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Alterations in gene expression in vitamin D-deficiency: Down-regulation of liver Cyp7a1 and renal Oat3 in mice

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Abstract

The vitamin D-deficient model, established in the C57BL/6 mouse after 8 weeks of feeding vitamin D-deficient diets in the absence or presence of added calcium, was found associated with elevated levels of plasma parathyroid hormone (PTH) and plasma and liver cholesterol, and a reduction in cholesterol 7α-hydroxylase (Cyp7a1, rate-limiting enzyme for cholesterol metabolism) and renal Oat3 mRNA/protein expression levels. However, there was no change in plasma calcium and phosphate levels. Appraisal of the liver revealed an up-regulation of mRNA expressions of the small heterodimer partner (Shp) and attenuation of Cyp7a1, which contributed to hypercholesterolemia in vitamin D-deficiency. When vitamin D-sufficient or D-deficient mice were further rendered hypercholesterolemic with 3 weeks of feeding the respective, high fat/high cholesterol (HF/HC) diets, treatment with 1α,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$], active vitamin D receptor (VDR) ligand, or vitamin D (cholecalciferol) to HF/HC vitamin D-deficient mice lowered the cholesterol back to baseline levels. Cholecalciferol treatment partially restored renal Oat3 mRNA/protein expression back to that of vitamin D-sufficient mice. When the protein expression of protein kinase C (PKC), a known, negative regulator of Oat3, was examined in murine kidney, no difference in PKC expression was observed for any of the diets with/without 1,25(OH)$_2$D$_3$/cholecalciferol treatment, inferring that VDR regulation of renal Oat3 did not involve PKC in mice. As expected, plasma calcium levels were not elevated by cholecalciferol treatment of vitamin D-deficient mice, while 1,25(OH)$_2$D$_3$ treatment led to hypercalcemia. In conclusion, vitamin D-deficiency resulted in down-regulation of liver Cyp7a1 and renal Oat3, conditions that are alleviated upon replenishment of cholecalciferol.

KEYWORDS

cholecalciferol and calcitriol, liver Cyp7a1, renal Oat3, vitamin D-deficiency, vitamin D receptor

1 | INTRODUCTION

Vitamin D-deficiency is the consequence of inadequate exposure to sunlight, insufficient dietary intake (Mithal et al., 2009), or genetic variations of the enzymes for synthesis (liver CYP27A1 and CYP2R1, then renal 1α-hydroxylase/CYP27B1) or degradation (CYP24A1) (Berry & Hypponen, 2011). Clinically, the vitamin D status is determined by circulating levels of 25-hydroxyvitamin D_3 [25(OH)D_3] and not by the natural, active ligand metabolite of the vitamin D receptor (VDR), calcitriol or 1α,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$], which exists at extremely low levels (Hollis, 1996). The increasing prevalence

of vitamin D-deficiency poses a major health concern in humans and there is growing evidence that suggests nutritional deficit as a contributing factor to the pathogenesis of cancer, metabolic syndrome and cardiovascular diseases (Dobnig et al., 2008; Ghanei et al., 2015; Gorham et al., 2005).

Typically, the activated-VDR tightly regulates endogenous 1,25(OH)$_2$D$_3$ levels by inhibiting the expression of the synthetic enzyme rodent Cyp27b1/human CYP27B1 (Murayama et al., 1999), while increasing the expression of the degradation enzyme, rodent Cyp24a1/human CYP24A1 (Meyer, Zella, Nerenz, & Pike, 2007), thereby controlling the levels of 1,25(OH)$_2$D$_3$. Binding of 1,25(OH)$_2$D$_3$ to the VDR results in an intricate interplay among the kidney, intestine, liver and the parathyroid gland for maintaining

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steady levels of plasma calcium, phosphate, parathyroid hormone (PTH) and $1,25(OH)_2D_3$ (Caniggia, Lore, di Cairano, & Nuti, 1987; Henry & Norman, 1984). In the intestine and kidney, $1,25(OH)_2D_3$ regulates plasma calcium and phosphate levels via the transient receptor potential cation calcium channels (TRPV5/6) and sodium-coupled phosphate cotransporter (NaP_i), respectively, upon binding to the respective vitamin D response elements (VDREs) (den Dekker, Hoenderop, Nilius, & Bindels, 2003; Dusso, Brown, & Slatopolsky, 2005; Hildmann, Storelli, Danisi, & Murer, 1982; Hoenderop et al., 2001; Jones, Strugnell, & DeLuca, 1998; Meyer, Watanuki, Kim, Shevde, & Pike, 2006; Takeda, Taketani, Sawada, Sato, & Yamamoto, 2004; Tatsumi et al., 1998). In addition, $1,25(OH)_2D_3$-liganded VDR may also exert translational regulation (Hoenderop et al., 2001). The secretion of PTH is regulated by serum ionized calcium acting via the parathyroid calcium-sensing receptor (CaSR) and the $1,25(OH)_2D_3$-liganded VDR (Brown et al., 1996). Decreased plasma calcium levels that are detected by the calcium sensing receptor (CaSR) that trigger the release of PTH into the bloodstream, resulting in induction of CYP27B1 (Healy, Vanhooke, Prahl, & DeLuca, 2005), thus increasing plasma $1,25(OH)_2D_3$ levels (Beckman et al., 1995; Jones et al., 1998). These intricate feedback controls act to maintain the homeostasis of calcium, PTH and $1,25(OH)_2D_3$.

Recently, the VDR was found to play a key role in cholesterol regulation. Cholesterol metabolism is rate-limited by cholesterol 7α-hydroxylase (CYP7A1) (Chiang, 2004), a liver enzyme that is positively up-regulated with VDR activation due to the presence of VDREs in mouse/human SHP promoters, resulting in de-repression of Cyp7a1/CYP7A1 (Chow et al., 2014). This new pathway adds to the well known negative regulatory roles of the farnesoid X receptor (FXR)-small heterodimer partner (SHP) cascade (Goodwin et al., 2000) and the intestinal FXR-fibroblast growth factor 15/19 (rodent Fgf15/human FGF19) pathway on Cyp7a1/CYP7A1 (Inagaki et al., 2005). The latter feedback pathways are regulated by bile acids, products of cholesterol metabolism and ligands of FXR (Makishima et al., 1999).

VDR activation could further bring changes of gene expressions towards drug absorption and disposition via direct or indirect mechanisms. Direct regulation of gene expressions by VDR occurs transcriptionally via dimerization to RXR, retinoid X receptor heterodimer, then as transcription factors to bind to VDREs of Cyp3a/CYP3A4 (Thompson et al., 2002; Thummel et al., 2001), MDR1 (multidrug resistance protein 1) (Saeki, Kurose, Tohkin, & Hasegawa, 2008), OATP1A2 (organic anion transporter polypeptide 1A2) (Eloranta, Hiller, Juttner, & Kullak-Ublick, 2012), and the proton-coupled folate transporter (Eloranta et al., 2009). Activated VDR was shown to increase the protein stability of the human multidrug resistance-associated protein 4 (MRP4) post-transcriptionally, thereby increasing transporter activity (Fan et al., 2009). In addition, VDR could trigger changes in mRNA or protein expressions of non-VDR target genes via indirect effects or cross-talk with other nuclear receptors such as FXR (Honjo, Sasaki, Kobayashi, Misawa, & Nakamura, 2006), hepatic nuclear factor 1α (HNF-1α) and HNF-4α (Chow, Sun, Khan, Groothuis, & Pang, 2010). For example, $1,25(OH)_2D_3$ treatment could directly target protein kinase C (PKC) to down-regulate the renal transporters, namely, the organic anion transporters 1 and 3 (Oat1 and Oat3) and the oligopeptide transporter 1 (PepT1) in rodents (Chow et al., 2010; Kim

et al., 2014; Muller et al., 1996; Takeda, Sekine, & Endou, 2000). Alteration of these genes by VDR activation would elicit functional changes and affect the pharmacokinetics of drugs *in vivo*: up-regulation of Mdr1 by $1,25(OH)_2D_3$ treatment led to increased renal clearance of digoxin in mice (Chow, Durk, Cummins, & Pang, 2011), while suppression of Oat1/Oat3 expressions by $1,25(OH)_2D_3$ treatment reduced the renal entry and therefore, the renal clearances of cefdinir and cefadroxil in rats (Kim et al., 2014). Although extensive studies have concentrated on VDR activation of gene expression, the influence of vitamin D-deficiency on gene expression, however, has not been systemically studied.

In this communication, a vitamin D-deficient mouse model was established to investigate the effects of vitamin D-deficiency on plasma calcium, PTH and $1,25(OH)_2D_3$ levels and bile acid pool sizes, as well as VDR-targeted and non-targeted gene expression, recognizing that bile acids may also affect FXR-activation of intestinal and liver genes. We wished to test the hypothesis that VDR-target genes would be reduced in vitamin D-deficiency. It was observed that the established vitamin D-deficient mouse model was devoid of changes in plasma calcium and phosphate levels, but plasma and liver cholesterol levels and Shp expression were elevated, accompanied by decreased expression of liver Cyp7a1. Moreover, vitamin D-deficiency resulted in a significant down-regulation of renal Oat3 expression in mice. $1,25(OH)_2D_3$ or cholecalciferol treatment in vitamin D-deficient mice reversed these changes and restored cholesterol and renal Oat3 expression back to normal levels.

2 | MATERIALS AND METHODS

2.1 | Materials

Cholecalciferol and $1,25(OH)_2D_3$ powders were purchased from Sigma-Aldrich (Mississauga, ON). Mouse Cyp7a1 (N-17), Oat3 (P-13), PepT1 (H-235) and PKC (MC5) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX), whereas antibodies for mouse Oat1 (cat. no. ab135924), Trpv5 (EPR8875), Trpv6 (cat. no. ab63094), Vdr (9A7), glyceraldehyde-3-phosphate dehydrogenase (Gapdh) (6C5) and human CYP7A1 (C-terminus) were purchased from Abcam (Cambridge, MA); donkey anti-goat (Jackson Immunoresearch Laboratories, West Grove, PA), goat anti-rat (Santa Cruz Biotechnology), goat anti-mouse, and goat anti-rabbit (Bio-Rad, Mississauga, ON) antibodies were also procured. The anti-Mrp3 antibody was a kind gift from Dr. Yuichi Sugiyama (RIKEN Innovation Center, Japan). All other reagents were obtained from Sigma-Aldrich or Fisher Scientific (Mississauga, ON).

2.2 | Animal studies

2.2.1 | Diets

The compositions of the different diets (Harlan Laboratories, Madison, WI) are summarized in Supplemental Table 1. Changes in the plasma concentrations of $25(OH)D_3$, plasma and liver $1,25(OH)_2D_3$ and cholesterol, and expression levels of VDR-related genes were examined among mice fed the different diets: the vitamin D-sufficient [2200 IU/kg vitamin D, 0.47% Ca^{2+}, and 0.3% phosphorus (P^{3+})] diet that normally contained 10% fat was known as the 'normal diet' that was used for the control vitamin D-sufficient group. There were two

vitamin D-deficient diets; one with 0.47% and one with added calcium (2.5% Ca^{2+}). These two 'normal' diets of vitamin D-deficiency (0 IU/kg vitamin D): one without additional Ca^{2+} (0.47%) and P^{3+} (0.3%) and one with calcium and phosphate (2.5% Ca^{2+} and 1.5% P^{3+}) supplementation, were used. The rationale for testing different dietary Ca^{2+} levels in the diets was to avoid the scenario that low Ca^{2+} levels would elevate Cyp27b1 expression and deplete $25(OH)D_3$ (Vieth, Fraser, & Kooh, 1987) to promote $1,25(OH)_2D_3$ synthesis and increase $1,25(OH)_2D_3$ levels in plasma (Goff, Reinhardt, Engstrom, & Horst, 1992; Song & Fleet, 2007). The vitamin D-sufficient or vitamin D-deficient diets were further modified with high-fat/high-cholesterol (HF/HC; 42% fat/0.2% cholesterol) in the diet for feeding mice for 3 weeks, a duration that was shown to elevate plasma and liver cholesterol without changing Cyp7a1 mRNA or protein expression (Chow et al., 2014); the 3 weeks of HF/HC diet was superposed onto the last 3 weeks of the vitamin D-sufficient or D-deficient diets.

2.2.2 | Animal housing and tissue collection

All protocols were approved by the Animal Care Committee at the University of Toronto. Male C57BL/6 mice (Charles River Canada; Saint-Constant, QC) were maintained in groups of 3 or 4 mice per cage and given food and water *ad libitum* under a 12 h light/dark cycle; the vitamin D-deficient mice were housed under incandescent light to minimize the endogenous production of vitamin D. At the end of treatment, mice were anesthetized with 150 mg/kg ketamine and 10 mg/kg xylazine by intraperitoneal (i.p.) injection prior to cardiac puncture for blood collection into a 1 ml heparinized syringe. All mice were euthanized at 12 p.m. to minimize the circadian rhythm effects of Cyp7a1 (Noshiro, Nishimoto, & Okuda, 1990). Tissue collection was performed, as previously described (Chow, Durk, et al., 2011) and samples were stored at −80°C until analyses.

2.3 | Study 1: Establishment of the vitamin D-deficient model

Mice were fed the normal vitamin D-sufficient diet or vitamin D-deficient diet with 0.47% or 2.5% Ca^{2+} for 8 weeks. The mice were killed at 0, 2, 4, 6 or 8 weeks (n = 3–4 or 6–7 per group) and assayed for $25(OH)D_3$, $1,25(OH)_2D_3$, cholesterol, PTH, calcium and Vdr genes. At the end of the study, the mice were 11 weeks old (28.5 ± 0.4 g). Among all control groups (vitamin D-sufficient or D-deficient diet controls, with or without Ca^{2+} supplementation), changes in mRNA expression levels of Vdr, Shp and Cyp7a1 and Cyp7a1 protein expression were correlated with changes in liver cholesterol and $1,25(OH)_2D_3$ concentrations. Similar correlations were sought among SHP, CYP7A1 and cholesterol and $1,25(OH)_2D_3$ in human liver tissue.

2.4 | Study 2: Intervention studies with $1,25(OH)_2D_3$ or vitamin D (cholecalciferol) in vitamin D-deficient mice

Since Study 1 confirmed that Ca^{2+} replenishment in the vitamin D-deficient diets failed to perturb plasma $25(OH)D_3$ or $1,25(OH)_2D_3$ levels at the end of 8 weeks, intervention studies were conducted in mice fed the diets without additional Ca^{2+}. Preliminary studies showed

that treatment with 20 μg/kg cholecalciferol i.p. every other day for 1 week did not fully restore the elevated plasma cholesterol levels back to baseline levels. Hence, a longer intervention regimen (4 weeks) was used for cholecalciferol treatment. The mice were fed either the normal vitamin D-sufficient or vitamin D-deficient (0.47% Ca^{2+}) diet for the entire 8 weeks ($1,25(OH)_2D_3$ intervention group) or 11 weeks (cholecalciferol intervention group). For both HF/HC groups, the normal vitamin D-sufficient or normal vitamin D-deficient mouse groups were fed their normal vitamin D-sufficient or D-deficient diet for the first 5 or 8 weeks, followed by the HF/HC vitamin D-sufficient or HF/HC vitamin D-deficient diet for another 3 weeks for a total of 8 or 11 weeks.

2.4.1 | $1,25(OH)_2D_3$ intervention

Beginning on week 7 of the $1,25(OH)_2D_3$ intervention group, control mice on the vitamin D-sufficient diets or the vitamin D-deficient diets were treated i.p. with vehicle control (corn oil) (n = 3–4 per group), whereas mice on the vitamin D-deficient diet were treated with 2.5 μg/kg $1,25(OH)_2D_3$ (n = 5–6 per group) every other day for 1 week at 10 a.m., and both groups euthanized at 50 h after the last dose at 12 p.m. These mice were 11 weeks old (29.5 ± 0.4 and 32.6 ± 0.7 g for normal and HF/HC diet-fed mice, respectively) at the end of the study.

2.4.2 | Cholecalciferol intervention

Beginning on week 7 of the cholecalciferol intervention group, control mice on the vitamin D-sufficient and vitamin D-deficient diets were treated i.p. with vehicle (corn oil) (n = 5 per control group) and mice on vitamin D-deficient diets were treated with 20 μg/kg cholecalciferol (n = 6 per treated group) every other day during the last 4 weeks of diet. Mice were euthanized for tissue collection at 50 h after the last dose. At the end of the study, the mice were 14 weeks old (35.1 ± 1.2 and 35.5 ± 0.9 g for normal and HF/HC diet-fed mice, respectively).

2.5 | Study 3. Bile acid pool sizes in vitamin D-deficient vs. sufficient mice

Mice were examined for bile acid pool sizes during the period of established vitamin D-deficiency. Mice placed on the respective diets were treated with $1,25(OH)_2D_3$ or cholecalciferol (n = 4 per group). Amounts of the bile acids were determined by LC-MS/MS, and the values normalized to the body weight. For determination of bile acid pool sizes, mice were feed-deprived for 4 h (from 8:00 a.m. to 12:00 p.m.) prior to anesthesia, and the intact gallbladder, liver and intestine were removed altogether for analyses at the end of the diet, as described (Chow et al., 2014).

2.5.1 | Murine tissue analyses

Plasma was obtained at sacrifice for analyses. Intestinal, liver and renal tissues were obtained at sacrifice and assayed for mRNA or protein expression levels of nuclear receptors, transporters and enzymes, and VDR-related genes. Results for the correlations obtained for the liver in mice were compared against those in human liver tissue.

2.6 | Human liver tissue

Human livers (n = 11) were provided by In Vitro ADMET Laboratories from the International Institute for the Advancement of Medicine (Edison, NJ) and the National Disease Research Interchange (Philadelphia, PA). These donor tissues were the same as those examined previously in another report, wherein information on the donors was described (Chow et al., 2017).

2.7 | Analysis

2.7.1 | Analysis of plasma $25(OH)D_3$, $1,25(OH)_2D_3$, calcium and PTH levels

The concentrations of $25(OH)D_3$ (cat#AC-57SF1) and $1,25(OH)_2D_3$ (cat#AC-62F1) were assayed by enzyme-immunoassay kits from the Immunodiagnostics Systems, obtained from Inter Medico (Markham, ON), as per the manufacturer's protocols. The extraction procedure and quantification of $1,25(OH)_2D_3$ were identical to those described (Chow, Quach, Vieth, & Pang, 2013). Plasma calcium was assayed by inductively coupled plasma atomic emission spectroscopy (Optima 3000 DV, Perkin Elmer Canada, Woodbridge, ON) (Chow, Durk, et al., 2011). The mouse PTH 1-84 ELISA kit (cat#60-2305) from Immutopics was used to determine PTH concentrations (Joldon Diagnostics, Burlington, ON) (Chow, Quach, et al., 2013).

2.7.2 | Determination of plasma and liver cholesterol levels

Plasma cholesterol levels were determined by the total cholesterol kit (Wako Diagnostics, Richmond, VA). Cholesterol in mice and human liver tissue was determined from extracts using Infinity Cholesterol Reagents (Thermo Scientific, cat# TR13421, Rockford, IL), as described (Folch, Lees, & Sloane Stanley, 1957).

2.7.3 | Determination of bile acids by LC-MS/MS

The LC-MS/MS method employed was identical to that described by Chow et al. (2017). Bile acids and internal standards were obtained from Sigma-Aldrich, Steraloids (Newport, RI), and CDN Isotopes (Pointe-Claire, QC). The extraction procedures for the bile acids and the internal standards [a mixture of 0.25 mg/ml of $CA-d_4$, $DCA-d_4$, $CDCA-d_4$ and $LCA-d_4$] used for the determination of bile acid pool size were identical to those previously described (Chow et al., 2014). Bile acids were quantified based on the area under the curve of the peak, corrected by the appropriate internal standard, using calibration curves based on authentic standards.

2.8 | Quantitative real-time polymerase chain reaction (qPCR)

The mRNA expression levels of nuclear receptors, transporters and enzymes, particularly those responsive to Vdr (Chow et al., 2009, 2010; Chow, Durk, Maeng, & Pang, 2013; Chow, Quach, et al., 2013; Chow, Sondervan, Jin, Groothuis, & Pang, 2011) and highly expressed in mouse tissues (Alnouti & Klaassen, 2006; Alnouti, Petrick, & Klaassen, 2006; Buckley & Klaassen, 2007; Buist & Klaassen, 2004; Cheng, Maher, Chen, & Klaassen, 2005; Lu & Klaassen, 2006) were examined. Total RNA extraction and cDNA synthesis procedures were performed as described (Chow et al., 2009; Chow, Durk, et al., 2011);

specific primer sequences (Supplemental Table 2) were obtained from the literature (Chow, Durk, et al., 2013; Chow, Quach, et al., 2013; Chow et al., 2014; Chow et al., 2016; Chow et al., 2017; Durk et al., 2014; Gibson, Hossain, Richardson, & Aleksunes, 2012; Gong et al., 2011; Lee et al., 2009) or designed using Primer-BLAST (Chow et al., 2016). The critical threshold cycle (C_T) values of target genes for mouse liver and kidney were normalized to that for cyclophilin while those of intestine (ileum) were normalized to that of villin, then expressed as the relative mRNA expression of the 0-week of the normal vitamin D-sufficient diet controls. For human liver genes, the mRNA expression was normalized to that of GAPDH.

2.9 | Western blotting for relative protein analysis

Previously reported (Chow, Quach, et al., 2013; Chow et al., 2014; Kim et al., 2014) or newly validated primary antibodies were used in this study. Western blotting analysis was performed to determine the relative protein expression in mouse kidney (Trpv5, Trpv6, PepT1, Oat1, Oat3 and PKC), intestine (PepT1) and liver (Mrp3), as well as Cyp7a1/CYP7A1 in mouse and human livers. Protein expression was normalized to that of Gapdh/GAPDH (Chow et al., 2009; Chow, Durk, et al., 2011).

2.10 | Statistical analysis

Data are expressed as mean ± SEM. For Study 1, the mean at each time point (0 or 8 weeks post diet) for the vitamin D-deficient mice (0.47% or 2.5% Ca^{2+}) were compared with those for the normal vitamin D-sufficient mice using a one-way ANOVA and a post hoc Tukey honest significant difference test. For Study 2, the unpaired Student's t-test was used for comparison of the means for the normal vs. HF/HC vitamin D-sufficient diets. A one-way ANOVA and a post hoc Tukey honest significant difference test were used to compare the effects of diet (vitamin D-deficient vehicle controls vs. vitamin D-sufficient vehicle controls) and treatment. The unpaired Student's t-test was used to compare renal protein expressions between vehicle and $1,25(OH)_2D_3$ treated groups. The ANOVA models for assumptions of homogeneity of variance were assessed using the Brown-Forsythe test, but no outlier was found to significantly influence the model. Correlations were analysed by Pearson's correlation coefficient. Statistical analyses were performed with IBM SPSS Statistics (version 21.0, IBM Inc., Armonk, NY) and GraphPad Prism (version 6.01, GraphPad Software Inc., La Jolla, CA). Means were considered to be significantly different at $p < 0.05$.

3 | RESULTS

3.1 | Establishment of vitamin D-deficient mouse model (Study 1)

3.1.1 | Reduced levels of $25(OH)D_3$ and $1,25(OH)_2D_3$

The vitamin D-deficient diets (0.47% or 2.5% Ca^{2+}) resulted in progressively lower levels of plasma $25(OH)D_3$, plasma $1,25(OH)_2D_3$ and liver $1,25(OH)_2D_3$ levels on 0, 2, 4, 6 and 8 weeks post diet, compared with the corresponding vitamin D-sufficient control (intervening data not shown); maximum differences were observed at 8 weeks (Figures 1a–c). Additional Ca^{2+} supplementation (2.5% Ca^{2+}) in the diets

did not alter levels of 25(OH)D$_3$ nor 1,25(OH)$_2$D$_3$ after 8 weeks of the vitamin D-deficient diets (Figures 1a–c). Subsequent intervention studies (Study 2, with 1,25(OH)$_2$D$_3$ and cholecalciferol) were therefore conducted in mice fed the vitamin D-deficient diet with 0.47% Ca^{2+}.

Despite the vitamin D-deficient mice displaying lower plasma and liver 1,25(OH)$_2$D$_3$ levels, the liver-to-plasma tissue partitioning ratio (K$_p$ or C$_{liver}$/C$_{plasma}$) of 1,25(OH)$_2$D$_3$ remained unchanged compared with that for the vitamin D-sufficient mice (K$_{liver}$ = 0.08 ± 0.02 vs. 0.09 ± 0.01, p > 0.05), inferring that liver tissue partitioning was unaffected by the vitamin D status and that 1,25(OH)$_2$D$_3$ passively and rapidly equilibrated between liver and plasma. However, the apparent partitioning ratio for the kidney differed: K$_{kidney}$ [synthesis rate constant/(efflux and degradation rate constant)] = 0.39 ± 0.04 for the vitamin D-sufficient mice vs. 0.63 ± 0.07 for the vitamin D-deficient mice (p < 0.05). This is due to the higher, apparent intrarenal synthesis of 1,25(OH)$_2$D$_3$ by the higher Cyp27b1 and the reduced Cyp24a1 levels for degradation of 1,25(OH)$_2$D$_3$ in vitamin D-deficiency (see later).

3.1.2 | Changes in calcium, PTH, and cholesterol levels

Plasma Ca^{2+} levels were similar in mice fed either vitamin D-deficient diet (0.47% or 2.5% Ca^{2+}) (Figure 1d). But the plasma PTH levels (Figure 1e) and plasma and liver cholesterol levels at the end of the 8-week diet (Figures 1f, g) were considerably higher in the vitamin D-deficient mice compared with the vitamin D-sufficient controls.

3.1.3 | Changes in cholesterol-regulating gene expression in vitamin D-deficiency

Since elevated plasma and liver cholesterol levels in vitamin D-deficiency are consequences of the degradation or synthetic pathways, cholesterol-regulatory gene expressions in livers of vitamin D-

sufficient and D-deficient mice were compared. Mice fed the vitamin D-deficient diet without added Ca^{2+} showed significantly lower Vdr mRNA expression, although the reduction in Vdr relative protein expression was not statistically significant (Figure 2a). Both Fxr and Shp mRNA expression levels were higher with added 2.5% Ca^{2+} in the vitamin D-deficient diet at week 8, with correspondingly diminished Cyp7a1 mRNA and protein expression (Figure 2a). However, the hepatic mRNA expression of the sterol regulatory element-binding protein (Srebp2) that regulates the cellular uptake of cholesterol and fatty acids, and HMG-CoA reductase (Hmgcr) that regulates cholesterol synthesis, remained unchanged after 8 weeks of the vitamin D-deficient diet (Supplemental Figures 1a, b), while increased mRNA expression of 7-dehydrocholesterol reductase (Dhcr7), the enzyme that converts 7-dehydrocholesterol (7-DHC), the common precursor of both cholesterol and vitamin D, was observed with the 2.5% Ca^{2+} vitamin D-deficient diet (Supplemental Figure 1c). In the ileum, where regulatory elements of Cyp7a1 exist, changes in Vdr and Fxr mRNA expression were examined. The changes were insignificant, although higher ileal Fgf15 mRNA expression levels were oberved in mice fed the vitamin D-deficient diet with added calcium (Figure 2b).

3.1.4 | Correlation of liver cholesterol vs. 1,25(OH)$_2$D$_3$ levels and genes associated with cholesterol degradation (Shp and Cyp7a1) or transport/synthesis (Srebp2, Hmgcr and Dhcr7)

For understanding of the correlation between 1,25(OH)$_2$D$_3$ and cholesterol-regulating gene expression levels in the liver (Shp and Cyp7a1), the mRNA expression of each gene among all untreated mice fed the different diets (vitamin D-sufficient and D-deficient, with 0.47% or 2.5% Ca^{2+}) was plotted against its corresponding liver 1,25(OH)$_2$D$_3$ concentration (Figure 3a). A significant and positive correlation was
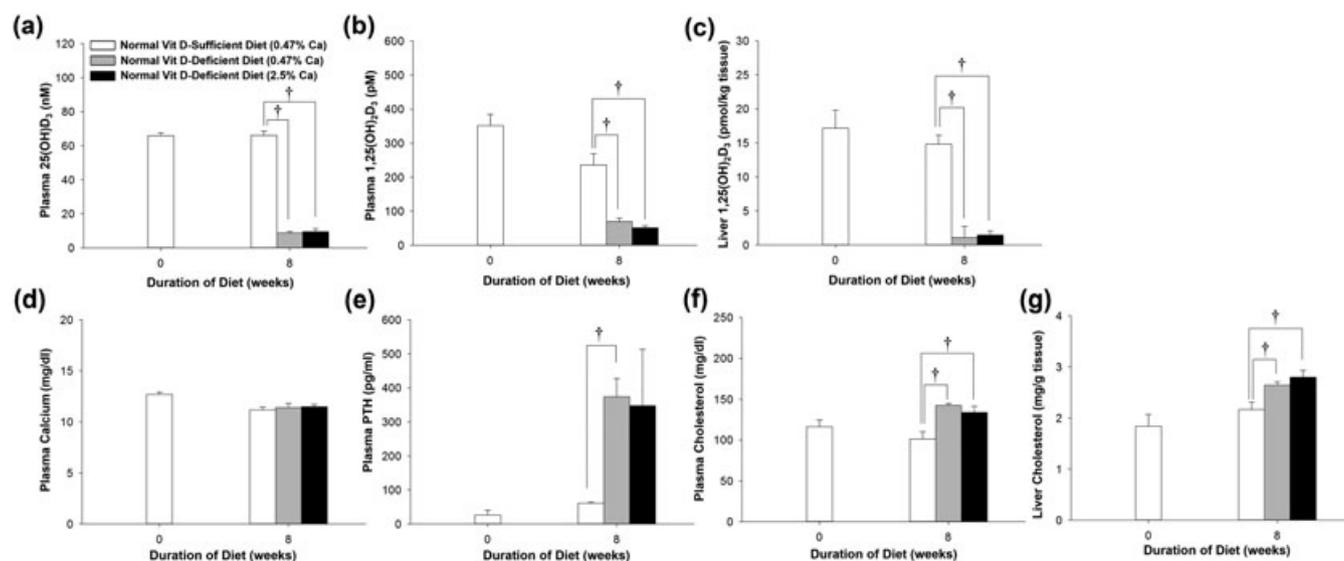
FIGURE 1 Reduced 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ levels and elevated cholesterol levels in vitamin D-deficiency at week 8 after diets (Study 1). Mice were fed the normal vitamin D-deficient diets with 0.47% (grey column) or 2.5% (black column) Ca^{2+} supplementation and levels were assayed at 0, 2, 4, 6 and 8 weeks to compare with levels for mice fed the normal vitamin D-sufficient (white column) to establish the vitamin D-deficient mouse model. (a) Plasma 25(OH)D$_3$, and (b) plasma and (c) liver 1,25(OH)$_2$D$_3$ levels were significantly reduced at the end of 8 weeks of both vitamin D-deficient diets compared with those after vitamin D-sufficient diet (8 weeks); (d) plasma Ca^{2+} levels remained unchanged regardless of diet, while (e) plasma PTH as well as (f) plasma and (g) liver cholesterol levels were elevated after 8 weeks of both vitamin D-deficient diets. Data are expressed as mean ± SEM (n = 3–7 per group); †p < 0.05 for vitamin D-sufficient vs. vitamin D-deficient controls
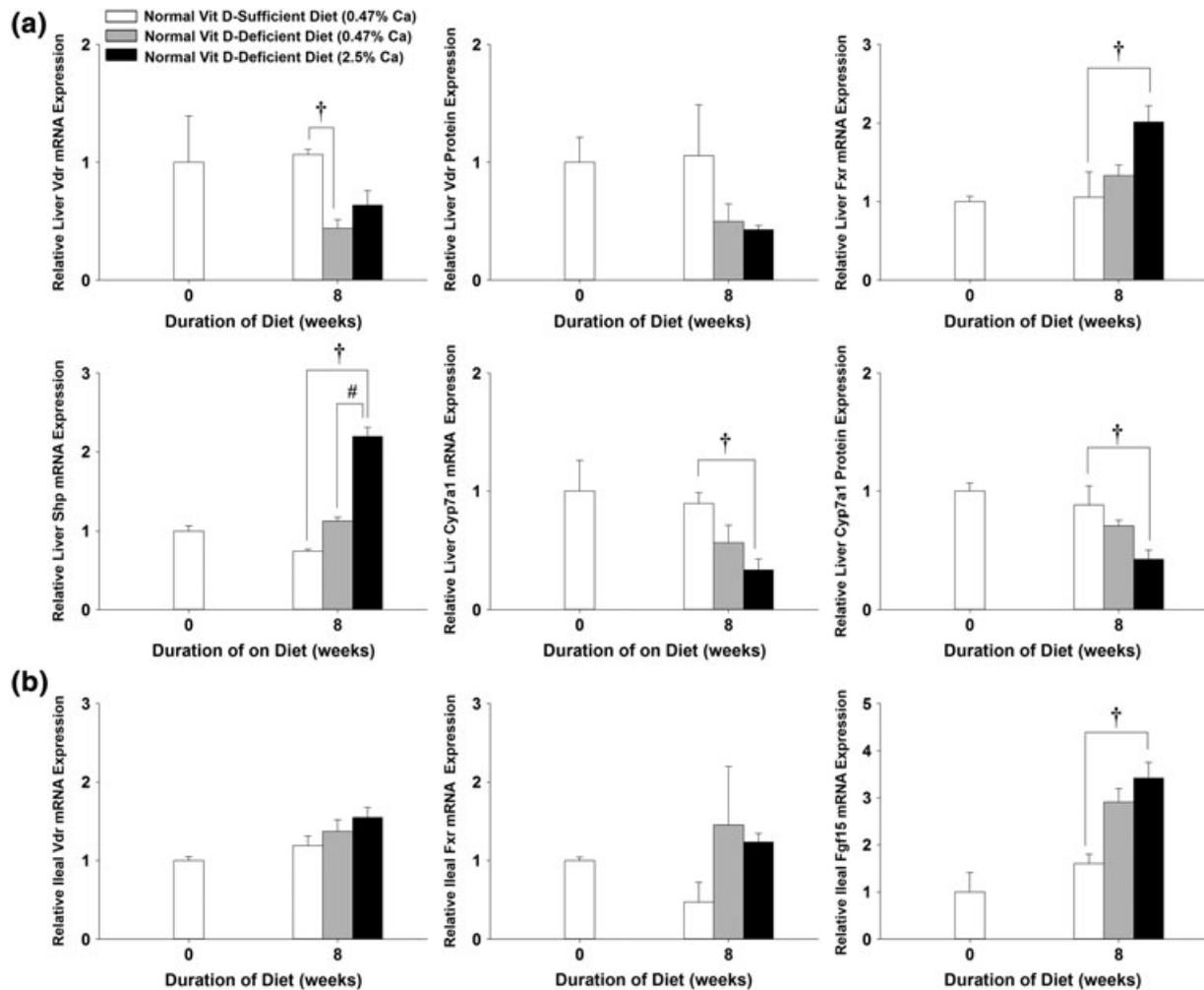
FIGURE 2 Changes in cholesterol-regulating genes in the liver and ileum in vitamin D-deficiency (Study 1). Mice were fed the normal vitamin D-deficient for 8 weeks to establish vitamin D-deficient mouse model. (a) Mice fed the vitamin D-deficient diet with 0.47% (grey column) or 2.5% Ca^{2+} supplementation (black column) had lower liver Vdr mRNA, higher Fxr and Shp mRNA expression, and lower Cyp7a1 mRNA and protein expression after 8 weeks of diet compared with mice fed the vitamin D-sufficient control diet (white column). (b) In ileum, the mRNA expression levels of Vdr and Fxr remained unchanged, while that for Fgf15 was increased after 8 weeks of vitamin D-deficient diets. Data are expressed as mean ± SEM (n = 3–7 per group); [†]p < 0.05 for vitamin D-sufficient vs. vitamin D-deficient controls; [#]p < 0.05 for vitamin D-deficient controls, 0.47% vs. 2.5% Ca^{2+} supplementation

observed between Vdr and Cyp7a1 mRNA and protein expression vs. liver $1,25(OH)_2D_3$ levels (upper panel). Furthermore, similar inverse relationships were observed between liver cholesterol levels vs. $1,25(OH)_2D_3$, CYP7A1 mRNA and CYP7A1 protein in human liver tissue (Figure 3b), although these correlations were non-significant. Inverse correlations were noted between mouse Shp mRNA vs. liver $1,25(OH)_2D_3$ and Cyp7a1 mRNA and protein expression levels vs. Shp mRNA, albeit the correlation between Cyp7a1 mRNA and Shp mRNA was not significant (p = 0.07) (Figure 3a; middle panel). In contrast, the mRNA expressions of cholesterol transport or synthetic genes such as Srebp2, Hmgcr and Dhcr7 were not correlated with liver $1,25(OH)_2D_3$, nor with cholesterol levels in mice fed vitamin D-sufficient or D-deficient diets (Supplemental Figures 1d–i). Taken together, these results suggest that the Shp-mediated suppression of Cyp7a1 is directly influenced by vitamin D-deficiency and levels of liver $1,25(OH)_2D_3$, and the hypercholesterolemia observed in vitamin D-deficient mice is primarily attributed to the altered expression of

cholesterol-regulatory genes in the degradative (Shp and Cyp7a1) and not synthetic (Hmgcr and Dhcr7) pathway.

3.2 | Changes associated with vitamin D-deficiency, before and after $1,25(OH)_2D_3$ or cholecalciferol treatment (Study 2)

3.2.1 | Changes in cholesterol

For examination of the effects of $1,25(OH)_2D_3$ or cholecalciferol treatment on the vitamin D-deficient mouse, wherein reduction of plasma $25(OH)D_3$ and plasma and liver $1,25(OH)_2D_3$ levels (Table 1) and elevation of cholesterol were observed in normal (Figures 1f, g) and HF/HC diets (Figure 4), intervention with $1,25(OH)_2D_3$ was found to restore plasma and liver cholesterol levels back to baseline levels of vitamin D-sufficient diet controls (Figure 4a; upper panel). Similarly, treatment with cholecalciferol in the HF/HC vitamin D-deficient mice also reduced the plasma and liver cholesterol levels (Figure 4a; lower panel).
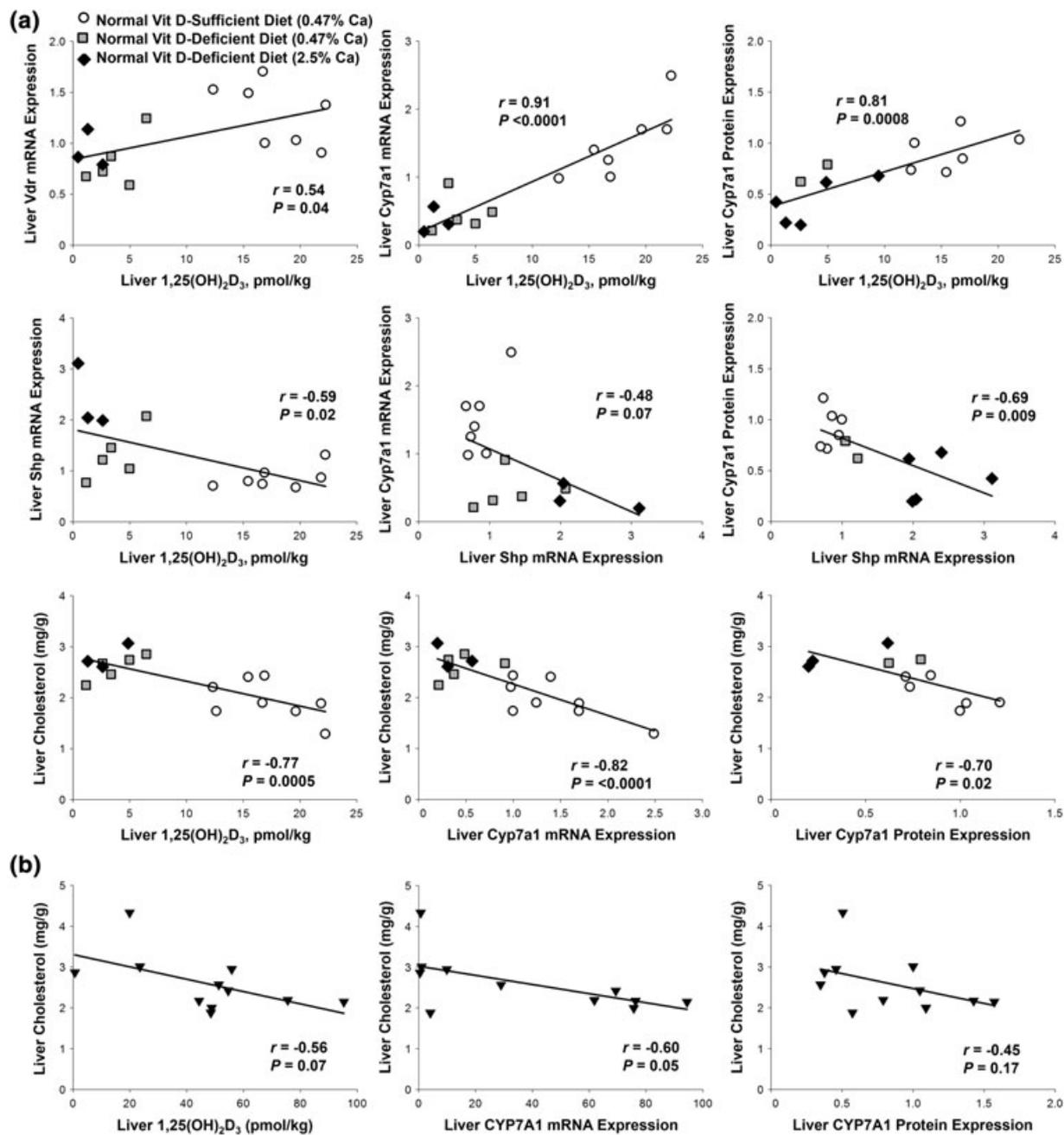
FIGURE 3 Correlation among liver 1,25(OH)$_2$D$_3$, cholesterol and regulatory gene expressions to elucidate the impact of vitamin D-deficiency in mice (Study 1) (a), and correlation in human liver tissue (b). At the end of the 8-week vitamin D-sufficient (white circle) or vitamin D-deficient diets with 0.47% (grey square) or 2.5% (black diamond) Ca^{2+} supplementation diets, (a) liver 1,25(OH)$_2$D$_3$ levels among all the diets were found positively and significantly correlated to Vdr mRNA and Cyp7a1 mRNA, and Cyp7a1 protein expression, while Shp mRNA expression was inversely and significantly correlated. The correlation between Shp mRNA and Cyp7a1 mRNA was not significant, although a significant, positive correlation existed between Shp mRNA and Cyp7a1 protein expression. Liver cholesterol was negatively correlated to liver 1,25(OH)$_2$D$_3$ concentration, Cyp7a1 mRNA and Cyp7a1 protein expression among all mice. Each symbol represents datum from one individual mouse. (b) In human liver tissue, liver cholesterol concentration was inversely associated with liver 1,25(OH)$_2$D$_3$ concentration, CYP7A1 mRNA, and CYP7A1 protein expression levels; each point (inverted solid triangle) represents one, untreated human liver sample

Plasma calcium levels were unchanged with vitamin D-deficiency and the diets, but levels were increased upon intervention with 1,25(OH)$_2$D$_3$ but not cholecalciferol (Table 1).

3.2.2 | Changes in regulatory cholesterol-gene expression

Cyp7a1 protein expression remained unaltered in mice fed the HF/HC diet for 3 weeks, verifying that the HF/HC diet would not increase Cyp7a1 protein expression (Figure 4b). Also, liver Vdr and Fxr mRNA

expression of the normal or HF/HC vitamin D-sufficient and D-deficient groups remained mostly unchanged (Figure 4b). Shp mRNA expression levels in vitamin D-deficient mice were generally higher than those in vitamin D-sufficient mice (Figure 4b). Cyp7a1 mRNA expression that bore a reciprocal relation to Shp, was reduced significantly for the vitamin D-deficient diet control vs. the vitamin D-sufficient diet control for both the normal and HF/HC diets (Figure 4b; lower panel). The overall pattern inferred that Shp mRNA was elevated

TABLE 1 Plasma (25(OH)D$_3$, 1,25(OH)$_2$D$_3$, calcium and PTH) and liver (1,25(OH)$_2$D$_3$) levels associated with vitamin D-deficiency in mice fed the normal and HF/HC diets, before and after treatment with vehicle, 2.5 μg/kg 1,25(OH)$_2$D$_3$ every other day during the last week of diet, or 20 μg/kg cholecalciferol every other day during the last 4 weeks of diet (Study 2)[a]; these were shown against data on mice fed the vitamin D-sufficient diets

Parameter	Normal diet		HF/HC diet[b]	
	Vit D-sufficient	Vit D-deficient	Vit D-sufficient	Vit D-deficient
Plasma 25(OH)D$_3$ (nM)				
+ vehicle (for 1 week)	62.3 ± 2.19	12.3 ± 0.270[†]	63.6 ± 0.884	12.5 ± 0.281[†]
+1,25(OH)$_2$D$_3$ (for 1 week)		11.3 ± 0.477		11.6 ± 0.414
+ vehicle (for 4 weeks)	63.4 ± 1.77	1.81 ± 0.663[†]	71.0 ± 1.83	3.27 ± 1.01[†]
+ Cholecalciferol (for 4 weeks)		73.6 ± 1.2*		86.3 ± 3.51*
Plasma 1,25(OH)$_2$D$_3$ (pM)				
+ vehicle (for 1 week)	179 ± 12.8	55.6 ± 11.2[†]	147 ± 26.8	44.8 ± 3.80[†]
+1,25(OH)$_2$D$_3$ (for 1 week)		24.5 ± 2.24*		53.6 ± 19.8
+ vehicle (for 4 weeks)	200 ± 35.7	83.8 ± 15.0[†]	241 ± 10.9	59.6 ± 4.96[†]
+ Cholecalciferol (for 4 weeks)		221 ± 23.0*		261 ± 4.90*
Liver 1,25(OH)$_2$D$_3$ (pmol/kg)				
+ vehicle (for 1 week)	14.8 ± 1.30	1.11 ± 1.65[†]	18.5 ± 2.60	3.65 ± 1.54[†]
+1,25(OH)$_2$D$_3$ (for 1 week)		3.96 ± 1.13		21.3 ± 3.74*
+ vehicle (for 4 weeks)	18.5 ± 2.60	3.65 ± 1.54[†]	27.6 ± 10.6	0.562 ± 0.611[†]
+ Cholecalciferol (for 4 weeks)		21.3 ± 3.7*		31.2 ± 4.10*
Plasma calcium (mg/dl)				
+ vehicle (for 1 week)	11.2 ± 0.271	11.4 ± 0.472	11.5 ± 0.473	11.8 ± 0.0944
+1,25(OH)$_2$D$_3$ (for 1 week)		13.8 ± 0.225*		17.4 ± 1.49*
+ vehicle (for 4 weeks)	12.9 ± 0.171	12.3 ± 0.323	11.8 ± 0.797	11.7 ± 0.112
+ Cholecalciferol (for 4 weeks)		12.2 ± 1.01		12.5 ± 0.554
Plasma PTH (pg/ml)				
+ vehicle (for 1 week)	60.8 ± 3.53	374 ± 62.1[†]	105 ± 22.8	274 ± 43.3[†]
+1,25(OH)$_2$D$_3$ (for 1 week)		1.01 ± 0.906*		16.3 ± 12.6*
+ vehicle (for 4 weeks)	36.7 ± 26.1	184 ± 18.6[†]	74.7 ± 13.1	313 ± 4.99[†]
+ Cholecalciferol (for 4 weeks)		60.2 ± 19.6*		106 ± 23.5*

[a]Parameters were measured 50 h after the last dose on week 8 (1,25(OH)$_2$D$_3$ intervention study) or week 11 (cholecalciferol intervention study) of the diets; values are mean ± SEM (n = 3–7 per group).

[b]Mice were fed a normal diet for 5 weeks (1,25(OH)$_2$D$_3$ intervention study) or 8 weeks (cholecalciferol intervention study) and then switched over to HF/HC diet for the remaining 3 weeks.

[†]$p < 0.05$ for vitamin D-sufficient vs. vitamin D-deficient vehicle controls in respective diets.

*$p < 0.05$ for vitamin D-deficient vehicle control vs. vitamin D-deficient + 1,25(OH)$_2$D$_3$ (1,25(OH)$_2$D$_3$ intervention study) or vitamin D-deficient vehicle control vs. vitamin D-deficient + cholecalciferol (cholecalciferol intervention study) in respective diets.

with deficiency while Cyp7a1 mRNA was down-regulated relative to the vitamin D-sufficient counterpart (Figure 4b).

Following treatment of 1,25(OH)$_2$D$_3$ (2.5 μg/kg every other day for 1 week i.p.), there was an attenuation of Shp mRNA, non-significantly for the normal diet but significantly for the HF/HC diet, and a concomitant elevation of Cyp7a1 mRNA and protein expression, with significant changes for the HF/HC diet (Figure 4b; upper panel). Similarly, the vitamin D deficiency-induced changes in Shp and Cyp7a1 mRNA expression in both normal and HF/HC vitamin D-deficient diets were reversed by the 4 week treatment with cholecalciferol (20 μg/kg every other day for 4 weeks i.p.). Cyp7a1 mRNA expression was increased significantly back to normal, while protein expression was increased, though non-significantly, back to basal levels after treatment of the vitamin D-deficient mice fed the normal and HF/HC diets (Figure 4b; lower panel). In contrast, ileal Fgf15 mRNA expression was unchanged upon treatment with 1,25(OH)$_2$D$_3$ as well as cholecalciferol (Figure 4b). Other factors that could influence

cholesterol homeostasis, namely mediators of cholesterol uptake (LDL receptor, scavenger receptor class B member 1, and Niemann-Pick C1 Like 1 transporter) and cholesterol efflux (Abca1, Abcg5 and Abcg8 transporters) (van der Wulp, Verkade, & Groen, 2013) were examined. 1,25(OH)$_2$D$_3$ treatment generally decreased the hepatic and ileal expression of Abca1, Abcg5 and Abcg8 in vitamin D-deficient mice fed the HF/HC diet; cholecalciferol treatment to vitamin D-deficient mice fed the HF/HC diet did not perturb the expression levels of Abca1, Abcg5, and Abcg8 much, and levels remained similar to those of mice on the vitamin D-sufficient diet (Supplemental Figure 2). No definitive pattern was found.

3.2.3 | Effects of vitamin D-deficiency on mRNA expression of liver, ileum, and kidney genes

Changes of the liver, intestine and renal nuclear receptors, transporters and enzymes in vitamin D-deficiency, before and after treatment, were compared. The notable changes in liver were the 2.33-fold higher
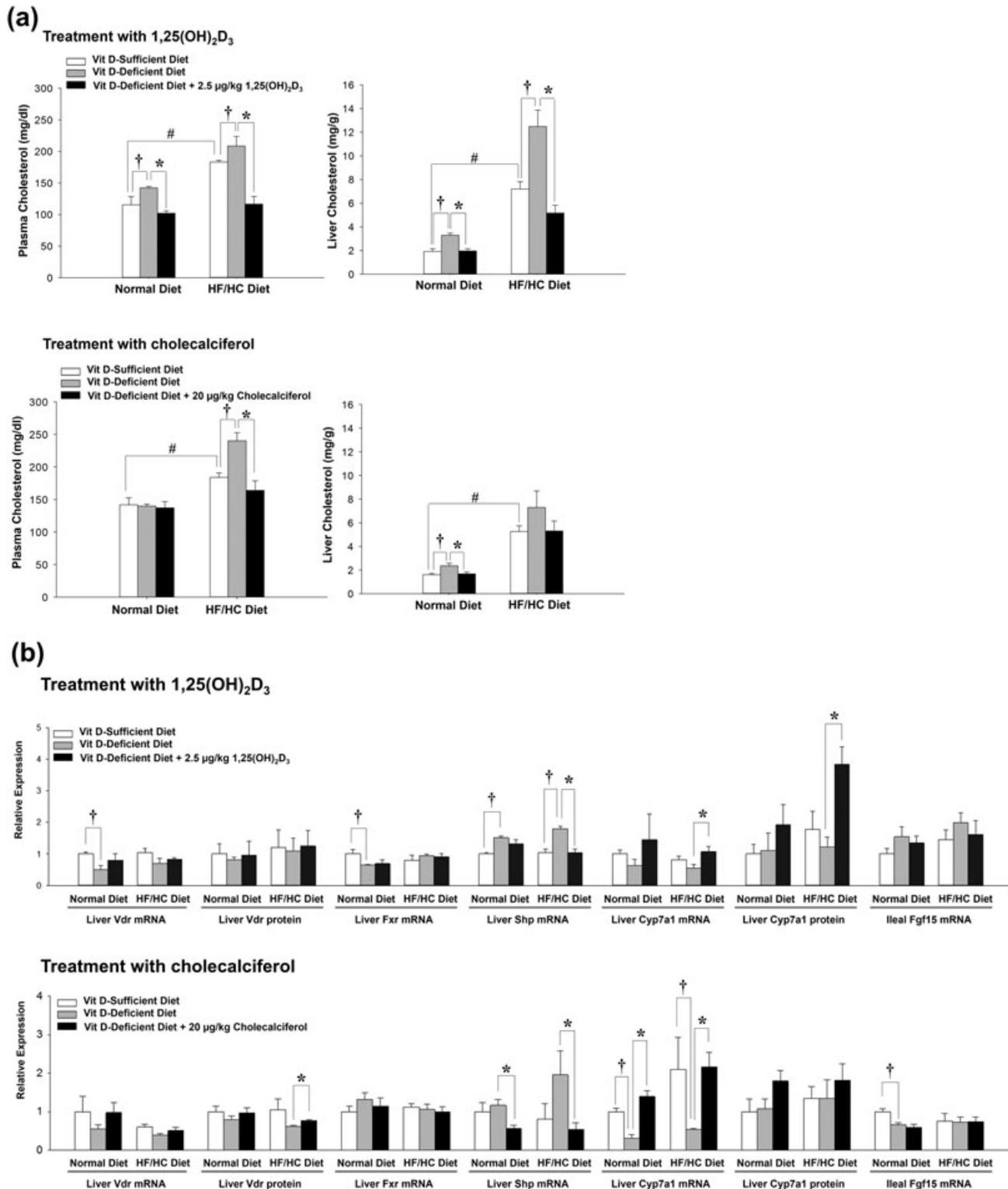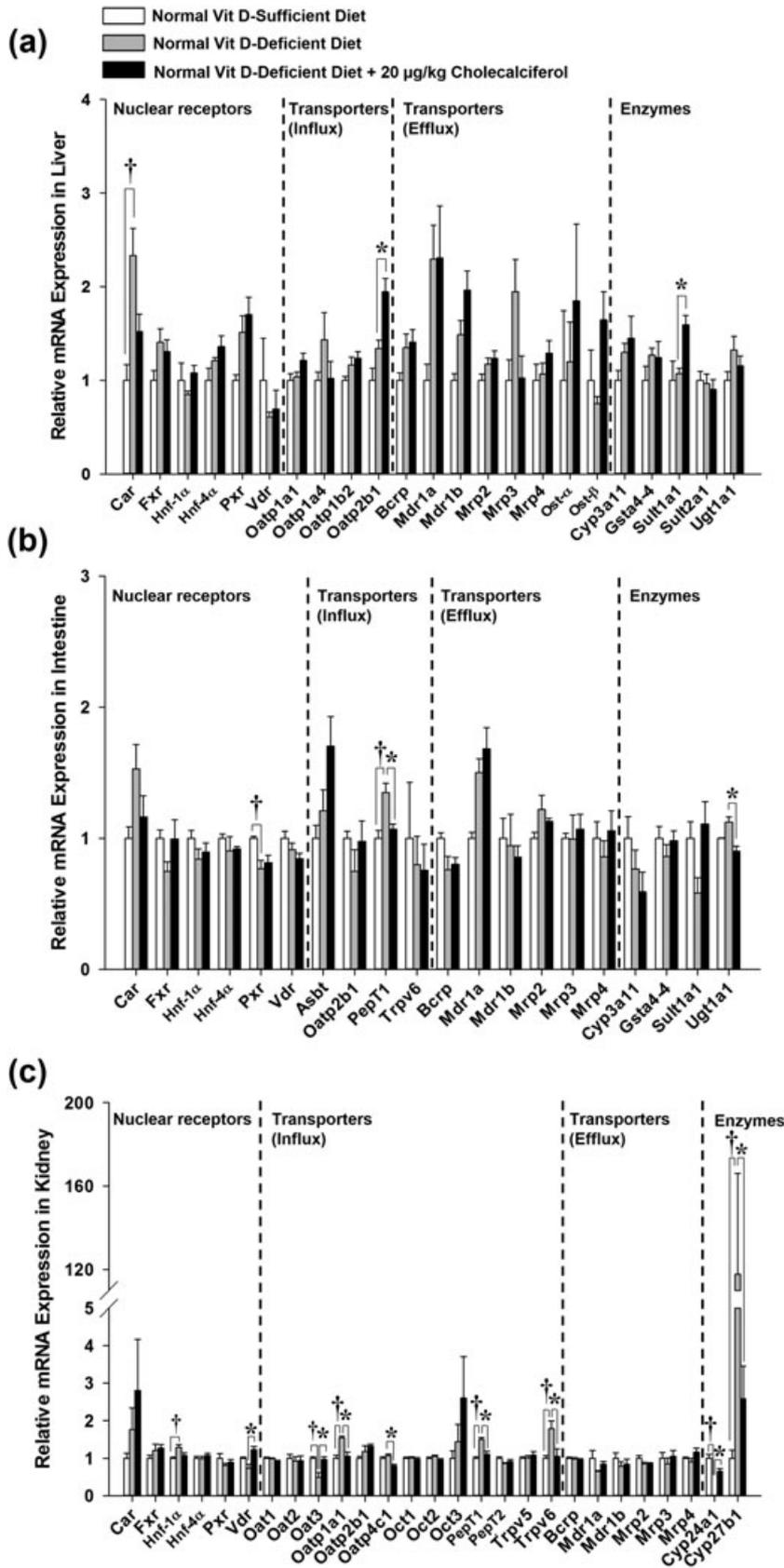
FIGURE 4 Impact of vitamin D-deficiency in mice fed the normal and HF/HC diet, before and after treatment of 1,25(OH)$_2$D$_3$ or cholecalciferol on plasma and liver cholesterol levels and expression levels of cholesterol-regulating genes (Study 2). (a) Vitamin D-deficiency in mice fed the normal or HF/HC diets generally showed increased plasma/liver cholesterol levels compared with those of corresponding, vitamin D-sufficient mice. Treatment with 1,25(OH)$_2$D$_3$ (1 week) or cholecalciferol (4 weeks) decreased plasma and liver cholesterol in vitamin D-deficient mice back to control (vitamin D-sufficient) levels. Patterns of change in plasma and liver cholesterol levels among the diets with cholecalciferol were similar to those with 1,25(OH)$_2$D$_3$, although the change for liver cholesterol was not significant. (b) Among the mice, there was a decrease in liver Vdr and Fxr mRNA expression but not Vdr protein expression in vitamin D-deficiency; the mRNA expression levels of Shp were elevated with the vitamin D-deficiency, but levels reduced upon 1,25(OH)$_2$D$_3$ or cholecalciferol treatment; opposite trends were observed for Cyp7a1 mRNA and protein expression. No definitive trend was observed for ileal Fgf15 mRNA. Data is expressed as mean ± SEM (n = 4–6 per group); [†]p < 0.05 for vitamin D-sufficient vs. vitamin D-deficient controls; *p < 0.05 for vitamin D-deficient control vs. vitamin D-deficient + 1,25(OH)$_2$D$_3$ or cholecalciferol; [#]p < 0.05 for vitamin D-sufficient vehicle controls, normal diet vs. HF/HC diet

constitutive androstane receptor (Car) (p = 0.005) in vitamin D-deficient mice vs. vitamin D-sufficient mice, and the higher Oatp2b1 (p = 0.011) and Sult1a1 mRNA expression following cholecalciferol treatment (Figure 5a). There was no other notable change in hepatic mRNA expression of liver Fxr, Hnf-1α, Hnf-4α, Pxr, Vdr, Oatp1a1, Oatp1a4, Oatp1b2,

Oatp2b1, Bcrp, Mdr1a, Mdr1b, Mrp2, Mrp3, Mrp4, Ost-α, Ost-β, Cyp3a11, Gsta4-4, Sult2a1 and Ugt1a1 when compared with corresponding expression in vitamin D-sufficient controls (Figure 5a).

In the intestine, there were only minimal changes in intestinal mRNA expression of nuclear receptors, transporters and enzymes. With vitamin

FIGURE 5 Impact of vitamin D-deficiency on gene expressions of nuclear receptor, enzyme and transporter mRNA expression levels in mice, before and after cholecalciferol treatment (Study 2). (a) Mice fed the normal vitamin D-deficient diet displayed significantly increased Car mRNA expression in liver when compared with vitamin D-sufficient controls; cholecalciferol treatment of vitamin D-deficient mice led to significant induction of Oatp2b1 and Sult1a1 mRNA expressions. (b) The intestine of vitamin D-deficient mice displayed significantly lower Pxr but increased PepT1 and Ugt1a1 mRNA expressions; cholecalciferol treatment returned levels of PepT1 and Ugt1a1 mRNA expressions back to the control levels of the vitamin D-sufficient group. (c) The kidney of the vitamin D-deficient mice showed significantly increased mRNA expression of Hnf-1α, Oatp1a1, PepT1, Trpv6 and Cyp27b1 but decreased Oat3 and Cyp24a1 mRNA expression compared with vitamin D-sufficient mice; cholecalciferol treatment led to partially or fully restored mRNA expression of these genes compared to control levels of the vitamin D-sufficient group. Data are expressed as mean ± SEM (n = 4–6 per group); †p < 0.05 for vitamin D-sufficient vs. vitamin D-deficient controls; *p < 0.05 for vitamin D-deficient control vs. vitamin D-deficient + cholecalciferol

D-deficiency, Pxr mRNA expression was slightly lower (23%) compared with the vitamin D-sufficient mice (p = 0.035), and cholecalciferol treatment did not restore Pxr mRNA expression fully back to baseline (Figure 5b). Only a small elevation in mRNA expression of PepT1 and Ugt1a1 mRNA expression was observed in vitamin D-deficient mice vs. the vitamin D-sufficient mice, and levels were restored to baseline after cholecalciferol treatment (Figure 5b). No change was observed for Asbt, Oatp2b1, Trpv6, Bcrp Mrp2, Mrp3, Mrp4, Mdr1a Mdr1b, Cyp3a11, Gsta4-4 and Sult1a1 mRNA expression in vitamin D-deficient mice when compared with their corresponding vitamin D-sufficient controls.

The greatest changes occurred in the kidney. Vdr mRNA expression was lower, though insignificantly, by 27% in the vitamin D-deficient mice vs. the vitamin D-sufficient controls, and levels were restored to baseline following cholecalciferol treatment (Figure 5c). Cyp24a1 mRNA expression was markedly reduced in the vitamin D-deficient mice (p < 0.001), although cholecalciferol treatment only partially restored Cyp24a1 expression back to baseline. In contrast, the mRNA expression of Cyp27b1 was increased by 117-fold in vitamin D-deficient mice vs. the D-sufficient mice (p = 0.041), and levels were restored to baseline following the 4 week treatment with cholecalciferol (p = 0.026). Oat3 mRNA expression levels in vitamin D-deficient mice were significanlty lower (51% those of vitamin D-sufficient mice), though these were increased back to baseline with supplementation of cholecalciferol. The mRNA expression levels of Oatp1a1, PepT1 and Trpv6 were significantly increased in the vitamin D-deficient mice (1.5–1.8-fold), and values were restored to baseline levels following cholecalciferol treatment. There was no change for Car, Fxr, Hnf-4α Pxr and Oatp4c1 mRNA expression levels in vitamin D-deficient mice when compared with their corresponding vitamin D-sufficient controls. Hnf-1α mRNA expression was slightly increased in vitamin-D deficient mice (p = 0.033) and again, levels returned back to baseline following cholecalciferol treatment.

3.2.4 | Effects of vitamin D-deficiency on protein expressions of liver, intestine and kidney genes

Due to the observed changes in mRNA expression of Vdr target genes with vitamin D-deficiency (Figure 5), corresponding changes in protein expression levels were further examined. No change in the relative protein expression was observed for hepatic Mrp3, intestinal PepT1 and renal Trpv5, Trpv6, PepT1 and Oat1 in vitamin D-deficiency mice, with or without treatement compared with the vitamin D-sufficient controls. In contrast, a dramatically lower (74%) renal Oat3 protein

FIGURE 6 Impact of vitamin D-deficiency on protein expressions of transporters and enzymes that exhibited notable changes in mRNA expression levels (Figure 5) (a), and changes in PKC, a negative regulator of renal Oat3, in renal subcellular fractions (b), before and after cholecalciferol treatment. (a) Insignificant changes were observed for the relative protein expression of liver Mrp3, intestinal PepT1 and renal Trpv5, Trpv6, PepT1 and Oat1 in mice fed the vitamin D-deficient diet compared with the vitamin D-sufficient diet controls, and levels remained relatively constant after treatment with cholecalciferol. Only renal Oat3 protein expression was significantly decreased with vitamin D-deficiency, and treatment with cholecalciferol partially restored Oat3 relative protein values to basal levels. (b) PKC relative protein levels in nuclear (9000 × *g* pellet), microsomal (100000 × *g* pellet) and cytosolic (100000 × *g* supernatant) fractions of the kidney in vitamin D-deficient and D-sufficient mice were similar; the values remained unchanged for the vitamin D-deficient mice before and after treatment with cholecalciferol. Data are expressed as mean ± SEM (n = 4–6 per group); [†]p < 0.05 for vitamin D-sufficient vs. vitamin D-deficient controls
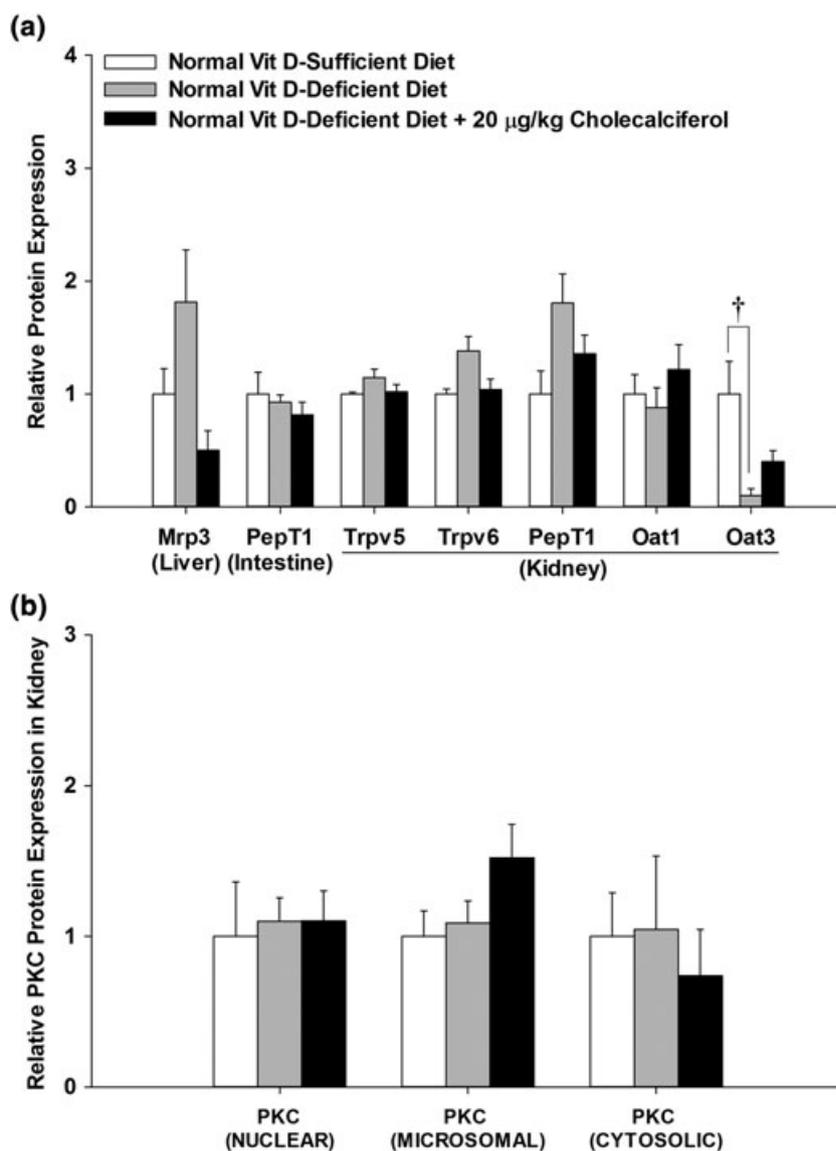
expression was observed with vitamin D-deficiency ($p = 0.026$), and treatment with cholecalciferol only partially restored the level to baseline levels (Figure 6a). Changes of renal Oat3 relative protein expression paralleled those of its mRNA expression (see Figure 5c). Since protein kinase C (PKC) is known to down-regulate Oat3 expression in rodents (Takeda et al., 2000), the relative protein expression levels of PKC were also determined among different fragments of the kidney. There was no change in PKC protein expression among the nuclear ($9000 \times g$ pellet), microsomal ($100000 \times g$ pellet) or cytosolic ($100000 \times g$ supernatant) fractions of the kidney among the vitamin D-deficient controls, vitamin D-deficient treated group and vitamin D-sufficient controls. Therefore, vitamin D-deficiency and intervention with cholecalciferol did not significantly alter PKC protein expression (Figure 6b).

3.3 | Bile acid pool sizes in vitamin D-deficiency, before and after treatment (Study 3)

Since vitamin D-deficiency raised cholesterol levels and reduced Cyp7a1 expression, the bile acid pool size was examined to determine if vitamin D-deficiency had altered the bile acid pool size or bile acid composition. Compared with their respective vitamin D-sufficient diet controls, vitamin D-deficiency did not dramatically alter the total bile acid pool sizes, although the HF/HC diet increased the total bile acid pool size over that of the normal controls (Figure 7). Treatment with $1,25(OH)_2D_3$ in vitamin D-deficient mice fed the normal and not HF/HC diets (Figure 7a) significantly elevated the bile acid pool size, as expected of Cyp7a1 induction and higher cholesterol metabolism. The same was observed with cholecalciferol treatment (Figure 7b). The overall bile acid composition remained relatively unchanged among the untreated and treated groups of different diets (Table 2). In the mouse, taurocholic acid (t-CA) as well as the tauroconjugated forms α, ß and ω- muricholic acids (MCA) remained relatively unchanged (Table 2).

4 | DISCUSSION

A vitamin D-deficient mouse model was developed to investigate vitamin D-deficiency on gene expressions. Progressive decreases in plasma $25(OH)D_3$ and $1,25(OH)_2D_3$ levels were observed at 4, 6, and 8 weeks in mice fed the vitamin D-deficient diets (with 0.47% and 2.5% Ca^{2+}) (data not shown). Discrepancies due to the different levels of plasma Ca^{2+}, $25(OH)D_3$ and $1,25(OH)_2D_3$ levels disappeared following 8 weeks of the diets (Figure 1). A time window was identified whereby levels of $25(OH)D_3$ and $1,25(OH)_2D_3$ remained well below baseline levels after 6–8 weeks of feeding of the vitamin D-deficient diet. Both $25(OH)D_3$ and $1,25(OH)_2D_3$ levels in the vitamin D-deficient mice were almost depleted to 13–29% of that of the vitamin D-sufficient controls after 8 weeks of diet. It was further verified that these levels were unaffected with Ca^{2+} supplementation (Figures 1a–d), as found by others (Goff et al., 1992; Song & Fleet, 2007; Vieth et al., 1987). Seemingly, the low $1,25(OH)_2D_3$ levels in mice were accompanied by high PTH levels (Figure 1e), and the
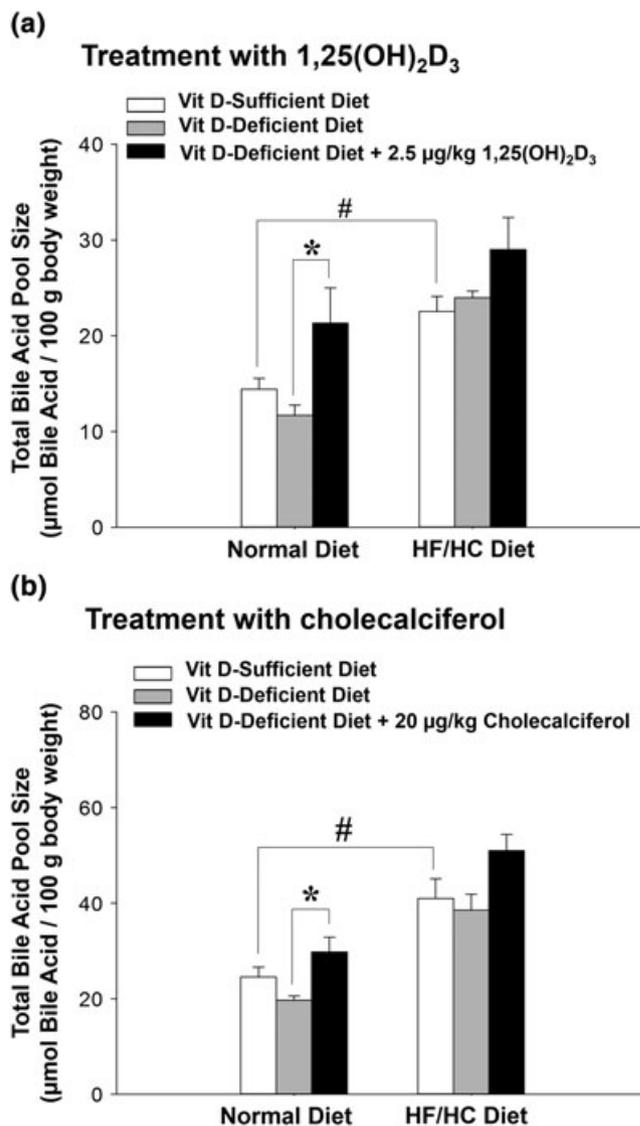
FIGURE 7 Impact of vitamin D-deficiency on bile acid pool sizes in vitamin D-sufficient vs. vitamin D-deficient diets with (a) $1,25(OH)_2D_3$ or (b) cholecalciferol treatment (Study 3). The total bile acid pool size (comprised t-CA, t-βMCA, t-αMCA, t-ωMCA, and CA) was higher for the HF/HC vs. normal vitamin D-sufficient groups, but remained unchanged between the vitamin D-sufficient and vitamin D-deficient groups under normal diet or HF/HC diet. Vitamin D-deficient mice fed the normal diet showed slightly but insignificantly lower bile acid pool size compared with the vitamin D-sufficient controls. Upon treatment with (a) $1,25(OH)_2D_3$ or (b) cholecalciferol of the vitamin D-deficient mice, higher bile acid pool sizes were observed as expected due to higher Cyp7a1 protein levels, though significance was observed only for mice fed the normal diet; vitamin D-deficient treated vs. non-treated groups ($p < 0.05$), and HF/HC vitamin D-deficient treated vs. non-treated groups ($p > 0.05$). Data are expressed as mean ± SEM (n = 3–6 per group); *$p < 0.05$ for vitamin D-deficient control vs. vitamin D-deficient + cholecalciferol; #$p < 0.05$ for vitamin D-sufficient vehicle controls, normal diet vs. HF/HC diet

changes in serum Ca^{2+} were minimal (Figure 1d). This model is similar to a vitamin D-deficient model established in the rat (Li et al., 2016).

After validation of the vitamin D-deficient mouse model, we proceeded to evaluate the significance of vitamin D-deficiency on VDR on- and off-target changes. First, the vitamin D-deficient livers were examined to elucidate the mechanism that contributed to

TABLE 2 Percent composition of total bile acid pool sizes in mice fed normal or HF/HC vitamin D-sufficient or vitamin D-deficient diets for a total of 8 or 11 weeks, to appraise vitamin D-deficiency and 1,25(OH)$_2$D$_3$/cholecalciferol treatment on cholesterol metabolism on bile acid formation; mice were treated with vehicle (corn oil) or 2.5 µg/kg 1,25(OH)$_2$D$_3$ every other day during the last week of diet, or 20 µg/kg cholecalciferol every other day during the last 4 weeks of diet (Study 3)[a,b]

	% Bile acid composition			
	Normal diet		HF/HC diet	
	Vit D-sufficient	Vit D-deficient	Vit D-sufficient	Vit D-deficient
t-CA				
+ vehicle (for 1 week)	49 ± 2.0	42 ± 3.4	52 ± 1.0	49 ± 1.6
+1,25(OH)$_2$D$_3$ (for 1 week)		41 ± 4.3		33 ± 2.8*
+ vehicle (for 4 weeks)	63 ± 3.4	42 ± 3.6	53 ± 1.8	54 ± 1.5
+ Cholecalciferol (for 4 weeks)		48 ± 8.1		54 ± 2.5
t-βMCA				
+ vehicle (for 1 week)	31 ± 1.5	40 ± 3.7[†]	35 ± 1.1	34 ± 1.7
+1,25(OH)$_2$D$_3$ (for 1 week)		46 ± 4.2		55 ± 2.8*
+ vehicle (for 4 weeks)	24 ± 2.3	34 ± 2.7	32 ± 1.9	32 ± 1.5
+ Cholecalciferol (for 4 weeks)		37 ± 9.5		34 ± 2.7
t-αMCA				
+ vehicle (for 1 week)	11 ± 0.39	9.2 ± 1.6	8.4 ± 1.4	10 ± 1.1
+1,25(OH)$_2$D$_3$ (for 1 week)		6.1 ± 0.79*		6.8 ± 0.95
+ vehicle (for 4 weeks)	7.7 ± 0.57	15 ± 1.4[†]	11 ± 1.5	10 ± 0.48
+ Cholecalciferol (for 4 weeks)		9.2 ± 0.66*		7.8 ± 0.029
t-ωMCA				
+ vehicle (for 1 week)	7.5 ± 0.52	8.4 ± 0.77	4.1 ± 0.35[#]	5.4 ± 0.79
+1,25(OH)$_2$D$_3$ (for 1 week)		4.4 ± 0.93*		4.1 ± 0.27
+ vehicle (for 4 weeks)	5.4 ± 1.0	9.3 ± 0.54[†]	4.7 ± 0.31	3.9 ± 0.23
+ Cholecalciferol (for 4 weeks)		6.1 ± 2.5*		3.6 ± 0.23
CA				
+ vehicle (for 1 week)	1.7 ± 1.7	0.0 ± 0.0	0.29 ± 0.29	0.35 ± 0.35
+1,25(OH)$_2$D$_3$ (for 1 week)		2.1 ± 1.3		1.2 ± 0.58
+ vehicle (for 4 weeks)	0.15 ± 0.065	0.13 ± 0.002	0.25 ± 0.14	0.21 ± 0.048
+ Cholecalciferol (for 4 weeks)		0.14 ± 0.037		0.16 ± 0.014

[a]Study 3 (1,25(OH)$_2$D$_3$ intervention study): mice were fed normal vitamin D-sufficient or vitamin D-deficient diets for the entire 8 weeks, or for the first 5 weeks and then switched over to the respective HF/HC vitamin D-sufficient or vitamin D-deficient diets for the remaining 3 weeks; vitamin D-deficient mice were treated with 1,25(OH)$_2$D$_3$ (2.5 µg/kg every other day during the last week).

[b]Study 3 (cholecalciferol intervention study): mice were fed normal vitamin D-sufficient or vitamin D-deficient diets for the entire 11 weeks, or for the first 8 weeks and then switched over to the respective HF/HC vitamin D-sufficient or vitamin D-deficient diets for the remaining 3 weeks; vitamin D-deficient mice were treated with cholecalciferol (20 µg/kg every other day during the last 4-weeks).

[†]$p < 0.05$ for vitamin D-sufficient vs. vitamin D-deficient vehicle controls in respective studies and diets.

*$p < 0.05$ for vitamin D-deficient vehicle control vs. vitamin D-deficient + 1,25(OH)$_2$D$_3$, or vitamin D-deficient vehicle control vs. vitamin D-deficient + cholecalciferol in respective diets.

[#]$p < 0.05$ for D-sufficient vehicle controls, normal diet vs. HF/HC diet in respective studies.

hypercholesterolemia (Figures 1f, g) in vitamin D-deficiency. Critical and significant changes (Figure 2) and correlations (Figure 3) were detected in the 1,25(OH)$_2$D$_3$, cholesterol and mRNA expression levels of Vdr, Shp, and Cyp7a1. With vitamin D-deficiency, there was reduced Vdr expression and lower 1,25(OH)$_2$D$_3$ levels in murine livers: Shp expression was elevated, leading to reduced Cyp7a1 mRNA and protein expression and higher cholesterol levels. Significant, negative associations were found with cholesterol and 1,25(OH)$_2$D$_3$, Cyp7a1 mRNA and Cyp7a1 protein expression in mouse livers (Figure 3). These observations support the notion that there is a link between cholesterol and 1,25(OH)$_2$D$_3$ levels. Interestingly, a similar but non-significant trend was observed between cholesterol and 1,25(OH)$_2$D$_3$ levels, as well as cholesterol and CYP7A1 expression in human livers

(Figure 3b), suggesting that the relationship between vitamin D-deficiency and elevated cholesterol could potentially exist in humans. It is surmised that a bigger sampling size would support this notion.

For viewing changes in hypercholesterolemia in the vitamin D-deficient model and treatment (Study 2), the HF/HC diet was superposed on the existing, vitamin D-deficient diets. Plasma PTH levels were found to be notably higher with the HF/HC vitamin D-deficient diets (Table 1), albeit non-significantly, possibly due to a protective role in which excess PTH stimulates the breakdown of fat (Bousquet-Melou, Galitzky, Lafontan, & Berlan, 1995) or merely a homeostatic result of the lower plasma Ca^{2+} levels. Plasma and liver cholesterol levels were 60% and 2.7-fold higher than those of normal diet controls for the HC/HC diet-fed mice, and levels were exacerbated with vitamin D-

deficiency (Figure 4a). Consistent with previous findings (Chow et al., 2014), $1,25(OH)_2D_3$ treatment was effective in resulting in a prompt increase in Cyp7a1 protein expression via down-regulation of hepatic Shp (Figure 4b), events that led to decreased plasma and liver cholesterol levels in mice fed the normal and HF/HC vitamin D-deficient diets (Figure 4a). Collectively, this study has provided unequivocal evidence that liver $1,25(OH)_2D_3$ is positively linked to the Vdr and Cyp7a1 expression and inversely related to Shp expression in mice (Figure 3).

Expectedly, a longer treatment period of vitamin D-deficient mice with physiological doses of the inactive precursor, cholecalciferol, was also found to be satisfactory. A 4 week intervention with cholecalciferol triggered down-regulation of Shp and induction of Cyp7a1 to reduce cholesterol levels back to baseline (Figure 4b). Cholecalciferol was able to increase levels of $25(OH)D_3$ and $1,25(OH)_2D_3$ back to those of vitamin D-sufficient mice (Table 1). Interestingly, both mouse and human liver cholesterol levels exhibited an inverse association between $1,25(OH)_2D_3$ and Cyp7a1/CYP7A1 expression, albeit further studies in human liver are required to confirm these observations (Figure 3). The key regulators of the cholesterol metabolism pathway appear to be altered by vitamin D-deficiency and contribute to elevated levels of cholesterol, observations that are reversed upon replenishment of cholecalciferol (Figure 4b). The therapeutic potential of cholecalciferol in the reversal of high cholesterol levels is particularly interesting, as the correction occurred without hypercalcemia (Table 1), a toxic event that delimits the therapeutic use of $1,25(OH)_2D_3$.

Although changes in enzyme and transporter expression were observed from in vitro and in vivo studies with $1,25(OH)_2D_3$ treatment or VDR activation (Chow, Durk, et al., 2011; Chow, Durk, et al., 2013; Durk et al., 2015; Maeng, Chapy, Zaman, & Pang, 2012), the impact of vitamin D-deficiency on these proteins or their functions was virtually unknown. Our investigation showed that, in addition to the known suppression of Cyp24a1 and elevated Cyp27b1 expression with vitamin D-deficiency due to feedback control and elevated plasma PTH concentrations in response to the lowered plasma $1,25(OH)_2D_3$ concentration (Table 1; Figure 5c) (Bikle, 2000; Turunen, Dunlop, Carlberg, & Vaisanen, 2007), the expression of Trpv6 and Trpv5, the alternate

transporter for Ca^{2+} re-absorption in the kidney (Bikle, 2000), remained unaltered in vitamin D-deficiency. However, a notable change rested with renal Oat3, whose mRNA and protein expression levels were greatly depressed with vitamin D-deficiency (Figures 5c and 6a); these levels returned towards baseline levels with cholecalciferol intervention. Since Hnf-1α and Hnf-4α are transcriptional factors that could be involved in the up-regulation of Oat1 as well as Oat3 (Kikuchi et al., 2006; Martovetsky, Tee, & Nigam, 2013; Saji et al., 2008), the observed changes in renal Oat3 could be attributed to changes in Hnf-1α mRNA expression in vitamin D-deficient mice. But renal Hnf-4α mRNA levels remained unchanged in vitamin D-deficient mice or upon replenishment of cholecalciferol (Figure 5c). Another likely explanation is the consideration that $1,25(OH)_2D_3$ may trigger renal PKC activation (Simboli-Campbell, Franks, & Welsh, 1992), which down-regulates renal Oat3 expression (Soodvilai, Chatsudthipong, Evans, Wright, & Dantzler, 2004; Takeda et al., 2000). Upon further probling, changes in PKC were not observed for the mouse kidney (Figure 6b).

To confirm these findings, we re-assayed, among the samples of Chow, Quach, et al. (2013) for changes in Oat3 expression. In this study, mice were fed the normal vitamin D-sufficient diets and treated with the same dosing regimen of $1,25(OH)_2D_3$. Renal mRNA and protein expression levels of Oat3, which is highly expressed in the kidney in mice (Hwang, Park, Kim, Yang, & Kim, 2010), increased significantly by 2- and 4.3-fold, respectively, after $1,25(OH)_2D_3$ treatment (Figure 8). Again, the PKC protein expression levels in the nuclear, microsomal and cytosolic fractions of the mouse kidney were not affected by $1,25(OH)_2D_3$ treatment. The lack of change is likely due to the observation that conventional PKC isoenzymes such as PKC-α, PKC-βI and PKC-βII are not expressed in the murine proximal tubules (Redling, Pfaff, Leitges, & Vallon, 2004), although Oat3 is highly expressed in the rat (Breljak, Brzica, Sweet, Anzai, & Sabolic, 2013; Kojima et al., 2002) and mouse (Breljak et al., 2013; Kojima et al., 2002) renal proximal tubules. Taken together, these results imply that Vdr might be modulating Oat3 expressions directly or indirectly via gene transcriptional regulation in mice, independent of PKC
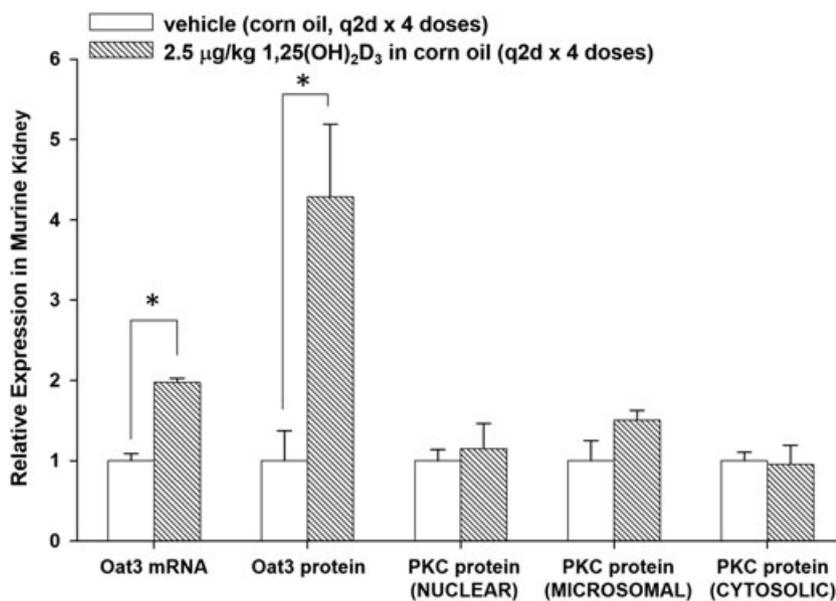
FIGURE 8 Impact of $1,25(OH)_2D_3$ treatment on renal Oat3 mRNA and protein expression in vitamin D-sufficient mice. Renal Oat3 mRNA and protein expression and PKC subcellular fragments of the kidney were appraised with samples of Chow, Quach, et al. (2013) wherein mice were fed the normal diet and treated with corn oil (vehicle control) or 2.5 μg/kg $1,25(OH)_2D_3$ i.p. every other day for four doses. The $1,25(OH)_2D_3$ treatment significantly increased renal Oat3 mRNA and protein expression in mice, but there was no net change in PKC expression for the nuclear ($9000 \times g$ pellet), microsomal ($100000 \times g$ pellet) and cytosolic ($100000 \times g$ supernatant) fractions of the kidney, with or without $1,25(OH)_2D_3$ treatment. Data are mean ± SEM (n = 4–7 per group); *p < 0.05 for vehicle vs. $1,25(OH)_2D_3$ treatment

(Figure 7); indeed, putative VDREs could be identified for murine Oat3 with the program, MatInspector (Genomatix, Munich, Germany). These results are in contrast to those observed for rat renal Oat3 mRNA and protein expression levels, which were found to be reduced by $1,25(OH)_2D_3$ treatment (Chow, Durk, et al., 2011). The stark contrast for the mouse and rat Oat3 responses to $1,25(OH)_2D_3$ treatment may exemplify a species difference of PKC localization in the kidney of these rodents. For mouse, conventional PKC isoenzymes such as PKC-α, PKC-βI and PKC-βII are not expressed in the murine proximal tubules (Redling et al., 2004) where Oat3 is localized (Breljak et al., 2013; Kojima et al., 2002). For the rat, PKC-α and PKC-βI were found highly expressed in the brush border of the proximal tubes (Pfaff, Wagner, & Vallon, 1999) and these co-localize with rat Oat3 at the proximal tubules, thus enabling PKC, upon VDR-mediated activation, to exert an inhibitory role on rat Oat3 function. Moreover, it is recognized that PKC/alpha inhibits whereas PKC/zeta stimulates Oat3 activity, and Oat3 activtors may activate or inhibit Oat3, depending on the concentration (discussion with Dr. Guofeng You, Rutgers University, New Jersey). Our vitamin D-deficient mouse model revealed that a reduction in mouse kidney Oat3 and elevated levels of cholesterol via reduction in Cyp7a1 are the major changes for vitamin D-deficiency.

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CONFLICT OF INTEREST

No conflicts of interest are declared by the authors.

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