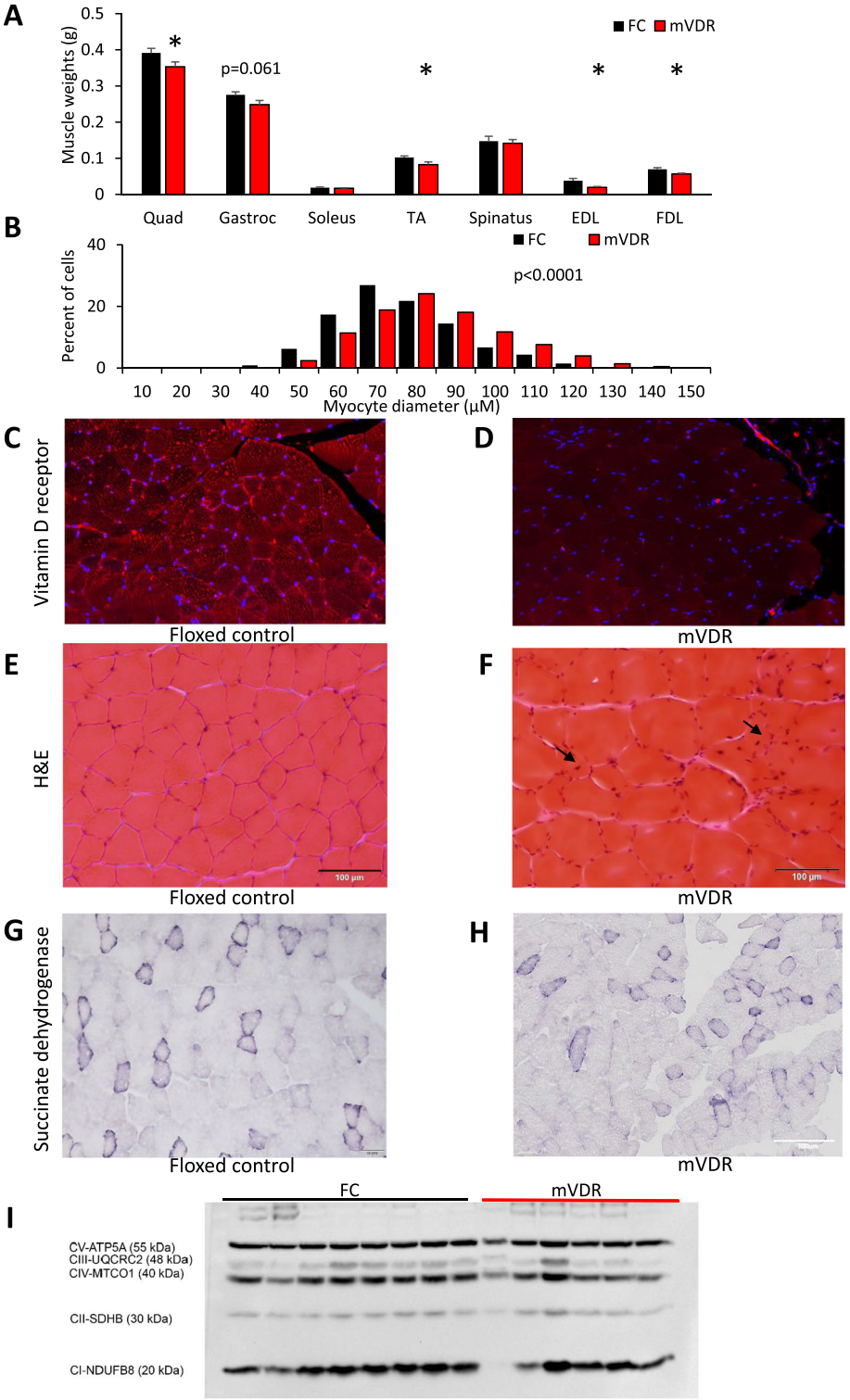
Figure 2 Muscle weights and fibre analysis (A) wet weight of muscles at sacrifice in 8–10 male mice at 13 weeks of age. EDL, extensor digitorum longus; FDL, flexor digitorum longus; gastroc, gastrocnemius; TA, tibialis anterior; Quad, quadriceps. (B) Myocyte diameter was measured in quadriceps in at least 900 fibres per genotype. (C) Vitamin D receptor immunostaining of quadriceps in floxed control and (D) mVDR mice. (E) Haematoxylin and eosin staining of quadriceps in floxed control and (F) mVDR mice. (G) Succinate dehydrogenase staining (SDH) of quadriceps in floxed control and (H) mVDR mice. (I) Western blotting of quadriceps muscle probed with 'mitomix' antibody showing mitochondrial subunits as labelled. FC, floxed control; mVDR, myocytes vitamin D receptor null. * = $P < 0.05$.



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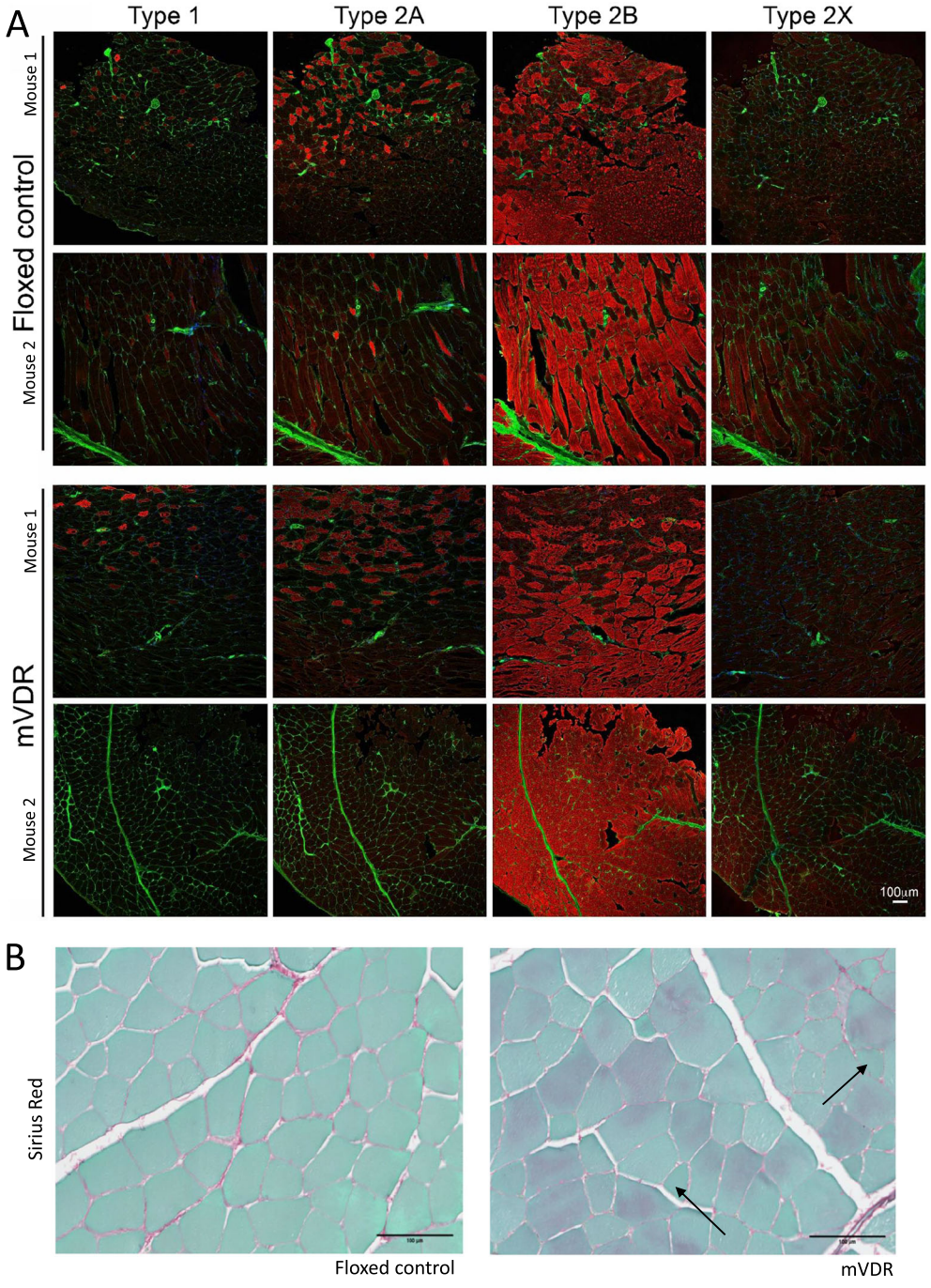
Probes, 1:300) for 2 h at room temperature protected from light. Sections were then washed and fixed with 3% paraformaldehyde (PFA) for 10 min. To label membranes, sections were incubated with wheat germ agglutinin-Alexa488 (ThermoFisher, 5 μg/mL in HBSS) for 10 min. Finally, sections were washed, incubated with DAPI (1 μg/Ml) for 5 min at room temperature, washed again, and mounted with coverslips using Immu-Mount mounting reagent (Thermo Scientific). Images were captured on a Leica SP5 scanning confocal microscope with a 20×/0.7NA objective.

Figure 3 Fibre typing of floxed control and mVDR mice (A) representative images are shown for two mice per genotype. Red colour in each image is myosin, antibody of the type indicated at the top of the column. Green is wheat germ agglutinin. All images are taken at the same magnification (see scale bar at bottom right). (B) Representative images of Sirius red histology from floxed control and myocyte vitamin D receptor-null (mVDR) mice. Collagen stains red.



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Real-time PCR

Real-time PCR was performed as previously described.[12] RNA was extracted from muscle using Qiagen RNEasy kits. cDNA was synthesized using the Invitrogen kit, and real-time PCR was performed using primers as shown in *Table* 1 and SybrGreen. Cyclophilin was used as the housekeeping gene, and it did not differ between groups (raw cross threshold (CT) values are shown in the section). The data are expressed as proportion of the value in floxed controls.[13,19,20]

Statistics

Unless otherwise specified, unpaired Student's *t*-tests were performed to assess significance. A *P*-value of <0.05 was considered signficiant. Statistical analysis was performed with either Microsoft Excel (Student's *t*-tests) or Prism Graphpad (version 6), which was used for all other tests. Unless otherwise described, data are presented as means ± standard error.

Results

Generation of myocyte-specific vitamin D receptor mice

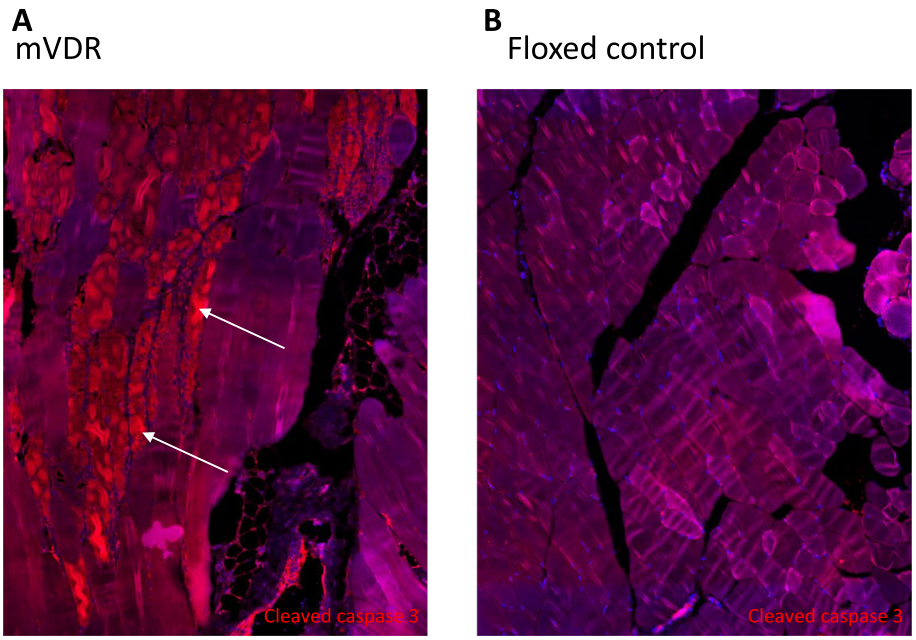
The mVDR strain was generated by crossing *HSA-Cre* and *Vdr*$^{flox/flox}$ strains over two generations of breeding. The resultant mice were viable and showed no changes in gross morphology. Expression of *Vdr* mRNA was significantly decreased in the soleus, tibialis anterior, and quadriceps in mVDR mice compared with littermate controls (*Figure* 1A–C). Whole muscle contains a range of other cell types that would be expected to continue to express normal *Vdr* so a ~50% decrease is appropriate.

Phenotype and muscle function

Whole-body VDR-null mice have reduced body size. In contrast, the mVDR mice showed no decrease in body weight and at older ages showed a trend to increased weight (*Figure* 1D). Analysis of body composition by DEXA at 3 months of age (13 weeks) revealed a decreased percentage of lean mass, at 70.3% vs. 77.9%, as shown in *Figure* 1E.

On grip strength testing, mVDR mice had a lower grip strength at each of the ages tested from 10 to 17 weeks. The decrease was 7–16% in magnitude (*Figure* 1F). Mice were placed in cages with voluntary running wheels. Per cent of time running was not significantly different in mVDR mice (daytime 13.4% vs. 9.7% in mVDR and night-time 28.2% vs. 25.4% in mVDR). However, running speed was decreased in mVDR mice (*Figure* 1G), and total running distance was decreased by 22% in mVDR mice (*Figure* 1H, $P = 0.009$). Fat mass was increased in mVDR mice at 20% of body weight compared with 13% in controls (*Figure* 1I).

Figure 4 Cleaved caspase 3 staining in quadriceps from (A) mVDR and (B) floxed control mice at 13 weeks of age. Cleaved caspase 3 is in red. White arrows indicate apoptotic myofibres in the mVDR sample. mVDR, myocyte vitamin D receptor.



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At sacrifice, muscle wet weight was significantly decreased for mVDR quadriceps, tibialis anterior, extensor digitorum longus, and flexor digitorum longus (*Figure* 2A). Gastrocnemius showed a trend towards a decreased size, and other muscles were non-significantly lighter in mVDR mice.

Muscle fibre analysis

We have previously reported smaller myocyte diameter in mice with whole-body VDR deletion.[1] It was therefore surprising to find that myocytes were slightly larger in mVDR mice than in their control littermates (*Figure* 2B). There was low but detectable staining for VDR in floxed control muscle (quadriceps, *Figure* 2C), and less VDR staining in mVDR mice (*Figure* 2D). H&E staining is shown in *Figure* 2E (floxed control) and 2f (mVDR). As well as the increase in myofibre size, H&E staining of the mVDR muscle showed more cells with angular fibres and some centralized nuclei suggesting ongoing regeneration (black arrows). In combination with the smaller muscle weights, the increased muscle fibre size suggests

Figure 5 Real-time PCR gene expression of myogenic genes and transforming growth factor β (*Tgfβ1*). FC, floxed control; mVDR, myocytes vitamin D receptor null. *N* = 6–12 per group.* = $P < 0.05$, ** = $P < 0.01$.

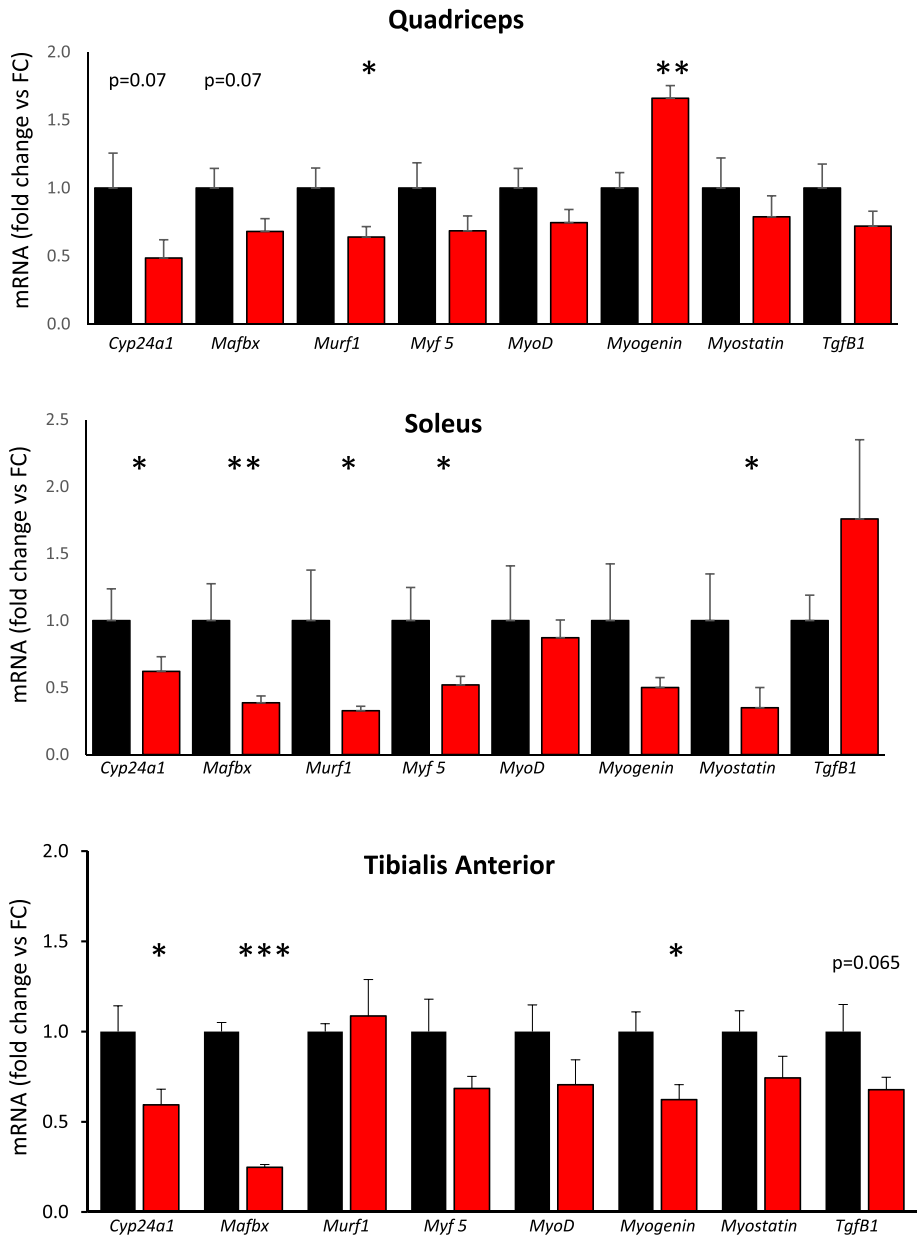

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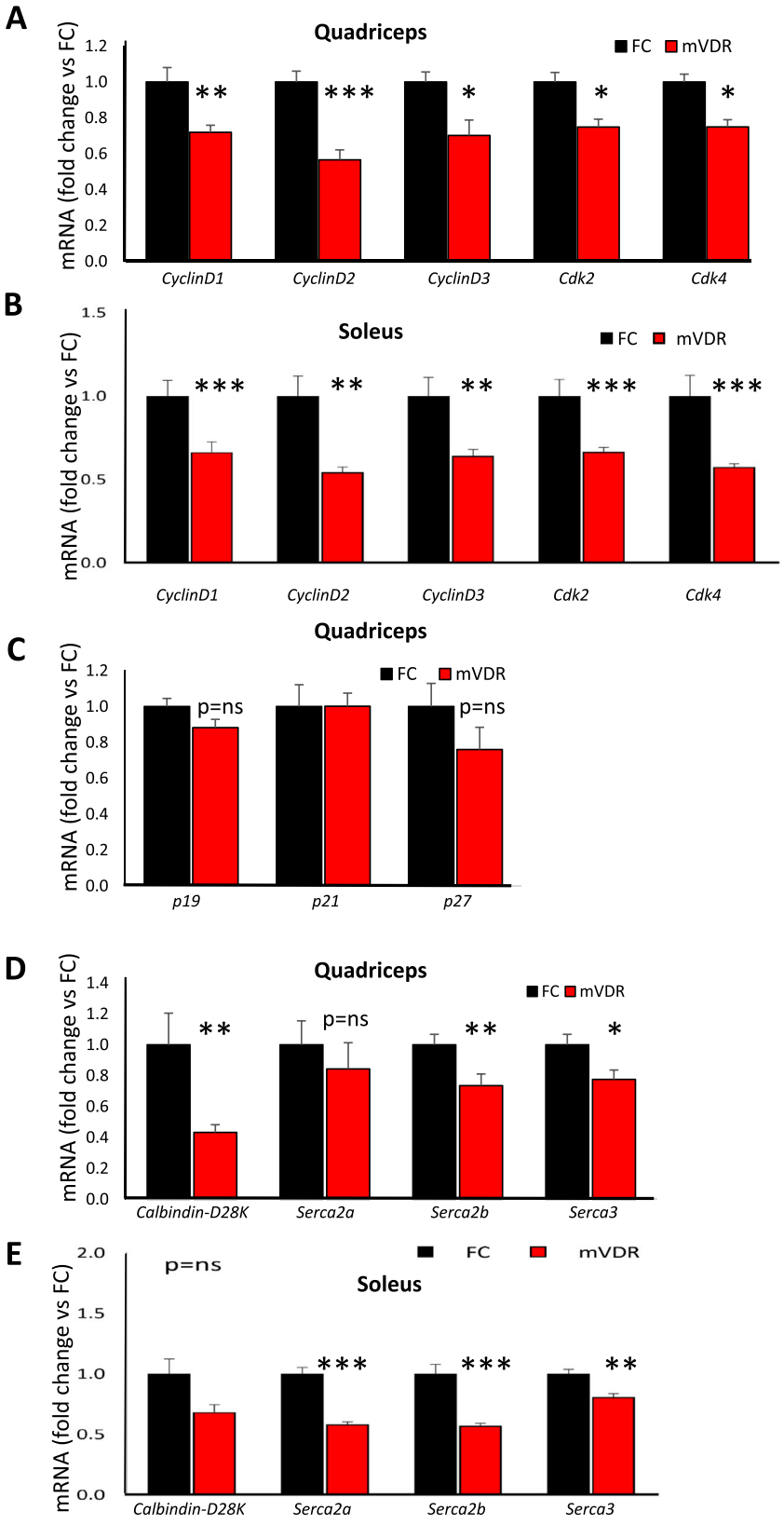
Figure 6 Real-time PCR gene expression in quadriceps of cell cycle and calcium handling genes *Cdk*, cyclin-dependent kinase; *Serca*, sarcoplasmic/endoplasmic reticulum calcium ATPase. FC, floxed control; mVDR, myocytes vitamin D receptor null. *N* = 6–12 per group. * = $P < 0.05$, ** = $P < 0.01$.



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fewer fibres per muscle, and this was seen on counting fibres; floxed controls had a median of 2525 fibres in a cross section at mid-level of quadriceps compared with 1412 in mVDR mice ($P < 0.05$). The full cross section of the quadriceps was examined.

No clear differences in muscle metabolism was seen when fibre type was examined by SDH staining (*Figure* 2G and H). Similarly, western blotting did not indicate any significant differences in the mitochondrial subunits, including SDH which is complex II (*Figure* 2I). Formal fibre typing of quadriceps muscle was examined. Type 2B fibres were the most common in both genotypes, with similar, low proportions of other fibre types seen (Type 1, Type 2A, and Type 2X, *Figure* 3). This suggests that neither a shift in fibre type nor gross mitochondrial abnormalities are responsible for the defects in mass or muscle function in mVDR mice. We have previously reported increased fibrosis in liver of mice lacking VDR[6] so we examined muscle using Sirius red staining. There were no obvious differences between floxed control and mVDR mice (*Figure* 3B). Again, despite overall larger fibres, there are more small, angular fibres present in the mVDR slides (black arrows).

Apoptosis is a potential cause of smaller muscles. We examined sections of quadriceps in floxed control and mVDR mice. In one mVDR mouse, there was a block of myocytes that had condensed cytoplasm that were strongly positive for cleaved caspase 3 (*Figure* 4A), indicating apoptosis. However, this was not seen in any other mVDR or any floxed control (*Figure* 4B) mice so does not seem to be an active process in the mice, at least at 13 weeks of age.

Gene expression changes

Vitamin D receptor is a transcription factor, and so we examined changes in gene expression by real-time PCR. We focused on genes that we have previously reported to be altered in either whole-body VDR deletion or vitamin D deficiency[1,8,21] as the mVDR model allows examination of whether these changes in muscle are direct via myocyte VDR or indirect via other mechanisms in those whole-body models.

Raw expression of the housekeeping gene *Cyclophilin* did not differ between floxed control and mVDR mice, with Cq values of 21.01 ± 0.10 vs. 20.85 ± 0.11 ($P > 0.2$). Results in the figures show gene expression corrected for *Cyclophilin*. *Cyp24a1* is part of the vitamin D signalling pathway. *Cyp24a1* expression was decreased in mVDR muscles (*Figure* 5).

We then measured mRNA expression of a range of myogenic factors and transforming growth factor β1 (*TgfB1*), many of which are altered in muscle from whole-body VDR knockout mice (*Figure* 5). There was decreased expression of *Mafbx* and *Murf1*. This is in contrast to the whole-body VDR mice, which show increased expression. Decreases in *Mafbx* and *Murf1* could contribute to the lesser reduction in muscle size in mVDR mice in comparison with whole-body VDR knockout (VDRKO) mice. Another potential contributor to the smaller decrease in muscle mass in mVDR mice is the trend towards a decrease seen in *Myostatin* expression, which is increased in whole-body VDRKO mice. This was statistically significant in soleus. Myostatin inhibits muscle hypertrophy and hyperplasia, so lower levels permit increased muscle growth.[22]

Several genes that are important in cell cycle progression showed decreased expression in quadriceps from mVDR mice.[23,24] These included cyclins D1, D2, and D3 and cyclin-dependent kinases (*Cdk*)2 and 4 (*Figure* 6A). *Cyclins D1*, *D2*, and *D3* and *Cdk2* and *Cdk4* all showed significantly decreased expression in soleus muscle (*Figure* 6B). Expression of the cell cycle inhibitors *p19*, *p21*, and *p27* were all unchanged (*Figure* 6C).

The function of mVDR muscle was impaired, and vitamin D in other tissues regulates a number of cellular calcium handling genes, so we assessed expression of intracellular calcium handling genes. There was no significant change in *Serca2a* (sarcoplasmic/endoplasmic reticulum calcium ATPase) in quadriceps. However, expression of *Calbindin*, *Serca2b*, and *Serca3* were all lower in mVDR quadriceps (*Figure* 6D). In soleus, there were significant decreases in expression of each of the Sercas tested: *Serca2a*, *Serca2b*, and *Serca3* (*Figure* 6E).

Conclusions

Loss of muscle mass and function, most commonly associated with age, is called sarcopenia. It results in greater risks of falls, fracture, disability, and death.[25–28] Vitamin D deficiency is associated with sarcopenia and lower muscle mass in most studies in older people.[29–36] However, a direct role in muscle has not been able to be shown in people.

The results presented in this study confirm that *Vdr* is expressed in myocytes in mice. We now demonstrate that myocyte VDR plays a significant role; deletion affects muscle function and gene expression. mVDR mice have significantly decreased grip strength, which persists to at least 4 months of age. It would be interesting to age these mice and determine whether they develop more severe changes with middle and older age. As well as decreased lean mass, mVDR mice had a significant increase in fat mass. There was a significant decrease in voluntary exercise in the mice, and a more than 20% decrease in voluntary exercise would be consistent with the increase in fat mass seen.

In people with colorectal cancer, physical activity measured by accelerometer correlated with serum vitamin D levels.[37] This association was also seen in the NHANES study.[38] While increased exercise is likely in many people to be associated with increased sun exposure, it is interesting

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to hypothesize that lower vitamin D may associate, as in mVDR mice, with lower voluntary exercise.

Interestingly, the effects of myocyte VDR deletion showed some important differences from those seen with whole-body VDR deletion.[1] The reduction in size of individual muscles in mVDR mice is significant and of clinically relevant magnitude, but it appears to be less than the effect seen in whole-body VDR-null mice. This suggests that VDR in other cells types may play a role in muscle development in early life and in individual myofibre size and that these may partially negate the atrophic effects of VDR deletion in myocytes. As there was only a 50% decrease in *Vdr* mRNA in whole muscle, there may also be a contribution of incomplete deletion to the milder phenotype.

Murf1 and *Mafbx* are ubiquitin ligases that regulate proteosomal degradation. In muscle, they are associated with myonuclear apoptosis and reduced fibre size.[39–41] MAFbx additionally regulates protein synthesis and muscle regeneration via targeting of regulators including MyoD and eIF3F.[41,42] Their expression is increased in whole-body VDR-null mice, in which there is decreased fibre size. There was decreased expression of *Murf1* in quadriceps and soleus, and significantly decreased expression of *Mafbx* in two muscles with a trend towards a decrease in the third ($P = 0.07$). Decreases in *Murf1* and *Mafbx* in mVDR mice would support better maintenance of fibre size with inhibition of the ubiquitin–proteosome pathway. Interestingly, these were also decreased in human myotubes treated with vitamin D,[43] suggesting the possibility that in mVDR mice, the decrease could be mediated by effects of another cell type with intact vitamin D signalling.

Another potential contributor to the smaller decrease in muscle mass in mVDR mice is *Myostatin*, which is increased in whole-body VDRKO mice. Myostatin inhibits muscle hypertrophy and hyperplasia, so lower levels permit increased muscle growth.[22] In contrast to whole-body VDR-null mice, myostatin was significantly decreased in soleus, and it is interesting to note that there was no decrease in weight of this muscle in mVDR mice.

Because the whole muscles are lighter, but individual fibres are not smaller, it follows that there must be fewer myofibres in mVDR mice, and this was confirmed in quadriceps by counting total fibres at the muscle midpoint for the whole muscle. The decreased expression of cyclin D genes 1–3 and decreased expression of *Cdk2* and *Cdk4* could also contribute to decreased myofibre number as they would decrease cell cycle progression. Decreased myofibre number and muscle mass would both contribute to decreased strength. Impaired muscle function was shown by two modalities: grip strength and running speed. These techniques test different muscles, and acute vs. more chronic effort; both were significantly affected.

As discussed earlier, calcium handling is important for normal muscle function[44] and calcium handling in the whole body is regulated by vitamin D signalling.[45,46] We examined expression of intracellular calcium handling genes and found significant changes in expression of Serca2b and 3 in quadriceps. These sarcoplasmic/endoplasmic reticulum calcium ATPase proteins are calcium pumps in the ER and muscle sarcoplasmic reticulum membrane that help to concentrate calcium in the lumen of the ER.[47] This permits muscle relaxation after contraction. Calbindin acts both as a calcium buffer and in some tissues as a calcium sensor. Its buffering function would lower the available cytosolic calcium and also help with normal muscle relaxation. It is known to be regulated by vitamin D signalling in other tissues including kidney.[48] It is therefore possible that reduced grip strength in these mice was the cause not only of reduced muscle mass but also alterations in contraction–relaxation via alterations in the calcium handling apparatus.

We therefore report significant phenotypic changes in muscle following tissue-specific deletion of VDR. VDR is present in muscle, deletion in myocytes impairs muscle function, muscle size is reduced, but myofibres are slightly larger. The larger myofibres contrast to the findings in whole-body knockout VDR mice. This suggests that as well as the role for myocyte VDR in muscle size and function, vitamin D signalling in other tissues or cell types plays a role in initial muscle development.

The results suggest that maintenance of normal vitamin D signalling is important for preservation of muscle bulk and function. These findings also suggest that therapies targeting VDR could impact skeletal muscle mass and function and may present a novel strategy in addressing or preventing age-related sarcopenia and other disorders of muscle function.

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The authors certify that they comply with the ethical guidelines for publishing in the Journal of Cachexia, Sarcopenia and Muscle: update 2017.[49]

Conflict of interest

The authors declare that they have no relevant conflicts of interest.

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