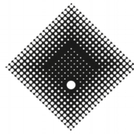

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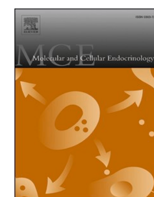

Detecting the vitamin D receptor (VDR) protein in mouse and human skeletal muscle: Strain-specific, species-specific and inter-individual variation

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ABSTRACT

Vitamin D, and its receptor (VDR), play roles in muscle development/function, however, VDR detection in muscle has been controversial. Using different sample preparation methods and antibodies, we examined differences in muscle VDR protein abundance between two mouse strains and between mice and humans. The mouse D-6 VDR antibody was not reliable for detecting VDR in mouse muscle, but was suitable for human muscle, while the rabbit D2K6W antibody was valid for mouse and human muscle. VDR protein was generally lower in muscles from C57 B l/6 than FVB/N mice and was higher in human than mouse muscle. Two putative VDR bands were detected in human muscle, possibly representing VDR isoforms/splice variants, with marked inter-individual differences. This study provides new information on detecting VDR in muscle and on inter-mouse strain and inter-human individual differences in VDR expression. These findings may have implications for future pre-clinical and clinical studies and prompt further investigation to confirm possible VDR isoforms in human muscle.

1. Introduction

Vitamin D (Vit D) plays a fundamental role in the maintenance of bone mineral homeostasis and, therefore, bone structure and function (Henry, 2011). Vit D can be obtained through dietary intake, and through exposure of the skin epidermis to ultraviolet B (UVB) radiation which stimulates the photoconversion of 7-dehydrocholesterol to Pre-Vit D_3 (DeLuca, 2004). Pre-Vit D_3 then spontaneously isomerizes to Vit D_3 (cholecalciferol), which is subsequently converted to calcifediol [25(OH)D_3] in the liver. In turn, calcifediol is transported in the plasma to the kidney, bound to the Vit D binding protein (VDBP), where it is converted to the active form of Vit D, calcitriol [1,25$(OH)_2D_3$], by the enzyme, 1-α-hydroxylase (CYP27B1). Calcitriol is then able to exert its biological effects by binding to the intracellular Vit D receptor (VDR) in target tissues. Finally, after its action in target tissues, active calcitriol can then be inactivated by Vit D_3 24-hydroxylase (CYP24A1) to calcitetrol (DeLuca, 2004).

The VDR belongs to the nuclear steroid receptor family and is expressed in most tissues. The calcitriol-bound VDR forms a heterodimer with the retinoid X receptor (RXR), which binds to DNA Vit D response elements and regulates gene expression by interacting with other regulatory proteins (Thompson et al., 1998). In addition to the genomic action of calcitriol/VDR complexes, which typically takes place over the

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Abbreviations

Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
EDL	extensor digitorum longus
KO	knock-out
MAPK	mitogen-activated protein kinase
MARRS	membrane-associated rapid response steroid binding protein
PI3K	phosphoinositide 3-kinase
PIP_3	phosphatidylinositol (345)-triphosphate
PKA	protein kinase A
PLA_2	phospholipase A_2
PLC	phospholipase C
PLT	plantaris
Pre-Vit D_3	pre-vitamin D_3
RXR	retinoid X receptor
SOL	soleus
TA	tibialis anterior
HRT	heart
UVB	ultraviolet B
VDBP	vitamin D binding protein
VDR	vitamin D receptor
Vit D	vitamin D
Vit D_3	vitamin D3

time frame of hours to days, there is evidence that calcitriol can exert rapid (sec-min) non-genomic actions in cells (Carlberg, 2018) via the activation of various signalling molecules and second messengers including, PLC, PLA_2, PI3K, Ras, Ca^{2+}, cAMP, PIP_3, PKA, SRC, MAPKs (for a detailed review see (Hii and Ferrante, 2016)). However, it remains to be determined which of these non-genomic actions of Vit D require the classical VDR or another type of Vit D-binding protein/receptor (e.g. the membrane-associated rapid response steroid binding protein; MARRS) (Nemere et al., 2004). Nonetheless, Vit D has the potential to impact a range of cellular responses via genomic and non-genomic mechanisms.

In addition to its well described role in bone biology, there is increasing evidence that Vit D plays important roles in other tissues, including the development of skeletal muscle (for reviews see (Bouillon et al., 2018; Girgis and Brennan-Speranza, 2021)). For example, VDR is highly expressed in cultured proliferating myoblasts and then decreases during differentiation into myotubes (Girgis et al., 2014), and the developmental knockout of the VDR in mice results in dysregulation of myogenic transcription factors (Endo et al., 2003). To date, a significant body of knowledge has accumulated regarding the non-genomic functions of Vit D in skeletal muscle cells (for review see (Boland, 2011)); however, the genomic role of Vit D in mature skeletal muscle has been somewhat controversial, in part, due to the relatively low expression of the VDR in muscle compared to other tissues, such as the liver and kidney (Wang et al., 2012). Using a mouse monoclonal antibody (clone D-6) that had been previously validated for the detection of the VDR in mouse tissues with high VDR expression (e.g. duodenum and kidney (Wang et al., 2010);), Wang and Deluca concluded that the VDR protein was undetectable in mouse skeletal muscle via immunohistochemistry or Western blot (Wang and DeLuca, 2011).

More recently, however, using the D-6 VDR antibody and a specific sample lysis buffer and sample preparation protocol, combined with Western blotting, Girgis et al. (2014) reported the detection of a faint band at a molecular weight corresponding with that of the VDR in mature mouse skeletal muscle (Girgis et al., 2014). This finding re-affirmed that the VDR protein is indeed expressed, at least in mouse skeletal muscle, and at very low levels compared to other tissues (e.g. the kidney). In contrast to these mouse data, using the same lysis buffer and D-6 antibody, we recently reported relatively robust Western blot signals for VDR protein in human skeletal muscle samples (Brennan-Speranza et al., 2017), suggesting the possibility of species-related differences in the level of VDR expression in skeletal muscle. Indeed, there are significant differences in the location, structure and regulation of the mouse and human VDR genes (for review see (Marcinkowska, 2020)).

In addition to potential species-related differences in VDR expression and, thus, Vit D action, recent studies have reported mouse strain-related differences with respect to Vit D levels and Vit D metabolism. For example, black C57 B l/6 mice have higher circulating levels of calcitriol [$1,25(OH)_2D_3$] compared to white BALB/c mice when fed the same Vit D sufficient diet (Misharin et al., 2009a). Others have also shown higher levels of plasma calcifediol [$25(OH)D_3$] in C57 B l/6 mice compared to white BALB/c and KK/HlJ mice (Berndt et al., 2011; Groves et al., 2017). Furthermore, C57 B l/6 mice have ~17-fold higher expression of kidney CYP27B1 mRNA compared to white BALB/c mice (Misharin et al., 2009b). Finally, black mouse strains have a higher susceptibility to UVB-induced immunosuppression compared to white strains of mice (Noonan and Hoffman, 1994), and Vit D protects against UVB-induced immunosuppression in black C57 B l/6 mice but not in white BALB/c mice (Malley et al., 2013). When combined, these data suggest the possibility of differences in Vit D signalling in skeletal muscle of different mouse strains, which may have implications for mouse-based pre-clinical studies on the action and metabolism of Vit D in skeletal muscle.

Therefore, the overall aim of this study was to compared two commercially available VDR primary antibodies (the mouse D-6 clone vs the more recently available rabbit D2K6W clone) and three published sample preparation methods for their suitability for detecting the VDR protein via Western blot in mouse and human skeletal muscle, and to investigate possible inter-species and inter-mouse strain differences in the expression of the VDR protein in mouse and human skeletal muscle.

2. Methods

2.1. Animal studies

All experimental procedures were approved by the Victoria University Animal Ethics Committee and conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th ed, 2013).

Female black C57 B l/6 and white FVB/N mice were purchased from Animal Research Centre (Western Australia, Australia) and housed at the Western Centre for Health, Research and Education (Sunshine Hospital, Victoria, Australia) in a light- and temperature-controlled room (12 h light/dark cycle, 21 °C) with *ad libitum* access to food and water. Mice were deeply anesthetised with isoflurane and the extensor digitorum longus (EDL), soleus, plantaris, and tibialis anterior (TA) muscles, and the kidney and the heart, were collected and snap frozen in liquid nitrogen for later analysis.

Eight-week-old Fischer 344 male rats were obtained from the Animal Resource Centre (Western Australia, Australia) and housed in pairs at the Western Centre for Health, Research and Education (Sunshine Hospital, Victoria, Australia) in a light- and temperature-controlled room (12 h light/dark cycle, 21 °C). Rats were deeply anaesthetised with an intraperitoneal injection of pentobarbital (60 mg/mL) and the PLT, EDL and SOL were collected and snap frozen in liquid nitrogen for later analysis. Rats were killed by removal of the heart under while still under deep anaesthesia.

Knock-out (KO) kidney tissue was received as a generous gift from

Prof. JE Gunton (University of Sydney). The original VDRKO mouse was generated by Li et al. (1997) on a C57 B l/6 background.

2.2. Human study

All experimental procedures were approved by, and conducted in accordance with, the Victoria University Human Research Ethics Committee and was registered with the Australian New Zealand Clinical Trials Registry (www.anzctr.org.au; ACTRN12615000755538). Verbal and written explanations about the study were provided prior to obtaining written informed consent. The study protocol for obtaining human biopsy samples has been previously published and methods used for muscle tissue for sampling in this study are described in (Parker et al., 2019). Briefly, muscle samples from healthy young men aged 28 ± 2 yr were obtained from the vastus lateralis under local anaesthesia (xylocaine 1%; AstraZeneca, Macquarie Park, NSW, 2113, Australia) utilizing a Bergstrom needle with suction. The samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

2.2.1. Serum 25 hydroxyvitamin D3 ($25(OH)D_3$) analysis

A blood sample was collected from an antecubital vein after an overnight fast of at least 10 h. Blood was centrifuged and serum was stored at −80 °C until analysis of circulating $25(OH)D_3$ (calcifediol) at Austin Pathology, Melbourne, Victoria, on the Modular Analytics E170 (Roche, Germany) according to manufacturer's instructions (Brennan-Speranza et al., 2017).

2.3. Western blotting

Western blot samples were prepared using one of three different homogenization protocols/buffers. Specifically, some samples were prepared using our standard protocol (Method A (Tabbaa et al., 2021);) of 20 s homogenization with a handheld Omni homogenizer (Model #TH220) in ice-cold buffer A, consisting of 40 mM Tris, pH 7.5; 1 mM EDTA; 5 mM EGTA; 0.5% Triton X-100; 25 mM β-glycerolphosphate (β-GP); 25 mM NaF; 1 mM Na_3VO_4; 10 μg/mL leupeptin; and 1 mM phenylmethylsulfonyl fluoride (PMSF). Some samples were processed using the protocol and buffer of Girgis et al. (Method B (Girgis et al., 2014);), which consisted of 20 s homogenization in ice-cold hyperosmolar buffer comprising 10 mM Tris; 6.7 M urea; 10% glycerol; 1% sodium dodecyl sulfate (SDS); 1 mM dithiotreitol (DTT); 1 mM PMSF; 25 mM β-GP; 25 mM NaF; 1 mM Na_3VO_4; and 10 μg/mL leupeptin. Following homogenization, these particular samples were then subjected to sonication with a tip sonicator (Model HD, 2070) for 20 s at 30% power. Lastly, other samples were prepared using the methodology of Camperi et al. (Method C (Camperi et al., 2017);) which involved 20 s homogenization in an ice-cold buffer composed of 80 mmol/L Tris-HCl, pH 6.8; 1 mmol/L DTT; 70 mmol/L SDS; 1 mmol/L glycerol; 25 mM β-GP; 25 mM NaF; 1 mM Na_3VO_4; 10 μg/mL leupeptin; and 1 mM PMSF. Regardless of the sample preparation method, whole homogenates were used for further Western blot analysis.

Sample protein concentrations were determined with a detergent compatible (DC) protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), and equivalent amounts of protein from each sample were dissolved in Laemmli buffer, heated for 5 min at 95 °C. Samples were then subjected to electrophoretic separation on SDS polyacrylamide gel electrophoresis (SDS-PAGE) gels. Following electrophoretic separation, proteins were transferred to a polyvinylidene fluoride membrane (300 mA, 1 h), blocked with 5% powdered milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h followed by an overnight incubation at 4 °C with primary antibody dissolved in TBST containing 1% BSA. After overnight incubation, the membranes were washed for 30 min in TBST and then probed with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Following 30 min of washing in TBST, the blots were developed with a DARQ CCD camera mounted to a Fusion FX imaging system (Vilber Lourmat, Eberhardzell, Germany) using ECL Prime reagent (Amersham, Piscataway, NJ, USA). Membranes were then stained for total protein with Coomassie Blue. The signal for the band of the protein of interest was then normalized to the signal for total protein in each lane. Densitometric measurements were carried out using Fusion CAPTAdvance software (Vilber Lourmat) and quantification of Coomassie Blue images was completed on Image J. Complete blots for each main figure presented in the Results section are provided as Supplementary Figs. 3–9.

2.4. Antibodies

VDR mouse D-6 (Santa Cruz, SC-13133; 1:1000); VDR rabbit D2K6W (Cell Signalling Technology, 12,550; 1:1000); Anti-mouse HRP IgG (Vector Laboratories, PI-1000; 1:5000); Anti-rabbit HRP IgG (H + L) (Vector Laboratories, PI-2000; 1:20,000); Anti-mouse HRP IgG_{2a} (Jackson ImmunoResearch, 115-035-206; 1:50,000); Anti-mouse HRP IgG_{2c} (Jackson ImmunoResearch, 115-035-208; 1:50,000).

2.5. Statistical analysis

Data are presented as Mean ± Standard Error of Mean (SEM). Statistical significance was determined by conducting a Student's unpaired, 2-tailed *t*-test between the same muscles of the FVB/N and C57/Bl6 murine strains. Linear regression analysis was used to calculate the correlation between the vitamin D proteins analysed in the human samples. Differences between groups were considered significant when $p < 0.05$. All statistical analyses were performed on GraphPad Prism Version 9 software (La Jolla, CA).

3. Results

Potential issues when using the mouse D-6 primary antibody to detect VDR expression in murine skeletal muscle.

Using a mouse monoclonal antibody (clone D-6) that had been validated for the detection of the VDR in mouse tissues with high VDR expression (e.g. duodenum and kidney (Wang et al., 2010);), Wang and Deluca concluded that the VDR protein was undetectable in mouse skeletal muscle via immunohistochemistry or Western blot (Wang and DeLuca, 2011). However, more recently, Girgis et al. (2014), also using the D-6 antibody, combined with a sample preparation protocol thought to maximise the extraction of the nuclear hormone receptors, concluded that the VDR protein was detectable in mature mouse skeletal muscle, albeit at very low levels. Since then, using the D-6 antibody, combined with a different Western blot sample homogenization buffer to that used by Girgis et al., Camperi et al. (2017) reported relatively robust Western blot signals for the VDR protein in mouse skeletal muscle and that the level of VDR expression was increased by Vit D supplementation and in a model of cancer cachexia.

Given these reported differences in the strength of VDR detection, we first validated the mouse D-6 VDR primary antibody in our hands in a tissue with relatively high VDR abundance (i.e. kidney) using three different sample preparation methods. Briefly, Method A involved homogenization with our standard Western blot homogenization buffer (Tabbaa et al., 2021; Goodman et al., 2011), Method B used the homogenization/sonication protocol and buffer of Girgis et al. (2014), while Method C involved homogenization with the buffer of Camperi et al. (2017). As shown in Fig. 1A, when combined with a general anti-mouse IgG (heavy and light chain) secondary antibody, all three sample preparation methods detected a band in the WT kidney sample at the expected size for the VDR (~50 kDa) at a relatively short exposure time (1 min). Importantly, this band was absent in a VDR knockout kidney sample that was prepared using the same three protocols. Interestingly, when the exposure time was increased substantially to 30 min, ~50 kDa bands were detected in the lanes of VDR KO samples (Fig. 1B), suggesting that the primary and/or secondary antibodies bound to another, less abundant, protein species in the VDR KO kidney.

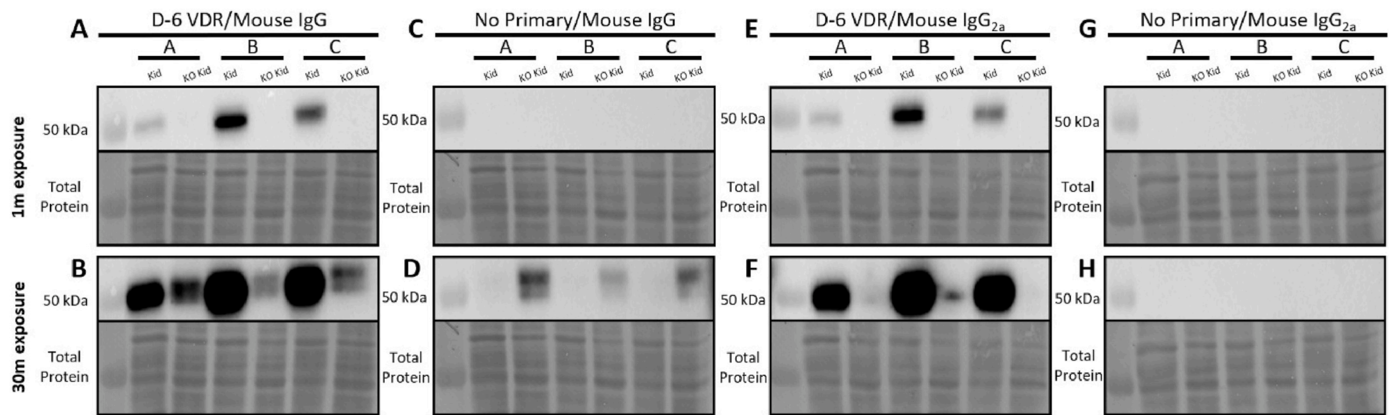

Fig. 1. VDR expression in WT and VDR knockout mouse kidney using the mouse D-6 primary antibody. Kidney (Kid) samples from C57 B l/6 wild-type (WT) and VDR knockout (KO) mice were homogenised using either Method A (A), Method B (B) or Method C (C; see Methods for details), and equivalent amounts of protein were separated via SDS-PAGE as described in the methods. Western blots show vitamin D receptor (VDR) protein expression using the mouse D-6 VDR antibody and a general mouse IgG secondary antibody at (A) 1 min exposure and (B) 30 min exposure. The primary antibody was omitted, and blots were imaged at (C) 1 min exposure and (D) 30 min exposure. Western blots utilizing the mouse D-6 VDR antibody and a specific mouse IgG_{2a} secondary antibody were also imaged at (E) 1 min exposure and (F) 30 min exposure. The primary antibody was omitted, and blots were imaged at (G) 1 min exposure and (H) 30 min exposure.

One important consideration that arises when using a mouse primary antibody for detection of a protein in mouse tissue is the potential for the secondary antibody to detect endogenous IgG, in addition to the primary antibody bound to the protein of interest (POI). This is especially important when the POI is approximately the same molecular mass as the endogenous IgG heavy or light chains. For example, the molecular mass of the IgG heavy chain is ~50 kDa, while the VDR molecular mass is reported to be between 48 and 53 kDa (https://www.uniprot.org/uniprot/P11473). Thus, there is potential for the signal from the endogenous IgG heavy chain to interfere with the detection of the VDR in mouse tissue, especially if the VDR expression is relatively low and/or long exposure times are required. Therefore, to investigate whether this non-specific binding in the VDR KO tissue was due to the primary or secondary antibody, we ran the WT and KO samples again without the primary antibody, while still incubating with the general anti-mouse IgG secondary antibody. As expected, at the short exposure time, the VDR signal in the WT samples was absent (Fig. 1C), however, at the longer exposure, the bands in the VDR KO lanes remained (Fig. 1D), suggesting that the general anti-mouse secondary was reacting with another protein, possibly endogenous IgG heavy chain. Indeed, when we repeated the analysis using the D-6 primary antibody and an anti-mouse IgG 2a-specific secondary antibody (Note: the D-6 antibody is IgG 2a isotype and C57 B l/6 mice do not express IgG 2a endogenously but express IgG 2c instead (Morgado et al., 1989; Martin et al., 1998)), the non-specific binding at the long exposure time was markedly reduced but not completely eliminated (compare Fig. 1B and 1F). Moreover, when the IgG 2a-specific secondary antibody was used without the primary VDR antibody, the signal was eliminated at the long exposure time (compare Fig. 1D and 1H). These data suggest that the 'non-specific' signal in the VDR knockout samples is predominantly coming from the anti-mouse secondary antibody detecting endogenous mouse IgG. Thus, while the D-6 antibody may be used with confidence in mouse tissue with high VDR expression (i.e. kidney), it may lead to issues when being used in tissues with low VDR expression, such as mature skeletal muscle.

To highlight the potential problem of using the D-6 antibody in mouse skeletal muscle, we compared the VDR signal in EDL muscles from FVB/N and C57 B l/6 mice. To decide on which sample preparation method to use from this point onwards, we first compared the VDR signals obtained when muscle samples were prepared using Methods B and C, which gave the strongest signals in Fig. 1A. Furthermore, to avoid the issue of using a mouse primary in mouse tissue, we used rat skeletal muscle. As shown in Suppl. Fig. 1, the signal from the D-6 VDR antibody was comparable between Methods B and C and, importantly, the signal was eliminated in the absence of the primary antibody. Based on this analysis, we chose to use Method C (Camperi et al., 2017) for all remaining VDR analysis due to its relative simplicity compared to Method B. When we attempted to compare the VDR signal in EDL muscles from FVB/N and C57 B l/6 mice using the D-6 primary antibody, combined with a general anti-mouse IgG secondary, it appeared that VDR protein abundance was greater in C57 B l/6 muscle (Fig. 2A); however, when we eliminated the primary antibody, the 'VDR' signal remained, indicating that the signal was predominantly coming from detection of endogenous IgG (Fig. 2B). To reduce this non-specific IgG signal, we next used the IgG 2a-specific secondary, which gave a lower VDR signal (Fig. 2C) in the C57 B l/6 EDL (which doesn't contain endogenous IgG 2a) that was eliminated in the absence of the primary antibody (Fig. 2D); however, the IgG 2a secondary gave an enhanced 'VDR' signal in the FVB/N muscle (Fig. 2C) which, as expected (FVB/N mice express IgG 2a), remained in the absence of the primary antibody (Fig. 2D). To further demonstrate that the 'VDR' signal obtained in the FVB/N muscle using the IgG 2a secondary is indeed non-specific (Fig. 2C), we combined the D-6 primary antibody with an IgG 2c-specific secondary (Note: IgG 2c is not expressed in FVB/N mice but is expressed in C57 B l/6 mice). Using this combination, we were now unable to detect a VDR signal in the FVB/N muscle (Fig. 2E) but, as expected, detected a robust signal in the C57 B l/6 muscle (Fig. 2E), which was not eliminated in the absence of the primary antibody (Fig. 2F), indicating non-specific binding. When combined, these data demonstrate that the D-6 VDR primary antibody is relatively unreliable for detecting VDR protein levels in mouse skeletal muscle, especially when comparing across different strains.

3.1. Validation of the rabbit D2K6W primary antibody for detecting VDR protein

Given the unsuitability of the D-6 antibody for study in mouse muscle tissue, we next tested a more recently available rabbit monoclonal VDR antibody (clone D2K6W) with the WT and KO kidney samples prepared using Methods A, B and C. As shown in Fig. 3, this antibody, combined with a general anti-rabbit secondary antibody, also detected the VDR at the appropriate molecular mass in the WT, but not KO, samples at relatively short exposure times (i.e. 10 sec) (Fig. 3A), and that this signal was eliminated when the primary antibody was omitted (Fig. 3C). Interestingly, like the D-6 antibody (Fig. 1B), when a longer exposure was used (i.e. 10 min), a faint band also appeared at ~50 kDa in the lanes containing VDR KO kidney samples prepared using Methods A and

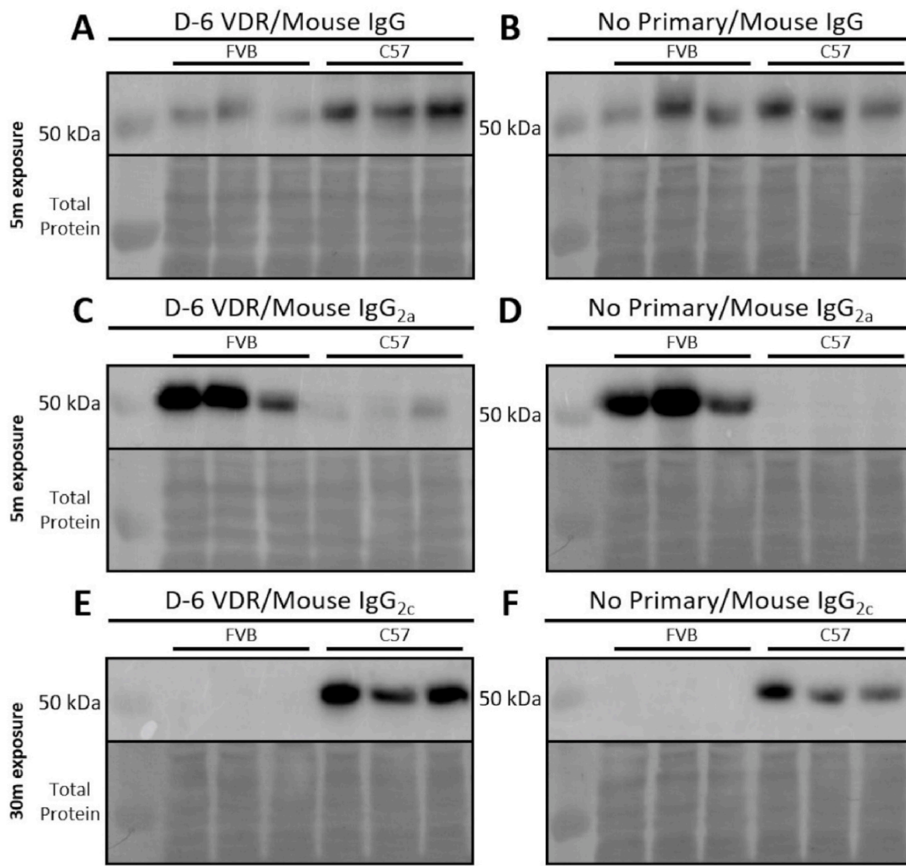

Fig. 2. *VDR expression in mouse skeletal muscle using the mouse D-6 primary antibody. Extensor digitorum longus (EDL) muscles of FVB/N (FVB) and C57 B l/6 (C57) mice were homogenised using Method C, and equivalent amounts of protein were separated via SDS-PAGE as described in the Methods. Western blots show vitamin D receptor (VDR) protein expression using a general mouse IgG secondary antibody (A) with and (B) without the primary mouse D-6 VDR antibody, a specific mouse* IgG_{2a} *secondary antibody (C) with and (D) without the primary D-6 VDR antibody or a specific mouse* IgG_{2c} *secondary antibody (E) with and (F) without the primary D-6 VDR antibody. N=3* per group. Note: the total protein stain for are the same as the membrane used for Fig. 2B was stripped and reprobed with the mouse IgG2c antibody (Fig. 2F). Furthermore, the total protein stain for Fig. 2D and 2E and are also the same as the membrane used for Fig. 2D was stripped and repobed for D-6 VDR (Fig. 2E).

B, but not Method C (Fig. 3B). The reason that both the D-6 and D2K6W antibodies detected faint bands in the VDR KO samples at longer exposure times (Figs. 1F and 3B) remains to be determined. Importantly, all the bands detected at this longer exposure time were eliminated in the absence of the primary antibody, indicating that these bands were specific for the primary antibody. Thus, based on these data we proceeded to compare VDR expression in skeletal muscles from FVB/N and C57 B l/6 mice prepared using Method C.

Differences in VDR protein abundance in muscles from white FVB/N and black C57 B l/6 mice.

As shown in Fig. 4, the D2K6W antibody produced two bands at, or just above, 50 kDa, likely corresponding to splice variant isoforms (https://www.uniprot.org/uniprotkb/P11473/entry). For quantification, the intensity of both bands was used and, as shown in Fig. 4, VDR protein abundance was higher in FVB/N SOL, EDL, PLT and TA muscles compared to those from C57 B l/6 mice. This analysis shows that VDR protein abundance is generally higher in muscles from white FVB/N mice compared to those from black C57 B l/6 mice, suggesting the possibility of strain-related differences in Vit D action in skeletal muscle.

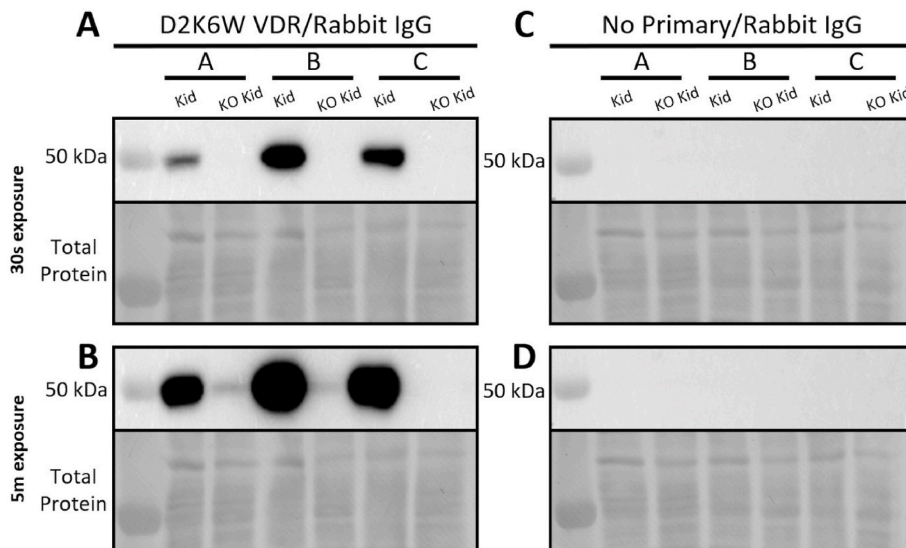

Fig. 3. VDR expression in WT and KO mouse kidney using the rabbit D2K6W primary antibody. Kidney (Kid) samples from C57 B l/6 wild-type (WT) and VDR knockout (KO) mice were homogenised using either Method A (A), Method B (B) or Method C (C), and equivalent amounts of protein were separated via SDS-PAGE as described in the methods. Western blots show vitamin D receptor (VDR) protein expression with the D2K6W VDR primary antibody combined with a general rabbit IgG secondary antibody at (A) 30 s exposure and (B) 5 min exposure. The primary antibody was omitted, and blots were imaged at (C) 30 s exposure and (D) 5 min exposure.

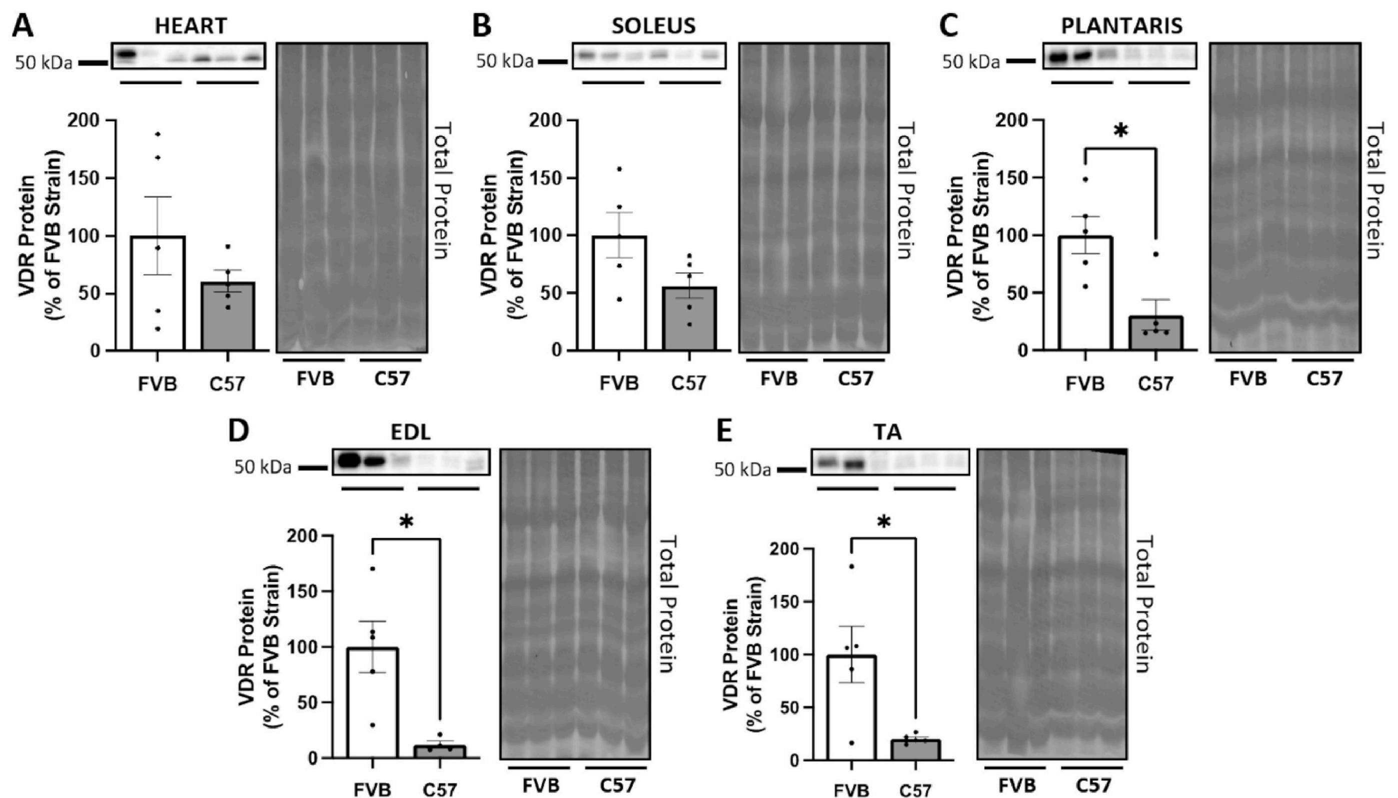

Fig. 4. VDR protein expression in mouse skeletal muscle using the D2K6W primary antibody. Muscles from FVB/N (FVB) and C57 B l/6 (C57) mice were homogenised using Method C, and equivalent amounts of protein were separated via SDS-PAGE as described in the Methods. Western blots show vitamin D receptor (VDR) protein expression in the (A) heart, (B) soleus, (C) plantaris, (D) extensor digitorum longus (EDL) and (E) tibialis anterior (TA) muscles using the D2K6W VDR primary antibody. C57Bl/6 values expressed as a percentage of FVB/N values. N = 5 per group. *$p < 0.05$.

3.1.1. *VDR protein abundance in human compared to mouse skeletal muscle*

Next, we examined VDR protein abundance in human skeletal muscle and compared these to mouse skeletal muscle. For this analysis, 8 healthy young men underwent biopsies of the vastus lateralis muscle and venous blood sampling to determine serum Vit D concentration. The mean characteristics of these participants are shown in Table 1. As shown in Fig. 5A and B, the D2K6W and D-6 VDR antibodies detected 2 bands at or just above 50 kDa. To determine whether any of the signal was non-specific, the human samples were also run without the primary antibody to determine whether the secondary antibody would produce any non-specific signal and, as expected, no non-specific signal was observed (Suppl. Fig. 2). Interestingly, there was marked inter-individual variation in the overall intensity and in the ratio of the upper and lower bands in the human samples. Moreover, when compared to the EDL muscles of FVB/N mice, the level of VDR protein detected by the D2K6W antibody (both bands combined; Fig. 5A) in the human samples was markedly higher (Fig. 5C). (Note: due to the strong signal for the VDR in the FVB/N samples caused by the presence of endogenous IgG 2a, we did not quantify the species difference in VDR protein detected by the D-6 antibody). Upon closer inspection, there also appeared to be differences in the intensity of the upper VDR band detected by the 2 antibodies in the human samples, potentially due to differences in epitopes (D-6 detects the VDR C-terminal vs D2K6W detects the VDR N-terminal), epitope affinity and/or the abundance of different isoforms/splice variants. Indeed, while the intensities of the lower bands detected by the 2 primary antibodies were positively correlated, there was no significant correlation between the upper band intensities (Fig. 5D and E). Overall, these data reveal species-related differences in muscle VDR protein abundance, and the possibility of differential expression of VDR isoform/splice variants.

Table 1
Human participant characteristics.

	Age (yr)	Weight (kg)	Height (cm)	BMI (kg/m^2)	Resting Heart Rate (bpm)	$VO_{2\,Peak}$ (ml/kg/ml)	Serum 25 $(OH)D_3$ (mmol/l)
1	21	72.9	174.5	23.9	68	53.8	100
2	25	86.9	191.8	23.6	68	51.2	63
3	24	76.9	175.4	25.0	79	46.4	89
4	26	89.4	187.7	25.4	65	46.9	97
5	38	81.3	176.2	26.2	78	39.8	31
6	31	85.4	191.0	23.4	58	51.4	72
7	26	95.4	180.4	29.3	57	35.3	151
8	30	57.0	163.0	21.5	74	60.0	25
Ave	**27.6**	**80.7**	**180.0**	**24.8**	**68.4**	**48.1**	**78.5**

4. Discussion

This study compared two commercially available VDR primary antibodies (the mouse D-6 clone vs the more recently available rabbit D2K6W clone) and three published sample preparation methods for their suitability for detecting the VDR protein via Western blot in mouse and human skeletal muscle, and to investigate possible inter-mouse strain (black vs white), inter-species (mouse vs human) and inter-individual (human) differences in VDR protein abundance in skeletal muscle. We found that, while the mouse monoclonal VDR D-6 antibody is valid for detecting VDR in tissues with high relative VDR expression (e.g. kidney), it is not reliable for VDR analysis in mouse skeletal muscle due to the potential for non-specific binding by the required anti-mouse IgG secondary antibody to endogenous mouse IgG. Instead, we show that the rabbit D2K6W antibody is a more appropriate option for use in

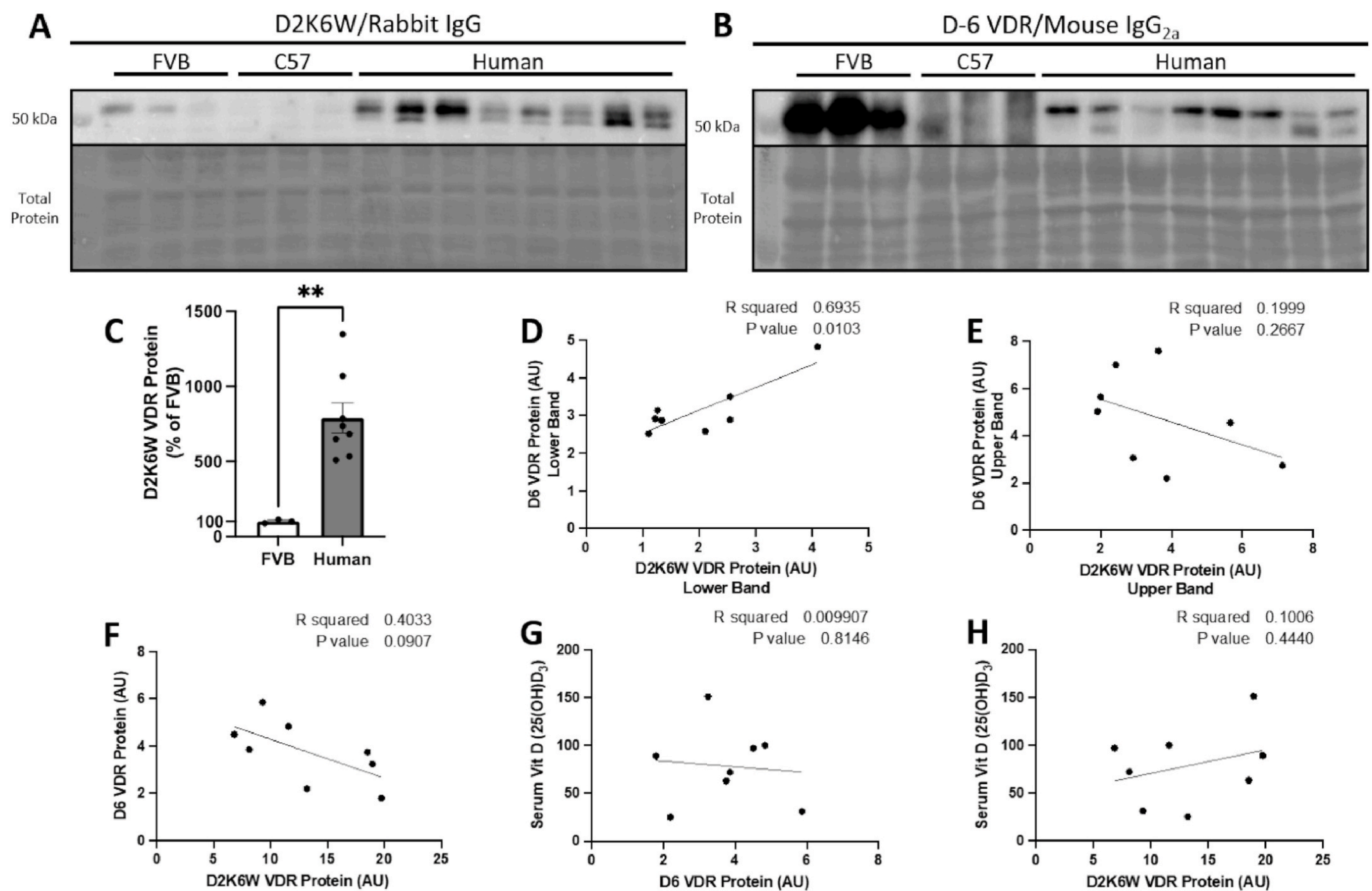

Fig. 5. VDR protein expression in mouse and human muscle. Extensor digitorum longus (EDL) muscles from FVB/N (FVB) and C57 B l/6 (C57) mice, and vastus lateralis muscle biopsy samples from human subjects were homogenised using Method C (see Methods for details), and equivalent amounts of protein were separated via SDS-PAGE as described in the Methods. Westerns blots show (A) vitamin D receptor (VDR) protein using the D2K6W VDR antibody, (B) VDR protein using the D-6 antibody, and (C) the quantification of FVB muscle VDR protein compared to human muscle sample using the D2K6W VDR antibody. N = 3 per murine groups; n = 8 human group. $^{**}p < 0.01$. Correlation graphs depicting the relationship between: (D) the lower bands of the VDR detected by the D-6 and D2K6W primary antibodies, (E) the upper bands of the VDR detected by the D-6 and D2K6W primary antibodies, (F) total signal of the VDR detected by the D-6 and D2K6W primary antibodies, (G) the serum vitamin D (Vit D; $25(OH)D_3$) and the VDR detected by the D6 primary antibody, and (H) the serum Vit D and the VDR detected by the D2K6W primary antibody. Correlation coefficients (r^2) and p values are shown on each correlation graph.

mouse muscle. Using this antibody, we found that VDR is expressed in skeletal muscle, with its abundance generally being lower in muscles from black C57 B l/6 mice compared to white FVB/N mice. In addition, we showed that both the D-6 and D2K6W antibodies can be reliably used to detect the VDR in human skeletal muscle, albeit with slightly different results, which may be related to differences in epitope or isoform specificity. The VDR was detected in significantly higher abundance in human skeletal muscle compared to mouse muscle, and there was marked inter-individual variability in total human VDR abundance and in the relative ratios of putative VDR isoforms. Overall, these findings suggest that the rabbit D2K6W VDR antibody is more appropriate than the mouse D-6 antibody for detecting the VDR protein via Western blot in mouse skeletal muscle. Moreover, the identified mouse strain-dependent differences in the abundance of VDR protein suggests the possibility of strain-dependent differences in VDR signalling, which may have implications for the choice of mouse strain to be used in pre-clinical studies, as it is not yet clear whether differences in the abundance of VDR results in differences in Vit D signalling basally and/or under conditions of Vit D depletion or supplementation. Finally, our results suggest the possible presence of different VDR isoforms/splice variants in human skeletal muscle with significant inter-individual differences in their abundance; however, this data requires further work to definitively confirm that the two bands that are detected by the VDR antibodies are indeed both VDR protein. If so, this would open new avenues of research to further investigate the regulation and physiological significance, if any, of VDR isoforms/variants in human skeletal muscle.

To enable us to investigate mouse strain-related differences in the abundance of muscle VDR protein, we needed to establish which commercially available antibody was the most appropriate to use. The mouse D-6 antibody had previously been validated by Wang et al. (2010) in mouse tissues with relatively high VDR expression; however, using the D-6 antibody, the same group failed to detect the VDR in skeletal muscle (Wang and DeLuca, 2011). More recently, a very low level of VDR protein was reported in mature male mouse muscle using the D-6 antibody combined with a specific sample preparation protocol (Girgis et al., 2014) (Method B in the current study). In contrast, another study, also using the D-6 antibody but with different sample preparation protocol (Method C in this study), reported that the VDR was upregulated in muscle from cachectic mice and Vit D-treated mice (Camperi et al., 2017). One potential problem associated with using a mouse primary antibody in mouse tissue, especially if the POI is lowly expressed, is that the secondary antibody will not only detect the primary antibody bound to the POI, but also the endogenous IgG heavy and light chains. This is particularly important if the POI is a similar size to IgG heavy (~50 kDa) or light chains (~25 kDa). Given that the mouse VDR has a reported molecular mass of between 48 and 53 kDa (https://www.uniprot.org/uniprot/P11473), the possible simultaneous detection of the VDR and endogenous IgG heavy chain has the potential

to confound the results, especially in experimental conditions associated with increased inflammation, such as cancer cachexia (Zimmers et al., 2016). Depending on the isotype of the primary antibody, this issue may be minimised or eliminated with an isotype-specific secondary antibody; however, as shown in this study, this may also depend on the strain of mouse used. We recommend that if investigators need to use the D-6 antibody with mouse muscle tissue that a secondary antibody only blot is also run at the same exposure time to rule out the possibility that the secondary antibody is also detecting endogenous mouse IgG. Better still, our results show that a simpler solution is to use the rabbit D2K6W antibody with mouse muscle tissue, as an anti-rabbit secondary IgG will not detect endogenous mouse IgG. Interestingly, we found that the D2K6W antibody (and D-6) detected a faint band at longer exposure times in VDR KO tissue using sample preparation methods A and B, but not method C (see Figs. 3 and 1). Given that this signal was eliminated in the absence of the primary VDR antibody, this suggests the presence of some unknown non-specific binding. Thus, we recommend using the preparation Method C of Campari et al. for Western blot-based VDR protein analysis (Camperi et al., 2017).

Prompted by a previous study that had reported very low levels of VDR protein in mature male mouse skeletal muscle (Girgis et al., 2014) and our human study which showed seemingly robust VDR levels in adult human muscle (Brennan-Speranza et al., 2017) (both studies using the mouse D-6 primary antibody), we performed the first direct comparison of VDR protein between mouse and human muscle. Our results show that the signal for VDR protein (using the D2K6W antibody) is indeed higher in human muscle compared to mouse muscle. The differences in the abundance of VDR protein suggests that there may be differential regulation of the VDR gene expression, mRNA translation and/or protein stability between mice and humans. Indeed, there are significant differences in the location, structure and regulation of the mouse and human VDR genes (for review see (Marcinkowska, 2020)). Other factors not examined in this study that could also play a role in the observed species-dependent variability in VDR expression include differences in dietary Vit D intake, muscle/muscle fiber-type, sex and physical activity levels. Whether these inter-strain differences have implications for relating data from pre-clinical mouse studies to human clinical trials on factors that regulate VDR abundance and activity in skeletal muscle remains to be determined in future studies.

An interesting feature of our analysis of human muscle is the detection of at least two VDR bands, with both the D2K6W and D-6 antibodies, suggesting the possible presence of multiple VDR isoforms or splice variants. Indeed, it has been known for some time that the human VDR gene contains multiple alternate promoters that, when combined with alternate splicing, produces up to 14 different VDR mRNA transcripts (Crofts et al., 1998). Of these, 3 transcripts lead to the production of the major protein isoforms/variants with the following amino acid (aa) numbers and molecular weights: 1) 427 aa, ~48 kDa (considered the canonical sequence); 2) 450 aa, ~51 kDa (contains an extra 23 aa at the N-terminus compared to sequence #1); and 3) 477 aa, 54 kDa (contains an extra 50 aa at the N-terminus compared to sequence #1) (Crofts et al., 1998). Upon close inspection of our human VDR blots for both the D2K6W (Fig. 5A) and D-6 (Fig. 5B) antibodies, the upper band is located above the 50 kDa molecular weight marker, indicating that this band could represent the 54 kDa form of the VDR, while the lower band runs almost the same as the 50 kDa molecular weight marker, suggesting that this band may be the 51 kDa form or the 48 kDa form with a mobility shift due to a post-translation modification, such as phosphorylation (see https://www.phosphosite.org). One fascinating finding amongst these data is that there appears to be marked inter-individual variation in the ratio between the upper and lower bands (Fig. 5A and B). However, while these data are interesting, further work is now required to definitively confirm that these two bands are, in fact, the VDR.

In conclusion, this study provides important new information on sample preparation and antibody selection for the detection of the VDR in mouse skeletal muscle via Western blot, with the rabbit D2K6W antibody being more appropriate than the mouse D-6 antibody. Furthermore, the identified mouse strain-dependent differences in the abundance of the VDR protein suggesting the possibility of strain-dependent differences in Vit D metabolism and VDR signalling, which may have implications for the choice of mouse strain used in pre-clinical studies. Finally, our results suggest the possible presence of different VDR isoforms/splice variants in human skeletal muscle with significant inter-individual differences in their abundance; however, this data requires further work to definitively confirm that the two bands that are detected by the VDR antibodies are indeed both VDR protein.

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CRediT authorship contribution statement

Hannah Lalunio: Conceptualization, Data curation, Investigation, Methodology, Roles/, Writing – original draft. **Lewan Parker:** Data curation, Investigation, Writing – review & editing. **Erik D. Hanson:** Data curation, Investigation, Writing – review & editing. **Paul Gregorevic:** Funding acquisition, Resources, Writing – review & editing. **Itamar Levinger:** Data curation, Funding acquisition, Resources, Writing – review & editing. **Alan Hayes:** Conceptualization, Data curation, Resources, Funding acquisition, Supervision, Writing – review & editing. **Craig A. Goodman:** Conceptualization, Data curation, Funding acquisition, Resources, Investigation, Methodology, Supervision, Roles/, Writing – original draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Lewan Parker reports financial support was provided by National Health and Medical Research Council. Paul Gregorevic reports financial support was provided by National Health and Medical Research Council.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2023.112050.

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