Published in final edited form as:

Gastroenterology. 2009 April ; 136(4): 1317–e2. doi:10.1053/j.gastro.2008.12.051.

Intestinal Vitamin D Receptor is Required for Normal Calcium and Bone Metabolism in Mice

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Abstract

Background & Aims—Vitamin D Receptor (VDR)-knockout mice develop severe hypocalcaemia and rickets, accompanied by disruption of active intestinal calcium absorption. To specifically study the effects of VDR in intestinal Ca absorption, we investigated whether restoration of intestinal VDR is sufficient to recover the phenotype of VDR-knockout mice.

Methods—We generated mice with intestine-specific transgenic expression of human VDR and crossed them to VDR knockout mice. The intestine, kidney and bone phenotypes of the VDR-knockout mice with intestine-specific expression of human VDR (KO/TG) were analyzed.

Results—Transgenic expression of VDR in the intestine of VDR-knockout mice normalized duodenal vitamin D-regulated calcium absorption as well as vitamin D-regulated calbindin D_{9k} and TRPV6 gene expression in the duodenum and proximal colon. As a result, animal growth and the serum levels of calcium and parathyroid hormone were normalized in KO/TG mice. Other phenotypes were revealed when calcium metabolism was normalized in KO/TG mice: serum 1,25 dihydroxyvitamin D levels were higher in KO/TG mice than normal mice, due to reduced renal expression of the vitamin D-degrading enzyme CYP24; urinary calcium excretion was higher and associated with lower renal calbindin D_{9k} and D_{28k} than normal mice; and bone density and volume increased in KO/TG compared with normal mice, due to increased mineral apposition rate and osteoblast number.

Conclusions—Intestinal VDR and vitamin D-regulated intestinal calcium absorption are critical for controlling whole-body calcium metabolism in growing mice. Normalizing intestinal calcium absorption and metabolism reveals essential roles for VDR in control of bone formation, renal control of serum 1,25(OH)₂D and urinary calcium excretion.

Introduction

The active hormonal form of vitamin D, 1,25 dihydroxyvitamin D₃, (1,25(OH)₂D), is a major regulator of calcium (Ca) metabolism.¹ Elevated circulating 1,25(OH)₂D levels activate the vitamin D receptor (VDR) leading to increased intestinal Ca absorption, reduced renal Ca excretion, and elevated bone resorption.^{2, 3} VDR is a nuclear hormone receptor superfamily member that mediates 1,25(OH)₂D function by heterodimerizing with the retinoid X receptor

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Conflicts of interest: There are no conflicts of interest to disclose.

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(RXR) and interacting with response elements on target genes whose protein products mediate Ca metabolism, e.g. an apical membrane calcium channel, the transient receptor potential cation channel, subfamily V, member 6 (TRPV6) and the calcium binding protein calbindin D_{9K},^{3, 4} proposed to be involved in intestinal Ca absorption.⁵

Intestinal Ca absorption occurs through both passive paracellular and active transcellular pathways; only the active pathway is regulated by 1,25(OH)₂D.^{6, 7} We⁹ and others¹⁰ have previously shown that deletion of VDR in mice leads to a dramatic loss in active intestinal Ca absorption efficiency (>70%) as well as reduced expression of the molecular markers of intestinal Ca absorption, calbindin D_{9K} (down 55%) and TRPV6 (down 90%). However, feeding a rescue diet with 2% Ca, 1.25% phosphate, and 20% lactose that bypasses the transcellular Ca absorption pathway can partially prevent the phenotype of VDR knockout (KO) mice⁸⁻¹². This suggests that a primary role for VDR and 1,25(OH)₂D in the control of Ca metabolism is to maintain high rates of active intestinal Ca absorption. To directly test this hypothesis we created a transgenic mouse with expression of a hemagglutinin (HA)-tagged human VDR (HA-hVDR) limited to the intestine using the villin promoter/enhancer.¹³ We found that transgenic hVDR expression restores Ca homeostasis and vitamin D-related intestinal functions as well as prevents the rachitic phenotype of KO mice. In addition, by normalizing Ca metabolism, our data reveal important functions for VDR in the kidney and bone. These studies support the hypothesis that intestinal VDR and vitamin D-regulated intestinal Ca absorption are central for controlling whole body Ca metabolism during growth.

Materials and Methods

Animals

All animal experiments were approved by the Purdue Animal Care and Use Committee. Mice were housed individually, exposed to a 12-h light/12-h dark cycle, and given standard chow diet containing 0.72% calcium and water *ad libitum*. VDR knockout mice (KO)¹⁴ were maintained as heterozygous females (VDR +/-) and KO males for breeding.¹¹

Generation of HA-hVDR Expressing Transgenic Mice

The transgene construct and the methods for generating the transgenic mice are included in the supplementary information online at www.gastrojournal.org.

The transgene was detected in tail genomic DNA by conventional PCR and the transgene copy number was determined using real-time PCR.

Characterization of Transgenic Mice

Transgene Tissue Distribution and Expression Level—Total RNA was isolated with Tri-reagent (Molecular Research Center Inc, Cincinnati, OH) from various tissues of 2-month-old wild type (WT) mice and transgenic mice (TG +/-) with low (TG(L)) or high (TG(H)) transgene expression and reverse-transcribed into cDNA as previously described.¹⁵

VDR protein levels from intestinal segments were evaluated in samples extracted (PRO-PREP™ protein extraction buffer, iNtRON Biotechnology, Inc., Gyeonggi-do, Korea) from 2 centimeters of each intestinal segment in WT and TG(H) mice after culture in DMEM plus 10% FBS for 2 h at 37°C, in 5% CO₂, 95% air. Western blot analysis using an anti-VDR primary antibody was used to detect the natural and transgenic VDR protein (see supplementary information online at www.gastrojournal.org).

Evaluation of the Phenotype of Transgenic Mice—Serum calcium, phosphate, and 1,25(OH)₂D, and bone mineral density (BMD) from 2-month-old, gender-matched WT and

TG(L) or TG(H) mice fed chow diet were determined as described in the supplementary information online at www.gastrojournal.com (n=8 each genotype).

Development and Characterization of VDR Knockout Mice Expressing the Villin-HA-hVDR Transgene—The experimental mice of four genotypes were obtained by pairing KO mice (VDR^{-/-}, TG^{-/-}) and VDR^{+/-}, TG^{+/-} mice: VDR^{+/-}, KO, or KO mice with intestine-specific TG expression (KO/TG(L) and KO/TG(H)). VDR^{+/-}, KO or KO/TG mice used for experiments were littermates and VDR^{+/-} mice were used as controls because they are phenotypically normal when fed a nutritionally adequate diet.^{14, 18} Experimental mice were fed the standard chow diet for either 8 wk (female) or 12 wk (male).

12-wk-old male mice were used for basal analysis (n=8 per group). Serum was prepared for analysis of calcium, phosphate, parathyroid hormone, and 1,25(OH)₂D levels. Urine was taken directly from the bladder for assessment of calcium to creatinine ratio. Mucosal scrapings from duodenum and proximal colon, and kidneys were harvested for analysis of gene expression (see below). Thyroid-parathyroid tissue and femora was harvested, fixed in 10% neutral buffered formalin for 12 h and 7 days, respectively, and stored in 75% ethanol at 4°C until the samples were processed.

8-wk-old female mice were fasted overnight and then treated with vehicle (a 1:9 mixture of ethanol:propylene glycol) or 1,25(OH)₂D (25 ng or 100 ng /100 g body weight in 0.1 ml vehicle) by i.p. injection. 9 h later mice were studied for intestinal calcium absorption (25 ng dose only) using *in situ* loops of duodenum (2 cm from pyloric sphincter, 2 mM total calcium),¹⁶ as well as renal and intestinal gene expression (n=6-8 per treatment per genotype group).

Bone modeling dynamics were assessed by double calcein labeling in 8-wk-old female VDR^{+/-}, KO and KO/TG(H) mice injected i.p. with 20 mg calcein /g body weight in PBS (Sigma Chemical, St Louis, MO) on the 10th day and 3rd day before termination of the experiment (n= 7 per genotype).¹⁷ Femurs were removed and cleaned of all soft tissue, fixed in 75% ethanol at 4°C until embedding in methyl methacrylate at low temperature.

Primers and PCR cycle conditions, analysis of tissues (bone densitometry, tomography, histology) and serum (calcium, phosphate, 1,25(OH)₂D, PTH), image quantification, and statistical analysis are included in the supplemental online information.

Results

Generation and Characterization of Intestine-specific hVDR Transgenic Mice

Transgenic mice expressing HA-hVDR under control of the villin promoter/enhancer were generated (Figure 1A). Four founders were identified (three are shown in Figure 1B) and the lines with 10 (TG(L)) and 50 (TG(H)) copies of the transgene were characterized further. Transgene mRNA was detectable throughout the intestine (Figure 1C, 1D); total VDR mRNA level in duodenum was increased by 4- and 20-fold in TG(L) and TG(H), respectively (Figure 2A). A small amount of transgene mRNA was expressed in the kidney but this had no impact on total VDR mRNA levels (Figure 2B). All other tissues were transgene negative (Figure 1D).

VDR was present in all segments of the intestine with the highest levels seen in the cecum and colon (Figure 2D). Transgene expression increased VDR protein level throughout the intestine with the highest expression in the small intestine (Figure 2D; e.g. 2 and 8- fold higher in TG (L) and TG(H) mice than wild type (WT) mice in duodenum, respectively Figure 2C). At 2 mo, all transgenic mice were healthy, fertile and grew normally. No differences were observed between the TG lines and WT mice for serum Ca, phosphate, 1,25(OH)₂D or bone mineral density (data not shown) .

Gross Appearance, Growth Curves, and Serum Phenotype

12-wk-old male KO mice had alopecia and were growth arrested when grown on a chow diet. The growth arrest of KO mice was prevented in both KO/TG(L) and KO/TG(H) transgenic lines but the alopecia remained (Figure 3A, 3B). KO mice also had hypocalcemia (44% lower than VDR+/-, Figure 3C a) and hyperparathyroidism (84-fold higher serum PTH, Figure 3C b, enlarged parathyroid glands, Figure 3C c). In KO/TG(L) and KO/TG(H), serum Ca, serum PTH, and parathyroid gland size were normalized (Figure 3C).

Intestinal Phenotype

Basal duodenal Ca absorption was 53% lower in 8-wk-old female, chow-fed KO mice than VDR+/- mice. In KO/TG(H) mice, Ca absorption was not only restored, it was 26% higher than VDR+/- mice (Figure 4A a). Similarly, duodenal calbindin D_{9k} and TRPV6 mRNA levels were significantly reduced in KO mice (i.e. by 93% and 99% compared to VDR+/- mice, respectively) and transgenic recovery of intestinal VDR increased their levels above that seen in VDR+/- mice by 150% (calbindin D_{9k}) and 67% (TRPV6)(Figure 4A c). 1,25(OH)₂D treatment increased duodenal Ca absorption by 70% in VDR+/- mice (to 51.7±2.9%), had no impact in KO mice, and increased Ca absorption by 40% in KO/TG(H) mice (to 52.5±4.0%, Figure 4A a). Both 25 ng and 100 ng/100 g body weight (BW) 1,25(OH)₂D doses significantly induced duodenal CYP24, calbindin D_{9k} and TRPV6 mRNA expression in VDR+/- but not in KO mice. KO/TG(H) mice responded to treatment but the induction was blunted for CYP24 and TRPV6 mRNA (Figure 4A b, c).

Similar findings to those in the duodenum were observed in the proximal colon. Calbindin D_{9k} and TRPV6 mRNA levels were nearly eliminated in KO mice and their expression was recovered in the proximal colon of KO/TG mice (Figure 4B a). Like we observed in the duodenum, 1,25(OH)₂D-responsive expression of calbindin D_{9k} and TRPV6 mRNA was restored in KO/TG but the response was blunted compared to VDR +/- mice (Figure 4B b).

Renal Phenotype

Chow-fed KO mice had higher serum 1,25(OH)₂D (98-fold) and renal CYP27B1 mRNA (400-fold) than VDR+/- mice at 12 wk. Renal CYP24 mRNA was undetectable in KO mice. Compared to KO mice serum 1,25(OH)₂D was significantly reduced in KO/TG(L) and KO/TG(H) mice but it was still 40- and 6-fold higher than VDR +/- mice, respectively (Figure 5A a). Renal CYP27B1 mRNA was 12-fold higher in KO/TG(L) than VDR +/- mice but was normalized in KO/TG(H) mice. In contrast, renal CYP24 mRNA was 95% and 75% lower than VDR+/- mice in KO/TG(L) and KO/TG(H) mice, respectively (Figure 5A b).

Renal Ca excretion was lower in chow-fed KO mice, presumably due to reduced serum Ca levels. Normalizing serum Ca revealed a significant increase in urinary Ca (248% higher in KO/TG(H) mice than VDR+/- mice, Figure 5A c). Renal calbindin D_{9k} and calbindin D_{28k} mRNA levels were significantly reduced in KO mice (by 90% and 50% compared to VDR+/- mice, respectively) and their levels were not recovered in KO/TG(H) mice (Figure 5A d). Neither renal TRPV5 nor TRPV6 mRNA levels were altered in either KO or KO/TG(H) mice (data not shown).

In VDR +/- mice 1,25(OH)₂D-treatment (25 ng/100g BW) had no impact on renal calbindin D_{9k}, calbindin D_{28k}, or TRPV6 mRNA (data not shown). Renal CYP24 mRNA was strongly induced in VDR+/- mice (270-fold), was not affected in KO, and was blunted in KO/TG(H) (4-fold increase, Figure 5B a). 1,25(OH)₂D suppressed renal CYP27B1 gene expression by 99% and induced renal TRPV5 gene expression by 2.8-fold in VDR+/- mice but these effects were not observed in either KO or KO/TG(H) mice (Figure 5B b, c).

Skeletal Phenotype

Femurs were significantly shorter in KO mice but length was normalized in KO/TG(L) and KO/TG(H) mice (data not shown). Femur bone mineral density (BMD) was decreased by 56% in KO mice. In KO/TG(L) and KO/TG(H) mice, BMD was not only restored, it was increased 14% above the level seen in VDR+/- mice (Figure 6A). Similarly, radiographs reveal that the density in the epiphysis, metaphysis and diaphysis of KO mouse femur was reduced and this was prevented in KO/TG(H) mice (Figure 6B). pQCT analysis of the femur midshaft showed that total bone area, cortical bone area, and polar moment of inertia (IP) were decreased by 33%, 90% and 42%, respectively, in KO mice. In the distal femur, total bone area and trabecular bone area were decreased by 32% and 39%. In KO/TG(H) mice total bone area, cortical bone area and IP in the midshaft of femur were increased (by 18%, 13% and 37%, respectively) and total bone area and trabecular bone area in the distal femur was elevated (by 17% and 18%, respectively) relative to VDR+/- mice (Table 1).

KO mice had disorganized, widened and expanded growth plates and this was completely prevented in KO/TG(H) mice (H&E staining not shown but collagen staining reveals this in Figure 7A). Trabecular bone volume (by total collagen staining) was increased by 113% in KO mice compared to VDR+/- mice. In KO/TG(H) mice trabecular bone volume was still 23% higher than that seen in VDR+/- mice (Figure 7A, 7F a). Osteoid volume (by von Kossa staining) was 13-fold higher in KO mice compared to VDR+/- mice but this was normalized in KO/TG(H) mice (Figure 7B, 7F b). Thus, while the increased trabecular bone volume in KO was due to more unmineralized bone, the increase in KO/TG(H) was due to new bone formation. This conclusion was supported by our examination of mineral apposition rate (MAR). While there was a lack of distinct calcein labeling in KO mice, distinct labeling was restored in KO/TG(H) mice and MAR was 2-fold higher than in VDR+/- mice (Figure 7C, 7F c).

Tartrate Resistant Acid Phosphatase (TRAP) positive osteoclast number was not influenced by VDR status (Figure 7E, 7F e). However, there was a 2-fold increase in osteoblast number in KO mice compared to VDR+/- mice. In KO/TG(H) mice, osteoblast number was reduced compared to KO mice but was still 40% higher than VDR+/- mice (Figure 7D, 7F d).

Discussion

The creation of VDR KO mice confirmed the critical role that VDR plays in the control of whole body Ca metabolism.^{14, 18, 19} Using these mice, we¹¹ and others¹⁹ found that transcellular intestinal Ca absorption was disrupted (e.g. 70% lower at 2 months) and was accompanied by lower levels of the molecular markers for active Ca absorption (e.g. calbindin D_{9k} mRNA 50% lower; TRPV6 mRNA >90% lower). By-passing active, vitamin D-dependent Ca absorption with high Ca, high lactose diets can prevent hypocalcemia and development of osteomalacia and rickets seen in KO mice.⁸⁻¹⁰ Collectively, these data suggest that controlling active intestinal Ca absorption is the central role that VDR plays in Ca metabolism during growth. Our research directly tests this hypothesis; we have now demonstrated that the restoration of VDR throughout the intestine of KO mice is sufficient to recover the phenotype of disrupted Ca metabolism in these animals. In addition, our research reveals several interesting aspects regarding the regulation of Ca metabolism across the three tissue axis of intestine, kidney, and bone whose activities are normally coordinated to regulate Ca metabolism.

Our data suggest that the lower bowel is a more important site for vitamin D-regulated Ca absorption than previously appreciated. Earlier work by Pansu *et al.* found vitamin D-regulated, transcellular Ca absorption in the duodenum but not the distal ileum of rats,⁷ thereby

identifying the proximal small intestine as the most important site for vitamin D-regulated Ca absorption. However, intestinal VDR levels are high in the cecum and colon of mice (Figure 2D) and this is accompanied by vitamin D regulated expression of TRPV6 and calbindin D_{9k} mRNA (Figure 4B and data not shown). In KO mice, calbindin D_{9k} and TRPV6 mRNA levels in the colon and cecum are severely reduced (Figure 4B, data not shown) and transgenic recovery of VDR in the large intestine of KO mice restored expression of these genes in the colon (Figure 4B a). Also, Fujita *et al.*²⁰ have recently reported that vitamin D-regulated expression of claudins-2 and 12 in the jejunum, ileum, and colon may modulate paracellular Ca absorption. These findings suggest that Ca absorption is regulated by vitamin D-mediated mechanisms in the lower intestinal segments, where long residence times²¹ would permit a significant amount of Ca absorption even if the rate of active/passive transport or the solubility of Ca was low. This is consistent with reports that net Ca absorption increases in the ileum²² and colon²³ of people treated with 1,25(OH)₂D and that active 1,25(OH)₂D-regulated Ca absorption is seen in isolated segments of the cecum²⁴ and colon^{25, 26} from rats. The importance of active 1,25(OH)₂D-regulated Ca absorption in the lower intestine is also consistent with our earlier report showing that although the adenosine deaminase (ADA) promoter-driven transgenic expression of hVDR to the distal duodenum and proximal jejunum of KO mice could restore 1,25(OH)₂D-mediated Ca absorption in those sections, this was not sufficient to maintain whole body Ca homeostasis on a commercial chow diet (27 and additional unpublished data).

Recent knockout mouse studies show that calbindin D_{9k} is not essential for vitamin D-regulated intestinal Ca absorption,^{28, 29} but our studies show that it continues to correlate well with it; both duodenal calbindin D_{9k} mRNA and Ca absorption levels were elevated in KO/TG(H) mice (150% and 26% higher than VDR+/-, respectively) and both were significantly elevated by 1,25(OH)₂D treatment (Figure 4A a, c). In contrast, the response of duodenal TRPV6 and CYP24 mRNA to 1,25(OH)₂D treatment was blunted in intestinal segments from KO/TG mice relative to VDR +/- mice. Although we cannot account for this effect, the disconnect between induction of Ca absorption by exogenous 1,25(OH)₂D and TRPV6 mRNA is consistent with several reports that show vitamin D-regulated Ca absorption can occur in the absence of TRPV6.^{30, 31}

Our HA-hVDR transgene was also expressed in the kidney. However, transgene expression did not increase total VDR mRNA in the kidney of normal mice suggesting the level of expression was low. Others have shown that the villin promoter drives gene expression in the epithelial cells of the renal proximal tubule but not distal tubule^{32, 33} where VDR,^{34, 35} CYP27B1,³⁶ and 1,25(OH)₂D-mediated renal Ca reabsorption are observed.³⁷ The lack of 1,25(OH)₂D-mediated regulation of CYP24, TRPV5, and CYP27B1 mRNA in the kidney of KO/TG mice indicates that these mice are still VDR-deficient in the normally vitamin D-responsive distal tubule and that they can be used to reveal other interesting phenotypes related to renal vitamin D action and metabolism. For example, we found that serum 1,25(OH)₂D levels were not fully recovered in KO/TG mice even though serum Ca and PTH were normalized. In KO mice, VDR inactivation increased renal CYP27B1 mRNA and serum 1,25(OH)₂D level. However, even when CYP27B1 mRNA was normalized in KO/TG(H) kidney, serum 1,25(OH)₂D was still elevated. We believe that this is because renal CYP24 mRNA expression was still 75% lower than normal in KO/TG(H). These data suggest that circulating 1,25(OH)₂D levels are dependent upon the balance between 1,25(OH)₂D production mediated by CYP27B1 in the kidney and CYP24-mediated degradation there.

Another interesting renal phenotype we observed in KO/TG mice was that the urinary Ca:creatinine ratio was increased by nearly 3-fold compared to VDR +/- mice. This is consistent with Li *et al.*³⁸ who found that urinary Ca excretion was 2-fold higher in KO mice compared to wild type mice when both were fed a high Ca rescue diet. These data suggest that the

normalization of serum Ca in KO mice reveals a defect in the Ca reabsorption machinery of KO mouse kidney. We saw that renal calbindin D_{9k} and calbindin D_{28k} mRNA levels were significantly reduced in KO mice (by 95% and 50%, respectively) and that this was not restored in the KO/TG(H) mice (Figure 5A d). These proteins have previously been shown to be critical for renal Ca reabsorption.³⁹⁻⁴¹

The final VDR function revealed by our KO/TG mouse is related to bone. We found that bone density and volume in KO/TG(H) mice fed a high calcium chow diet were not only normalized but were increased relative to VDR^{+/+} mice. This was due to a significant increase in MAR and osteoblast number in the KO/TG mouse bone (Figure 7). This suggests that the predominant role for VDR in the growing mouse bone is to suppress bone formation. This conclusion is consistent with previous studies by Tanaka *et al.* who reported that femur from VDR knockout mice formed more bone when transplanted into wild type mice⁴² as well as Sooy *et al.*⁴³ who found that calvarial osteoblasts from KO mice displayed enhanced osteogenesis *in vitro*. As Sooy *et al.* speculated in their report, this is likely due to the suppressive effects that 1,25(OH)₂D has on expression of the runt-related transcription factor 2 (Runx2),⁴⁴ a critical regulator of osteoblast differentiation.⁴⁵ The increase of bone mineralization and volume we observed in KO/TG has not been observed in VDR knockout mice after normalization of ion homeostasis by feeding a high Ca/high lactose rescue diet.⁸⁻¹² We believe that this is because the rescue diet is traditionally fed after the development of secondary hyperparathyroidism in VDR knockout offspring.

In summary, our data show the critical importance of intestinal VDR and vitamin D-regulated Ca absorption for the control of whole-body Ca metabolism during growth, a time when Ca needs are maximal. Intestine-specific recovery of VDR completely normalized serum Ca, serum PTH, bone growth, and bone mineralization. In addition, our model reveals critical functions of VDR in bone and kidney, i.e. suppression of osteoblast formation and bone growth, renal control of circulating 1,25(OH)₂D levels and urinary Ca excretion. Because we have normalized Ca metabolism and growth in our KO/TG mice without the need to feed a special diet, we also believe our model will be a valuable tool to study the action of vitamin D in non-classical target organs. In addition to bone, intestine, and kidney, VDR is present in many other tissues including prostate, breast, pancreas, skin, reproductive organs, adipose tissue, and muscle (both cardiac and skeletal).^{46, 47} Using our KO/TG(H) mouse to study the role of VDR in these tissues without the confounding influence of disrupted Ca metabolism or growth will permit researchers to directly test the mechanistic foundation for the proposed relationships between vitamin D status and the prevention of cancer, diabetes, hypertension, and other chronic diseases.⁴⁸

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH awards DK054111 and CA101113 to JCF.

The authors would like to thank: Purdue University: Ms. Katherine Barzan-Smith and Ms. Rebecca McCreedy (Department of Foods and Nutrition, organ harvests), Ms. Yan Sun and Dr. Steve Konieczny (Department of Biology, histology), Dr. John Turek (School of Veterinary Medicine, collection of histological images). Indiana University School of Medicine: Mr. Qiwei Sun and Dr. Charles Turner (bone densitometry and pQCT); University of Tokyo: Dr. Shigeaki Kato for mVDR knockout mice; University of Michigan: Dr. Debra Gumucio for pUC12.4kb-villin plasmid, and Case Western Reserve University: Dr. Paul N. McDonald for pCDNA3.1-HA-hVDR plasmid.

Abbreviations

calbindin D_{9k}	calcium binding protein D _{9k}
calbindin D_{28k}	calcium binding protein D _{28k}
KO	VDR knockout
PTH	parathyroid hormone
TG	transgenic
TRPV6	transient receptor potential cation channel, subfamily V, member 6
TRPV5	transient receptor potential cation channel, subfamily V, member 5
VDR	vitamin D receptor
WT	wild type
1,25(OH)₂D	1 alpha, 25 dihydroxyvitamin D ₃

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Figure 1. Generation of intestine-specific, human VDR expressing, transgenic mice

(A) Transgene construct. A 12.4 kb villin promoter/enhancer was used to drive expression of a hemagglutinin (HA)-tagged-human VDR cDNA transgene in the intestine. Primers P1 and P2 were used for genotyping; primers P3 and P2 were used for assessing transgene mRNA levels. (B) Genotyping of transgenic offspring yields a 347 bp PCR product. Wild type mice = W; transgenic mice = T. (C) Transgene mRNA expression in intestinal segments of wild type (WT) and high transgene expressing mice (TG(H)) (Dd, duodenum; Je, jejunum; IL, ileum; Ce, cecum; CoP, proximal colon; CoD, distal colon). Transgene mRNA = 392 bp PCR product. (D) Transgene mRNA expression in a survey of mouse tissues.

Figure 2. Characterization of transgene mRNA and protein levels in two transgenic lines
 Transgene (TG) and total VDR mRNA levels in duodenum (A) and kidney (B) of wild-type (WT), low transgene-expressing, TG(L), and high transgene-expressing, TG(H), mouse lines were determined. Bars represent the mean $\pm$ SEM, $n=4$. * $p<0.05$ compared with WT, + $p<0.05$ compared with TG(L). (C) Total VDR protein levels in duodenum and (D) in different segments of intestine in WT and TG(H) mice were determined using a rat anti-VDR antibody by Western blot analysis as described in Materials and Methods. The band density values were measured by image J and are shown on the Western blot image relative to the values for the WT duodenum. (VDR STD: vitamin D receptor protein standard).

Figure 3. Gross appearance, growth curves and serum chemistries of VDR+/-, KO, KO/TG mice (A) Physical appearance and (B) Growth curves of 12-wk-old, male heterozygotes (VDR+/-), VDR knockout (KO), and KO mice recovered with high or low transgene expression, KO/TG (L) or KO/TG(H). Symbols represent the mean $\pm$ SEM, n=8, * p<0.05 compared with VDR+/- mice. (C) (a) serum calcium (Ca) and (b) parathyroid hormone (PTH) levels. Each value is the mean $\pm$ SEM (n=8 per genotype). * p<0.05 compared with VDR+/- mice. (c) Representative H&E stained histologic sections of parathyroid glands (arrows) and adjacent thyroid tissue. Original magnification=10X, scale bar = 254 μ m.

Figure 4. Intestinal phenotype of VDR+/-, KO and KO/TG mice

(A) Duodenal Ca absorption (a), CYP24 (b), calbindin D_{9k} (CaBP9K), and TRPV6 (c) mRNA levels in 8-wk-old female VDR+/-, KO and KO/TG(H) mice 9 h after treatment with 25 ng or 100 ng 1,25(OH)₂D/100 g body weight or vehicle (control). Bars represent the mean ± SEM, n=6-8. (B) CaBP9K, and TRPV6 mRNA levels in the proximal colon of 12-wk-old male VDR+/-, KO and KO/TG(H) mice (a) and in 8-wk-old female VDR+/-, KO and KO/TG(H) mice (b) 9 h after treatment with 25 ng 1,25(OH)₂D/100 g body weight or vehicle (control). Each value is the mean ± SEM (n=6-8 per genotype). * *p*<0.05 compared with control-treated VDR+/-, # *p*<0.05 compared with 1,25(OH)₂D-treated VDR+/- mice, + *p*<0.05 compared with control-treated KO/TG(H), \$ *p*<0.05 compared with 25 ng/100g BW 1,25(OH)₂D treated KO/

TG(H). mRNA levels were assessed by real time PCR analysis, normalized to the GAPDH mRNA level, and expressed relative to levels of VDR^{+/-} mice.

Figure 5. Renal phenotypes of VDR+/-, KO and KO/TG mice

(A) 12-wk old male VDR+/-, KO and KO/TG(L) and KO/TG(H) were examined for basal levels of: (a) serum 1,25(OH)₂D, (b) renal CYP27B1 and CYP24 mRNA levels, (c) Urine calcium (Ca)/creatinine ratio, and (d) Renal calbindin D_{9k} (CaBP9K) and calbindin D_{28k} (CaBP28K) mRNA level. Each value is the mean $\pm$ SEM (n=8 per genotype). * $p < 0.05$ compared with VDR+/- mice, # $p < 0.05$ compared with KO mice, + $p < 0.05$ compared with KO/TG(L) mice. (B) 8-wk-old female VDR+/-, KO and KO/TG(H) mice treated with 25 ng 1,25(OH)₂D/100 g body weight or vehicle (control) for 9 h and renal RNA was examined for: (a) CYP24, (b) CYP27B1 and (c) TRPV5 mRNA levels by real-time RT-PCR analysis. Values were normalized to GAPDH mRNA level and are expressed relative to levels in control-treated VDR+/- mice. Bars represent the mean $\pm$ SEM, n=6-8. * $p < 0.05$ compared with control-treated VDR+/- mice, # $p < 0.05$ compared with 1,25(OH)₂D-treated VDR+/- mice, + $p < 0.05$ compared with control-treated KO/TG(H).

Figure 6. Skeletal phenotype of VDR+/-, KO, KO/TG(L) and KO/TG(H) mice

(A) Femoral bone mineral density (BMD) of 12-wk-old male of VDR+/-, KO and KO/TG(L) and KO/TG(H) was determined using dual energy x-ray absorption (DEXA). Bars represent the mean $\pm$ SEM, n=8. * $p < 0.05$ compared with VDR+/- mice. (B) Representative contact radiographs of the femurs. Arrows are pointing to epiphysis, metaphysis and diaphysis (left to right).

Figure 7. Static and dynamic histomorphometric analysis of VDR+/-, KO and KO/TG(H) mice
Decalcified paraffin-embedded femoral sections from 12-wk-old male VDR+/-, KO and KO/TG(H) were examined for (A, F a) trabecular bone volume (trabecular bone volume/total tissue volume) by total collagen staining; (D, F d) osteoblast number (cell number/tissue area, #/mm²) from H&E stained sections; and (E, F e) osteoclast number (cell number/tissue area, #/mm²) from TRAP staining. Calcified, plastic-embedded femoral sections from 8-wk-old female VDR+/-, KO and KO/TG(H) mice treated twice with calcein were used for (B, F b) mineralization (osteoid volume/total bone volume, OV/BV) from von Kossa staining and (C, F c) mineral apposition rate (MAR). Representative micrographs of histological sections are shown in the left panels (A, B, C, D, E). The original magnification in these sections is: 4, 40, 20, 60 and 40X, respectively and the scale bars represent 254, 127, 100, 127, and 127 μm, respectively. Arrows are pointing to growth plate (A), osteoid (B), calcein labeling (C), osteoblast (D) and osteoclast (E). (F) Quantification of these data is presented in the graphs. Bars represent the mean ± SEM, n=8. * p<0.05 compared with VDR+/- mice, # p<0.05 compared with KO mice.

Table 1**Mid-shaft and Distal Femur pQCT Parameters**

	VDR+/-	KO	KO/TG(H)
Midshaft			
Total Area (mm ²)	1.60±0.06	1.07±0.03 [*]	1.88±0.08 ^{*#}
Cortical Area (mm ²)	0.76±0.04	0.07±0.01 [*]	0.86±0.03 ^{*#}
IP (mm ⁴)	0.45±0.03	0.26±0.02 [*]	0.62±0.06 ^{*#}
Distal (30%)			
Total Area (mm ²)	1.91±0.05	1.28±0.13 [*]	2.25±0.11 ^{*#}
Trabecular Area(mm ²)	1.07±0.12	0.65±0.15 [*]	1.27±0.06 ^{*#}

VDR knockout (KO), and KO mice recovered with high level transgene expression (KO/TG(H)). Values represent the mean ± SEM, n=8.

^{*} p<0.05 compared with VDR+/- mice,

[#] P<0.05 compared with KO mice.

IP: polar moment of inertia