Vitamin D receptor targets hepatocyte nuclear factor 4α and mediates protective effects of vitamin D in nonalcoholic fatty liver disease

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Epidemiological studies have suggested a link between vitamin D deficiency and increased risk for nonalcoholic fatty liver disease (NAFLD); however, the underlying mechanisms have remained unclear. Here, using both clinical samples and experimental rodent models along with several biochemical approaches, we explored the specific effects and mechanisms of vitamin D deficiency in NAFLD pathology. Serum vitamin D levels were significantly lower in individuals with NAFLD and in high-fat diet (HFD)-fed mice than in healthy controls and chow-fed mice, respectively. Vitamin D supplementation ameliorated HFD-induced hepatic steatosis and insulin resistance in mice. Hepatic expression of vitamin D receptor (VDR) was up-regulated in three models of NAFLD, including HFD-fed mice, methionine/choline-deficient diet (MCD)-fed mice, and genetically obese (ob/ob) mice. Liver-specific VDR deletion significantly exacerbated HFD- or MCD-induced hepatic steatosis and insulin resistance and also diminished the protective effect of vitamin D supplementation on NAFLD. Mechanistic experiments revealed that VDR interacted with hepatocyte nuclear factor 4α (HNF4α) and that overexpression of HNF4α improved HFD-induced NAFLD and metabolic abnormalities in liver-specific VDR-knockout mice. These results suggest that vitamin D ameliorates NAFLD and metabolic abnormalities by activating hepatic VDR, leading to its interaction with HNF4α. Our findings highlight a potential value of using vitamin D for preventing and managing NAFLD by targeting VDR.

Nonalcoholic fatty liver disease (NAFLD) is characterized by overdeposition of lipids in hepatocytes and is acknowledged as the hepatic manifestation of metabolic syndrome (1, 2). NAFLD progresses from simple steatosis to nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (3, 4). Excessive lipid accumulation leads to inflammation and insulin resistance (IR), which in turn aggravates hepatic steatosis, thus creating a vicious cycle that promotes progression of NAFLD (5). NAFLD not only causes hepatic bullous steatosis and affects liver metabolic function, but also further develops into cirrhosis and participates in the pathogenesis of metabolic diseases such as diabetes, obesity, and dyslipidemia (6).

Recent studies suggest that vitamin D plays a role in the development of metabolic disorders, including diabetes, NAFLD, and metabolic syndrome (7, 8). Vitamin D deficiency has been linked to the diagnosis of NASH and histological features consisting of ballooning, lobular inflammation, and fibrosis (9). Serum vitamin D levels have been suggested to represent a marker for predicting the severity of NAFLD (10). However, the mechanisms underlying the association between vitamin D deficiency and NAFLD remain unclear. The biological functions of vitamin D are primarily mediated via activation of the vitamin D receptor (VDR) (11), which is a member of the nuclear receptor (NR) family (12). A growing number of NRs and their cross-talk have been implicated in regulating the development of NAFLD (13), suggesting that vitamin D may modify NAFLD by targeting VDR and other NRs. Further elucidating the mechanisms underlying the association between vitamin D and NAFLD may provide a better understanding of the pathogenesis of NAFLD and guide novel treatments for its prevention/management.

In recent years, an increasing number of studies have investigated NR cross-talk. NRs are transcriptional factors that contain a DNA-binding domain at the N terminus and a ligand-
binding domain at the C terminus (14). In addition to regulating gene expression in a DNA-binding manner, NRs can also affect physiological functions via cross-talk with one another (15, 16). For example, a direct interaction between VDR and hepatocyte nuclear factor 4α (HNF4α) in a VDR ligand–dependent manner has previously been demonstrated (17). HNF4α also belongs to the NR family; its structural features are similar to those of general NRs, but it has no specific ligands (18). A previous study has demonstrated that HNF4α expression is down-regulated in NAS and diabetes, and HNF4α controls the expression of genes related to triglyceride (TG) transport, including microsomal TG transfer protein (MTTP) and apolipoprotein B (ApoB) (19). Loss of HNF4α leads to NAFLD development through impairment of lipid transport (20).

In this study, we discovered that vitamin D supplementation alleviated NAFLD by activating VDR, whereas hepatic-specific knockout of VDR abolished the ameliorative effects of vitamin D on NAFLD. Mechanistically, we found that VDR directly regulated HNF4α and thereby further ameliorated NAFLD.

Results

Vitamin D supplementation attenuates high-fat diet–induced hepatic steatosis

To explore the association between vitamin D with NAFLD, serum 25-hydroxyvitamin D (25–VitD) levels were evaluated in NAFLD patients and in high-fat diet (HFD)-fed mice. As shown in Fig. 1A, serum 25-VitD levels were decreased in NAFLD patients and in HFD-fed mice compared with those in healthy controls and standard chow diet (SCD)-fed mice, respectively.

To explore whether vitamin D supplementation is beneficial for NAFLD, 2000 IU of vitamin D per 4057 kcal were added to SCD-fed or HFD-fed mice for 8 weeks. As expected, HFD-fed mice had significantly higher body weights than those of SCD-fed mice after 8 weeks of feeding (Fig. 1B). Vitamin D supplementation significantly elevated serum 25-VitD levels (Fig. 1A) and reduced body weights in HFD-fed mice (Fig. 1B), although both groups of mice consumed comparable amounts of food during 8 weeks of feeding (Fig. S1A,B). Vitamin D supplementation also significantly reduced intrahepatic TG contents in HFD-fed mice (Fig. 1C). The ameliorated HFD-induced hepatic steatosis by vitamin D supplementation was further confirmed by hematoxylin and eosin (H&E) and Oil Red O staining (Fig. 1D).

Moreover, we found that vitamin D-treated HFD-fed mice showed lower hepatic protein expression of fatty acid synthesis–related genes (SREBP1c, FAS, ACC, andSCD1) but higher hepatic expression levels of fatty acid β-oxidation–related proteins (CPT1α and PPARα) compared with these parameters in HFD-fed mice without vitamin D supplementation (Fig. 1F). Ketone bodies are the product of fatty acid β-oxidation; we found that vitamin D supplementation increased total ketone body levels (the sum of acetoacetate and β-hydroxybutyrate) in the serum of HFD-fed mice (Fig. 1F), indicating that fatty acid β-oxidation was activated via vitamin D treatment. The anti-steatotic effect of vitamin D was further confirmed by an in vitro experiment showing that vitamin D treatment significantly ameliorated palmitic acid (PA)-induced lipid accumulation in both QSG-7701 and L02 human hepatocyte cell lines (Fig. S2, A–C).

Vitamin D supplementation alleviates HFD-induced insulin resistance

We next evaluated the effect of vitamin D supplementation on insulin resistance, a common manifestation of NAFLD. We found that vitamin D supplementation significantly attenuated HFD-induced hyperglycemia and hyperinsulinemia in mice (Fig. 2, A–C). Vitamin D supplementation also significantly improved HFD-induced glucose and insulin intolerance, as determined by the glucose tolerance test (GTT) and insulin tolerance test (ITT) (Fig. 2, D and E). To examine the molecular mechanisms by which vitamin D improves insulin intolerance, we examined the activation of phosphatidylinositol 3-kinases (PI3K) and protein kinase B (AKT)–both of which are essential factors that mediate insulin signaling (21)–as well as the expression of forkhead box protein O1 (FOXO1), the expression of which is known to be attenuated by insulin through the activation of the PI3K/AKT–signaling pathway (22). As shown in Fig. 2, F, vitamin D supplementation significantly up-regulated hepatic expression of PI3K and phosphorylation of AKT but down-regulated hepatic expression of FOXO1 in insulin-treated HFD-fed mice, compared with these parameters in insulin-treated HFD-fed mice without vitamin D supplementation.

Phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, two key gluconeogenic enzyme genes under the transcriptional control of FOXO1 (23), were also down-regulated following vitamin D supplementation (Fig. 2F). Finally, in vitro results recapitulated our in vivo findings, such that incubation with vitamin D slightly enhanced PA-induced PI3K and AKT activation in QSG-7701 cells (Fig. S3A).

VDR expression is up-regulated in steatotic livers

The biological functions of vitamin D are mediated via VDR binding/signaling, and hepatic VDR expression has been reported to be up-regulated in NAFLD mice and in NAFLD patients (24). Therefore, we investigated the involvement of VDR in hepatic steatosis in both diet-induced and genetically obese mouse models. As illustrated in Fig. 3A, HFD-fed mice showed significantly higher hepatic nuclear expression of VDR than did SCD-fed mice. Similar up-regulation of VDR was observed in methionine/choline-deficient diet (MCD)-fed mice (Fig. 3B) and in genetically obese (ob/ob) mice (Fig. 3C). In vitro experiments showed that nuclear expression of VDR was significantly up-regulated in primary mouse hepatocytes or in the human hepatocyte QSG-7701 cell line after palmitic acid stimulation (Fig. 3, D and E).

Hepatic-specific VDR deletion exacerbates HFD-induced steatosis and insulin resistance

A previous study reported that global VDR knockout mice had decreased HFD-induced fatty livers compared with those in wildtype (WT) mice provided with a HFD (24). However, global VDR knockout may lead to systemic metabolic abnormalities, such as hypocalcemia, impaired bone formation, growth retardation, a lean phenotype, and resistance to diet-induced obesity, which may be attributed to reduced liver ste-
**Figure 1. Vitamin D supplementation attenuates HFD-induced hepatic steatosis in mice.** Mice were fed with a SCD or HFD for 8 weeks with or without adding 2000 IU of vitamin D per 4057 kcal (n = 5–7 mice/group). A: Left, serum 25-VitD levels of nonsteatosis controls (NC) (n = 798) and NAFLD patients (n = 1596); *, p < 0.05 versus the NC group (p values calculated by Student’s t test). Right, serum 25-VitD levels from mice. B, body weights of mice. C, liver TG contents in the indicated groups. D, H&E and Oil Red O staining of liver sections of mice (100-fold magnification and scale bar = 200 μm). E, representative Western blot analyses of the protein levels of genes related to lipid synthesis (SREBP-1, FAS, SCD1, and ACC) and β-oxidation (CPT-1α and PPARα) in mouse livers. F, serum ketone body levels from mice: left, acetoacetate; middle, β-hydroxybutyrate; and right, total ketone bodies. *, p < 0.05, compared with the SCD group; #, p < 0.05, compared with the HFD group (p values calculated by one-way ANOVAs). SCD + VD indicates SCD with added 2000 IU of vitamin D (VD) per 4057 kcal; HFD + VD indicates HFD with added 2000 IU of vitamin D per 4057 kcal.
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Figure 2. Vitamin D supplementation improves HFD-induced insulin sensitivity in HFD-fed mice. Mice were fed with SCD or HFD for 8 weeks with or without adding 2000 IU of vitamin D per 4057 kcal (n = 5–7 mice/group). A, fasting blood glucose levels in mice. B, fasting serum insulin levels in mice. C, HOMA-IR indices in the indicated groups. D and E, GTT (D) and ITT (E) with the corresponding areas under the curve (AUCs) in mice. F: left, representative Western blot analyses; right, quantification of hepatic protein levels of PI3K, as well as the phosphorylation levels of AKT, FOXO1, PCK2, and G6P of 8-week-old HFD-fed mice with or without vitamin D supplementation and insulin administration (n = 3 mice/group without insulin injection; n = 3 mice/group with insulin injection). *, p < 0.05, compared with the SCD group; #, p < 0.05, compared with the HFD group (p values calculated by one-way ANOVAs). SCD + VD indicates SCD with added 2000 IU of vitamin D per 4057 kcal; HFD + VD indicates HFD with added 2000 IU of vitamin D per 4057 kcal.
Figure 3. VDR expression is up-regulated in fatty livers. A: left, representative Western blot analyses, and right, quantification of livers from mice fed with an SCD or HFD for 8 weeks (n = 6 mice/group). B: left, representative Western blot analyses, and right, quantification of livers from mice fed with an SCD or MCD for 5 weeks (n = 5–6 mice/group). C: left, representative Western blot analyses, and right, quantification of livers in ob/ob mice and lean controls (n = 4 mice/group). D: left, representative Western blot analyses, and right, quantification of VDR expression in primary cultured hepatocytes that were treated with BSA or PA (0.3 mmol/liter) (n = 3/group). E: left, representative Western blot analyses, and right, quantification of VDR expression in QSG-7701 cells that were treated with BSA or PA (0.3 mmol/liter) (n = 3/group). *, p < 0.05, compared with the corresponding control group (p values calculated by Student’s t tests).
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The pathogenesis of NAFLD, we generated hepatic-specific VDR knockout mice (HKO) (Fig. 54, A–C). VDR<sub>Flox/flox</sub> mice (Flox) served as the littermate WT control group. As illustrated in Fig. 4A, HKO mice showed higher body weight gain than did Flox mice, although both groups of mice consumed comparable amounts of food during 8 weeks of HFD feeding (Fig. S1B). HKO mice also had higher intrahepatic TG contents than those of Flox mice after HFD feeding (Fig. 4B), which was further confirmed by H&E and Oil Red O staining (Fig. 4C). Exacerbated steatosis in HKO mice versus Flox control mice was also observed in another model of NAFLD induced by feeding with an MCD diet (Fig. S5, A and B), indicating a protective role of VDR in NAFLD.

Moreover, compared with those of Flox mice, HKO mice had higher levels of hepatic protein expression of lipogenic genes (SREBP1c, FAS, ACC, and SCD1) but lower levels of hepatic fatty acid β-oxidation proteins (CPT1α and PPARα) after HFD feeding (Fig. 4D). HKO mice also had decreased ketone body levels (the sum of acetoacetate and β-hydroxybutyrate) in the serum compared with those in Flox mice after HFD feeding (Fig. 4E). HKO mice showed up-regulated hepatic SREBP1c and ACC but down-regulated hepatic CPT1α after MCD feeding compared with those of WT mice (Fig. S5C). Furthermore, knockdown of VDR via transfection with siRNA significantly aggravated PA-induced lipid accumulation in QSG-7701 and L02 cell lines (Fig. S6, A–C).

In addition to aggravated hepatic steatosis, HKO mice also showed higher fasting serum glucose, insulin, and homeostatic model assessment of insulin resistance (HOMA-IR) values compared with those of Flox mice after 8 weeks of HFD feeding (Fig. 4, F–H), indicating that hepatic-specific VDR deletion promoted insulin resistance. Furthermore, GTT and ITT analyses showed that HKO mice developed greater glucose and insulin intolerance than did Flox mice after 8 weeks of HFD feeding (Fig. 4, I and J). Insulin-induced PI3K/AKT activation was lower and the levels of insulin-induced gluconeogenesis-associated genes were higher in the livers of HFD-fed HKO mice, compared with those in Flox controls (Fig. 4K). Knockdown of VDR via siRNA also attenuated insulin-induced PI3K/AKT activation in QSG-7701 cells (Fig. S6D).

Hepatic VDR is required for the inhibitory effects of vitamin D on the development of NAFLD

To explore whether the benefit of vitamin D in inhibiting NAFLD is mediated by hepatic VDR, we supplemented VDR–HKO mice with 2000 IU of vitamin D per 4057 kcal during their HFD regimen. Vitamin D supplementation failed to reduce the HFD-induced body weight gain in VDR–HKO mice after 8 weeks of HFD feeding (Fig. 5A). VDR–HKO mice showed similar intrahepatic TG contents with or without vitamin D supplementation (Fig. 5B). H&E and Oil Red O staining revealed that hepatic-specific VDR knockout diminished vitamin D amelioration of hepatic lipid accumulation in HFD-fed mice (Fig. 5C). Moreover, VDR–HKO mice supplemented with vitamin D showed comparable hepatic levels of proteins mediating fatty acid synthesis (SREBP1c, FAS, ACC, and SCD1) and fatty acid β-oxidation (CPT1α and PPARα) compared with those of HFD-fed VDR–HKO mice without vitamin D supplementation (Fig. 5D). Moreover, vitamin D supplementation did not affect fasting serum glucose, insulin, or HOMA-IR values in HFD-fed HKO mice (Fig. 5, E–G). GTT and ITT analyses showed that HKO mice had similar HFD-induced glucose and insulin intolerance with or without vitamin D supplementation (Fig. 5, H and I). Vitamin D failed to affect insulin-induced PI3K/AKT activation or up-regulation of gluconeogenesis-related genes in the livers of HFD-fed HKO mice (Fig. 5J). These results suggest that hepatic-specific VDR deletion eliminates the beneficial effects of vitamin D in HFD-induced metabolic syndromes.

Activation of VDR up-regulates hepatic HNF4α expression

The downstream regulatory mechanisms of VDR in NAFLD remain unclear. VDR is an NR that interacts with HNF4α, a liver-specific transcriptional factor that plays an important role in the pathogenesis of NAFLD (17, 19, 20); thus, we hypothesized that activation of VDR ameliorates NAFLD via the regulation of HNF4α. As illustrated in Fig. 6, A and B, vitamin D supplementation significantly up-regulated hepatic HNF4α expression at the protein and mRNA levels in HFD-fed mice. Vitamin D supplementation also up-regulated hepatic MTTP and ApoB—which are two downstream molecules of HNF4α that export TGs from the liver into the circulation—in HFD-fed mice. In addition, vitamin D elevated serum very low-density lipoprotein (VLDL) levels in HFD-fed mice (Fig. 6C). In contrast, hepatic-specific deletion of VDR significantly down-regulated hepatic expressions of HNF4α, MTTP, and ApoB, as well as decreased serum VLDL levels in HFD-fed mice (Fig. 6, A–C). We found similar results in in vitro studies (Fig. S7).

Immunofluorescent results showed that VDR and HNF4α co-localized in the nuclei of hepatocytes (Fig. 6D). Co-immunoprecipitation (Co-IP) experiments showed that HNF4α directly interacted with VDR in QSG-7701 cells overexpressing VDR and HNF4α (Fig. 6E). Because further investigation of the binding sites of these two proteins would be of great value for future functional studies, we next explored the domains required for VDR–HNF4α interaction. To this aim, a series of HNF4α-deletion and VDR-deletion mutants were structured, and domain-mapping experiments were performed (Fig. 6, F and G). Co-IP experiments showed that amino acids (aa) 1–122 of VDR and aa 142–377 of HNF4α were required for the VDR–HNF4α interaction (Fig. 6, F and G). The 1–122-aa domain of VDR is the DNA-binding domain, and the 142–377-aa domain of HNF4α is the ligand-binding domain (28, 29), suggesting that VDR may affect the ability of HNF4α to bind to its endogenous ligands (i.e. fatty acids) via its DNA-binding ability. In addition, the interaction between VDR and HNF4α in QSG-7701 cells was enhanced by vitamin D treatment (Fig. 6H). Furthermore, luciferase assays showed that vitamin D enhanced the transcription of HNF4α to induce the expression of MTTP and ApoB (Fig. 6I).

Overexpression of HNF4α in the liver ameliorates steatosis and insulin resistance in HFD-fed VDR–HKO mice

To further explore whether HNF4α mediated the regulatory effects of VDR in NAFLD, a rescue experiment was performed by tail-vein injection of an HNF4α-overexpressing AAV into
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**A**

B

D

C

E

F

G

H

I

J

K

**D**

| Protein      | Flox HFD | HKO HFD | MW (kD) |
|--------------|----------|---------|---------|
| SREBP1       |          |         | 56      |
| FAS          |          |         | 170     |
| ACC          |          |         | 170     |
| SCD1         |          |         | 40      |
| CPT1α        |          |         | 70      |
| PPARα        |          |         | 55      |
| GAPDH        |          |         | 35      |

**E**

**F**

**G**

**H**

**I**

**J**

**K**

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VDR–HKO mice. Injection of HNF4α-overexpressing AAV significantly up-regulated hepatic expressions of HNF4α and its downstream molecules, MTTP and ApoB, in mice (Fig. 7A). Overexpression of HNF4α significantly restored fasting serum glucose, insulin, and HOMA-IR values (Fig. 7, B–D) and reduced intrahepatic TG contents in HFD-fed VDR–HKO mice (Fig. 7E). Serum VLDL levels were also restored via overexpression of HNF4α (Fig. 7F). The alleviation of hepatic steatosis in HNF4α-overexpressed VDR–HKO mice was confirmed by H&E and Oil Red O staining (Fig. 7G). These results suggest that HNF4α mediates the regulatory effects of VDR on NAFLD.

Discussion

In this study, we investigated the effects and mechanisms of vitamin D on the pathogenesis of NAFLD. We found that vitamin D supplementation played a protective role in HFD-induced NAFLD via activation of VDR. Hepatic-specific knockout of VDR aggravated HFD- or MCD-induced liver steatosis. In addition, HFD or MCD treatment led to a compensatory increase in VDR expression, which interacted with HNF4α to protect the liver from damage caused by lipid deposition.

NAFLD is a widespread disease that affects 25.24% of the global population (30). The prevalence of NAFLD in China has currently reached 29.2% and has continued to increase year after year (31). It has been widely accepted that vitamin D deficiency promotes insulin resistance and NAFLD (9, 32, 33). Based on clinical findings revealing an association between vitamin D levels and NAFLD, vitamin D has been suggested as a therapeutic option for NASH (34). However, whether vitamin D supplementation improves NAFLD has remained controversial in clinical trials (35–37). Here, we explored the role of vitamin D in the pathogenesis of NAFLD, and we found that vitamin D supplementation decreased HFD-induced lipid accumulation and insulin resistance in mice, suggesting a potential therapeutic effect of vitamin D for the treatment of NAFLD.

Vitamin D exerts its regulatory role primarily through VDR binding and signaling (38). A previous study has suggested that VDR expression is negatively associated with the severity of liver histology in NASH patients (39). More importantly, expression of liver VDR is induced in mouse models of NAFLD and NAFLD patients (24). Collectively, these findings strongly indicate that VDR plays a critical role in NAFLD. However, a previous study suggested that global VDR knockout decreases HFD-associated liver steatosis in mice (24), which contradicts the results of this study. However, global VDR knockout can lead to systemic change because VDR is widely expressed in various tissues, and ~3% of the human genome is regulated by VDR (40); hence, HFD-induced hepatic phenotypes induced by global VDR knockout may be caused by these aforementioned systemic changes. Furthermore, it has been reported that VDR null mice exhibit a lean phenotype and growth retardation, and global VDR knockout mice are resistant to diet-induced obesity (25, 27). Therefore, whole-body knockout of VDR may not represent the best model for exploring the effects of hepatic VDR on NAFLD. In this study, we used hepatic-specific VDR knockout mice—which showed no differences in growth or development characteristics compared with those of WT mice—to explore the hepatocyte-specific effects of VDR deletion on NAFLD. The use of hepatic-specific VDR knockout mice allowed us to decipher the specific roles of hepatic VDR in the pathogenesis of NAFLD. Our data showed that hepatic-specific VDR knockout mice exacerbated HFD-induced and MCD-induced liver steatosis, suggesting that VDR has an important role in NAFLD. We also found that hepatic-specific VDR deletion diminished the rescuing effect of vitamin D on hepatic steatosis and insulin resistance in mice, indicating that the regulatory role of vitamin D primarily depends on the activation of VDR.

It is well-established that HNF4α is a central transcription factor that regulates expression levels of genes involved in the progression of NAFLD in the liver (19, 41, 42). Previous studies have reported that loss of HNF4α leads to liver steatosis by reducing hepatic expression of MTTP and ApoB, both of which are essential to VLDL secretion (20, 43, 44). HNF4α, which belongs to the NR family, has been demonstrated to interact with VDR (17). In this study, we demonstrated that hepatic VDR deletion in mice led to decreased protein and mRNA levels of HNF4α, whereas vitamin D supplementation in WT mice restored these levels, as well as downstream levels of MTTP and ApoB (19). We also identified co-localization of VDR and HNF4α in the nucleus and confirmed their interaction as direct binding partners. Furthermore, it is noteworthy that we reported for the first time that the ligand-binding domain of HNF4α and the DNA-binding domain of VDR participated in this binding; the identification of this VDR–HNF4α interaction provides a foundation for future functional studies. We also demonstrated that vitamin D enhanced the binding of VDR and HNF4α and up-regulated the transcriptional activity of HNF4α. Moreover, exacerbation of HFD-induced hepatic steatosis and IR in VDR–HKO mice was diminished by overexpression of HNF4α via AAV injections. Therefore, we speculate that the regulation of VDR on the progression of NAFLD and its associated complications depends on HNF4α-mediated TG exporting. Hence, it may be worthwhile for future studies to investigate the correlation between the expression levels of VDR and HNF4α in the livers of NAFLD patients.

Figure 4. Hepatic-specific VDR knockout mice are more susceptible to HFD-induced steatosis. VDR–Flox control mice and VDR–HKO mice were fed with a HFD for 8 weeks (n = 3–6 mice/group). A, body weights of mice. B, liver TG contents in the indicated groups. C, H&E and Oil Red O staining of liver sections of the mice (100-fold magnification and scale bar = 200 μm). D, representative Western blot analyses of the protein levels of genes related to lipid synthesis (SREBP-1, FAS, SCD1, and ACC) and β-oxidation (CPT-1α and PPARα) in mouse livers. E, serum ketone body levels from mice: left, acetoacetate; middle, β-hydroxybutyrate; and right, total ketone bodies. F, fasting blood glucose levels in mice. G, fasting serum insulin levels in mice. H, HOMA-IR indices in the indicated groups. I, and J, GGT (I) and ITT (J) with the corresponding AUCs in mice. K, left, representative Western blot analyses, and right, quantification of protein levels of PPARγ and PPARα. L, Western blot analyses and quantification of protein levels of Pi3K, as well as the phosphorylation levels of AKT, FOXO1, PCK2, and G6P in the livers of 8-week HFD-fed VDR–Flox and VDR–HKO mice with or without insulin administration (n = 3 mice/group without insulin injection and n = 3 mice/group with insulin injection). *, p < 0.05, compared with the SCD group; #, p < 0.05, compared with the HFD group (p values calculated by one-way ANOVAs). Flox indicates the VDR–Flox group; HKO indicates the VDR–HKO group.
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**A**

![Body Weight (g)]

**B**

![Liver Weight (g)]

**C**

![H&E](HKO HFD) ![H&E](HKO HFD+VD)

![Oil Red](HKO HFD) ![Oil Red](HKO HFD+VD)

**D**

| Protein      | 55 kD | 170 kD | 40 kD | 70 kD | 55 kD | 35 kD |
|--------------|-------|--------|-------|-------|-------|-------|
| SREBP1       |       |        |       |       |       |       |
| FAS          |       |        |       |       |       |       |
| ACC          |       |        |       |       |       |       |
| SCD1         |       |        |       |       |       |       |
| CPT1α        |       |        |       |       |       |       |
| PPARα        |       |        |       |       |       |       |
| GAPDH        |       |        |       |       |       |       |

**E**

![Fasting Blood Glucose (mmol/L)]

**F**

![Fasting Serum Insulin (mIU/L)]

**G**

![LIPID](HKO HFD) ![LIPID](HKO HFD+VD)

**H**

![Blood Glucose (mmol/L)]

**I**

![Blood Glucose (mmol/L)]

**J**

![Protein/GAPDH (Fold change)]

- HKO HFD+VD
- HKO HFD
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Based on our present findings, further studies are needed to clarify the mechanisms of VDR regulation in NAFLD. This study cannot fully exclude the role of VDR itself in the progression of NAFLD, because ligand-independent regulations of VDR have been found in some studies (45, 46). Future studies testing hepatic VDR overexpression are needed to verify this distinction. Additionally, because we identified the interaction sites of VDR and HNF4α, further investigation should be performed to elucidate the precise mechanisms by which VDR regulates the HNF4α activity via protein interactions. Our data indicate that the DNA-binding domain of VDR participates in the interaction of VDR and HNF4α. As the most important part of VDR, the DNA-binding domain is sufficient for target gene discrimination and transcriptional regulation (47). Studies on the regulation of other transcriptional factors that modulate transcriptional activity of VDR have also been reported (48). It is possible that the interaction of VDR and HNF4α alleviates hepatic steatosis by affecting DNA-binding activity of VDR. For further confirmation, future studies should mutate the essential amino acids of the DNA-binding domain of VDR to decrease its ability to bind to VDR response elements of its target genes without affecting the interaction of VDR and HNF4α. In this study, we found that the effect of vitamin D on FOXO1 was only evident in the modeling groups and not in the control groups. A possible explanation is that PA stimulation in the modeling groups may have perturbed cellular homeostasis, and vitamin D treatment may facilitate the homeostasis. In contrast, cellular homeostasis in the control groups was already achieved under

Figure 6. VDR regulates HNF4α-mediated TG transportation via interaction with HNF4α. A: left, representative Western blot analyses of the protein levels of HNF4α, MTP, and ApoB in the livers of mice fed a HFD for 8 weeks with or without vitamin D added (n = 3 mice/group), and right, in the livers of the VDR–Flox and VDR–HKO mice after administration of a HFD for 8 weeks (n = 3 mice/group). B: representative quantitative real-time PCR analyses of the mRNA levels of HNF4α in the livers of the mice fed a HFD for 8 weeks with or without vitamin D added (n = 5–6 mice/group), and right, in the livers of VDR–Flox and VDR–HKO mice after administration of HFD for 8 weeks (n = 5–6 mice/group). C: serum VLDL levels from left, mice fed a HFD for 8 weeks with or without vitamin D added (n = 3–6 mice/group), and right, in the livers of the VDR–Flox and VDR–HKO mice after administration of HFD for 8 weeks (n = 3–6 mice/group). D: representative immunofluorescent images of DAPI, VDR, and HNF4α and the merge of all stainings in QSG-7701 cells (scale bar = 10 μm). E: Co-IP and Western blot analyses showing the interaction between VDR and HNF4α in QSG-7701 cells transfected with Flag-VDR or Flag-HNF4α. F and G: top, schematics of the HNF4α (F) and VDR (G) constructs. Bottom, interaction domains of HNF4α and VDR were explored using full-length and truncated HNF4α (F) and VDR (G) expression constructs based on co-immunoprecipitation assays in QSG-7701 cells. H: Co-IP and Western blot analyses showing the interaction between VDR and HNF4α in QSG-7701 cells transfected with or without Flag-VDR and 100 nm/liter vitamin D3, I, relative luciferase activity in L02 cells transfected with PGL3-basic vector or the HNF4α-promoter luciferase reporter plasmids for 48 h with or without 100 nm/liter vitamin D3 added (n = 3/group). * p < 0.05, compared with the SCD group; #, p < 0.05, compared with the HFD group or HNF4α promoter + NC group (p values calculated by one-way ANOVAs). SCD + VD indicates SCD with added 2000 IU of vitamin D per 4057 kcal; HFD + VD indicates HFD with added 2000 IU of vitamin D per 4057 kcal. Flox indicates the VDR–Flox group; HKO indicates the VDR–HKO group.
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**A**

|          | Flox ovNC | HKO ovNC | Flox ovHNF4α | HKO ovHNF4α |
|----------|-----------|-----------|--------------|-------------|
| HNF4α    | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) |
| MTTP     | ![Image](image5) | ![Image](image6) | ![Image](image7) | ![Image](image8) |
| ApoB     | ![Image](image9) | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| GAPDH    | ![Image](image13) | ![Image](image14) | ![Image](image15) | ![Image](image16) |

**B**

- Fasting Blood Glucose (mmol/L)
  - Flox HFD ovNC: ![Image](image17)
  - HKO HFD ovNC: ![Image](image18)
  - Flox HFD ovHNF4α: ![Image](image19)
  - HKO HFD ovHNF4α: ![Image](image20)

**C**

- Fasting Serum Insulin (ng/ml)
  - Flox HFD ovNC: ![Image](image21)
  - HKO HFD ovNC: ![Image](image22)
  - Flox HFD ovHNF4α: ![Image](image23)
  - HKO HFD ovHNF4α: ![Image](image24)

**D**

- HOMA-IR (mmol·min·ml⁻¹)
  - Flox HFD ovNC: ![Image](image25)
  - HKO HFD ovNC: ![Image](image26)
  - Flox HFD ovHNF4α: ![Image](image27)
  - HKO HFD ovHNF4α: ![Image](image28)

**E**

- Intracellular TG Contents (mg/g protein)
  - Flox HFD ovNC: ![Image](image29)
  - HKO HFD ovNC: ![Image](image30)
  - Flox HFD ovHNF4α: ![Image](image31)
  - HKO HFD ovHNF4α: ![Image](image32)

**F**

- Serum VLDL (ng/ml)
  - Flox HFD ovNC: ![Image](image33)
  - HKO HFD ovNC: ![Image](image34)
  - Flox HFD ovHNF4α: ![Image](image35)
  - HKO HFD ovHNF4α: ![Image](image36)

**G**

- H&E
  - Flox HFD ovNC: ![Image](image37)
  - HKO HFD ovNC: ![Image](image38)
  - Flox HFD ovHNF4α: ![Image](image39)
  - HKO HFD ovHNF4α: ![Image](image40)

- Oil Red O
  - Flox HFD ovNC: ![Image](image41)
  - HKO HFD ovNC: ![Image](image42)
  - Flox HFD ovHNF4α: ![Image](image43)
  - HKO HFD ovHNF4α: ![Image](image44)
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normal conditions, such that vitamin D supplementation may not have exhibited any further effect on such homeostasis. Future studies clarifying the different effects of vitamin D/VDR signaling in normal and NAFLD conditions may help to better elucidate the mechanisms and treatments for NAFLD. In this study, we also found an effect of VDR deletion in MCD models, which exhibit similar histological characteristics to those of human NASH (49). It has been widely accepted that vitamin D plays an important role in inflammatory processes (50). Consequently, further studies focusing on the role of vitamin D in inflammation are required. In this study, because vitamin D supplementation or VDR ablation had no effect on food intake, the observed decrease in body weight may be associated with increased energy expenditure. This hypothesis is partially supported by our present evidence that vitamin D supplementation and VDR ablation ameliorated hepatic steatosis and improved insulin resistance, the latter of which may consequently increase energy expenditure and inhibit body weight gain (51–53). Therefore, studies investigating the effect of vitamin D on energy expenditure are needed in the future. We found that vitamin D supplementation and VDR knockout did not change the serum nonesterified fatty acid (NEFA) concentration. The main source of serum NEFA is via lipolysis in adipocytes, and this process is regulated by several factors, such as the interplay of hormonal, neurological, and pharmacological stimuli (54). In addition, NEFA is metabolized by many metabolic pathways (55). Hence, the effects of vitamin D supplementation and VDR knockout on serum NEFA levels might be compensated by other factors that are related to NEFA metabolism; further studies are needed to clarify this issue.

In conclusion, we identified a novel mechanism by which vitamin D ameliorates NAFLD via the activation of hepatic VDR and its interaction with HNF4α (summarized in Fig. 8), providing a novel insight into improving treatment of NAFLD.

Experimental procedures

Patients

This study initially enrolled employees who were attending their annual health examinations (during the period between January 1, 2017, and June 31, 2019) at Zhejiang University Tai-zhou Hospital. A total of 2394 participants (1635 males and 759 females; between 16 and 86 years old) were included in the analysis. NAFLD was diagnosed by abdominal ultrasound by a hepatologist (Biocytogen). Venous blood samples were drawn after an overnight fasting for at least 8 h. A Sciex API 3200 mass spectrometer was used to detect serum 25-VitD levels. The study protocol was approved by the Ethics Committee of Zhejiang University Tai-zhou Hospital (approval number K20160603) and abided by the principles of the Declaration of Helsinki.

Mice and treatments

All mouse procedures were approved by the Animal Care and Use Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University. Male C57BL/6 mice (6–8 weeks old) were housed on a 12-h light/dark cycle in a temperature-controlled environment (23 ± 2°C) with ad libitum access to food and water. Mice were fed with a HFD (Research Diet, New Brunswick, NJ) for 8 weeks to establish an NAFLD model or an MCD (MPBio, Santa Ana, CA) for 5 weeks to establish a NASH model. For vitamin D administration, 2000 IU of vitamin D per 4057 kcal was added into the diet after weaning and maintaining until the end of the 8-week HFD. A vitamin D-supplemented diet and the corresponding controlled diet were purchased from Shuyishuo Bio (Changzhou, China). At the end of the mouse experiments, mice were fasted overnight for 16 h prior to being sacrificed. After an overnight fasting for 16 h, the mice were injected intraperitoneally with insulin (1 unit/kg) at 10 min prior to being sacrificed in order to investigate insulin signaling. VDRfl°x/+ mice from a C57BL/6J background were generated utilizing the CRISPR/CAS9 system by Biocytogen Biological Technology Co., Ltd. (Beijing, China). The third exon was flanked by loxP sites, and thus two single guide RNAs (sgRNA1 and sgRNA2) targeting VDR introns 2–3 and 3–4 were designed. The schematic diagram is shown in Fig. S3A. The PCR primers used for identification are listed in Table S1. Hepatocyte-specific VDR knockout mice (VDR–HKO) were generated by crossing VDRfl°x/+ mice with albumin-Cre mice (Biocytogen). The ob/ob mice were purchased from the Model Animal Research Center of Nanjing University and were fed normal chow. To overexpress HNF4α into the liver of

Figure 7. Overexpression of HNF4α ameliorates HFD-induced NAFLD in VDR–HKO mice. Eight-week-old HFD-fed VDR–Flox and VDR–HKO mice were injected with HNF4α overexpressing AAV or ovNC AAV through the tail vein (n = 6–10 mice/group). A, representative Western blot analyses of the protein levels of HNF4α, MTTP, and ApoB in the livers of mice. B, fasting blood glucose levels in mice. C, fasting serum insulin levels in mice. D, HOMA-IR indices in the indicated groups. E, liver TG contents in the indicated groups. F, serum VLDL levels in mice. G, H&E and Oil Red O staining of liver sections from mice (100-fold magnification and scale bar = 200 μm). *, p < 0.05, compared with the Flox HFD ovNC group; 8, p < 0.05, compared with the HKO HFD ovNC group (p values calculated by one-way ANOVAs). Flox HFD ovNC indicates the HFD-fed VDR–Flox group with ovNC AAV injected; HKO HFD ovNC indicates the HFD-fed VDR–HKO group with ovNC AAV injected; Flox HFD ovHNF4α indicates the HFD-fed VDR–Flox group with HNF4α overexpressing AAV injected; HKO HFD ovHNF4α indicates the HFD-fed VDR–HKO group with HNF4α overexpressing AAV injected.

Figure 8. Summary of pathological and underlying regulatory mechanisms of vitamin D and VDR in NAFLD.
VDR–HKO mice, VDR–HKO mice were injected with AAVs through the tail vein at a dose of $4 \times 10^{11}$ genome copies per mouse.

**Production of AAVs**

For viral production, 293T cells were maintained in 150-mm plates. For each transfection, 15 μg of AAV plasmid carrying the construct of interest, 15 μg of AAV helper plasmid, and 15 μg of pAAVDJ serotype-packing plasmid were added to 4.5 ml of serum-free DMEM. Then, 90 μl of Turbofect (Invitrogen) was added to the mixture and incubated at room temperature for 15–20 min. After incubation, the mixture was added to media and was mixed gently yet thoroughly. Cells were harvested between 48 and 72 h post-transfection. Subsequently, the recombinant AAV was purified via a ViraBind™ AAV purification kit (Cell Biolabs, San Diego, CA) according to the manufacturer’s protocol. Purified AAV was quantified via the QuickTiter™ AAV quantitation kit (Cell Biolabs).

**Measurement of biochemical indices and metabolic analysis**

Blood glucose was measured with a glucometer (Onetouch, LifeScan Inc., Milpitas, CA). Serum insulin levels were measured via ELISAs (Millipore, Billerica, MA). Hepatic TG contents were assayed by corresponding kits (Applygen, Beijing, China). Serum ketone bodies were assayed by corresponding kits (Applygen, Beijing, China). Serum VLDL levels were measured by ELISA kits (Sigma). Serum insulin levels were measured by ELISA kits (Applygen) was used to determine the concentrations of proteins. Then the extracted protein samples were separated with SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). After blocking in 5% nonfat milk, specific proteins were detected with corresponding antibodies. Details of antibodies were as follows: antibodies from Abcam included VDR (ab3508), FAS (ab128856), SREBP1 (ab3259), Gapdh (ab86759), ApoB (ab20737), and His (ab9108); antibodies from Cell Signaling Technology included ACC (4249), P-AKT (4060), AKT (4691), FOXO1 (2880), GAPDH (2118), and LaminB1 (12586); and an antibody from Sigma included FLAG (F7425).

**Quantitative real-time PCR**

RNAs were isolated by the TRIzol method and were reverse-transcribed into cDNAs. Quantitative real-time PCR was conducted using SYBR Green (Takara Shuzo, Otsu, Japan) with a Bio-Rad CFX384 system. The primers for quantitative real-time PCR were listed in Table S2. The relative expression levels of target RNAs were normalized using Gapdh as an internal control.
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Luciferase assay

L02 cells were transfected with firefly luciferase reporter plasmids (pGL3-basic vector) driven by the HNF4α promoter (HanyinBio, Shanghai, China). Renilla luciferase-expressing plasmids were co-transfected and used for normalization. Both firefly and Renilla luciferase activities were measured by using the Dual-Luciferase assay kit (Promega, Madison, WI) for 48 h after plasmid transfection. Firefly luciferase units were normalized against Renilla luciferase controls. Results are presented as firefly/Renilla luciferase activities.

Statistical analysis

Values are reported as the mean ± S.D. Statistical differences were determined via unpaired, two-tailed Student’s t tests or one-way analyses of variance (ANOVA) with Tukey’s correction when appropriate. All tests were performed in Prism 8 (GraphPad). p < 0.05 was considered statistically significant.

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