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FOXO1 Mediates Vitamin D Deficiency-induced Insulin Resistance in Skeletal Muscle

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Abstract

Prospective epidemiological studies have consistently shown a relationship between vitamin D deficiency, insulin resistance, and type 2 diabetes mellitus (DM2). This is supported by recent trials showing that vitamin D supplementation in prediabetic or insulin-resistant patients with inadequate vitamin D levels improves insulin sensitivity. However, the molecular mechanisms underlying vitamin D deficiency-induced insulin resistance and DM2 remain unknown. Skeletal muscle insulin resistance is a primary defect in the majority of patients with DM2. While sustained activation of forkhead box O1 (FOXO1) in skeletal muscle causes insulin resistance, a relationship between vitamin D deficiency and FOXO1 activation in muscle is unknown. We generated skeletal muscle-specific vitamin D receptor (VDR)-null mice and discovered that these mice developed insulin resistance and glucose intolerance accompanied by increased expression and activity of FOXO1. We also found sustained FOXO1 activation in the skeletal muscle of global VDR-null mice. Treatment of C2C12 muscle cells with 1,25-dihydroxyvitamin D (VD3) reduced FOXO1 expression, nuclear translocation, and activity. The VD3-dependent suppression of FOXO1 activation disappeared by knockdown of VDR, indicating that it is VDR-dependent. Taken together, these results suggest that FOXO1 is a critical target mediating VDR-null signaling in skeletal muscle. The novel findings provide the conceptual support that persistent FOXO1 activation may be responsible for insulin resistance and impaired glucose metabolism in vitamin D signaling-deficient mice, as well as evidence for the utility of vitamin D supplementation for intervention in DM2.

Keywords

Vitamin D; Gene Deletion; FOXO1; Insulin Resistance; Skeletal Muscle

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Introduction

The prevalence of type 2 diabetes mellitus (DM2) is rapidly escalating. Because of chronic and progressive micro- and macrovascular complications, DM2 is not only the leading cause of renal failure, non-traumatic lower-limb amputation, and new cases of blindness, but also a major cause of coronary artery disease and stroke in the United States. DM2 predominately results from an interaction between genetic factors and epigenetic influences, leading to insulin resistance and relative lack of insulin. Genome-wide association studies have shown that genetic variants account for only 5–10% of DM2 cases [1,2], suggesting that the growing prevalence of DM2 is largely due to relevant epigenetic risk factors.

One potential epigenetic risk factor related to DM2 is vitamin D deficiency. Vitamin D is synthesized in the skin by UV light exposure or absorbed from food. Subsequently, it is hydroxylated in the liver to generate 25-hydroxyvitamin D, which is hydroxylated in the proximal renal tubule to produce the most biologically active form, 1,25-dihydroxyvitamin D (VD3). VD3 as a nuclear hormone regulates gene transcription and exerts its effects through binding to the vitamin D receptor (VDR). Plasma level of 25-hydroxyvitamin D (25(OH)D) has been widely accepted as the most reliable marker for determining vitamin D status. Currently, more than 1 billion people are at risk for vitamin D deficiency worldwide [3]. Long-term prospective studies have consistently shown that high plasma vitamin D levels predict reduced risk of DM2 in high-risk individuals [4–6]. This is supported by recent trials [7–11] showing that vitamin D supplementation in pre-diabetic or insulin-resistant patients with vitamin D deficiency improves insulin sensitivity.

DM2 is primarily characterized by insulin resistance. Skeletal muscle is responsible for 80% of insulin-stimulated whole-body glucose disposal [12]; hence it plays an important role in the pathogenesis of insulin resistance. Insulin resistance in muscle has been demonstrated to be a primary characteristic, pathophysiological defect in the majority of patients with DM2 [13,14]. To compensate for insulin resistance and maintain euglycemia, beta cells increase insulin secretion. Insulin resistance is typically present for some years preceding the onset of DM2. When insulin levels decline because of decreased beta cell number and function, DM2 develops [15]. Observational studies have shown a significant inverse relationship between plasma 25(OH)D and insulin resistance [16–18].

Forkhead box O1 (FOXO1) is a key insulin signaling downstream negative regulator and plays a significant role in regulating glucose homeostasis [19]. FOXO1 activity can be regulated by post-translational modification and/or the change in transcriptional levels. At physiological conditions, insulin inhibits FOXO1 activity through activation of the insulin receptor (IR)/phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway. Activated Akt phosphorylates FOXO1 at three highly conserved phosphorylation sites (T24, S256, and S319), resulting in its nuclear exclusion and suppression of its transcriptional activity. During fasting, FOXO1 promotes gluconeogenesis in the liver by inducing phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase) expression and a transition from carbohydrate oxidation to lipid oxidation in muscle by stimulating pyruvate dehydrogenase kinase 4 (PDK4) expression. In the fed state, hepatic and skeletal muscle FOXO1 activity is inhibited by insulin, which suppresses hepatic

gluconeogenesis and stimulates muscle glucose oxidation. In contrast to the negative effect of insulin/PI3K/Akt signaling on FOXO1 activity, activation of c-jun N-terminal kinase (JNK) drives FOXO1 from the cytoplasm to the nucleus by phosphorylation of FOXO1 at specific sites that are distinct from the Akt phosphorylation sites or phosphorylation of 14-3-3 protein, which promotes dissociating from FOXO1 and enhances FOXO1-induced gene expression [20–22].

In addition to phosphorylation, the other major FOXO1 post-translational modification is acetylation/deacetylation. A number of studies have shown that FOXO deacetylation inhibits mammalian FOXO activities [23,24]. On the pathophysiological function, sustained over-activation of FOXO1 plays a critical role in the pathogenesis of metabolic dysfunction or insulin resistance. Molecular defects in insulin signaling that result in decreased Akt expression or activity (e.g., insulin receptor substrate 1 (IRS-1) deletion [25]) promote excess FOXO1 nuclear translocation through post-translational modifications, leading to insulin resistance and/or DM2. On the other hand, under conditions of catabolic metabolism, such as starvation, sepsis, and severe diabetes, selectively increased FOXO1 transcriptional levels in muscle induce its target gene PDK4 transcription [26]. Increased muscle PDK4 expression is associated with insulin resistance [27], and PDK4 deletion improves glucose tolerance and insulin sensitivity [28]. Furthermore, increased FOXO1 expression in skeletal muscle-specific FOXO1 transgenic mice displays decreased insulin sensitivity and impaired glucose tolerance [29]. FOXO1 haploinsufficiency protects mice against obesity-related insulin resistance in liver and skeletal muscle [30].

Taken together, these data indicate that increased FOXO1 activity by overexpression and/or excess nuclear translocation of FOXO1 in skeletal muscle leads to insulin resistance. Of note, severe over-activation of FOXO1 in muscle also triggers muscle atrophy by inducing ubiquitin ligases (e.g., muscle RING-finger protein-1 (MuRF1) [31]), and liposomal proteases (e.g., cathepsin L [32]). Although insulin sensitivity has been reported to correlate positively with muscle mass, a recent elegant study has shown that muscle atrophy is not causally related to muscle glucose homeostasis [33], suggesting that insulin resistance and muscle atrophy are two distinct phenotypes induced by muscle FOXO1 activation through FOXO1-PDK4, and FOXO1-MuRF1/FOXO1-cathepsin L mechanisms, respectively. Here we discovered VD3/VDR-dependent suppression of FOXO1 expression and activation in muscle cells *in vitro*, and persistently increased FOXO1 protein levels and activation in skeletal muscle of both $VDR^{-/-}$ and skeletal muscle-specific VDR-null ($SMVDR^{-/-}$) mice, which displayed insulin resistance and glucose intolerance. The novel findings provide the conceptual support that FOXO1 may be a major target to mediate muscle vitamin D signaling deficiency-induced insulin resistance and impaired glucose metabolism in $SMVDR^{-/-}$ mice.

Materials and Methods

Generation of $VDR^{-/-}$ and $SMVDR^{-/-}$ mice

We generated mice possessing loxp sites flanking exon 4 in the VDR gene ($VDR^{loxp/loxp}$). Cre-mediated deletion of VDR exon 4 introduces a stop codon in the mRNA, leading to complete ablation of VDR protein expression [34]. The floxed VDR mice were mated with

female Sox-2 Cre mice ubiquitously expressing Cre recombinase during embryonic development to generate $VDR^{-/-}$ mice [34]. At 3 weeks of age, all $VDR^{-/-}$ mice were administered a special diet enriched with calcium and phosphorus to maintain normal plasma calcium, phosphorus, and PTH levels [35]. $SMVDR^{-/-}$ mice were generated by crossing $VDR^{loxp/loxp}$ mice with mice with muscle-specific Cre recombinase expression under the control of the myosin light chain 1f (MLC 1f) genomic locus (kindly provided by Dr. Steven J. Burden, New York University) [36]. MLC 1f Cre expression has been shown to be restricted to skeletal muscle throughout development and in adult mice [37]. The research carried out on animals was in compliance with the guiding principles in the *Guide for the Care and Use of Laboratory Animal: Eighth Edition*. The Institutional Animal Care and Use Committees at the University of California San Francisco and Creighton University, Omaha, Nebraska, approved the animal research protocols.

Cell culture and SiRNA transfection

C2C12 myoblasts were obtained from ATCC (Manassas, VA). Myoblasts were grown to 65–70% confluence in DMEM (Dulbecco's Modification of Eagles Medium) with 10% fetal bovine serum (FBS). After being exposed to DMEM containing 2% horse serum for 2 days to induce differentiation, the cells were then treated with vehicle or VD3 or paricalcitol for 48 hours in 2% horse serum media. At the end of treatment, they were used for immunofluorescence assay or lysed to collect protein or total RNA. For VDR siRNA transfection experiments, after one day in 2% horse serum, cells were transfected with 100 nM VDR or control siRNAs mixed with Lipofectamine RNAiMAX transfection reagent for 24 hours. The medium was changed and cells were treated with VD3 for 48 hours prior to the collection for Western blot analysis.

Nuclear and cytoplasmic protein preparation, gene array, genotyping, histology, and differential expression analysis

Mice were euthanized and their quadriceps were isolated, diced into 1–2 mm pieces with a clean scalpel, and homogenized on ice. The nuclear and cytoplasmic protein from the fresh mouse quadriceps was prepared for Western blot using the Nuclear Extract Kit (Active Motif, California) according to its protocol instruction. Gene array, genotyping, histology, total RNA and cell protein preparation, RT-PCR, and Western blot have been described previously [34].

Immunofluorescence

After fixing the cultured cells with 4% paraformaldehyde or after rehydration of the frozen skeletal muscle sections, antigen retrieval was performed prior to immunostaining. Sections were incubated for 2 hours in block/permeabilizing solutions containing PBS, 0.25 % Triton X-100, and 5% (v/v) goat serum at room temperature. The slides were subsequently incubated with primary antibodies including rabbit anti-FOXO1 antibody (Cell Signaling, #2880S) at 4°C overnight. After washing with PBS three times for 5 minutes each, a secondary antibody (Alexa Fluor 594 AffiniPure Goat Anti-Rabbit antibody, Jackson ImmunoResearch, 1:400) was applied to the sections for 2 hours in the dark. Negative controls were run in parallel with normal host IgG. Sections were washed with PBS four

times for 5 minutes. Nuclei were counterstained with DAPI. Pictures were taken using a Zeiss ApoTome microscope.

Glucose metabolic profile

Glucose tolerance test (GTT): After a 16-h fasting, mice were weighed and fasting blood glucose level was measured using the Contour Glucometer. Mice were injected intraperitoneally with glucose solution (2g/kg body weight). Blood glucose levels were then measured at 0, 15, 30, 60, and 90 minutes after the injection. Insulin measurement: Blood was collected after fasting and 30 minutes after glucose injection. Serum containing protease inhibitors (Roche, Indianapolis, IN) was stored at −80°C. Insulin concentration was calculated using the Insulin EIA kit (ALPCO) per the manufacturer's instructions. Insulin tolerance test (ITT): After 6-h fasting, the mice were injected intraperitoneally with insulin (1 U/kg body weight). Blood glucose was determined at 0, 15, 30, 90, and 120 minutes after insulin injection. Glucose levels after insulin injection were presented as the percentage of initial glucose concentrations.

Statistical analysis

Comparisons between multiple groups were performed by two-way analysis of variance using GraphPad Prism 6, followed by a *post hoc* Bonferroni test to determine the significance of differences between two groups. An unpaired two-tailed Student's t test was used to compare the differences between two groups. The data were presented as mean +/− SD. Values of $P \leq 0.05$ were considered significant.

Results

Persistent activation of FOXO1 in skeletal muscle of VDR$^{-/-}$ mice

To determine potential biological mechanisms of vitamin D deficiency in insulin resistance and DM2, we used VDR gene deletion mouse models to examine whether there is a skeletal muscle insulin signaling defect in vitamin D signaling-deficient mice. To identify the target genes involved in insulin signaling induced by VDR deficiency, we performed cDNA microarray analysis to compare the mRNA content of quadriceps in VDR$^{-/-}$ mice and control littermates (floxed VDR mice). The results from hybridization of muscle cDNA to Mouse Genome 1.0 ST array yielded 26,581 individual gene sequences. More than 95% of genes were expressed equally in VDR$^{-/-}$ mice as compared to controls. Expression levels of the genes related to this study, including those whose expression was significantly upregulated or markedly downregulated ($P<0.05$) in the VDR$^{-/-}$ group compared with control littermates, are listed in Fig. 1A. We identified that expression of FOXO1, a key downstream target of insulin signaling, and several of its target genes (e.g., PDK4, PEPCK, G6Pase, and MuRF1) was significantly increased in the quadriceps of VDR$^{-/-}$ mice compared to controls (Fig. 1A), indicative of sustained FOXO1 activation in VDR-null muscle. Interestingly, muscle transcription of MAPK phosphatase 1 (MKP-1), whose primary function is to dephosphorylate JNKs and inactivate them [38], was significantly decreased in VDR$^{-/-}$ mice compared to controls. Expression of MKK6, a p38 MAPK upstream kinase, was also markedly reduced in VDR$^{-/-}$ mice. MKP-1 has been documented to be an activated VDR direct target [39], and JNK and p38 activities displayed opposite roles

in skeletal muscle [40], suggesting that decreased MKP-1 and MKK6-mediated p38 levels could stimulate JNK activities in VDR$^{-/-}$ skeletal muscle that may contribute to FOXO1 activation (see below). In addition, expression of histone deacetylases 3 (HDAC3) and HDAC9 was reduced in quadriceps of VDR$^{-/-}$ mice by 2.84-fold and 1.69-fold (Fig. 1A), respectively, compared to controls. The reduction of the two HDAC levels could decrease FOXO1 deacetylation, which may participate in stimulating FOXO1 nuclear translocation and its activity in VDR-null muscle.

To validate the gene array results, we directly measured the mRNA levels of some of the genes by RT-PCR using skeletal muscle RNA extracted from VDR$^{-/-}$ mice and age- and sex-matched controls. Expression levels of FOXO1, PDK4, PEPCK, and MuRF1 were significantly elevated and expression of MKP-1 and MKK6 was dramatically downregulated when normalized to *GAPDH* RNA in skeletal muscle from the VDR$^{-/-}$ mice compared to controls (Fig. 1B). The RT-PCR results matched well with those of the gene array, confirming the gene array data. The increase in FOXO1 mRNA levels was accompanied by increased amounts of total FOXO1 protein (Fig. 2A). There was no significant difference in expression of FOXO1 upstream insulin signaling genes, including IR, IRS-1 and 2, PI3K, and Akt1 and 2, as well as glucose transporter type 4 (Glut4), lipoprotein lipase (L-lipase), translocase CD36, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), FOXO3a, and FOXO4 between VDR$^{-/-}$ and the control mice (Fig. 1A), some of which were confirmed by RT-PCR (data not shown). Several studies have shown that decreased activity of insulin-like growth factor 1 (IGF-1) or insulin/PI3K/Akt signaling reduces FOXO1 phosphorylation at the three sites (T24, S256, and S319), promoting its nuclear translocation and activity, leading to insulin resistance [41]. In the muscle of VDR$^{-/-}$ mice, neither total and phosphorylated Akt (S473) nor phosphorylated FOXO1 (Ser 256) levels were significantly changed (data not shown); however, nuclear FOXO1 levels were markedly increased (normalized to nuclear protein Lamin A/C levels (Fig. 2B)) compared to controls. Although the nuclear extracts in some samples displayed low levels of cytoplasmic protein contamination detected by α-Tubulin antibody, indicating that the Nuclear Extract Kit was not perfect to separate nuclear protein from cytoplasmic protein, Lamin A/C levels in cytoplasmic extracts were undetectable (Fig. 2B). Furthermore, increased nuclear FOXO1 in VDR-null muscle was confirmed by immunofluorescence assay (Fig. 2C).

Characterization of SMVDR$^{-/-}$ mice

VDR expression has been identified in human and rodent skeletal muscle cells [42,43]. To determine whether there is insulin resistance in vitamin D signaling-deficient skeletal muscle that is sufficient to induce glucose intolerance, we generated SMVDR$^{-/-}$ mice as described above. Six-month-old SMVDR$^{-/-}$ mice were euthanized and the myocytes were isolated from their quadriceps using a modified procedure [44]. DNA, RNA, and protein were extracted from the myocytes. As shown in Fig. 3A, floxed VDR allele recombination and efficient deletion of VDR from the myocytes were detected by PCR of genomic DNA. The deletion of VDR from the myocytes was quantified by measuring VDR mRNA expression using RT-PCR with GAPDH as an internal control (Fig. 3B). We observed ~85% excision of VDR in myocytes isolated from SMVDR$^{-/-}$ mice. Incomplete deletion was likely due to low levels of contamination from other cell types in the myocyte preparations. Consistent with

previous reports [42,43], VDR protein is expressed in skeletal muscle cells (Fig. 3C). VDR deletion in the myocytes from quadriceps was confirmed by Western blot with α-Tubulin as an internal control. VDR protein expression in the myocytes from SMVDR$^{-/-}$ mice was too low to be detected by Western blot (Fig. 3C).

These results suggest that the MLC 1f Cre driver line is highly efficient to delete VDR in skeletal muscle cells. SMVDR$^{-/-}$ mice are viable and fertile with normal serum calcium and phosphorus levels. While quadriceps weight (QW)/body weight (BW) ration in VDR$^{-/-}$ mice was significantly lower than controls (QW/BW: 4.76 +/− 0.68 vs. 5.65 +/− 0.85 mg/g, n=10, P<0.05), QW/BW in SMVDR$^{-/-}$ mice tended to be lower than that of controls, but this did not reach statistical significance (QW/BW: 6.3 +/− 0.60 vs. 7.04 +/− 0.84 mg/g, n=9, P=0.052). The quadriceps from some SMVDR$^{-/-}$ mice with their age- and sex-matched control littermates were rapidly frozen in liquid nitrogen-cooled isopentane for histology and immunofluorescence analyses. Muscle tissue sections were subjected to H&E staining or immunostaining with anti-myosin heavy chain fast type (MHCf) antibody to assess myocyte size in type II fibers. While muscle architecture assessed by H&E staining was normal (Fig. 3D), quadriceps myocyte size in SMVDR$^{-/-}$ mice was slightly, but significantly, smaller than controls (Fig. 3 E). However, as mentioned above, muscle atrophy is the other phenotype induced by FOXO1 overactivation [45], and it is not causally related to muscle glucose homeostasis [33].

The role of skeletal muscle-specific VDR deletion in glucose metabolism and FOXO1 activation

To determine the role of skeletal muscle-specific inactivation of VDR in glucose metabolism, we performed glucose metabolic tests in 6-month-old SMVDR$^{-/-}$ and age- and sex-matched control mice. There was no significant difference in fasting blood glucose levels between control and SMVDR$^{-/-}$ mice. However, an intraperitoneal glucose tolerance test (GTT) demonstrated that SMVDR$^{-/-}$ mice displayed significantly elevated plasma glucose levels at 15 minutes, which remained elevated until the end of this experimental time period (Fig. 4A), suggesting that they were markedly glucose intolerant compared with controls. Fasting insulin levels in SMVDR$^{-/-}$ mice were ~2-fold higher than those in controls (Fig. 4B). Despite glucose intolerance, 30 minutes after glucose injection, insulin levels in SMVDR$^{-/-}$ mice trended higher rather than lower than those in controls (Fig. 4B), indicating that glucose intolerance reflects impaired muscle glucose uptake and oxidation, and not defective beta cell insulin secretion. To determine whether SMVDR$^{-/-}$ mice had reduced insulin sensitivity, we performed an insulin tolerance test (ITT). As shown in Fig. 4C, ITT results showed a markedly blunted decline in glucose levels upon insulin injection in SMVDR$^{-/-}$ compared with control mice, indicative of peripheral insulin resistance in SMVDR$^{-/-}$ mice.

To explore the potential molecular mechanisms of skeletal muscle-specific VDR deletion-induced insulin resistance, we collected total RNA and protein in the quadriceps of SMVDR$^{-/-}$ and control mice. Real-time PCR and Western blot results demonstrated that FOXO1 expression was dramatically upregulated in the muscle of SMVDR$^{-/-}$ mice compared with controls (Fig. 5 A & B). To determine whether there is also increased

FOXO1 nuclear translocation and activity in SMVDR$^{-/-}$ muscle cells, we conducted immunofluorescence assay and PDK4 mRNA measurement. As shown in Fig. 5C, FOXO1 protein accumulated in muscle cell nuclei of SMVDR$^{-/-}$ mice, which was accompanied by increased PDK4 expression in SMVDR$^{-/-}$ muscle (Fig. 5 D). These data identify that, like VDR$^{-/-}$ mice, there is persistent FOXO activation in muscle of SMVDR$^{-/-}$ mice. The activation of FOXO1 has been shown to play a critical role in muscle insulin resistance [29]. The novel findings suggest that FOXO1 activation in VDR-null muscle may play a central role in muscle insulin resistance responsible for glucose intolerance.

VD3/VDR-dependent inhibition of FOXO1 expression, nuclear accumulation, activity, and JNK activity in C2C12 myotubes

Selective deletion of VDR in skeletal muscle cells *in vivo* leads to increased FOXO1 expression and activity, suggesting that VD3/VDR signaling negatively regulates FOXO1 gene transcription and promotes cytoplasmic translocation. We treated C2C12 myotubes with indicated doses of VD3 and its analogue, paricalcitol, for 48 hours. As shown in Fig. 6A&B, VD3 dose-dependently inhibited FOXO1 transcription and translation. Paricalcitol had similar effects. C2C12 myotubes maintained in 2% horse serum (a starvation status) accumulated high levels of FOXO1 in the nuclei (left panel of Fig. 6C). Treatment of VD3 for 48 hours markedly promoted FOXO1 from nucleus to cytoplasm in a dose-dependent manner (Fig. 6C). Notably, a lower dose of VD3 caused an incomplete FOXO1 cytoplasmic translocation. As shown in the right panel of Fig. 6C, FOXO1 protein was located in both the nucleus and cytoplasm in some cells. These results show that VD3 negatively regulates FOXO1 transcription and nuclear translocation.

To examine whether the inhibitory effect of VD3 on FOXO1 expression and activity is VDR-dependent, we suppressed VDR protein levels using a VDR siRNA approach. As shown Fig. 6D, the VDR siRNA effectively knocked down VDR protein expression. Knockdown of VDR had a limited role in basal FOXO1 levels, which were high in the medium with 2% horse serum. However, it led to the loss of a VD3-mediated inhibitory effect on FOXO1 and PDK4 expression (Fig. 6E & F), suggesting that VD3 negatively regulates FOXO1 expression and activity through binding its receptor. This supports the *in vivo* data that deletion of VDR resulted in increased over-activation of FOXO1. To determine whether VD3 inhibits JNK activity, we treated C2C12 myotubes with VD3 for 48 hours. VD3 dose-dependently reduced JNK activity, but not JNK1 expression, which was accompanied by increased VDR expression in a dose-dependent fashion (Fig. 7). These results suggest that VD3 enhances vitamin D signaling by amplification of VDR expression that suppresses JNK activity, and support the concept that vitamin D signaling deficiency triggers JNK activation, leading to FOXO1 nuclear translocation.

Discussion

Epidemiological prospective studies [4–6] and recent clinical trials [7–11] have clearly shown a relationship between vitamin D deficiency, insulin resistance, and DM2. However, mechanistic links between vitamin D signaling, insulin resistance, and the development of DM2 remain unclear and cannot be answered by these clinical studies. Skeletal muscle

insulin resistance has been implicated as a major contributor to human DM2 [13,14]. It is well known that FOXO1 activation in skeletal muscle causes insulin resistance. However, whether vitamin D-deficient signaling in muscle causes insulin resistance and impaired glucose metabolism and triggers FOXO1 activation is unknown. We discovered that SMVDR$^{-/-}$ mice displayed insulin resistance and glucose intolerance and increased expression and activity of FOXO1 in skeletal muscle. Persistently increased FOXO1 protein levels and activation were also seen in the skeletal muscle of VDR$^{-/-}$ mice. In the opposite way, enhanced VDR signaling by VD3 inhibited FOXO1 expression, nuclear translocation, and activity in C2C12 muscle cells. The VD3-dependent suppression of FOXO1 activation disappeared when VDR was knocked down. These results suggest that FOXO1 is a major target mediating VDR-null signaling in skeletal muscle. These novel findings indicate that persistent FOXO1 activation in skeletal muscle may be responsible for insulin resistance and impaired glucose metabolism in SMVDR$^{-/-}$ mice.

Recently, An et al. [46] reported that VD3 upregulates FOXO3a and FOXO4 in SCC25 cells, a cancer cell line. The different effect of VD3 on FOXO regulation may depend on different tissues and cells. However, VDR-null mice do not develop cancers. Upregulation of FOXOs in cancer cells may not be a VD3 physiological function. In contrast, Eelen et al. [47] showed that VD3 induced the expression of FOXO3a, but downregulated FOXO1 levels in osteoblasts, suggesting that VD3 regulates different isoforms of FOXOs with distinct mechanisms. Deletion of the VDR gene in both VDR$^{-/-}$ and SMVDR$^{-/-}$ mice led to increased FOXO1 transcription and translation and its target gene expression in skeletal muscle, and treatment of VD3 and its analogue inhibited FOXO1 expression and activity in C2C12 myotubes. Our results have clearly demonstrated that VD3 negatively regulates FOXO1 expression and activity in skeletal muscle (Fig. 8). However, it remains to be elucidated whether activated VDR inhibits FOXO1 transcription through direct binding to VDR elements in the FOXO1 promoter with recruitment of corepressors, or by indirect binding to DNA through contiguous transcription factors, or by indirectly regulating expression of other genes.

Increased FOXO1 activity can be regulated by enhanced transcription and/or post-translational regulation. Our SMVDR$^{-/-}$ mice displayed increased fasting insulin levels (Fig. 4B), which should promote FOXO1 nuclear exclusion. However, SMVDR$^{-/-}$ mice demonstrated significant FOXO1 nuclear accumulation (Fig. 5C), even though increased muscle FOXO1 expression makes a partial contribution (Fig. 5A & B). Furthermore, while treatment of C2C12 myotubes with 10^{-8} mol/L VD3 reduced FOXO1 protein level by ~65% (Fig. 6A & B), this dose of VD3 almost completely led to FOXO1 nuclear export (Fig. 6C). Blockade of VDR lost VD3-induced suppression of FOXO1 and its target gene PDK4 expression, indicating that the inhibitory role of VD3 is VDR-dependent.

These findings suggest that VDR deficiency induced FOXO1 nuclear translocation and activation cannot be completely explained by its effect on FOXO1 transcription in SMVDR$^{-/-}$ mice. Higher levels of nuclear FOXO1 in VDR$^{-/-}$ muscle (Fig. 2B) support the above statement. Data from our microarray showed significant reduction of MKP-1 and MKK6 expression in VDR-null muscle, which could increase JNK activity. Treatment of C2C12 myotubes with VD3 for 48 hours dose-dependently reduced JNK activity and

FOXO1 expression and activation. These results support the concept that vitamin D signaling deficiency may trigger JNK activation, leading to FOXO1 phosphorylation at specific sites, which participates in FOXO1 nuclear translocation, and provide direct evidence that activated VDR suppresses FOXO1 activity partially through reduction of JNK activation (Fig. 8). In support of these statements, recently decreased MKP-1 and persistent activation of JNK were seen in p38α-null myoblasts [48]. Furthermore, increased nuclear FOXO1 and FOXO3 were associated with decreased p38 activities in a dexamethasone-induced muscle atrophy model and a colorectal cancer model, respectively [49,50].

While JNK activation-dependent phosphorylation of FOXO1 as a post-translational regulation may participate in FOXO1 activation, a mechanistic link between activation of JNK and FOXO1 in vitamin D-deficient skeletal muscle remains to be determined. In addition, the reduction of HDAC3 and HDAC9 in VDR-null muscle, which could decrease FOXO1 deacetylation leading to FOXO1 activation, may participate in FOXO1 post-translational regulation (Fig. 8). Nonetheless, whether VDR-null-dependent reduction of HDAC3 and HDAC9 contributes to muscle FOXO1 activation needs further investigation.

As reported by Zeite et al. [51] , a global VDR knockout mouse model expressing a form of VDR with a partially truncated DNA binding domain [52] displayed glucose intolerance. Since these mice had normal fasting insulin levels, but impaired insulin secretion capacity, it is difficult to determine whether there is muscle insulin resistance that is largely responsible for the glucose intolerance in these mice. Increased FOXO1 expression and nuclear translocation, as well as the elevated downstream target gene transcription, provided convincing evidence to display persistent activation of FOXO1 in our $VDR^{-/-}$ skeletal muscle, indicating that there is peripheral insulin resistance in the $VDR^{-/-}$ mice. Our $SMVDR^{-/-}$ mice with intact VDR in islet β-cells showing sustained muscle FOXO1 activation and elevated fasting insulin levels confirm that VDR-null muscle is able to induce peripheral insulin resistance responsible for modest glucose intolerance (Fig. 4A). The VDR knockout mice developed by Zeite et al., similar to vitamin D-deficient rats [53], display impaired insulin secretory capacity [51]. Therefore, it is possible that the compensatory increase in fasting insulin levels against insulin resistance induced by prolonged FOXO1 activation is counteracted, leading to pseudo-normal fasting insulin levels. This explanation is supported by the fact that, because of peripheral insulin resistance and impaired insulin secretion, the mice developed by Zeite et al. displayed more severe glucose intolerance than $SMVDR^{-/-}$ mice (3-month-old $VDR^{-/-}$ mice: ~90 mg/dl difference compared to the control 60 minutes after 1.5 g/kg glucose injection when glucose-stimulated insulin effect reaches close to maximum levels (Fig. 2D in the article by Zeite et al [51]), vs. 6-month-old $SMVDR^{-/-}$ mice: 40 mg/dl difference compared to the control 60 minutes after 2.0 g/kg glucose injection (Fig. 4A)). However, the $SMVDR^{-/-}$ mouse is a novel and unique model to elucidate the molecular mechanism of skeletal muscle insulin resistance to shed the light on the understanding of tissue-specific roles in vitamin D deficiency-induced insulin resistance.

In conclusion, these studies identified FOXO1 as the molecular target for the role of vitamin D signaling deficiency in insulin resistance through the generation of global and tissue-specific genomic VDR deletion mouse models and *in vitro* muscle cell culture systems. By

understanding how VDR-null signaling stimulates FOXO1 activation through transcriptional and possible post-translational regulation and determining the role of FOXO1 in VDR-deficient insulin resistance leading to glucose intolerance (Fig. 8), our experiments provide critical information about an important metabolic pathway involved in DM2. This work will help to more effectively design clinical trials exploring the potential utility of vitamin D supplementation for the prevention and treatment of DM2.

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A

Gene Name	Fold Induction
FOXO1	2.25**
PDK4	2.19**
PEPCK	1.9**
G6Pase	1.5*
MuRF1	2.7**
Cathepsin L	2.09**
MKK6	-3.56**
MKP-1	-2.04**
HDAC3	-2.84**
HDAC9	-1.69*
FOXO3a	1.40
FOXO4	1.01
Glut4	-1.21
PGC-1α	1.11
Insulin-R	1.09
IRS-1	0.88
IRS-2	0.92
PI3-K catalytic-A	1.09
Akt1	0.89
Akt2	1.19
L-lipase	-1.11
CD36	1. 02

B

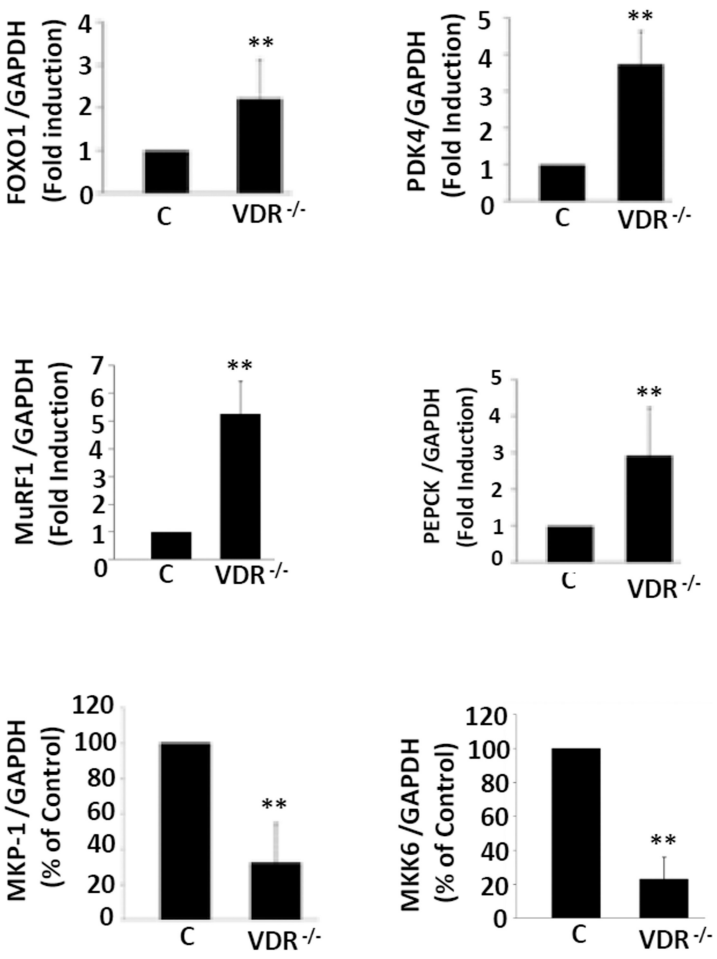

Fig. 1.

The expression levels of FOXO1 and its target genes, and genes related to glucose metabolism in quadriceps of VDR $^{-/-}$ mice compared to floxed VDR control (C). A. Gene array analysis (n=5, each group). B. Direct measurement of mRNA by RT-PCR analysis (n=8, each group). *P<0.05, **P<0.01 vs. control groups.

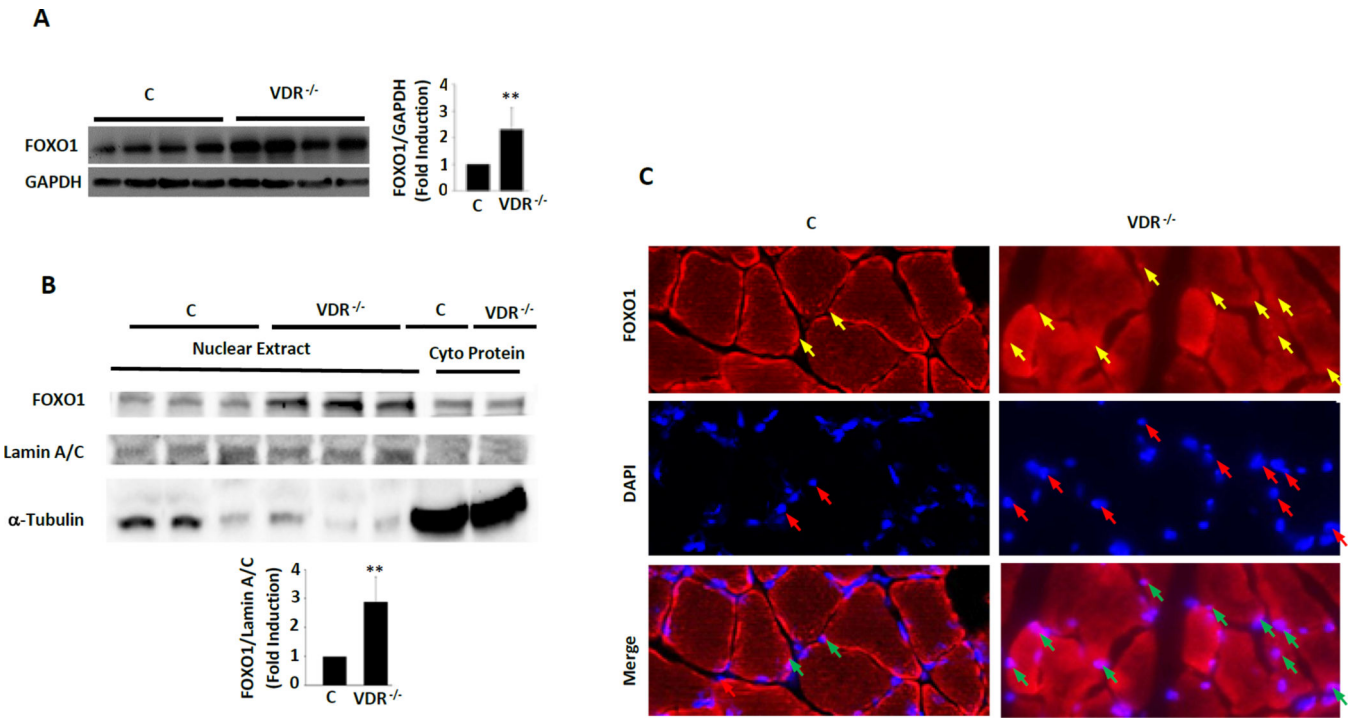

Fig. 2.

The effect of VDR deletion on total FOXO1 protein expression and nuclear FOXO1 protein levels. A. Total FOXO1 protein levels were significantly increased in the quadriceps of VDR $^{-/-}$ mice compared to the control group (quantified by densitometry in right panel, n=8 each group). B. Nuclear FOXO1 protein levels were dramatically enhanced in the skeletal muscle of VDR-null mice normalized by nuclear protein Lamin A/C levels (quantified by densitometry in lower panel, n=6 each group). In addition, a representative picture of a cytoplasmic protein, α-Tubulin levels in nuclear and cytoplasmic extracts is shown. C. Immunofluorescence assay displayed increased nuclear FOXO1 accumulation in the myocytes of VDR$^{-/-}$ mice compared with controls. Yellow and green arrows indicate FOXO1 nuclear staining, and red arrows indicate the nuclear staining. Cyto: cytoplasmic. **P<0.01 vs. control groups.

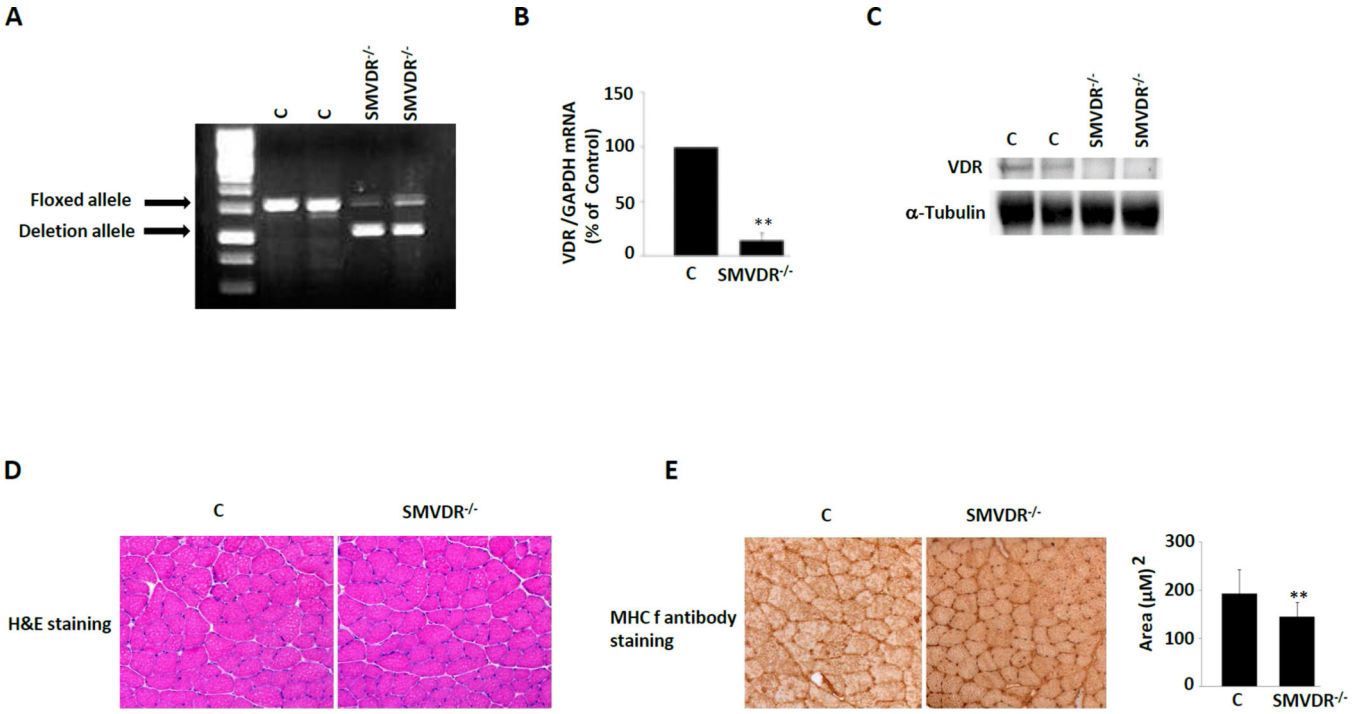

Fig. 3.

Characterization of SMVDR$^{-/-}$ mice. A. Recombinant and deletion alleles of VDR were characterized by PCR using genomic DNA from isolated quadriceps myocytes. B &C. VDR expression in isolated quadriceps myocytes was detected by RT-PCR and Western blot analyses (representative protein results are shown, n=4 each group). D. Muscle architecture in quadriceps sections was assessed by H&E staining. E. Representative images of myocytes from quadriceps by anti-MHCf antibody staining (brown for type II fibers, white for type I fibers) are shown; quantified myocyte size by calculated type II fiber area in SMVDR$^{-/-}$ mice was slightly, but significantly, smaller than controls (330 cells from n=3 each group). **P<0.01 vs. control groups.

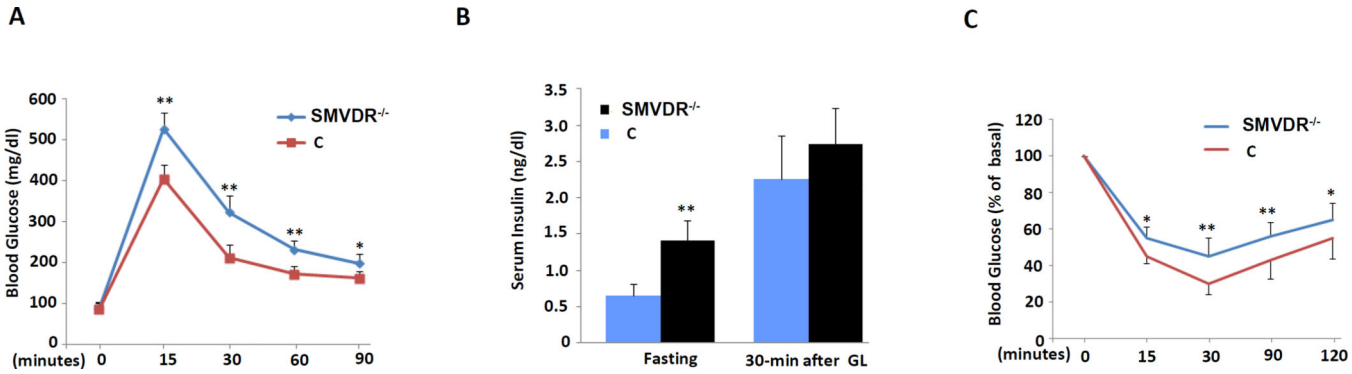

Fig. 4.

Glucose metabolic profiles in SMVDR$^{-/-}$ mice. A. GTT test. After 16-h fasting, mice were loaded with 2g/kg glucose intraperitoneally and blood glucose was measured using the Contour glucometer at the time intervals indicated. B. Serum insulin levels were determined after fasting for 16 hours and 2 g/kg glucose loading (GL) for 30 minutes. C. ITT test. After 6-h fasting, the mice were injected intraperitoneally with insulin (1 U/kg). Blood glucose was determined at indicated times. Glucose levels after insulin injection were presented as the percentage of initial glucose concentrations. Pooled data were obtained from the experiments at different times. N=6–8 each group, *P<0.05, **P<0.01 vs. control groups.

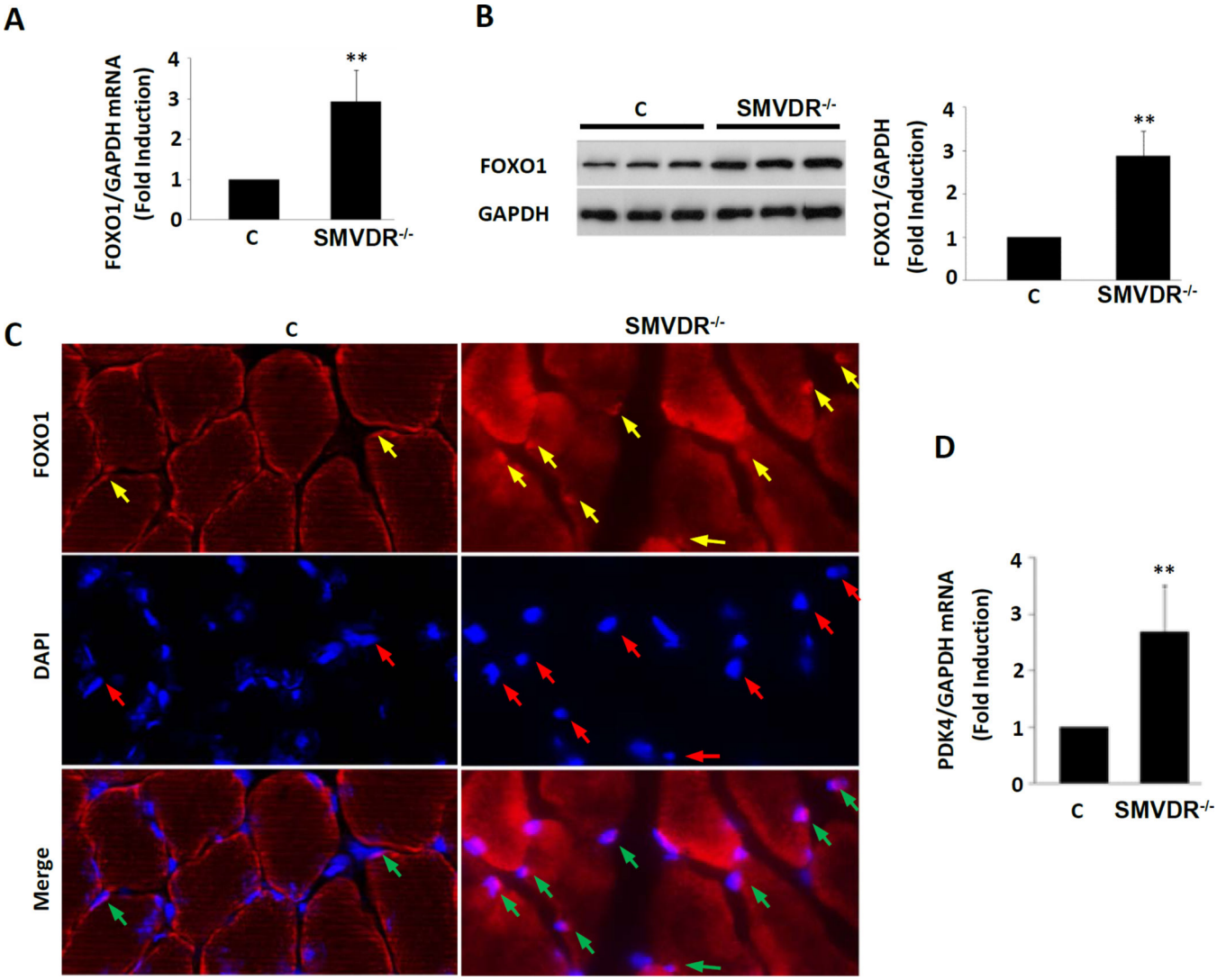

Fig. 5.

Increased FOXO1 expression, nuclear translocation, and activity in quadriceps of SMVDR$^{-/-}$ mice compared to controls. A. FOXO1 mRNA levels were three-fold higher in SMVDR$^{-/-}$ mice than controls. B. Total FOXO1 protein levels were significantly increased in SMVDR$^{-/-}$ mice compared to the control group (quantified by densitometry in lower panel). C: Deletion of VDR in skeletal muscle cells resulted in dramatically increased FOXO1 nuclear translocation in the myocytes of SMVDR$^{-/-}$ mice. Representative pictures are shown. Yellow and green arrows indicate FOXO1 nuclear staining, and red arrows indicate the nuclear staining. D. PDK4 mRNA levels were significantly higher in SMVDR$^{-/-}$ muscle than controls. N=6 per group. **P<0.01 vs. control groups.

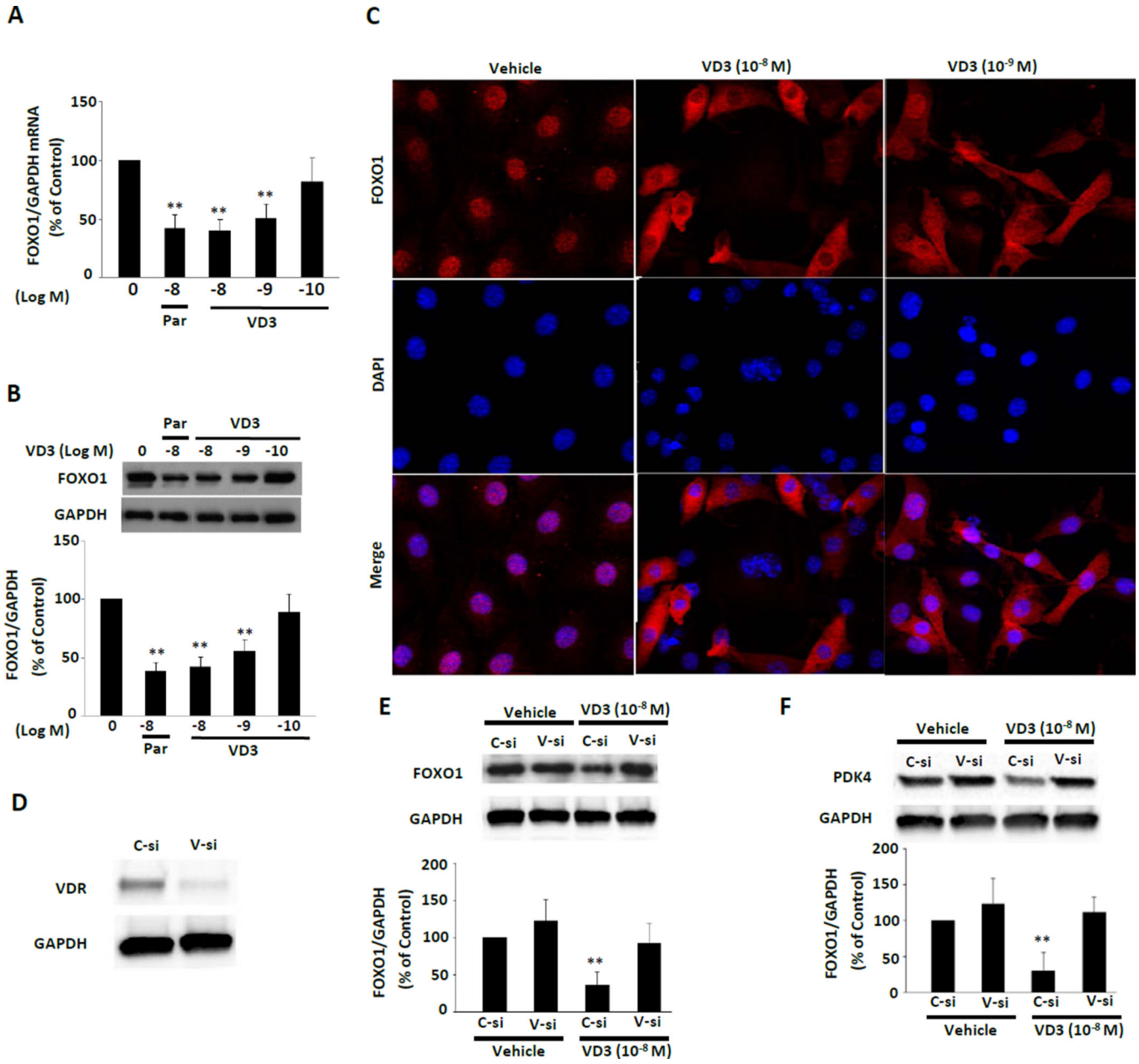

Fig. 6.

VDR mediated VD3 inhibition of FOXO1 expression and activity in C2C12 myotubes in a dose-dependent manner. A. VD3 mediated reduction of FOXO1 mRNA levels detected by RT-PCR B. VD3 inhibited FOXO1 protein levels, as determined by Western blot (quantified in lower panel). C. VD3 promoted FOXO1 translocation from nucleus to cytoplasm. D. VDR SiRNA effectively knocked down VDR protein expression. E&F. Knockdown of VDR resulted in loss of VD3 inhibitory effect on FOXO1 and PDK4 expression. Representative images are shown. Par: VD3 analogue, paricalcitol. C-si: Control siRNA. V-si: VDR siRNA. **P<0.01 vs. control groups (n=3–5 per group).

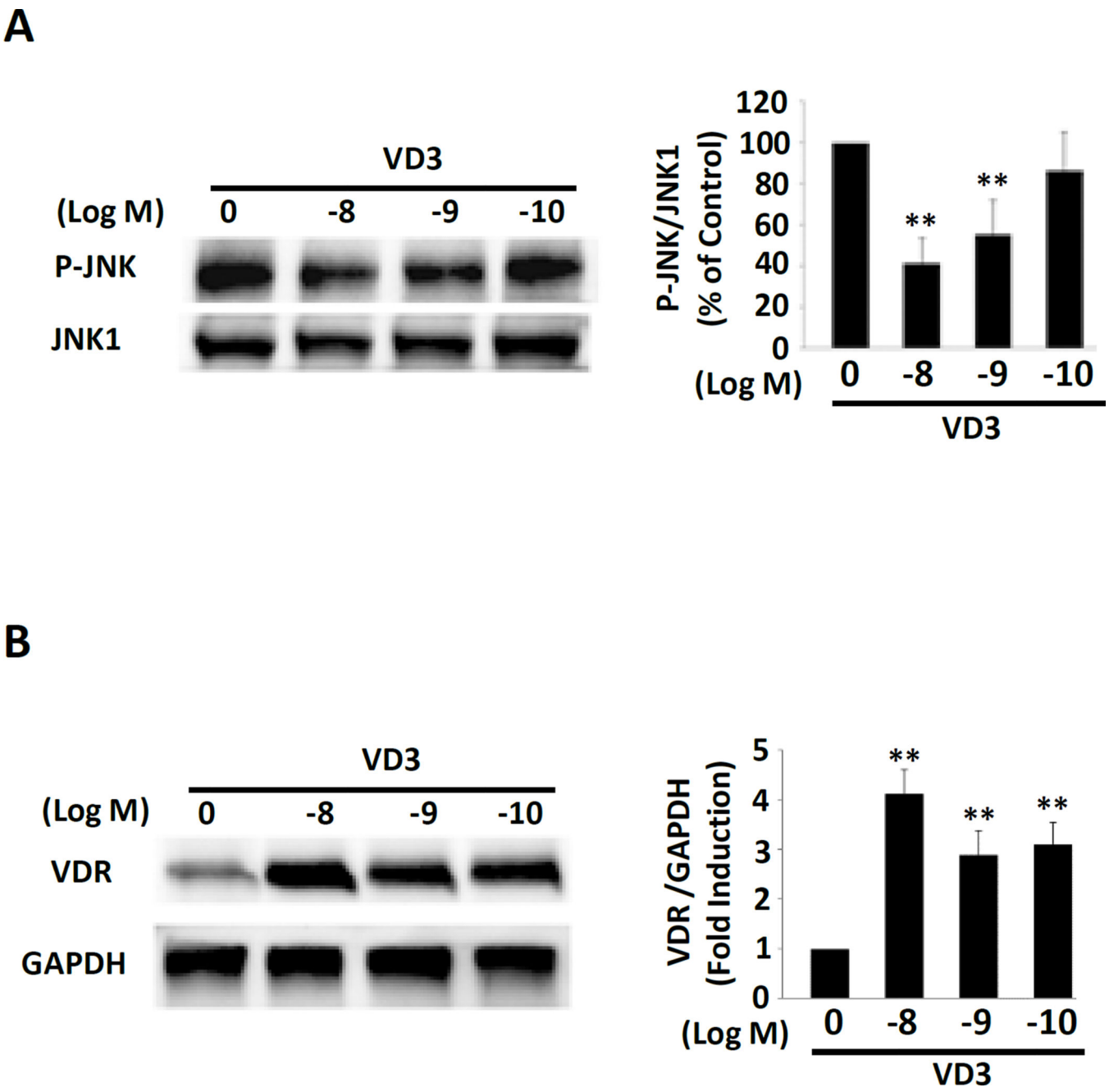

Fig. 7.

VD3 dose-dependently inhibited P-JNK activity and increased VDR expression in C2C12 myotubes. A. Treatment with VD3 dose-dependently reduced JNK activity, but not JNK1 protein levels. P-JNK levels were normalized by JNK1 expression. B. VD3 amplified VDR expression. VDR levels were normalized by GAPDH expression. Representative images are shown. **P<0.01 vs. control groups (n=3 per group).

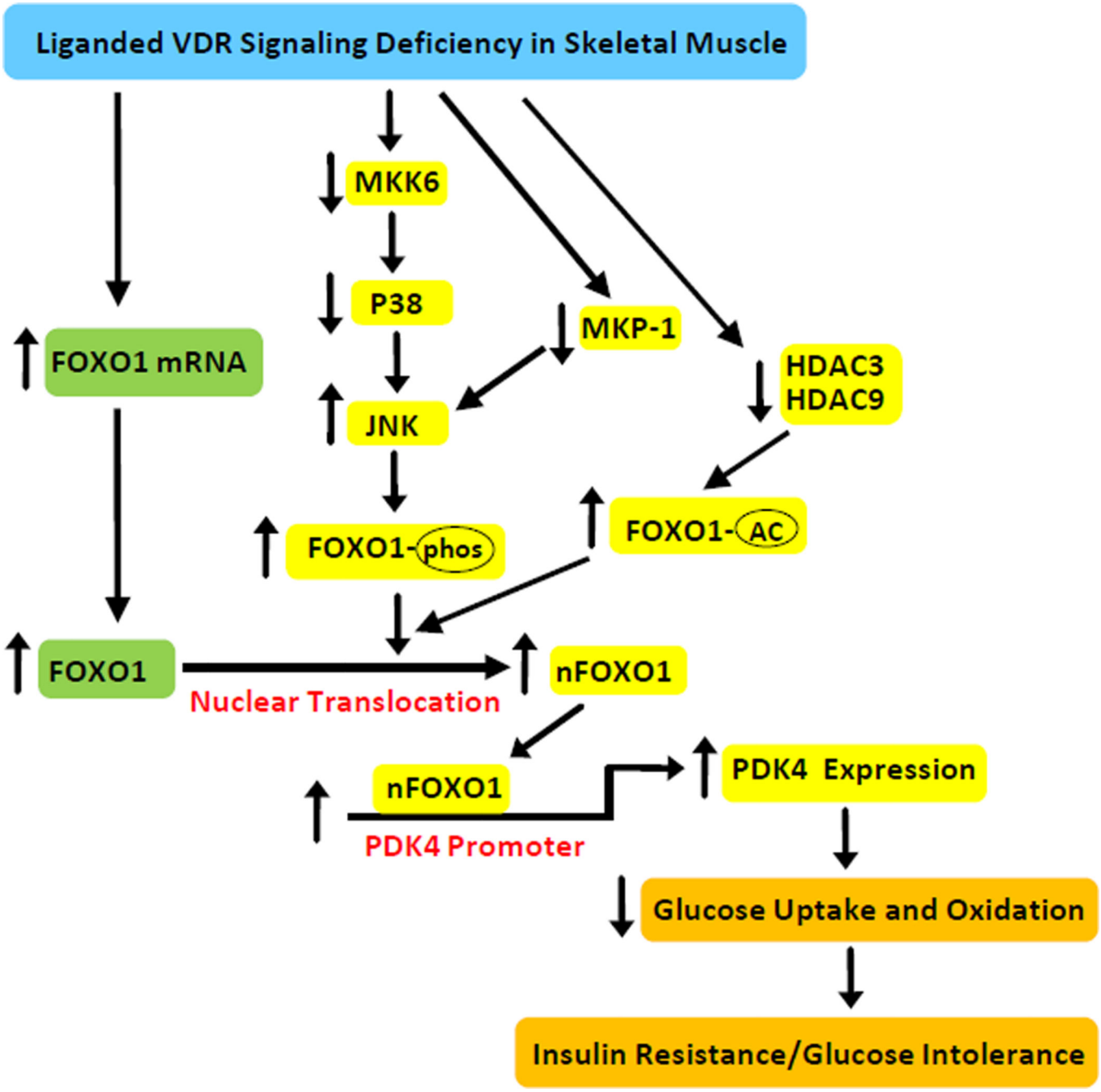

Fig. 8.

Schematic diagram shows the possible mechanisms of VD3/VDR signaling deficiency in skeletal muscle-induced insulin resistance leading to glucose intolerance. nFOXO1: nuclear FOXO1; Phos: Phosphorylation; AC: Acetylation.