Is the Vitamin D Receptor Found in Muscle?

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The active form of vitamin D, 1α,25-dihydroxyvitamin D_3, is critical for the regulation of serum calcium and phosphorus levels that in turn support bone mineralization and neuromuscular activity. It is well known that vitamin D deficiency causes rachitic/osteomalacic myopathy and cardiac disorder and the provision of vitamin D can reverse the symptoms. However, the underlying mechanisms remain unclear. The question of whether the vitamin D receptor is found in muscle has been debated but not settled. We recently studied all available antibodies against the vitamin D receptor and found that most antibodies used detect proteins other than the vitamin D receptor, and therefore, the utility of these antibodies may generate the false-positive results. Using antibodies that do not detect proteins in tissues from vitamin D receptor null mice, we have developed a specific and sensitive immunohistochemical assay. The results from this investigation show that the vitamin D receptor is undetectable in skeletal, cardiac, and smooth muscle, suggesting that the function of vitamin D on muscle is either of an indirect nature or does not involve the known receptor. **(*Endocrinology* 152: 354–363, 2011)**

Vitamin D_3 can be obtained from the diet or generated through photolysis of 7-dehydrocholesterol in the skin by UV light (1–3). Vitamin D is then activated by two sequential hydroxylation reactions. The first of these, 25-hydroxylation, takes place largely in the liver to produce 25-hydroxyvitamin D_3 (2). This molecule circulates bound to the vitamin D transport protein and is indicative of vitamin D status. The second hydroxylation takes place predominantly in the kidney to generate 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. The active metabolite, 1,25$(OH)_2D_3$, is a calcium/phosphorus-regulating hormone, indispensable for calcium and phosphate homeostasis. This function provides for normal skeletal mineralization and neuromuscular activity.

1,25$(OH)_2D_3$ acts by binding to the vitamin D receptor (VDR). VDR is a transcriptional factor that regulates gene expression in a ligand-dependent manner (2). It is classified as a member of the steroid hormone receptor superfamily (4). The human *VDR* gene located on chromosome 12q is composed of two promoters and regulatory regions (exons 1a-1f). The coding sequence found in exons 2–9 specifies the full-length VDR protein (5). The VDR contains several structurally and functionally important domains, including a nuclear localization signal, a hormone ligand-binding region, a DNA-binding sequence, a dimerization region, an activation function 2, and transactivation domains (6). 1,25$(OH)_2D_3$ is the physiological ligand for VDR in the nucleus of target cells and controls the transcription of target genes in a positive or negative fashion (5). VDR activation results in a stimulation of calcium absorption in intestine, calcium reabsorption in kidney, and calcium mobilization from bone (2). Interestingly, the activation of VDR can also control proliferation, differentiation, and survival of various cell lineages *in vitro* (7, 8), although the physiological relevance of these phenomena is not fully known.

There are three muscle types: skeletal, cardiac, and smooth. Their function is to produce force and cause motion. Muscles can cause either locomotion of the organism itself or movement of internal organs. Cardiac and smooth muscle contraction is controlled by the autonomic nervous system and is necessary for survival. Examples are heart contraction and intestinal peristalsis. Voluntary contraction of the skeletal muscles results in the movement of body parts and the organism as a whole. Impaired vitamin D actions, including vitamin D deficiency, genetic defects

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2010-1109 Received September 23, 2010. Accepted November 18, 2010.
First Published Online December 29, 2010

Abbreviations: DAPI, 4′,6′-Diamino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; KO, knockout; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; α-SMA, α-smooth muscle actin; VDR, vitamin D receptor; WT, wild type.

in the vitamin D-activating enzyme, 25-hydroxyvitamin D_3 1α-hydroxylase, or in the VDR lead to rickets or osteomalacia characterized by hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, and bone abnormalities due to mineralizing defects (9–12). Clinical evidence suggests that vitamin D plays a role in muscle metabolism and function (13–15). Progressive weakness and wasting of skeletal muscle have been demonstrated in patients with rickets or osteomalacia (16, 17). Plasma 25-hydroxyvitamin D_3 levels have been positively correlated with muscle power, force, velocity, and jumping height (13). Vitamin D supplementation is believed to improve muscle function and reduce falls in vitamin D-deficient, older adults (15). However, the mechanism of rachitic/osteomalacic myopathy is not fully understood, and it is currently unclear whether muscle abnormalities in those patients are a direct consequence of impaired vitamin D actions in muscle or a result of secondary systemic changes such as hypocalcemia, hypophosphatemia, and elevated PTH levels in the circulation. A loss of muscle strength occurs in vitamin D-deficient rats and clearly results from hypophosphatemia and not from an absence of vitamin D (18).

Recent epidemiological studies have shown that vitamin D deficiency is associated with death from heart failure and sudden cardiac death (14). In animal studies, vitamin D deficiency in Sprague Dawley rats has been reported to cause hypertension and cardiac hypertrophy (19). Treatment of Dahl salt-sensitive rats with the vitamin D analog, 19-nor-1,25-dihydroxyvitamin D_2, reverses cardiac hypertrophy in that model (20). In addition, the VDR knockout (KO) mouse displays hypertension, cardiac hypertrophy with enlargement of individual myocytes, and elevations in atrial natriuretic peptide expression (21, 22). 1α-Hydroxylase null mutant mice also show cardiac hypertrophy (23).

Thus, there is mounting evidence that vitamin D is involved in cardiac, skeletal, and perhaps smooth muscle function. Of major importance is the underlying mechanism. If vitamin D acts directly on muscle, VDR must be present. Before the availability of antibodies to the receptor, Stumpf *et al.* (24) studied by autoradiography the location of $1,25(OH)_2[^3H]D_3$ in the nuclei of target tissues. This approach failed to show such localization in muscle (25–28) but showed strong localization in targets such as intestinal enterocytes, osteoblasts, parathyroid cells, and distal renal tubules. Consistent with this is the failure to detect mRNA encoding VDR in heart and liver (29). Immunoassays on Western blot also did not support a finding of VDR in mature skeletal and heart muscle (30).

On the other hand, reports have appeared that VDR can be found in skeletal muscle cells, myoblasts, cardiomyocytes, and smooth muscle cells (19, 31). Low levels of VDR have also been reported in rat and mouse heart tissues on the basis of immunoblotting using chick monoclonal antibody 9A7 or rabbit polyclonal antibody C-20 (32–34). The first *in situ* detection of VDR in human skeletal muscle tissue by immunohistochemistry, again using VDR antibody 9A7 was reported by Bischoff *et al.* (35). However, the 9A7 and C-20 react with proteins on Western blot not related to VDR. Furthermore, these antibodies detect proteins in extracts prepared from *VDR* null mice (36) that have no VDR (37).

To address the critical issue of whether the VDR is present in mature muscle tissue *in vivo*, we determined the VDR in mouse, rat, and human muscle tissues including skeletal, cardiac, and smooth muscle with an antibody that does not react with tissue prepared from the Demay *VDR* KO mice. Our results show that the VDR is not detected in smooth muscle, heart muscle, or skeletal muscle.

Materials and Methods

Antibodies against VDR

The rat monoclonal monoclonal antibody 9A7 antibody was purchased from Affinity BioReagents (Rockford, IL). Mouse monoclonal antibody D-6 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse anti-α-smooth muscle actin (α-SMA) monoclonal antibody was purchased from Sigma (St. Louis, MO). The horseradish peroxidase (HRP)-conjugated goat antimouse IgG light-chain secondary antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA); Alexa fluor 594 goat antimouse or antirat IgG secondary antibodies were from Invitrogen (Carlsbad, CA).

Animals and maintenance

Six- to 7-wk-old C57BL/6 mice were used for our experiments. The Demay *VDR* KO mice and C57BL/6 littermates were purchased from Jackson Laboratory (36). After arrival in our facility, animals were maintained on chow diet for 72 h. Blood samples were collected for the measurement of serum calcium concentrations. Animals were euthanized with CO_2. Once the death was confirmed, the perfusion was immediately applied to reduce the blood contamination. Thirty milliliters of cold saline solution were injected into circulation through the left ventricle and perfusate exited from the right atrium. Duodenum, colon, kidney, liver, femoral skeletal muscle, heart, and aorta samples were collected, and half of each organ was used for the fixation and half for making tissue lysates. Both the wild-type (WT) and KO mice from the same littermates with identical age were used for each experiment.

Five 7-wk-old rats used in this study were male Wistar rats obtained from Harlan (Indianapolis, IN). The purified diet was used to mimic the dietary condition reported previously (18). Fat-soluble vitamins A, D, E, and K were added to maintain animals for the vitamin D sufficiency. Blood samples were collected for the measurement of serum calcium concentrations. Animals were euthanized with CO_2. Duodenum, femoral skeletal muscle, and heart were collected and fixed for immunohistochemical staining.

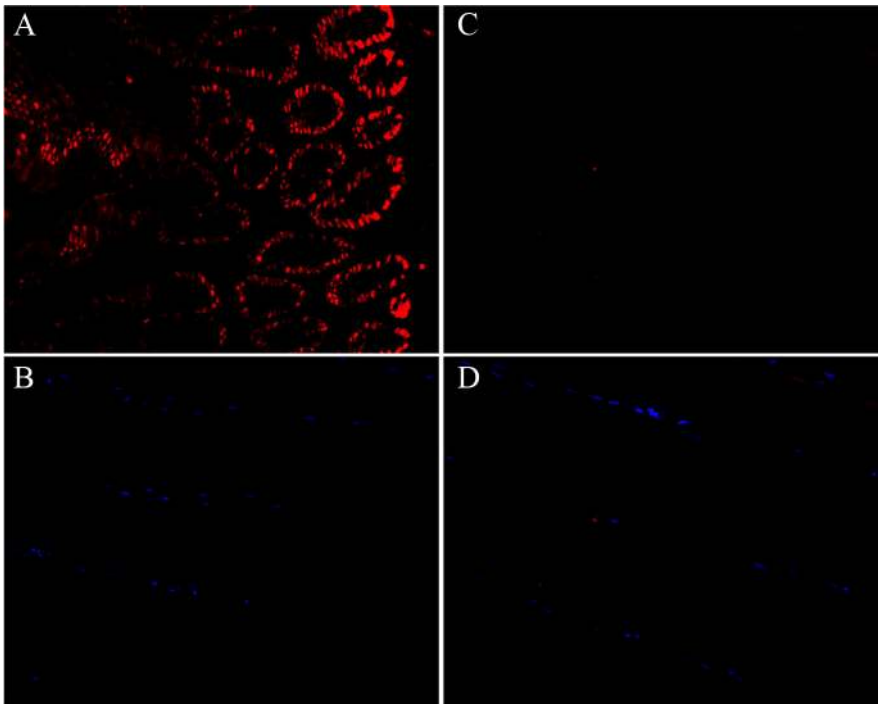

FIG. 1. VDR is undetectable in rat skeletal muscle by *in situ* immunohistochemical staining. The skeletal muscle sections were stained with the VDR antibodies (*red*) and DAPI (*blue*). The images of their colocalization are shown. A, D-6 staining in the sections of rat duodenum. The VDR signal (*red*) was selectively and highly expressed in the gut epithelial cells. B, Mouse isotype IgG/DAPI colocalization in the sections from rat skeletal muscle. C, D-6 antibody staining in the sections from rat skeletal muscle. D, D-6/DAPI colocalization in the sections from rat skeletal muscle. The images represent the identical results from five animals. Original magnification, ×200.

1α-Hydroxylase KO ($1\alpha^{-/-}$) mice (38) were generated through breeding of heterozygous mice and identified by PCR. The age-matched $1\alpha^{-/-}$ and WT littermates were used for experiments. After weaning, the $1\alpha^{-/-}$ mice were fed a rescue diet containing 2% calcium, 1.25% phosphorus, and 20% lactose (Harlan Teklad, Madison, WI) for 3–4 wk (39). For 10 subsequent days, animals received either vehicle or $1,25(OH)_2D_3$ at a dose of 50 ng/kg body weight per day in the rescue diet. Blood samples were collected for the measurement of serum calcium concentration. Animals were euthanized with CO_2 and perfusion with normal saline was used to reduce the blood contamination. Duodenum, kidney, femoral skeletal muscle, and heart were collected, and half of each organ was used for the fixation and half for making total RNA samples using Tri-Reagent (Sigma). The WT mice from the same littermates with identical age were fed a chow diet used as a normal control.

Experimental protocols were reviewed and approved by our Research Animal Care and Use Committee (University of Wisconsin, Madison).

Normal human tissue sections

Normal human heart left ventricle (HuFPT056) and skeletal muscle (HuFPT075) serial tissue sections were purchased from U.S. Biomax, Inc. (Rockville, MD). These tissues were collected from a 21-yr-old female donor and were paraffin embedded by the supplier.

Immunoblotting

Tissue lysates were prepared according to a published protocol (40) with several modifications. Tissues were washed, sliced, and boiled immediately for 10 min within lysis buffer containing 20 mM HEPES (pH7.4), 1 mM EDTA, 4% sodium dodecyl sulfate, and a protease inhibitor cocktail (Roche, Indianapolis, IN). Tissue samples were thoroughly homogenized by sonication. The homogenate was boiled for an additional 5 min and centrifuged for 15 min at 16,000 × *g* in a table centrifuge. The supernatant was transferred into a fresh tube, and the small pellet containing no detectable VDR was discarded (37). After measuring the protein concentration of the lysates, either 20 or 50 μg of protein samples was loaded onto a 4–20% SDS-PAGE gel. Immunoblotting was performed as described previously (37). The HRP signal was developed by incubation of the blot with enhanced chemiluminescence solution (Amersham, Piscataway, NJ) and captured by x-ray film for increments of 1, 5, and 40 min, depending on the intensity of signal.

Immunohistochemical staining

Tissues from mice and rats were collected and washed gently with PBS to remove contaminants. PBS was then replaced with 3.7% paraformaldehyde and allowed to incubate on a rocker overnight for tissue fixation. The detailed protocol was performed as described as previously (37). Images were captured using a fluorescence microscope (Nikon inverted microscope ECLIPSE TE2000-U; Nikon Instruments, Inc., Melville, NJ).

Quantitative RT-PCR

Transcription of *VDR* and *CYP24A1* genes was assessed by quantitative RT-PCR using the VDR and CYP24A1 TaqMan gene expression assays (Applied Biosystems, Foster City, CA). The amplification conditions for VDR, CYP24A1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 50 C for 2 min, 95 C for 10 min, 45 cycles of 95 C for 15 sec, and 60 C for 60 sec on the StepOne real-time PCR system (Applied Biosystems). Transcription of *VDR* and *CYP24A1* genes was normalized to the GAPDH and all samples were analyzed in triplicate.

Results

VDR is undetectable in skeletal muscle

As expected, strong VDR immunochemical staining was seen in the rat duodenal tissue. The VDR signal was selectively shown in the rat gut epithelial cells (Fig. 1A). The muscle sections from five adult rats were also stained using the same method. No VDR signal was seen in the mature skeletal muscle (Fig. 1, C and D).

Immunoblotting confirmed that the VDR was not detectable in the skeletal muscle tissue lysates from either

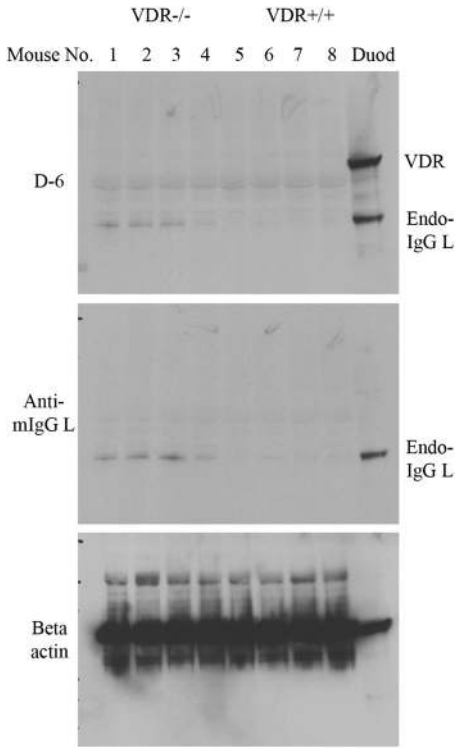

FIG. 2. VDR is undetectable in mouse skeletal muscle by immunoblotting. Fifty micrograms of skeletal muscle tissue lysates from *VDR* KO Demay (lanes 1–4) or WT (lane 5–8) mice and 20 μg duodenal tissue lysate from the WT mouse were used for immunoblotting. The blots were first incubated with VDR antibody D-6 and then with HRP-conjugated goat antimouse IgG light chain secondary antibody (Anti-mIgG L). For the blots used to evaluate nonspecific reactions of secondary antibodies with tissue, the primary antibody was omitted. The HRP signal was developed by incubation of the blot with enhanced chemiluminescence solution (Amersham) and captured by x-ray film for a period of 40 min. The bands of mouse VDR and endogeneous IgG light chain (Endo IgG L) were indicated in Fig. 2. The blots were also reprobed with an antibody to β-actin as a loading control.

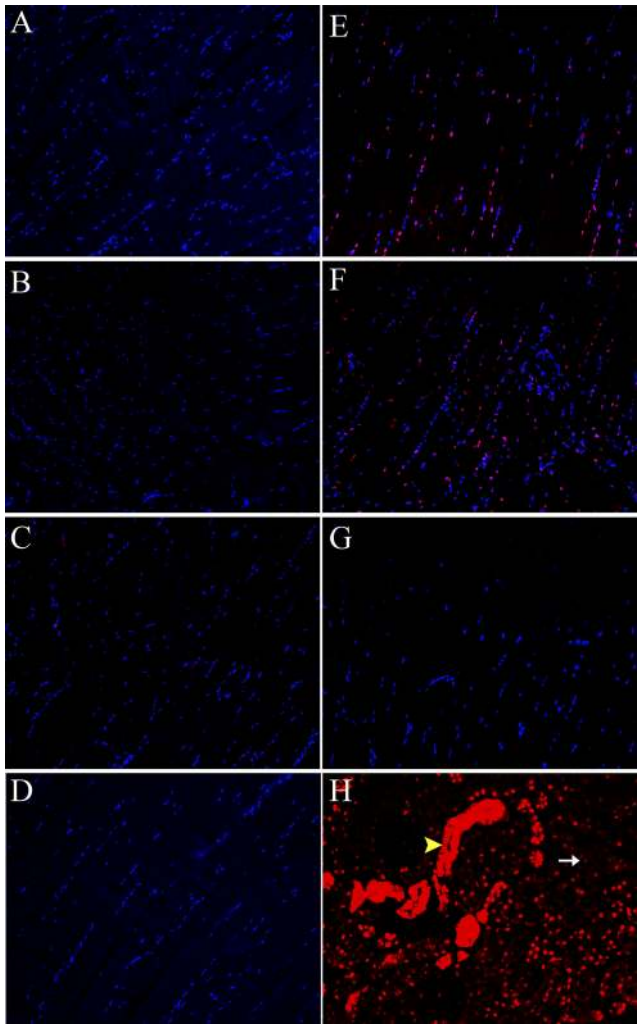

FIG. 3. VDR is undetectable in mouse skeletal muscle by *in situ* immunohistochemical staining. The skeletal muscle sections were stained with the VDR antibodies (*red*) and DAPI (*blue*). The images of their colocalization are shown. A, D-6/DAPI colocalization in the sections from the WT mice. B, D-6/DAPI colocalization in the sections from the Demay *VDR* KO mice. C, Mouse isotype IgG/DAPI colocalization in the sections from the WT mice. D, DAPI staining of the sections from the WT mice. E, 9A7/DAPI colocalization in the sections from the WT mice. F, 9A7/DAPI colocalization in the sections from the Demay *VDR* KO mice. G, Rat isotype IgG/DAPI colocalization in the sections from the WT mice. H, D-6 staining in the sections of WT mouse kidney. Distal convoluted tubule (*arrow*) of normal adult kidneys highly expressed VDR (*red*) but proximal tubule (*white arrow*) were positive for VDR expression. The images represent the identical results from three animals. Original magnification, ×200.

WT or Demay *VDR* mice. We used a long-time exposure to detect even a weak signal, but no significant signal appeared (Fig. 2). In contrast, the positive control, *i.e.* the lysates from WT mouse intestinal tissue clearly showed a VDR specific band. Because the band of the endogenous IgG heavy chain on immunoblots is identical to the VDR, we used a special secondary antibody made against the IgG light chain to avoid potential interference. This experimental setup eliminates effects of the endogenous IgG heavy chain on immunoblotting. As expected, the antimouse IgG secondary antibody control reacted only with the endogenous IgG light chain on the blot but not with the heavy chain (Fig. 2).

Immunohistochemistry of the skeletal muscle sections using D-6 antibody showed that the VDR was absent in either WT or Demay *VDR* KO mice (Fig. 3, A–D). The isotype IgG control occasionally generated a signal at the site of small vascular capillaries (not shown) but had no significant staining in the muscle cells. Therefore, the endogenous IgG did not interfere with the determination of VDR in the target cells under the experimental conditions (Fig. 3C). In contrast, the positive control, WT mouse kidney sections, clearly showed a VDR-specific signal in either the distal tubule or proximal tubule (Fig. 3H). Expression of VDR in the renal proximal tubule is usually very low compared with the distal tubule (Fig. 3H).

The VDR has been detected in the nuclei of human skeletal muscle cells by immunohistochemistry using the chick monoclonal VDR antibody 9A7 (35). We have dem-

onstrated that this antibody binds to VDR but also stains with other non-VDR proteins in either mouse duodenum or kidney (37). Using the 9A7 antibody, we found that it reacted with muscle from both the Demay *VDR* KO mice and WT mice (Fig. 3, E and F). The rat isotype IgG control had no significant staining, indicating that the immunosignal in the Demay *VDR* KO and WT mice was contributed by the primary antibody (Fig. 3G).

As seen in Fig. 4, C and F, D-6 and 9A7 antibodies both stained the nuclei of mouse colon epithelial cells. However, the D-6 antibody failed to stain human skeletal muscle tissue section (Fig. 4, A and B), whereas the 9A7 antibody significantly stained the human skeletal muscle sections (Fig. 4, D and E).

Failure to detect the VDR protein in the normal mature skeletal muscle raised a question whether the *VDR* gene is transcribed in this tissue. PCR analysis showed an extremely low level of *VDR* transcripts in the muscle tissue compared with mouse duodenum (Table 1). The ratio of the WT skeletal muscle to duodenum was 1.2:10,000, when the *VDR* gene transcription was normalized by *GAPDH*. Our pilot experiment showed that when the same amounts of RNA samples from the skeletal muscle and duodenum are used, the quantitative RT-PCR threshold cycle values of *GAPDH* are comparable.

We next determined whether exogenous $1,25(OH)_2D_3$ could enhance *VDR* gene transcripts in the muscle. 1α-Hydroxylase knockout ($1\alpha^{-/-}$) mice were chosen to do the experiment. These animals are unable to produce $1,25(OH)_2D_3$ and because serum calcium plays an important role in the ligand-induced transcription of *VDR* gene, the animals were fed the rescue diet. $1,25(OH)_2D_3$ treatment increased the serum calcium and the *VDR* gene transcription by 1.4- to 1.8-fold in mouse duodenum and kidney (data not shown). However, *VDR* gene transcription was still extremely low and there was no induction in the muscle tissue (Table 1). As expected, we failed to detect the receptor protein in either the vehicle or $1,25(OH)_2D_3$-treated muscle tissues (data not shown).

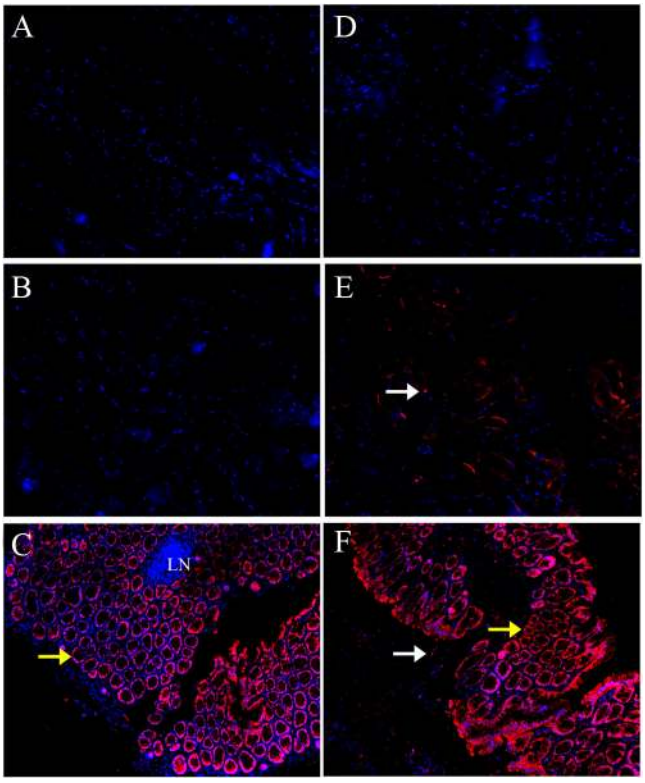

FIG. 4. VDR is undetectable in human skeletal muscle by *in situ* immunohistochemical staining. The skeletal muscle sections were stained with the VDR antibodies (*red*) and DAPI (*blue*). The images of their colocalization are shown. A, Mouse isotype IgG/DAPI colocalization in the human skeletal muscle. B, D-6/DAPI colocalization in the human skeletal muscle. C, D-6/DAPI colocalization in the WT mouse colon tissue sections. The receptor was selectively detected in the nuclei of mouse colon epithelial cells (*yellow arrow*). The VDR was undetectable in the lymphatic node (LN). D, Rat isotype IgG/DAPI colocalization in the human skeletal muscle. E, 9A7/DAPI colocalization in the human skeletal muscle. Immunosignals were denoted by *white arrow*. F, 9A7/DAPI colocalization in the WT mouse colon tissue sections. Immunosignals stained by the antibody 9A7 are visualized in the colon epithelial cells (high, *yellow arrow*) and nonepithelial cells (low, *white arrow*). The images represent the identical results from three repetitive experiments. Original magnification, ×200.

VDR is undetectable in cardiac muscle

A low level of VDR has been reported in heart using VDR antibodies 9A7 or C-20 (19, 32–34, 41). In agreement with these reports, the 9A7 antibody significantly stained the sections of human cardiac muscle tissue (Fig. 5, B and C). However, the D-6 antibody failed to reveal VDR in the human left ventricle (Fig. 5A). Because the 9A7 and C-20 antibodies have been shown to interact with proteins other than VDR, we decided to evaluate VDR expression in mouse cardiac muscle tissue using a highly specific and sensitive VDR antibody.

In situ immunohistochemistry of the cardiac muscle sections using the D-6 antibody showed that the VDR was absent in either WT or Demay *VDR* KO mice (Fig. 5, D–F). We examined both ventricles (left and right) and atriums (left and right) for the expression of VDR. The representative results from the left ventricle are shown Fig. 5, D–F. In contrast, the VDR signal was detected in the WT mouse kidney sections (Fig. 3H). The mouse isotype IgG control showed light staining in the small vascular capillaries but did not in the muscle cells. Therefore, the endogenous IgG did not interfere with the evaluation of VDR signal in the muscle cells under our experimental conditions (Fig. 5F).

Furthermore, extremely low levels of *VDR* transcript could be found by PCR analysis (Table 1). In addition, ligand-mediated induction of *VDR* gene transcription was not found (Table 1). Although the transcription in the cardiac muscle seemed slightly higher than that in the skel-

TABLE 1. Transcription of *VDR* gene in mouse skeletal muscle, cardiac muscle, and duodenum

Mouse strains[a]	Diet/treatments	Tissues	VDR/GAPDH	Ratios
Wild type	Chow	Duodenum	$6 \times 10^{-2} \pm 1.8 \times 10^{-2}$	10,000
Wild type	Chow	Skeletal muscle	$7.0 \times 10^{-6} \pm 1.5 \times 10^{-6}$	1.2
$1\alpha^{-/-}$	Purified diet/vehicle	Skeletal muscle	$1.8 \times 10^{-5} \pm 1.1 \times 10^{-5}$	3
$1\alpha^{-/-}$	Purified diet/50 ng $1,25D_3$	Skeletal muscle	$6.7 \times 10^{-6} \pm 3.4 \times 10^{-6}$	1.1
Wild type	Chow	Left ventricle	$4.5 \times 10^{-5} \pm 1.0 \times 10^{-5}$	7.5
$1\alpha^{-/-}$	Purified diet/vehicle	Left ventricle	$7.8 \times 10^{-5} \pm 1.4 \times 10^{-5}$	13
$1\alpha^{-/-}$	Purified diet/50 ng $1,25D_3$	Left ventricle	$5.0 \times 10^{-5} \pm 6.6 \times 10^{-6}$	8.4

[a] n = 4 mice.

etal muscle, no receptor could be detected in this tissue by the *in situ* immunohistochemistry (data not shown).

VDR is undetectable in aorta smooth muscle and endothelial cells

VDR could not be detected using the D-6 antibody by immunocytochemistry of aortic smooth muscle cells and vascular smooth muscle cells in either the WT or Demay *VDR* KO mice. In this experiment, we stained the consecutive sections with either the antibody to α-SMA, a marker for the smooth muscle cells, or the antibody to VDR (D-6). The α-SMA staining helped locate the smooth muscle tissue layer in the vascular vessels such as aorta. It also aided in differentiating endothelial cells from the smooth muscle tissue because the α-SMA is absent from the endothelial cells. We observed that the level of α-SMA in the aorta of Demay *VDR* KO mice was lower than that in the WT mice (Fig. 6, A and C). The endothelial cells were located in the apical end of the blood vessels, which lacked the expression of α-SMA (Fig. 6A). Our immunohistochemical staining showed that the VDR was not detected in the vascular endothelial cells. In addition, we did not see any VDR-positive staining in any vessels including major vein and smaller blood vessels (Fig. 6, E and F). The IgG control had no significant staining (not shown). In contrast, the positive control, WT mouse kidney sections, clearly showed a VDR-specific signal in either the distal tubule or proximal tubule (Fig. 2H).

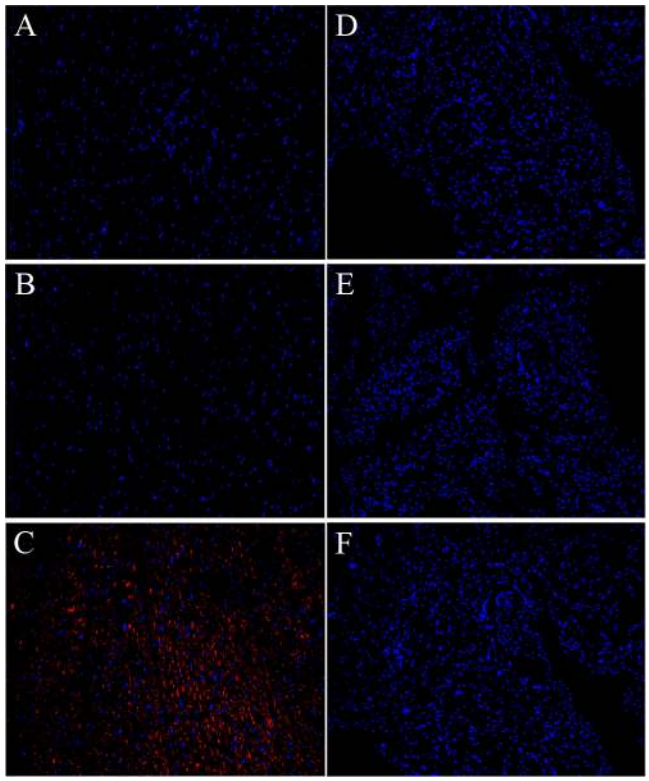

FIG. 5. VDR is undetectable in human and mouse cardiac muscle by *in situ* immunohistochemical staining. The cardiac muscle sections were stained with the D-6 VDR antibody (*red*) and DAPI (*blue*). A, D-6/DAPI colocalization in the human skeletal muscle. B, Rat isotype IgG/DAPI colocalization in the human skeletal muscle. C, 9A7/DAPI colocalization in the human skeletal muscle. The immunosignals were visualized when the 9A7 antibody was used. Please note that the mouse isotype IgG/DAPI colocalization in the human skeletal muscle were identical to D-6/DAPI colocalization (not shown). D, D-6/DAPI colocalization in the sections from the WT mice. E, D-6/DAPI colocalization in the sections from the Demay *VDR* KO mice. F, Mouse isotype IgG/DAPI colocalization in the sections from the WT mice. The images represent the identical results from five animals. Original amplification, ×200.

VDR is undetectable in intestinal smooth muscle

Immunohistochemistry of the intestinal sections showed that the VDR was present in the epithelial cells of mouse duodenal tissue. The VDR immunosignal was colocalized with the 4′,6′-diamino-2-phenylindole (DAPI) staining, indicating a nuclear location of the VDR (Fig. 7B). In contrast, the VDR was absent from the smooth muscle cells of intestinal muscularis externa (circular muscular layer and longitudinal muscular layer) from either WT or Demay *VDR* KO mice (Fig. 7, A–D). In this experiment, we stained the consecutive sections with either the antibody to α-SMA or the antibody to VDR (D-6). The α-SMA staining confirmed the location of the smooth muscle tissue layer. The mouse isotype IgG control generated some immunosignals, which were restricted to the sites of either lymphatic tissues or small vascular capillaries.

In contrast to the D-6 antibody, the 9A7 antibody stained both epithelium and smooth muscle tissue (Fig. 7F), whereas the rat isotype IgG control showed no significant staining (Fig. 7E).

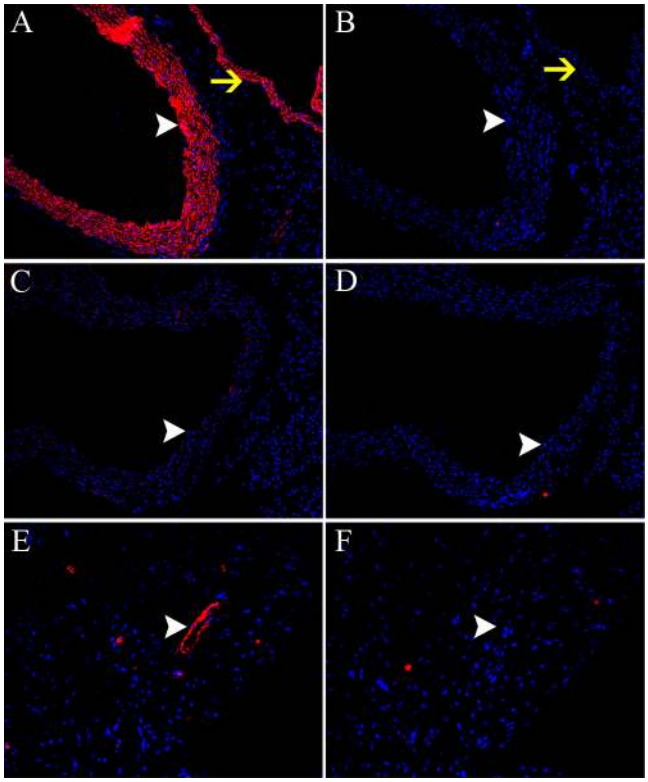

FIG. 6. VDR is undetectable in mouse aorta smooth muscle by *in situ* immunohistochemical staining. The aorta smooth muscle sections were stained with either the mouse antibody to α-SMA (*red*) and DAPI (*blue*) or mouse anti-VDR antibody D-6 (*red*) and DAPI (*blue*). The blood vessels were located according to the α-SMA staining. Therefore, the staining for the VDR and α-SMA was performed in serial sections. A, α-SMA/DAPI colocalization in the sections from the WT mice. The aorta is denoted by an *arrowhead*, whereas the big vein is denoted by a *yellow arrow*. B, D-6/DAPI colocalization in the sections from the WT mice. C, α-SMA/DAPI colocalization in the sections from the Demay *VDR* KO mice. Please note that the expression of α-SMA in the Demay mice was very low. D, D-6/DAPI colocalization in the sections from the Demay *VDR* KO mice. E, α-SMA/DAPI colocalization in the sections from the WT mice. The small blood vessel is denoted by an *arrowhead*. F, D-6/DAPI colocalization in the sections from the WT mice. The sections from the WT mice stained with the mouse isotype IgG had no significant immunosignal in the targets (not shown). The images represent the identical results from five animals. Original magnification, ×200.

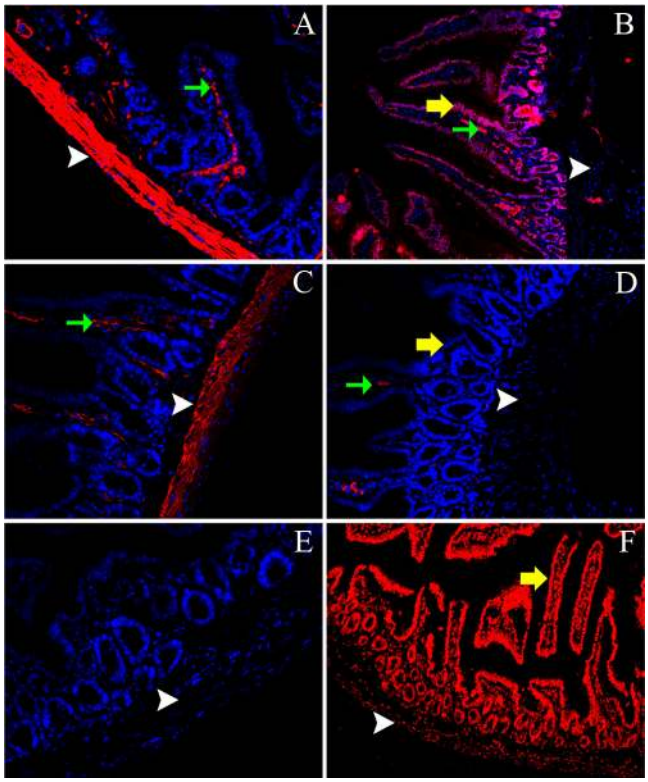

FIG. 7. VDR is undetectable in intestinal smooth muscle by *in situ* immunohistochemical staining. The intestinal smooth muscle sections were stained with either the mouse antibody to α-SMA (*red*) and DAPI (*blue*) or mouse anti-VDR antibody D-6 (*red*) and DAPI (*blue*). The smooth muscle tissues were located according to the α-SMA staining. A, SMA/DAPI colocalization in the sections from the WT mice. Smooth muscle tissue layer was denoted by an *arrow*, whereas the staining located in the interior of microvilli is denoted by a *green arrow*. Please note that there are smooth muscle cells in the interior of microvilli as well; therefore, some staining might be the endogenous IgG but some might be the smooth muscle cells. B, D-6/DAPI colocalization in the sections from the WT mice. The VDR-positive staining for the gut epithelial cells is denoted by a *broad arrow*. C, α-SMA/DAPI colocalization in the sections from the Demay *VDR* KO mice. D, D-6/DAPI colocalization in the sections from the Demay *VDR* KO mice. E, The sections from the WT mice stained with the rat isotype IgG had no significant immunosignal. Smooth muscle layer is denoted by an *arrow*. F, 9A7/DAPI colocalization in the sections from the WT mice. The VDR-positive gut epithelial cells are denoted by an *arrow*. The sections from the WT mice stained with the mouse isotype IgG showed the endogenous IgG staining (not shown) identical to A. The images represent the identical results from five animals. Original magnification, ×200.

Discussion

There is adequate reason to believe that a consequence of vitamin D deficiency is skeletal muscle weakness (13–15). Furthermore, strong evidence is available that vitamin D deficiency results in left ventricle hypertrophy and changes in heart structure (23). Exactly how vitamin D is involved in these functions is not understood.

Central to the issue of the role of vitamin D in skeletal, heart, and smooth muscle is whether the VDR is expressed *in vivo* in these tissues. Using physiological amounts of $1,25(OH)_2[^3H]D_3$, Stumpf *et al.* (24) could find no nuclear localization or even the presence of $1,25(OH)_2[^3H]D_3$ in skeletal, heart, and smooth muscle using autoradiography despite clear nuclear localization in proven target organs. With the availability of antibodies to the VDR came a rash of conflicting observations. Little or no attention was paid to the selectivity and sensitivity of the antibodies used. In 1985 Simpson *et al.* (31) reported a VDR-like protein in rodent skeletal muscle cell lines in culture. Boland *et al.* (42) reported VDR in monolayers of chick myoblasts, whereas Costa *et al.* (43) reported similar findings in cloned human skeletal muscle cells. These studies were carried out on cultured cells and *in vivo* studies were not reported.

Using the monoclonal antibody 9A7 raised against chick VDR, Bischoff *et al.* (35) reported *in situ* VDR in human skeletal muscle. However, 9A7 on Western blot binds not only VDR but other proteins as well (37). The present investigation demonstrates that the protein detected in the study by Bischoff *et al.* is likely not VDR. The signal generated by 9A7 was equally evident in WT and the Demay KO mice in which no VDR is produced (see Figs. 2 and 6) (37). The epitope of the monoclonal antibody 9A7, located in the second zinc finger of the VDR DNA binding domain, is deleted in the Demay *VDR* KO mice (36, 44, 45). Therefore, the observed staining with the 9A7 antibody in the Demay *VDR* KO mice cannot be attributed to VDR (Fig. 2, E and F).

In addition to the skeletal muscle, there has been a great effort on elucidating the effect of $1,25(OH)_2D_3$ on cardiac muscle (41, 46, 47). In culture, it has been reported that cardiomyocytes express VDR. Treatment of cultured cardiomyocytes with $1,25(OH)_2D_3$ leads to increased expression and nuclear localization of the VDR, increased expression of myotrophin, and decreased expression of atrial natriuretic peptide, c-myc (33) and human B-type natriuretic peptide (34). These peptides are up-regulated during hypertrophy. A recent study reported a low level of VDR in heart tissue by immunoblotting using a rabbit polyclonal antibody to VDR (SC-1008) (34). These investigators also reported that myocyte hypertrophy either *in vitro* or *in vivo* is associated with an increase in VDR mRNA and VDR levels. They conclude that the vitamin D system may act as an antihypertrophic system in cardiac muscle. However, the C-20 antibody used in that study also binds proteins in *VDR* KO mouse tissue (37).

In our present study, we used both immunoblotting and *in situ* immunohistochemical staining using a highly specific and sensitive VDR antibody to determine whether VDR is in mouse, rat, and human muscle tissues. Our results demonstrate that VDR is undetectable in normal mature skeletal, cardiac, and smooth muscle tissue. In contrast, the VDR is clearly detected in the renal proximal tubule and distal tubule and intestinal epithelial cells under the same experimental conditions. We previously demonstrated that the average VDR expression in the renal proximal tubules is 24-fold lower than in the distal tubules. Some tubular epithelial cells have a 69-fold lower level of VDR than does the distal tubule (Wang, Y., and DeLuca, H.F., unpublished results). Therefore, determination of VDR in the mouse renal proximal tubules (Fig. 2H) demonstrated that our immunostaining assay possesses not only high specificity but also high sensitivity.

The failure to find VDR on immunohistochemical staining with D-6 raised the question of whether a tightly bound protein in muscle might prevent D-6 from interacting with the VDR. However, Western analysis also failed to show the presence of VDR in muscle. Because the Western blots are under denaturing conditions, masking of the VDR could not explain our results.

Extremely low levels of *VDR* transcripts in the cardiac and skeletal muscle can be, at least in a part, an explanation for our failure to detect the receptor. It is possible that such low levels of *VDR* transcripts may not be translated into the protein. As further support, the transcription of *CYP24A1*, one of the most responsive VDR controlled genes, is extremely low or undetectable in the mouse cardiac and skeletal muscle, regardless the presence of $1,25(OH)_2D_3$. In contrast, $1,25(OH)_2D_3$ induces its transcription by 10-fold in the duodenum and 5.8-fold in the kidney (Wang, Y., and DeLuca, H.F., unpublished results).

Because skeletal muscle weakness of vitamin D deficiency is rapidly corrected by vitamin D (18), our study was directed to mature muscle response and whether it is mediated by VDR. We could not detect VDR in skeletal muscle by either Western blot or immunohistochemical staining by the specific antibody D-6. This suggests that the correction of mature muscle weakness by vitamin D may be indirect. Clinically, patients with PTH excess (as in hyperparathyroidism) share similar symptoms of muscle weakness and fatigue (48), and biopsies demonstrate atrophy of type II muscle fibers as in vitamin D deficiency (49). Low vitamin D levels stimulate PTH production and PTH may either have direct effects on skeletal muscle or may cause a lowering of plasma phosphate, giving rise to phosphate deficiency in muscle. Studies in rats have demonstrated that PTH induces muscle catabolism (50).

Cross-sectional clinical studies have shown an association between lower vitamin D levels, plasma renin activity (51), and blood pressure (52, 53). Studies in KO mice confirm that the absence of VDR activation leads to tonic up-regulation of the renin-angiotensin system and increased PTH, with the development of hypertension and left ventricular hypertrophy (2, 22, 34, 54, 55). $1,25(OH)_2D_3$ suppresses rennin and PTH (22, 54, 56). A strong inverse correlation has been observed between circulating $1,25(OH)_2D_3$ levels and plasma renin activity in patients with essential hypertension (51). These accumulating results suggest that the renin and PTH may be intermediate factors in the $1,25(OH)_2D_3$-mediated effects on muscle function.

Our findings of the absence of VDR in the mature muscle tissue are not directed to the question of whether VDR plays a role in the differentiation of the myocyte during development. This separate question needs to be addressed with the D-6-specific antibody and will be the subject of future investigations.

In summary, we used multiple immunoassays to detect VDR in the mouse muscle tissues. Our results showed that

VDR is not present in muscle tissue. Absence of VDR from these tissues suggests that the effect of $1,25(OH)_2D_3$ on muscle function is most likely indirect.

Acknowledgments

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This work was supported by the Wisconsin Alumni Research Foundation.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Holick MF** 2007 Vitamin D deficiency. N Engl J Med 357:266–281
2. **DeLuca HF** 2004 Overview of general physiologic features and functions of vitamin D. Am J Clin Nutr 80:1689S–1696S
3. **Jones G, Strugnell SA, DeLuca HF** 1998 Current understanding of the molecular actions of vitamin D. Physiol Rev 78:1193–1231
4. **Morrison NA, Shine J, Fragonas JC, Verkest V, McMenemy ML, Eisman JA** 1989 1,25-Dihydroxyvitamin D-responsive element and glucocorticoid repression in the osteocalcin gene. Science 246: 1158–1161
5. **Deeb KK, Trump DL, Johnson CS** 2007 Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. Nat Rev Cancer 7:684–700
6. **Haussler MR, Whitfield GK, Haussler CA, Hsieh JC, Thompson PD, Selznick SH, Dominguez CE, Jurutka PW** 1998 The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. J Bone Miner Res 13:325–349
7. **Stumpf WE** 1995 Vitamin D sites and mechanisms of action: a histochemical perspective. Reflections on the utility of autoradiography and cytopharmacology for drug targeting. Histochem Cell Biol 104:417–427
8. **Walters MR** 1992 Newly identified actions of the vitamin D endocrine system. Endocr Rev 13:719–764
9. **Hutchison FN, Bell NH** 1992 Osteomalacia and rickets. Semin Nephrol 12:127–145
10. **Pettifor JM** 2002 Rickets. Calcif Tissue Int 70:398–399
11. **Bouillon R, Carmeliet G, Verlinden L, van Etten E, Verstuyf A, Luderer HF, Lieben L, Mathieu C, Demay M** 2008 Vitamin D and human health: lessons from vitamin D receptor null mice. Endocr Rev 29:726–776
12. **Kato S, Yoshizazawa T, Kitanaka S, Murayama A, Takeyama K** 2002 Molecular genetics of vitamin D-dependent hereditary rickets. Horm Res 57:73–78
13. **Ward KA, Das G, Berry JL, Roberts SA, Rawer R, Adams JE, Mughal Z** 2009 Vitamin D status and muscle function in post-menarchal adolescent girls. J Clin Endocrinol Metab 94:559–563
14. **Pilz S, Marz W, Wellnitz B, Seelhorst U, Fahrleitner-Pammer A, Dimai HP, Boehm BO, Dobnig H** 2008 Association of vitamin D deficiency with heart failure and sudden cardiac death in a large cross-sectional study of patients referred for coronary angiography. J Clin Endocrinol Metab 93:3927–3935
15. **Giovannucci E** 2009 Expanding roles of vitamin D. J Clin Endocrinol Metab 94:418–420
16. **Ritz E, Boland R, Kreusser W** 1980 Effects of vitamin D and parathyroid hormone on muscle: potential role in uremic myopathy. Am J Clin Nutr 33:1522–1529
17. **Smith R, Stern G** 1967 Myopathy, osteomalacia and hyperparathyroidism. Brain 90:593–602
18. **Schubert L, DeLuca HF** 2010 Hypophosphatemia is responsible for skeletal muscle weakness of vitamin D deficiency. Arch Biochem Biophys 500:157–161
19. **Weishaar RE, Simpson RU** 1987 Vitamin D3 and cardiovascular function in rats. J Clin Invest 79:1706–1712
20. **Bodyak N, Ayus JC, Achinger S, Shivalingappa V, Ke Q, Chen YS, Rigor DL, Stillman I, Tamez H, Kroeger PE, Wu-Wong RR, Karumanchi SA, Thadhani R, Kang PM** 2007 Activated vitamin D attenuates left ventricular abnormalities induced by dietary sodium in Dahl salt-sensitive animals. Proc Natl Acad Sci USA 104:16810–16815
21. **Chen S, Nakamura K, Gardner DG** 2005 1,25-Dihydroxyvitamin D inhibits human ANP gene promoter activity. Regul Pept 128:197–202
22. **Xiang W, Kong J, Chen S, Cao LP, Qiao G, Zheng W, Liu W, Li X, Gardner DG, Li YC** 2005 Cardiac hypertrophy in vitamin D receptor knockout mice: role of the systemic and cardiac renin-angiotensin systems. Am J Physiol Endocrinol Metab 288:E125–E132
23. **Xue Y, Karaplis AC, Hendy GN, Goltzman D, Miao D** 2006 Exogenous 1,25-dihydroxyvitamin D3 exerts a skeletal anabolic effect and improves mineral ion homeostasis in mice that are homozygous for both the 1α-hydroxylase and parathyroid hormone null alleles. Endocrinology 147:4801–4810
24. **Stumpf WE, Sar M, Reid FA, Tanaka Y, DeLuca HF** 1979 Target cells for 1,25-dihydroxyvitamin D3 in intestinal tract, stomach, kidney, skin, pituitary, and parathyroid. Science 206:1188–1190
25. **Narbaitz R, Stumpf W, Sar M, DeLuca HF, Tanaka Y** 1980 Autoradiographic demonstration of target cells for 1,25-dihydroxycholecalciferol in the chick embryo chorioallantoic membrane, duodenum, and parathyroid glands. Gen Comp Endocrinol 42:283–289
26. **Stumpf WE, Sar M, O'Brien LP, Morin J** 1988 Pyloric gastrin-producing cells and pyloric sphincter muscle cells are nuclear targets for 3H 1,25(OH)2 vitamin D3. Studied by autoradiography and immunohistochemistry. Histochemistry 89:447–450
27. **Feldman D, McCain TA, Hirst MA, Chen TL, Colston KW** 1979 Characterization of a cytoplasmic receptor-like binder for 1α, 25-dihydroxycholecalciferol in rat intestinal mucosa. J Biol Chem 254: 10378–10384
28. **Pike JW, Goozé LL, Haussler MR** 1980 Biochemical evidence for 1,25-dihydroxyvitamin D receptor macromolecules in parathyroid, pancreatic, pituitary, and placental tissues. Life Sci 26:407–414
29. **Burmester JK, Maeda N, DeLuca HF** 1988 Isolation and expression of rat 1,25-dihydroxyvitamin D3 receptor cDNA. Proc Natl Acad Sci USA 85:1005–1009
30. **Sandgren ME, Bronnegärd M, DeLuca HF** 1991 Tissue distribution of the 1,25-dihydroxyvitamin D3 receptor in the male rat. Biochem Biophys Res Commun 181:611–616
31. **Simpson RU, Thomas GA, Arnold AJ** 1985 Identification of 1,25-dihydroxyvitamin D3 receptors and activities in muscle. J Biol Chem 260:8882–8891
32. **O'Connell TD, Simpson RU** 1996 Immunochemical identification of the 1,25-dihydroxyvitamin D3 receptor protein in human heart. Cell Biol Int 20:621–624
33. **Nibbelink KA, Tishkoff DX, Hershey SD, Rahman A, Simpson RU** 2007 1,25(OH)2-vitamin D3 actions on cell proliferation, size, gene expression, and receptor localization, in the HL-1 cardiac myocyte. J Steroid Biochem Mol Biol 103:533–537
34. **Chen S, Glenn DJ, Ni W, Grigsby CL, Olsen K, Nishimoto M, Law CS, Gardner DG** 2008 Expression of the vitamin D receptor is increased in the hypertrophic heart. Hypertension 52:1106–1112
35. **Bischoff HA, Borchers M, Gudat F, Duermueller U, Theiler R, Stähelin HB, Dick W** 2001 *In situ* detection of 1,25-dihydroxyvitamin D3 receptor in human skeletal muscle tissue. Histochem J 33:19–24
36. **Li YC, Pirro AE, Amling M, Delling G, Baron R, Bronson R, Demay MB** 1997 Targeted ablation of the vitamin D receptor: an animal

model of vitamin D-dependent rickets type II with alopecia. Proc Natl Acad Sci USA 94:9831–9835
37. **Wang Y, Becklund BR, DeLuca HF** 2010 Identification of a highly specific and versatile vitamin D receptor antibody. Arch Biochem Biophys 494:166–177
38. **Vanhooke JL, Prahl JM, Kimmel-Jehan C, Mendelsohn M, Danielson EW, Healy KD, DeLuca HF** 2006 CYP27B1 null mice with LacZ reporter gene display no 25-hydroxyvitamin D3–1α-hydroxylase promoter activity in the skin. Proc Natl Acad Sci USA 103:75–80
39. **Zhou C, Lu F, Cao K, Xu D, Goltzman D, Miao D** 2008 Calcium-independent and 1,25(OH)2D3-dependent regulation of the renin-angiotensin system in 1α-hydroxylase knockout mice. Kidney Int 74:170–179
40. **Li YC, Bolt MJ, Cao LP, Sitrin MD** 2001 Effects of vitamin D receptor inactivation on the expression of calbindins and calcium metabolism. Am J Physiol Endocrinol Metab 281:E558–E564
41. **Weishaar RE, Simpson RU** 1987 Involvement of vitamin D3 with cardiovascular function. II. Direct and indirect effects. Am J Physiol 253:E675–E683
42. **Boland R, Norman A, Ritz E, Hasselbach W** 1985 Presence of a 1,25-dihydroxy-vitamin D3 receptor in chick skeletal muscle myoblasts. Biochem Biophys Res Commun 128:305–311
43. **Costa EM, Blau HM, Feldman D** 1986 1,25-dihydroxyvitamin D3 receptors and hormonal responses in cloned human skeletal muscle cells. Endocrinology 119:2214–2220
44. **Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T, Kato S** 1997 Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. Nat Genet 16:391–396
45. **Erben RG, Soegiarto DW, Weber K, Zeitz U, Lieberherr M, Gniadecki R, Möller G, Adamski J, Balling R** 2002 Deletion of deoxyribonucleic acid binding domain of the vitamin D receptor abrogates genomic and nongenomic functions of vitamin D. Mol Endocrinol 16:1524–1537
46. **O'Connell TD, Giacherio DA, Jarvis AK, Simpson RU** 1995 Inhibition of cardiac myocyte maturation by 1,25-dihydroxyvitamin D3. Endocrinology 136:482–488
47. **Chen S, Ni XP, Humphreys MH, Gardner DG** 2005 1,25 Dihydroxyvitamin D amplifies type A natriuretic peptide receptor expression and activity in target cells. J Am Soc Nephrol 16:329–339
48. **Kristoffersson A, Boström A, Söderberg T** 1992 Muscle strength is improved after parathyroidectomy in patients with primary hyperparathyroidism. Br J Surg 79:165–168
49. **Mallette LE, Patten BM, Engel WK** 1975 Neuromuscular disease in secondary hyperparathyroidism. Ann Intern Med 82:474–483
50. **Garber AJ** 1983 Effects of parathyroid hormone on skeletal muscle protein and amino acid metabolism in the rat. J Clin Invest 71:1806–1821
51. **Resnick LM, Müller FB, Laragh JH** 1986 Calcium-regulating hormones in essential hypertension. Relation to plasma renin activity and sodium metabolism. Ann Intern Med 105:649–654
52. **Kristal-Boneh E, Froom P, Harari G, Ribak J** 1997 Association of calcitriol and blood pressure in normotensive men. Hypertension 30:1289–1294
53. **Lind L, Hänni A, Lithell H, Hvarfner A, Sörensen OH, Ljunghall S** 1995 Vitamin D is related to blood pressure and other cardiovascular risk factors in middle-aged men. Am J Hypertens 8:894–901
54. **Li YC, Kong J, Wei M, Chen ZF, Liu SQ, Cao LP** 2002 1,25-Dihydroxyvitamin D(3) is a negative endocrine regulator of the renin-angiotensin system. J Clin Invest 110:229–238
55. **Wang TJ, Pencina MJ, Booth SL, Jacques PF, Ingelsson E, Lanier K, Benjamin EJ, D'Agostino RB, Wolf M, Vasan RS** 2008 Vitamin D deficiency and risk of cardiovascular disease. Circulation 117:503–511
56. **2006 The role of calcium in peri- and postmenopausal women** 2006 position statement of the North American Menopause Society. Menopause 13:862–877; quiz 878–880