The vitamin D receptor is not required for fetal mineral homeostasis or for the regulation of placental calcium transfer in mice

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Abstract

We utilized a vitamin D receptor (VDR) gene knockout model to study the effects of maternal and fetal absence of VDR on maternal fertility, fetal-placental calcium transfer and fetal mineral homoeostasis. *Vdr* null mice were profoundly hypocalcemic, conceived infrequently, and had significantly fewer viable fetuses *in utero* which were also of lower body weight. Supplementation of a calcium-enriched diet increased the rate of conception in *Vdr* nulls but did not normalize the number or weight of viable fetuses. Among offspring of heterozygous (*Vdr*+/−) mothers (WT, *Vdr*+/− and *Vdr* null fetuses), there was no alteration in serum Ca, P, Mg, parathyroid hormone, placental 45Ca transfer, Ca and Mg content of the fetal skeleton, and morphology and gene expression in the fetal growth plates. *Vdr* null fetuses did have 3-fold increased 1,25-dihydroxyvitamin D levels accompanied by increased 1α-hydroxylase mRNA in kidney but not placenta; a small increase was also noted in placental expression of parathyroid hormone-related protein (PTHrP). Among offspring of *Vdr* null mothers, *Vdr*+/− and *Vdr* null fetuses had normal ionized calcium levels and a skeletal ash weight that was appropriate to the lower body weight. Thus, our findings indicate that VDR is not required by fetal mice to regulate placental calcium transfer, circulating mineral levels, and skeletal mineralization. Absence of maternal VDR has global effects on fetal growth that were partly dependent on maternal calcium intake, but absence of maternal VDR did not specifically affect fetal mineral homeostasis.

Key words: fetus, vitamin D receptor, calcitriol, placental calcium transfer, skeletal mineralization, mineral homeostasis, parathyroid hormone-related protein, parathyroid hormone, calbindin-D9k, Ca2+-ATPase, calcium transporter 1 and 2, 1α-hydroxylase, placenta
Introduction

It is well appreciated that vitamin D, and more importantly its active metabolite calcitriol or 1,25-dihydroxyvitamin D (1,25-D), is critically required for normal mineral and skeletal homeostasis in neonatal and adult mammals. For example, deficiency of vitamin D in children results in hypocalcemia and the characteristic skeletal deformities of rickets, whereas deficiency of vitamin D in adults results in hypocalcemia and osteomalacia.

In contrast to the adult, very little and conflicting information has been known about the possible role of 1,25-D in regulating fetal mineral metabolism (24,25). The receptor for 1,25-D (vitamin D receptor or VDR) is expressed early and widely during fetal development in a manner that would predict its importance for skeletal development: it appears on day 13 of gestation in the rat, in the mesenchyme that will subsequently condense to form the skeletal tissues, and by day 17 of gestation it is expressed in proliferating and hypertrophic chondrocytes, and osteoblasts of limb buds and the vertebral column (20). The VDR is also expressed in parietal cells of the intraplacental yolk sac in mice (27), where (analogous to its role in the adult intestine) it is well positioned to potentially regulate the expression of calcium transporters and binding proteins and, thereby, the flow of calcium from mother to fetus. The fetal kidneys and placenta both synthesize 1,25-D (57,62), and yet circulating 1,25-D levels are lower than the maternal level in late gestation of humans, rats and mice (13,18,28,52,63). This lower circulating level may be a response to the low parathyroid hormone (PTH) and high phosphorus levels that occur in fetuses (24,25), but may also indicate that it is not essential to the fetus. As in the adult, the fetal kidneys are capable of upregulating the synthesis of 1,25-D in response to PTH, because in the model of calcium sensing receptor gene (Castr) ablation, a step-wise increase in PTH levels was associated with a step-wise increase in 1,25-D levels (28).
Several experimental models have suggested that 1,25-D is required for normal fetal calcium and bone homeostasis. A 72 hr infusion of a crude polyclonal antiserum raised against 1,25-D decreased the ovine fetal blood calcium level (50). Bilateral nephrectomy in fetal sheep removed the main source of 1,25-D in the fetus, and resulted in reduced ionized and total calcium, and increased phosphorus and PTH levels; these changes were reversed by administration of 1,25-D to the fetus (44). In contrast to these studies, experimental vitamin D deficiency in rats and sheep caused maternal hypocalcemia, but the fetuses maintained normal blood calcium and phosphorus levels and had normally mineralized skeletons at term, as assessed by total weight, ash weight, and (in fetal sheep) calcium content of ashed femurs (6,14,15,38,43). Similarly, the 1-hydroxylase-deficient Hannover pig model (33) demonstrated that fetuses of homozygous 1,25-D-deficient sows maintained normal blood calcium and phosphorus levels and appeared to fully mineralize their skeletons by the time of birth (33). In both the vitamin D deficiency models and the Hannover pig model, the fetal skeletal response to vitamin D deficiency was not examined closely beyond measuring the ash weight and content, nor was placental calcium transport directly assayed.

Similar to the limited and conflicting animal data, there is only limited data from humans about whether vitamin D or 1,25-D is needed for normal fetal calcium and bone metabolism. At term, cord blood calcium and skeletal mineralization have appeared to be normal in the offspring of vitamin D-deficient mothers (7,48,56). Study of neonates in North America has suggested that it is only in the first or second week after birth that hypocalcemia develops in such offspring; skeletal demineralization and other rachitic changes are typically not detectable until one or two months of age (25,29). However, case series from countries in which profound vitamin D
deficiency is endemic have suggested that skeletal changes may be observable at birth (widely separated sutures, large anterior fontanelle, and wide wrists) (58)

In view of the limited and conflicting animal and human data about the role of vitamin D or 1,25-D in fetal mineral homeostasis, the purpose of these studies was to test the hypothesis that the lack of VDR expression in fetal tissues does not caused marked impairment of fetal mineral homeostasis. We utilized the murine knockout of the VDR gene (Vdr) for this approach. In this model, VDR is absent and resistance to the known genomic actions of 1,25-D is present; loss of VDR will not block actions of 1,25-D that might not rely on the VDR. This model allowed the assessment of fetal need for VDR (and, therefore, indirectly for 1,25-D) by contrasting the phenotypes of normal (WT), heterozygous-deleted (Vdr\textsuperscript{+/-}), and completely ablated (Vdr null) fetuses. In addition, because Vdr null females survive but are subfertile, we were able to determine if the fetus would be affected by maternal ablation of Vdr and the resultant hypocalcemia.
Methods

Animal Husbandry

Vdr null mice were obtained by targeted disruption of the murine gene in embryonic stem cells, as previously described (36). The original strain was back-crossed into Black Swiss (Taconic, Germantown, NY) for at least four generations, and the colony was maintained through breeding heterozygous mice together. Vdr\(^{+/-}\) males and females were mated to create pregnancies in which WT, Vdr\(^{+/-}\), and Vdr null fetuses were present. Vdr\(^{+/-}\) males and Vdr null females were also mated to yield pregnancies with Vdr\(^{+/-}\) and Vdr null fetuses. The Vdr\(^{+/-}\) and Vdr null females were first degree relatives of each other. Mice were mated overnight after demonstrating evidence of estrus in chosen females; the presence of a vaginal mucus plug on the morning after mating marked gestational day 0.5. Normal gestation in these mice is 19 days. All studies were performed with the prior approval of the Institutional Animal Care Committee of Memorial University of Newfoundland.

Genomic DNA was obtained from fetal tails, and genotyping was accomplished by PCR using primers that were specific to the Vdr gene sequence and the neomycin cassette, in a single-tube, 36-cycle PCR reaction utilizing a PTC-200 Peltier Thermal Cycler (MJ Research, Cambridge, MA). The nucleotide sequences for Vdr PCR primers were (forward and reverse, respectively): 5’ CTG CCC TGC TCC ACA GTC CTT 3’ and 5’ GCA GAC TCT CCA ATG TGA AGC 3’.

At the time of each cesarian section (day 17.5 or 18.5 of gestation), the uterus was completely excised and the number of viable fetuses present were counted. The number of viable fetuses in utero is normally higher than the litter size observed 6-24 hours after birth, due to early culling of the litter by the mother, and neonatal deaths due to other causes.
Diet

The mice routinely received a standard chow diet which contained 1% calcium (LabDiet 5P00, PMI Nutrition International, Richmond, IN). To determine the effect of increased calcium intake on fetal number and size, an enriched diet containing 2% calcium and 20% lactose (TekLad TD96348, Harlan Teklad, Madison, WI) was substituted for at least two weeks prior to mating, and this diet was maintained through the subsequent pregnancy. Lactose increases intestinal calcium absorption by means that do not require the VDR, and this diet has been previously been shown to be effective in Vdr null mice (35).

Assays of Hormones and Minerals

Whole blood, serum and amniotic fluid were collected using methods previously described (31). Ionized calcium was measured on whole blood using a Chiron Diagnostics 634 Ca++/pH Analyzer (Chiron Diagnostics, East Walpole, MA). Serum PTH was measured on embryonic day (ED) 18.5 fetuses using a rodent PTH 1-34 Elisa kit (Immutopics, San Clemente, CA); the stated detection limit of the assay was 1.6 pg/ml. Serum 1,25-D was measured using an RIA kit with sera from 4 or 5 genetically identical fetuses pooled together to obtain the 125 µl sample size (Immunodiagnostic Systems Ltd., Boldon, Tyne and Wear, UK). No pooling was required for maternal samples. Total calcium, magnesium and phosphorus were measured as previously described (31) using the respective calcium, magnesium and phosphorus colorimetric assay kits (Sigma-Aldrich, Oakville, ON) in which serum or amniotic fluid from each fetus constituted one sample (Sigma-Aldrich, Oakville, ON).
Placental Calcium Transfer

This procedure has been described in detail elsewhere (30). Briefly, pregnant dams on ED 17.5 were given an intracardiac injection of 50 µCi $^{45}$Ca and 50 µCi $^{51}$Cr-EDTA. Five minutes later, the dam was sacrificed, and each fetus was removed. The ratio of $^{45}$Ca to $^{51}$Cr radioactivity was determined for each fetus using a liquid scintillation counter, respectively. The data were normalized to the mean $^{45}$Ca/$^{51}$Cr activity ratio of the $Vdr^{+/−}$ fetuses in each litter, in order that the results from different litters could be compared.

Tissue Collection

For in situ hybridization and immunohistochemistry of fetal bones, whole fetuses from $Vdr^{+/−}$ mothers (ED 17.5 or 18.5) were placed in 10% formalin after first incising the abdomen to prevent its gaseous expansion. After 12-24 hours in the fixative, the lower limbs were removed and separately processed, embedded in paraffin, and cut into 5 micron sections.

WT and $Vdr$ null placentas were obtained from $Vdr^{+/−}$ mothers at ED 16.5 to 18.5, because this timeframe corresponds to the interval of rapid maternal-fetal transfer of calcium across the placenta (24,25). Placentas were obtained after placental perfusion with paraformaldehyde in order to minimize the degradation in mRNA or protein levels that might occur during fixation and processing of the RNase and protease-rich placental tissues (31). Following this, the placentas were removed and placed in 10% formalin for standard processing, embedding in paraffin, and sectioning.

For RNA studies and the Western blot, WT, $Vdr^{+/−}$ and $Vdr$ null placentas and fetal kidneys were obtained on ED 18.5, snap frozen in liquid nitrogen, and stored at –80°C until used. To harvest RNA, frozen tissue samples were placed directly into RNeAlater RNA
Stabilization Reagent (Ziagen, Mississauga, ON), and homogenized for 1 minute with a tissue homogenizer at high speed. Total cellular RNA isolation was performed using the RNeasy Midi Kit (Qiagen). Protein extraction for the Western blot was done as detailed below.

**Fetal Ash and Skeletal Mineral Assay**

Using methods previously described (26), intact fetuses (ED 18.5) were reduced to ash in a furnace (500°C x 24 hr), and the ash was assayed for calcium and magnesium on a Perkin Elmer 2380 Atomic Absorption Flame Spectrophotometer. Since fetal size varied from litter to litter and would affect the individual measurements (large litter, smaller fetuses; small litter, larger fetuses), the data were also analyzed after normalizing to the mean value of the heterozygotes within each litter. The heterozygotes were chosen as the baseline for this comparison since, on average, they accounted for 50% of the fetuses in a given litter.

**Riboprobe labeling**

For *in situ* hybridization, plasmids containing cDNAs were linearized with appropriate restriction enzymes, and labeled with 125 µCi of $^{35}$S-UTP using an SP6/T7 Transcription Kit (Promega/Fisher Scientific Ltd, Burlington, ON), and the appropriate polymerase. Unincorporated nucleotides were removed with NucTrap columns (Stratagene, La Jolla, CA).

Gene expression in the cartilaginous growth plate and adjacent bone (periosteum and endosteum) was examined using the following cDNAs: pro-1(I) chain of human type I collagen (4), pro-1(II) chain of rat type II collagen (23), H4 histone (53), and mouse type X collagen (2) (gifts of K. Lee, Massachusetts General Hospital [MGH]); mouse osteocalcin (10), rat osteopontin (45), rat alkaline phosphatase (59), and murine cartilage matrix protein (matrilin 1)
(3) (gifts of B. Lanske, MGH); murine interstitial collagenase (17), and murine 92-kDa gelatinase (type IV collagenase or MMP-9) (49) (gifts of S.M. Krane, MGH).

Placental cDNAs used included murine calbindin-D$_{9k}$ (42) (gift of S. Christakos, University of Medicine and Dentistry of New Jersey); human Ca$^{2+}$-ATPase (32) (gift of R. Kumar, Mayo Clinic); murine alpha-fetoprotein (gift of Margaret Baron, Mount Sinai School of Medicine); murine placental lactogen (19) and murine proliferin (39) (gifts of D. Linzer, Northwestern University); and murine nodal (66) (gift of M. Kuehn, National Institutes of Health).

**Real time PCR**

cDNA synthesis was performed using the Superscript III First-Strand Synthesis System (Invitrogen). Approximately 100 ng of total RNA, 1 mM dNTP mix, and 5 ng/µl random primers were incubated at 65°C for 5 minutes and chilled on ice. A reaction mixture was added to a final concentration of 1X RT Buffer (20 mM Tris-HCl, pH8.4; 5 mM KCl), 5 mM MgCl$_2$, 0.01 mM DTT, 2 U/µl RNaseOut and 20 U/µl Superscript III RT and incubated for 10 minutes at 25°C followed by a 50 minute incubation at 50°C. 1 µl of 2 U/µl Rnase H was added and incubated for 20 minutes at 37°C.

Real Time PCR data and analysis were obtained using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). 2.0 µl of cDNA reaction, 830 pmol/µl of sequence specific primers, and SYBR Green PCR Master Mix (Applied Biosystems) were incubated at 95°C for 15 seconds and 60°C for 1 minute for a total of 40 cycles. Nucleotide sequences for GAPDH PCR primers (forward and reverse, respectively) were: 5’ TTG TCA TCA ACG GGA AGC CCA TCA 3’ and 5’ TCT CGT GGT TCA CAC CCA TCA CAA 3’; for PTHrP were: 5’
In situ hybridization

In situ hybridization was performed on 5 micron tissue sections as described previously (34). Hybridization was performed in a humidified chamber (16h, 55°C) with the labeled riboprobe diluted 1:20 in the hybridization solution. Sections were successively washed, RNAse treated, and dehydrated in graded ethanol series. An overnight exposure of the slides to plain x-ray film enabled an estimate of exposure time for the liquid emulsion step. Slides were then dipped into NTB-2 liquid emulsion, dried, stored in light-tight boxes, and kept at 4°C until developed (2 to 6 weeks). The emulsion was developed using standard developer and fixer, and the sections were counterstained with hematoxylin-eosin.

All comparisons of WT to Vdr null were made between tissues obtained from within the same litter, and which had been processed, embedded and sectioned at the same time. All comparative sections were always hybridized together with the same probe, and washed together, in order to validate the comparison and to minimize artifacts. Assessments of signal intensity were determined in a blinded fashion (no knowledge of the genotype). The reproducibility of the results was confirmed independently on at least three separate litters.

Protein Extraction and Quantification
Placentas were rinsed with PBS, homogenized for 1 minute in 1 mL of lysis buffer (20 mM Tris pH 7.4-7.8, 10% glycerol, 137 mM NaCl, 0.1% SDS, 1% Triton X, 2 mM EDTA, 0.5% sodium deoxycholate, 1 mM aprotinin, 1 mM phenylmethylsulphonyl fluoride [PMSF]) and incubated on a shaker overnight at 4°C. The samples were then centrifuged at 10000 rpm for 15 minutes and the supernatant was collected and stored at –70°C. The protein samples were diluted 1:5 with deionized water and quantified using the Pierce BCA Protein Assay kit (MJS Biolynx, Brockville, ON). 25 µl of standard or sample was added to 200 µl of working reagent in a 96 well plate, incubated at 37°C for 30 minutes and the absorbance was obtained at 570 nm using a Vmax kinetic microplate reader (Molecular Devices, Sunnyvale, CA).

Western Blotting and Immunodetection

Electrophoresis and transfer were performed using equipment, buffers and gels associated with the NUPAGE electrophoresis system (Invitrogen, Burlington, ON). Sample buffer, antioxidant and 100 µg of protein were heated to 70°C for 10 minutes and run on a 4-12% Tris-Bis gel at 200V for 35 minutes. The gels were transferred onto a polyvinylidene difluoride (PVDF) membrane at 30 V for one hour. The membranes were air dried, wrapped in plastic and stored at room temperature.

The Western Breeze Chemiluminescent Immunodetection kit (Invitrogen) was used for protein detection. Washes and incubations were performed on a rotary shaker at 1 rev/sec at room temperature. Membranes were soaked for 30 seconds in methanol, rinsed twice with ultrapure water and blocked for 30 minutes in Blocking Solution. The membranes were washed twice for five minutes in water and incubated for one hour with either rabbit anti-PTHrP or rabbit anti-Actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500. Following
four washes with buffered saline, the blots were incubated with alkaline phosphatase (AP) conjugate anti-rabbit IgG antibodies for 30 minutes. Again, the blots were washed with buffered saline four times, followed by two water rinses. CDP-Star chemiluminescent substrate was added to the membrane and incubated for five minutes, blotted with filter paper, and exposed to Kodak Xomat LS x-ray film (Amersham) for 20 seconds to five minutes.

**Histology**

5 micron sections were deparaffinized, rehydrated in a graded ethanol series, and transferred to distilled water. For morphological assessment of the growth plate, sections were stained with hematoxylin and eosin, or 1% methyl green, then dehydrated and mounted. For von Kossa staining, the sections were transferred to 1% aqueous silver nitrate solution and exposed for 45 min under a strong light. They were then washed thrice in distilled water, placed in 2.5 % sodium thiosulphate (5 min), and washed thrice again in distilled water. Finally, they were counterstained with methyl green, dehydrated in 1-butanol and xylene, and mounted.

**Immunohistochemistry**

PTHrP immunohistochemistry was performed as previously described (31) using rabbit anti-PTHrP[1-14] (22) diluted 1:200 in PBS/NBS, and applied at 20 °C x 60 min (this step omitted from control sections). The primary antibody was detected using biotinylated goat anti-rabbit (Vector, Burlington, ON), ABC reagent (Vector), and DAB-Tris-peroxidase substrate (Vector). Sections were counterstained, washed, dehydrated, and mounted.

**Alizarin Red S and Alcian Blue Preparations**
Fresh fetuses from Vdr<sup>+</sup> mothers (ED 18.5) were obtained and the skin, viscera and adipose tissue were carefully removed. In individual scintillation vials, the fetuses were fixed in 95% EtOH for 5 days, followed by acetone for 2 days to remove the remaining fat and firm up the specimen. Following this, the fetuses were stained for 3 days in 10 ml of freshly prepared staining solution at 37 °C (1 volume 0.3% Alcian blue 8GS in 70% EtOH: 1 volume 0.1% Alizarin red S in 95% EtOH: 1 volume acetic acid: 17 volumes 70% EtOH). They were then washed in distilled water, and then immersed in 1% aqueous KOH until the fetal skeleton was clearly visible through the surrounding tissue (approximately 12-48 hr). They were cleared in 1% KOH containing increasing concentrations (20, 50 and 80%) of glycerine (7-10 days at each step). Finally, they were transferred into 100% glycerine for permanent storage.

**Statistical Analysis**

Data was analyzed using SYSTAT 5.2.1 for Macintosh (SYSTAT Inc, Evanston, IL). ANOVA was used for the initial analysis; Tukey’s test was used to determine which pairs of means differed significantly from each other. Real time PCR results were analyzed by the 2<sup>-ΔΔCt</sup> method where the target and reference are amplified in separate wells (40). Two-tailed probabilities are reported, and all data are presented as mean ± SE.
Results

*Maternal Effects of Vitamin D Receptor Ablation*

Despite mating as readily as their WT and Vdr\(^{+/−}\) siblings (as judged by the presence of a vaginal mucous plug on the morning after a timed mating), Vdr null mothers conceived at 5-10% of the frequency of their siblings. Furthermore, when Vdr null mice did conceive, they had significantly fewer viable fetuses *in utero* (Figure 1A). This result is consistent with experimental models of vitamin D deficiency in pregnancy (15,16). No difference in the number of resorption sacs or nonviable fetuses were noted between Vdr\(^{+/−}\) and Vdr null pregnancies, indicating that the decrease in gestational litter size occurs before the embryonic stage. Also, Vdr\(^{+/−}\) and Vdr null fetuses were present in the expected 1:1 Mendelian ratio, indicating that the reduction in gestational litter size was not specific to any genotype. To determine if the reduced gestational litter size could be corrected by improving the calcium intake, additional Vdr null mothers were placed on a calcium and lactose enriched diet at least two weeks prior to mating. On the enriched diet, the frequency of confirmed pregnancy following a confirmed mating was over 80%, no different from the overall frequency for WT mice in our laboratory. The mean number of viable fetuses from such calcium-supplemented Vdr null dams (Figure 1B) was intermediate between that of Vdr\(^{+/−}\) and Vdr null mothers who consumed the normal chow (Figure 1A), and not significantly different from either value.

Maternal ablation of VDR also resulted in fetuses of lower weight. Among Vdr\(^{+/−}\) mothers, the fetal weight was 1.10 ± 0.03 g in WT, 1.09 ± 0.03 g in Vdr\(^{+/−}\), and 1.11 ± 0.03 in Vdr null. Among Vdr null mothers, the fetal weight was 0.80 ± 0.03 g in Vdr\(^{+/−}\) and 0.74 ± 0.03 g in Vdr null (p<0.0001 vs. weight of counterpart from Vdr\(^{+/−}\) mothers). The fetuses from Vdr null mothers were phenotypically indistinguishable from each other and from their counterparts from
Vdr\(^{+/+}\) mothers, apart from their smaller size and body weight. Treatment of Vdr null mothers with a calcium-enriched diet resulted in an intermediate increase in the fetal weight, to 0.91 ± 0.02 g in Vdr\(^{+/+}\) and 0.93 ± 0.02 g in Vdr null (p<0.001 versus weight of counterpart from Vdr\(^{+/+}\) mothers, and p<0.01 versus weight of counterpart from Vdr null mothers).

As has been previously demonstrated (36), maternal ablation of the Vdr significantly reduced the serum ionized calcium to 0.71 ± 0.05 mmol/l as compared to WT and Vdr\(^{+/+}\) (1.23 ± 0.05 mmol/l). In our laboratory, normal mice in the Black Swiss background maintain a normal ionized calcium throughout pregnancy and lactation (data not shown). The maternal ionized calcium remained unchanged in WT or Vdr\(^{+/+}\) during late pregnancy, but dropped slightly in Vdr null to 0.62 ± 0.05 mmol/l (p = not significant). Despite the profound hypocalcemia in the Vdr null, no maternal seizures, tetany or deaths were noted to occur in late pregnancy.

**Placental Calcium Transfer**

Fetal absence of VDR did not impair placental calcium transfer at 5 minutes; instead, there was a non-significant trend for the rate of placental calcium transfer to increase modestly among the fetuses of Vdr\(^{+/+}\) mothers, from WT to Vdr\(^{+/+}\) to Vdr null (p<0.09 by ANOVA; p<0.05 on separate t-test comparing WT and Vdr null alone) (Figure 2). Due to far fewer available pregnancies, placental calcium transfer could only be measured in two Vdr null mothers. Again, a non-significant trend to increased placental calcium transfer was observed in Vdr null fetuses (169 ± 33.6% versus 100 ± 36.3% in Vdr\(^{+/+}\) fetuses). Analysis of pooled data from Vdr\(^{+/+}\) and Vdr null mothers resulted in a statistically significant difference among all three genotypes: WT 95.4 ± 9.2%, Vdr\(^{+/+}\) 101.5 ± 5.7%, and Vdr null 128.4 ± 7.9% (p< 0.01 by ANOVA).
Serum and Amniotic Fluid Mineral Levels

The minimal effect of VDR ablation on the fetus extended to serum mineral levels. WT, Vdr<sup>+/−</sup> and Vdr null fetuses had the same ionized calcium level, which was maintained above the ambient maternal ionized calcium level as is normal for mammalian fetuses (Figure 3A). Even in the severely hypocalcemic Vdr null mothers, Vdr<sup>+/−</sup> and Vdr null fetuses maintained a normal ionized calcium level, indistinguishable from that of the fetuses of Vdr<sup>+/−</sup> mothers (Figure 3B). Due to the maternal hypocalcemia, the corresponding maternal-fetal calcium gradient was significantly (p<0.001) increased in fetuses obtained from Vdr null mothers as compared to normal (Figure 3B).

Similar to the fetal ionized calcium levels, serum magnesium and phosphorus were unaltered among WT, Vdr<sup>+/−</sup> and Vdr null fetuses (Table 1). The normal phosphorus levels are a striking contrast to the low phosphorus levels seen in adult humans and mice that have inactive VDRs.

PTH is upregulated significantly in states of vitamin D insufficiency and deficiency, and in the absence of the VDR in both adult humans and adult Vdr null mice. Unlike the adult, Vdr null fetuses had normal serum PTH levels that were indistinguishable from WT and Vdr<sup>+/−</sup> siblings (Table 1).

Serum 1,25-D levels were unaltered in Vdr<sup>+/−</sup> as compared to WT fetuses, but were significantly increased in Vdr null fetuses (Figure 4). By comparison, adult mice show a step-wise increase in 1,25-D from WT to Vdr<sup>+/−</sup> to Vdr null (p<0.001), and the peak level achieved in adult Vdr null mice is almost twice that achieved in Vdr null fetuses (Figure 4). To determine if kidney or placenta was the source of the increase in 1,25-D, mRNA for 1-hydroxylase was
amplified using quantitative real time PCR on kidney and placenta of WT and Vdr null fetuses, and was found to be increased in Vdr null kidney (Figure 5A), but not in placenta (Figure 5B).

Amniotic fluid is made largely from fetal urine, and is a surrogate measure of mineral excretion by the fetal kidneys. No differences in the amniotic fluid volume or content of calcium, magnesium or phosphorus were noted (Table 2).

**Skeletal Morphology and Mineral Content**

The fetal skeleton is intimately involved in calcium metabolism and it mineralizes during late gestation. Impairment of fetal mineral homeostasis will lead to (or be associated with) abnormalities in skeletal mineralization, growth plate gene expression, and bone turnover, as we have previously demonstrated in other murine models (26,28,31,34). Therefore, the fetal skeleton was examined to determine if lack of VDR altered skeletal development, skeletal mineral content, or gene expression within the growth plate and adjacent bone.

The gross morphology and mineralization of the fetal skeleton were examined using intact but cleared fetuses that had been stained with alcian blue (for cartilage) and alizarin red S (for mineralized bone). The fetal skeletons exhibited no gross abnormalities, including normal length and morphology of the long bones in the appendicular skeleton (Figure 6A, B). The relative distribution of mineral also appeared to be normal.

The mineral content of fetal skeletons was determined by obtaining the ash weights of fetuses (which represents largely mineral), and then assaying the ash to determine the calcium and magnesium content of it. No differences were found in ash weights of fetuses of Vdr<sup>+/−</sup> mothers, which were 21.4 ± 0.7 mg in WT, 20.1 ± 0.6 mg in Vdr<sup>+/−</sup>, and 20.5 ± 0.8 mg in Vdr null. The ash weights of fetuses from Vdr null mothers were not significantly different from
each other, but were significantly lower than the corresponding genotypes of $Vdr^{+/c}$ mothers at 12.5 ± 1.3 mg in $Vdr^{+/c}$, and 12.2 ± 1.3 mg in $Vdr$ null (p < 0.001 vs. counterpart). When expressed as percent of fetal weight, the relative skeletal weights were not significantly different between $Vdr$ null fetuses of $Vdr^{+/c}$ mothers and $Vdr$ null fetuses of $Vdr$ null mothers (1.8 ± 0.1 % vs 1.7 ± 0.1 % respectively, p = not significant). Treatment of $Vdr$ null mothers with a calcium-enriched diet resulted in a fetal ash weight to 12.6 ± 0.5 mg in $Vdr^{+/c}$ and 13.9 ± 0.5 mg in $Vdr$ null fetuses (p= not significant versus counterpart on regular diet). Thus, absence of VDR within the fetus did not alter skeletal mineral content as compared $Vdr^{+/c}$ or WT siblings, whereas absence of VDR within the mother resulted in smaller fetuses with proportionately smaller ash weights, but no difference in ash weight between $Vdr^{+/c}$ and $Vdr$ null.

The ash of fetuses obtained from $Vdr^{+/c}$ mothers was further analyzed by atomic absorption spectroscopy to specifically determine its calcium and magnesium content (Table 3). Results were calculated in mg of calcium or magnesium per gram of ash. The calcium and magnesium content of the fetal skeletons did not differ among WT, $Vdr^{+/c}$ or $Vdr$ null fetuses, confirming the result of the ash weight.

**Growth plate morphology and gene expression**

The growth plates were examined histologically and the distribution of mineral within the long bones was examined semi-quantitatively using the von Kossa method. In this method, silver displaces calcium to create black deposits of silver phosphate and silver carbonate; since calcium is the only known cation that binds to these insoluble anions in organic tissue, the method is considered to be sufficiently specific for calcium (5,46). Using this method, the mineral present in $Vdr$ null growth plates appeared to be distributed normally as compared to
WT siblings (Figure 6C, D). The length of the growth plate, the morphology of the growth plate from the proliferative to the hypertrophic zones of chondrocytes, and the thickness of the periosteum also did not differ among WT or Vdr null fetuses (Figure 6C, D).

The growth plates and adjacent bones were further examined by in situ hybridization to determine if genes expressed by chondrocytes, osteoblasts, osteoclasts, and the matrix were altered by the absence of VDR. Representative sections are shown in Figure 6 E through N. The distribution pattern and intensity of expression of the mRNAs for type I, II and X collagens, H4 histone, cartilage matrix protein (matrilin 1), osteoblast markers (osteopontin, osteocalcin, interstitial collagenase, and alkaline phosphatase), and an osteoclast marker (92-kDa gelatinase or type IV collagenase or matrix metalloproteinase-9) were all examined. No differences were seen between WT and Vdr null growth plates (Fig 6 E through N, and data not shown).

Placental Morphology, Gene Expression, and Immunohistochemistry

To determine if absence of VDR affected placental structure or function, we examined placental morphology at the gross and cellular level, and further examined the relative expression of trophoblast specific markers, and calbindin-D_{9k}, Ca^{2+}-ATPase, by in situ hybridization. No gross abnormality of placental weight, structure or cellular morphology was evident. By light microscopic examination, the amount and distribution of the intraplacental yolk sac was normal (Figure 7 A and B versus E and F). Calbindin-D_{9k}, an important calcium binding protein that is required for placental calcium transfer, showed no alteration in location or intensity of expression in Vdr null placentas as compared to their wild type siblings (Figure 7 C versus G). Similarly, Ca^{2+}-ATPase showed no alteration in location or intensity of expression in Vdr null placentas when compared to their wild type siblings (Figure 7 D versus H). The relative
expression of the trophoblast markers placental lactogen, nodal, and proliferin, and the extraplacental yolk sac marker alpha-fetoprotein, were unaltered in Vdr null versus WT placentas (data not shown).

PTHrP is known to stimulate placental calcium transfer, and since the rate of placental calcium transfer showed a non-significant increase in Vdr null fetuses, we examined the expression of PTHrP within placenta to determine if it was altered in the absence of VDR. PTHrP was visibly but modestly increased in the Vdr null placenta as seen by immunohistochemistry, with the most intense expression noted in the intraplacental yolk sac (Figure 8 A-D). Quantitative real time PCR on placental mRNA confirmed a statistically significant increase in PTHrP mRNA in Vdr null versus WT placentas (Figure 8 E). Western blot of total placental protein showed no difference in PTHrP expression between WT and Vdr null. The intraplacental yolk sac could not be separately analyzed from the rest of the placenta.

To further assess for possible consequences of lack of placental expression of VDR, we examined the expression of calcium transporter 1 (CaT1) and calcium transporter 2 (CaT2 or ECaC), the two recently described apical calcium channels of which CaT1 is more abundantly expressed in placenta (47). The expression of CaT1 was significantly increased as assessed by quantitative real time PCR on RNA obtained from Vdr null versus wt placentas (Figure 9). The expression of CaT2 was 1,000-fold lower compared to CaT1 in WT and barely detectable; no difference was observed between WT and Vdr null expression of CaT2 (data not shown).
Discussion

We have examined the role of the VDR (and, indirectly, 1,25-D) in fetal mineral homeostasis by examining the effect of heterozygous and homozygous ablation of Vdr on the fetus, and on the mothers that carry these pregnancies. Pregnant Vdr null mothers have extremely low ionized calcium concentrations, reduced fertility, and smaller gestational litter sizes. In contrast, Vdr null fetuses, regardless of whether they are born of Vdr+/− or Vdr null mothers, have strikingly normal circulating mineral concentrations; have fully mineralized and normally developed skeletons; and have a normal (non-significantly increased) rate of transfer of calcium across normally developed placentas. The Vdr null fetus demonstrates the remarkable ability of the fetus and placenta to extract required mineral from the mother’s hypocalcemic circulation, and to do so by means that clearly do not require the VDR.

In vitamin D deficiency models in rats, maternal hypocalcemia has been noted to worsen during late pregnancy, with resultant maternal seizures, tetany, and up to 20% maternal deaths (15,16). In contrast, Vdr null mice were profoundly hypocalcemic and experienced a non-significant drop in ionized calcium in late pregnancy (ionized calcium about 50% of normal), but no seizures, tetany, or maternal deaths were observed. This finding may indicate that a model of vitamin D deficiency is more severe than one in which VDR is absent. However, it is relevant to note that normal rats experience a drop in ionized calcium during late pregnancy, whereas in our experience, normal Black Swiss mice do not. Therefore, the difference between the two models may reflect the differences between how rats and mice tolerate pregnancy, as opposed to differences between the relative severity of vitamin D deficiency and VDR ablation.

Vdr null mothers have reduced fertility in that they conceive less often than their siblings, and that they bear smaller litters. The reduced litter size of Vdr null mothers likely occurs at an
early time point, from ovulation to early embryonic development, given the lack of a difference in the numbers of resorption sacs or dead embryos among pregnancies of WT, Vdr\(^{+/-}\) and Vdr null mothers. A slightly reduced litter size has previously been observed before in vitamin D deficiency models in rats (15,16). Enrichment of the diet with extra calcium (2% versus 1%) resulted in an improved conception rate, and a gestational litter size of Vdr null mothers that was intermediate between that of Vdr\(^{+/-}\) and Vdr null mothers that consumed the 1% calcium diet. Thus, the reduced fertility is at least partly due to the hypocalcemia in the Vdr null mothers. It has been noted in another murine model of Vdr ablation that increasing the calcium content of the diet will improve the likelihood that a Vdr null will conceive and bear live pups; however, the effect on gestational litter size was not reported in that study (21). Instead, that study reported the number of pups alive the day after delivery. That measurement is a less precise indicator of fertility due to the confounding fact that the mother will kill and consume some pups (cull the litter), and other neonatal deaths will occur in the first few hours after delivery. That study reported an average of only 4 neonates per mother, as compared to the expected in utero number of 6-12 viable fetuses. Whether the calcium-enriched diet enabled more live births or fewer neonatal deaths cannot be differentiated from the presented data (21). The data that we report here indicates that the number of viable fetuses was significantly reduced in pregnancies of Vdr null mothers as compared to pregnancies in first-degree relative Vdr\(^{+/-}\) mothers, and that the number was not restored to normal by consumption of a 2% calcium diet.

The previously cited vitamin D deficiency models in rats and sheep had demonstrated normal fetal blood calcium and phosphorus levels and apparently fully mineralized skeletons at term (6,14,15,38,43). In each study, the interpretation of results was limited by the possibility that low levels of vitamin D or 25-hydroxyvitamin D had reached the fetus and had been
sufficient for fetal needs. The \textit{Vdr} null model conclusively shows that fetal loss of VDR and, thereby, the classical actions of 1,25-D, resulted in no effect on circulating mineral and phosphorus concentrations, endochondral bone development, and skeletal calcium and magnesium content.

Our results do differ in one respect from a recent study in the Leuven-strain model of \textit{Vdr} ablation. In that study (51), \textit{Vdr} null mothers were mated to WT males so that all fetuses were \textit{Vdr}^{+/−}, and those studies were controlled by pregnancies in WT mothers mated to WT males (the \textit{Vdr} null and WT female mice were not stated to be genetically related). The authors observed the fetal plasma calcium to be increased in \textit{Vdr}^{+/−} fetuses compared to unrelated WT fetuses; the ionized calcium was not measured. Our results indicate that the ionized calcium, the physiologically important fraction of the plasma calcium, was unaltered in \textit{Vdr}^{+/−} or \textit{Vdr} null fetuses as compared to WT fetuses within the same pregnancies. In the same study in Leuven-strain mice (51), fetal skeletal mineralization was observed to be impaired in \textit{Vdr}^{+/−} fetuses of \textit{Vdr} null mothers, and to be improved by maternal calcium supplementation. That study could not determine if fetal absence of VDR contributed to the skeletal phenotype because it did not examine WT, \textit{Vdr}^{+/−} and \textit{Vdr} null fetuses within the same pregnancy. By comparing WT, \textit{Vdr}^{+/−}, and \textit{Vdr} null fetuses within the same litters, and also comparing the progeny of \textit{Vdr}^{+/−}, and \textit{Vdr} null mothers, our results indicate that absence of maternal VDR can impair fetal size and growth, but also indicate that absence of fetal VDR has no discernible effect on skeletal size, development, mineralization, and other parameters such as skeletal expression of osteocalcin and osteopontin.

The fetus normally sets an ionized calcium level that is well above the ambient maternal concentration. We have previously shown that, in the \textit{Castr} knockout model, the fetus sets its
calcium concentration independently of the maternal ionized calcium concentration (28). Our findings in the Vdr null model confirm that the fetus sets its ionized calcium independently of the mother even when the mother’s ionized calcium is quite low, and that the fetus does not require the VDR to accomplish this.

Placental calcium transfer was not directly measured in the previously cited vitamin D deficiency models. Care et al noted that prior nephrectomy of fetal sheep reduced calcium transfer in an in situ placental perfusion model, and this effect could be partly restored by administering 1,25-D (8). However, due to the nature of the model (fetal nephrectomy with subsequent removal of the fetus before placental perfusion was performed), it could not be certain that the observed phenotype was solely due to the effect of reduced fetal 1,25-D. Others noted that pharmacological doses of 1,25-D or 1-cholecalciferol increased calcium transfer in placental perfusion models in rats, guinea pigs and sheep (9,11,12). Our examination of placental calcium transfer in Vdr null fetuses and mothers presents definitive and novel evidence that, notwithstanding its expression within murine intraplacental yolk sac (27), the VDR is not at all required to stimulate placental calcium transfer, or that absence of VDR can be completely compensated for.

PTHRP, calbindin-D9k and Ca²⁺-ATPase are expressed in the intraplacental yolk sac and are required for the transplacental transfer of calcium (8,27,29). In the absence of PTHrP (Pthrp null fetus), placental calcium transfer and the expression of calbindin-D9k within the placenta were both reduced (27,30). In the Vdr null fetus, in which we have demonstrated placental calcium transfer to be normal (or non-significantly increased), placental expression of calbindin-D9k and Ca²⁺-ATPase were normal while PTHrP and CaT1 mRNA were modestly upregulated. The Western blot demonstrated no difference in PTHrP expression in the total placenta, but the
technique lacks the sensitivity to determine if PTHrP is unregulated within the much smaller intraplacental yolk sac, wherein PTHrP is most intensely expressed (27). The modest upregulation of PTHrP and CaT1 mRNA in placenta may indicate that the non-significant increase in placental calcium transfer was a true increase, but that the physiological assay lacked the precision to confirm it. The analysis of pooled placental calcium data is consistent with this possibility, since the pooled data showed statistical significance among the three genotypes. Thus, if VDR has any involvement in controlling placental calcium transfer, it may have an inhibitory effect that is released by its absence, perhaps by permitting up-regulation of PTHrP, which in turn upregulates placental calcium transfer.

Calbindin-D_{9k} is, as its name implies, one of the vitamin D-dependent calcium binding proteins. This dependency on vitamin D clearly occurs after birth, when vitamin D deficiency or VDR ablation will severely reduce transcription and translation of Calbindin-D_{9k} (29,37). The normal expression of calbindin-D_{9k} in Vdr null placentas indicates that it is not dependent on the VDR. This observation is consistent with previous studies in fetal rats, which have noted that although pharmacological doses of 1,25-D will increase the placental content of calbindin-D_{9k}, vitamin D deficiency has no quantifiable impact on the placental content of calbindin-D_{9k} (14,41,61).

The 1-hydroxylase is expressed in placenta as well as kidney during fetal development, but 1,25-D normally circulates at low levels in the fetus. Our findings indicate that the renal 1-hydroxylase was modestly upregulated in the absence of VDR, while the placental 1-hydroxylase was not upregulated. The renal 1-hydroxylase is likely subject to VDR-mediated 1,25-dihydroxyvitamin D feedback inhibition in the fetus just as it is in the adult, which may
explain why the 1-hydroxylase is upregulated in the Vdr null fetuses despite having normal calcium, phosphorus and PTH levels. Recalling that normal for the fetus means hypercalcemia, hyperphosphatemia and lower PTH as compared to the mother, this may explain why Vdr null fetuses achieved only a modest increase in serum 1,25-D level as compared to Vdr null adults.

In any study involving gene ablation, the resulting phenotype is a result of a combination of effects, including absence of the gene product and compensation by other genes. Absence of VDR does not necessarily equate to absence of 1,25-D, due to possible non-classical actions of 1,25-D that are not VDR-mediated. It is conceivable that 1,25-D is still acting on placental calcium transfer and fetal bone development through non-genomic pathways that do not require the VDR. The VDR normally forms heterodimers with one of the three RXRs (, , & ), and it has been demonstrated that, postnatally, double mutant mice lacking VDR and RXR have more severe impairment of growth plate development as compared to mice that lack VDR alone (64). These results have been interpreted to indicate that there is a functionally redundant VDR in chondrocytes that forms heterodimers with RXR; whether a functionally redundant VDR is present in placenta is unknown. However, the absence of impaired mineral and skeletal homeostasis in the Vdr null fetus contrasts with the observation that absence of VDR causes a very striking phenotype soon after birth in all three published Vdr null models (hyperparathyroidism, rickets, hypocalcemia, hypophosphatemia) (36,60,65), which makes it clear that VDR is not required by the fetus for normal mineral and skeletal homeostasis.

The skeletal and biochemical phenotype of adult Vdr null mice has been shown to be largely prevented by dietary supplementation with calcium, phosphorus and lactose (1,35,64). These observations are consistent with the dominant role that 1,25-D and VDR play in enhancing
calcium and phosphorus absorption by the intestine, in particular by upregulating the expression of calbindins and calcium transporters (54,55,60). In turn, these observations may provide the clue that explains the difference between the marked skeletal phenotype of the adult and the normal skeletal phenotype of the fetus. It is not the intestine but the placenta that regulates delivery of calcium and phosphorus to the fetus, and it is now clear from our studies that the placenta does not require the VDR.

In summary, we have carefully and systematically examined Vdr null fetuses and have noted that absence of VDR does not disturb fetal mineral homeostasis, including maintenance of normal mineral and phosphorus concentrations in the fetal circulation, regulation of placental calcium transfer, and development and mineralization of the endochondral skeleton. Our results confirmed the earlier observations from vitamin D deficiency models that ionized calcium and skeletal mineral content were normal in such fetuses, but we have examined skeletal and placental structure, function and gene expression in much greater detail in order to more rigorously detect impaired physiology as a consequence of absence of VDR. While some of the factors that regulate fetal mineral homeostasis have been identified (including PTH and PTHrP), it remains to be determined how fetal mice are able to achieve their mineral requirements without relying on all of the same factors that the adult requires. Whether human fetuses similarly do not require the VDR remains to be confirmed by careful assessment of fetuses near term in utero, and of neonates at birth.

Acknowledgements

The authors gratefully acknowledge Dr. Marie Demay at Harvard Medical School/Massachusetts General Hospital for kindly permitting us to study the Vdr mice. The
additional technical assistance of Linda L. Chafe, Judy Foote and Claude Mercer is acknowledged. Part of the work described in this manuscript was done toward partial fulfillment of the MSc thesis requirement for Mandy L. Woodland, who received a Young Investigator Travel Award from the American Society for Bone and Mineral Research, and the Alfred Burness Graduate Student Award from Memorial University of Newfoundland. Current address of James K. Friel is Department of Human Nutritional Sciences, Faculty of Human Ecology, University of Manitoba, Winnipeg, Manitoba, R3T 2N2, Canada.

Grant support

Christopher S. Kovacs was supported by operating grants and a 5-year Scholarship (New Investigator Award/Scholarship) from the Canadian Institutes for Health Research (formerly Medical Research Council of Canada), and by funds from the Research and Development Committee, the Medical Research Foundation, and the Discipline of Medicine, all in the Faculty of Medicine at Memorial University of Newfoundland. James K. Friel was also supported by an operating grant from the Canadian Institutes for Health Research.
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63. **Wieland P, Fischer JA, Trechsel U, Roth HR, Vetter K, Schneider H, and Huch A.**


**Figure Legends**

1. Gestational litter size. In A, the number of viable fetuses *in utero* was lower in *Vdr* null (-/-) versus *Vdr*<sup>+/−</sup> (+/-) mothers when counted at C-section on ED 17.5 or 18.5. In B, the number of viable fetuses from *Vdr* null mothers consuming a calcium-enriched diet was intermediate between the values shown in panel A, and not significantly different from either value. The number of observations is indicated in parentheses.

2. Placental calcium transfer. The ratio of <sup>45</sup>Ca to <sup>51</sup>Cr radioactivity accumulated in each fetus at 5 minutes after maternal injection of the isotopes is a measure of the relative transfer of calcium across the placenta. Placental calcium transfer at 5 minutes was not significantly different among fetuses obtained from *Vdr*<sup>+/−</sup> mothers, but a trend to increased rate of transfer was observed in *Vdr* null fetuses (-/-) as compared to *Vdr*<sup>+/−</sup> (+/-) and WT. The number of observations is indicated in parentheses.

3. Ionized calcium and maternal-fetal calcium gradient. Ionized calcium was measured on whole blood in fetuses obtained from *Vdr*<sup>+/−</sup> mothers (A) and *Vdr* null mothers (B) on ED 17.5 and 18.5. No difference in ionized calcium level was noted among the fetal genotypes, and the mother’s genotype or ionized calcium level did not affect the fetal ionized calcium level. The maternal-fetal calcium gradient (double-headed arrows) is the difference between the fetal ionized calcium and the corresponding mean maternal ionized calcium level (line). The SE on the maternal values is indicated by errors bars on the far right of each line. The maternal-fetal gradient was strikingly increased in fetuses of *Vdr* null mothers as compared to fetuses of *Vdr*<sup>+/−</sup> mothers (p<0.001). The number of observations is indicated in parentheses.
4. Fetal serum 1,25-D. Serum obtained on ED 17.5 and 18.5 from fetuses of Vdr<sup>+/−</sup> mothers was analyzed for 1,25-D level by radioimmunoassay. 1,25-D level was increased only in Vdr null fetuses as compared to WT and Vdr<sup>+/−</sup> fetuses. For comparison, mean values of 1,25-D in pregnant adult WT, Vdr<sup>+/−</sup> (+/−), and Vdr null (−/−) mice are represented in this graph by the indicated horizontal lines, with SE indicated on the far right of each line by error bars. The SE for WT is too small to be represented. The maternal values were significantly different from each other (p<0.001). The number of observations is indicated in parentheses.

5. Real time PCR of 1-hydroxylase mRNA. The expression of 1-hydroxylase mRNA in WT and Vdr null kidney is shown in panel A, while the expression in placenta is shown in panel B. Values shown are the mean (±SE) normalized to GAPDH and WT. Numbers in parentheses indicate the number of placentas studied.

6. Skeletal morphology and gene expression in Vdr null fetuses. Panels A and B show images of fetal skeletons (ED 18.5) stained with alizarin red (for mineral) and alcian blue (for cartilage). Skeletal morphology of the Vdr null was consistently normal, as shown by the normal crown-rump length, lengths of long bones, and mineralization pattern of the Vdr null (B) and its WT sibling (A). Panels C and D are von Kossa preparations (counterstained with methyl green) of the upper halves of fetal tibias (ED 18.5), which include the growth plates and part of the tibial shafts. The overall morphology of the tibias, the lengths of the growth plates, and periosteal thickness were normal in Vdr null (D) as compared to WT (C), and a normal amount of mineral (black) was present in the shafts of the tibias. Panels E through N are in situ hybridization
studies of fetal tibias (ED 18.5) in WT (E-I) and Vdr null (J-N) siblings, which were performed using $^{35}$S-labeled riboprobes. Representative darkfield images are shown of the detection of mRNA for collagen II (COL-II), collagen X (COL-X), collagen I (COL-I), 92 kDa gelatinase (GEL), and osteopontin (OP). No difference was seen in the expression of each mRNA between WT and Vdr null tibias. Scale bars indicate 0.5 cm in A & B, and 100 microns in the remaining panels.

7. Placental morphology and gene expression. The upper panels display images of sections from WT placentas, and the lower panels show sections from Vdr null (/-) placentas (ED 17.5 and 18.5). Gross and microscopic morphology of placentas were unchanged between WT (A) and Vdr null (E), including the relative amount, distribution, and morphology of the intraplacental yolk sac (arrows) within the larger structure created by the labyrinthine trophoblasts (L). The bilayered structure of the intraplacental yolk sac is shown at higher magnification in panels B and F, with the parietal cells overlying Reichert’s membrane indicated by arrows, and the opposing columnar cells indicated by arrowheads. Panels C, D, G, H are in situ hybridization studies performed on placental sections to detect specific mRNAs by $^{35}$S-labeled riboprobes. Representative darkfield images are shown of the detection of mRNA for calbindin$_{9k}$-D (C, G) and Ca$^{2+}$-ATPase (D, H). Both mRNAs were most intensely expressed in the intraplacental yolk sac (arrows) with less intense expression observed in the labyrinthine trophoblasts (L). No difference in the expression of either mRNA was observed between WT and Vdr null placentas. Scale bars indicate 100 microns.
Figure 8. PTHrP expression in Vdr null placentas. Panels A-D indicate immunohistochemistry for PTHrP, with sections from WT placentas in A & B, and sections from Vdr null (-/-) placentas in C & D. Panels A and C are non-immune controls, while immunostained sections are shown in panels B and D. The bilayered structure of the intraplacental yolk sac visible in each panel, with the parietal cells overlying Reichert’s membrane indicated by arrows, and the opposing columnar cells indicated by arrowheads. WT and Vdr null placentas both show PTHrP immunoreactivity throughout the placenta, with the most intense expression in the columnar cells of the intraplacental yolk sac. PTHrP immunoreactivity was consistently increased in the Vdr null sections. The scale bars indicates 100 microns. Panel E indicates real time PCR results for PTHrP mRNA normalized to GAPDH and WT. PTHrP mRNA expression was significantly increased in Vdr null versus WT. Panel F is a representative Western blot of PTHrP and actin expression in total placenta. No difference in PTHrP expression at the protein level was observed in Vdr null placentas vs. WT. Numbers in parentheses indicate the number of placentas studied.

Figure 9. Real time PCR of CaT1 mRNA. CaT1 mRNA expression was upregulated in Vdr null placentas versus WT. Values shown are the mean (±SE) normalized to GAPDH and WT. Numbers in parentheses indicate the number of placentas studied.
Table 1

Serum magnesium, phosphorus and PTH in fetuses obtained from $Vdr^{+/}$ mothers. No statistically significant differences were seen.

<table>
<thead>
<tr>
<th></th>
<th>Mg (mmol/l)</th>
<th>P (mmol/l)</th>
<th>PTH (pg/ml)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>1.04 ± 0.09 (6)</td>
<td>2.70 ± 0.2 (6)</td>
<td>10.5 ± 1.6 (9)</td>
</tr>
<tr>
<td>$Vdr^{+/}$</td>
<td>1.04 ± 0.06 (12)</td>
<td>2.48 ± 0.2 (12)</td>
<td>9.2 ± 1.1 (18)</td>
</tr>
<tr>
<td>$Vdr$ null</td>
<td>1.04 ± 0.09 (6)</td>
<td>2.82 ± 0.3 (6)</td>
<td>9.3 ± 1.6 (9)</td>
</tr>
</tbody>
</table>
Table 2

Amniotic fluid calcium, magnesium, and phosphorus in fetuses obtained from $Vdr^{+/-}$ mothers.

No statistically significant differences were seen.

<table>
<thead>
<tr>
<th></th>
<th>Ca (mmol/l)</th>
<th>Mg (mmol/l)</th>
<th>P (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2.31 ± 0.14 (16)</td>
<td>1.34 ± 0.07 (15)</td>
<td>1.99 ± 0.14 (9)</td>
</tr>
<tr>
<td>$Vdr^{+/-}$</td>
<td>2.25 ± 0.08 (37)</td>
<td>1.38 ± 0.04 (41)</td>
<td>1.94 ± 0.08 (31)</td>
</tr>
<tr>
<td>$Vdr$ null</td>
<td>2.19 ± 0.14 (12)</td>
<td>1.32 ± 0.08 (13)</td>
<td>1.68 ± 0.14 (9)</td>
</tr>
</tbody>
</table>
Table 3

Absolute skeletal calcium and magnesium content of fetuses obtained from $Vdr^{+/-}$ mothers. No statistically significant differences were seen.

<table>
<thead>
<tr>
<th></th>
<th>Calcium (mg/g ash)</th>
<th>Magnesium (mg/g ash)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>82.7 ± 1.9 (14)</td>
<td>14.6 ± 0.3 (14)</td>
</tr>
<tr>
<td>$Vdr^{+/-}$</td>
<td>80.4 ± 1.5 (23)</td>
<td>14.2 ± 0.2 (23)</td>
</tr>
<tr>
<td>$Vdr$ null</td>
<td>80.4 ± 1.7 (17)</td>
<td>14.1 ± 0.2 (17)</td>
</tr>
</tbody>
</table>
Figure 1

A. Regular Chow

B. Calcium-enriched Chow

LITTER SIZE

p < 0.004

+/- (58)  -/- (10)  

-/- (6)
Figure 2

$^{45}\text{Ca}$ Transfer (% HET Mean)

- WT (20)
- +/- (47)
- -/- (20)
Figure 3

[Graph showing ionized calcium levels for different genotypes of mothers.]

**A** $Vdr^{+/−}$ Mothers

- WT (12)
- +/- (35)
- +/- (22)

**B** $Vdr$ null Mothers

- +/- (10)
- +/- (20)
Figure 4

The diagram shows the comparison of 1,25-D levels (pg/ml) in adult mice of different genotypes:

- **WT**
- **+/−**
- **−/−**

- **ADULT NULL**
- **ADULT +/−**
- **ADULT WT**

A statistical significance is indicated with *p < 0.0002*. The sample size for each group is denoted as (5).
Figure 5

- **A** KIDNEY
- **B** PLACENTA

WT -/-

\( p<0.05 \)
Figure 7
Figure 8

WT

/- (3)

p<0.05

PTHrP mRNA

E

F

WT (3)  +/- (3)

Actin

WT  +/-  -/-
Figure 9

WT (3)  

CaT1 mRNA

p<0.05

/- (3)  

Figure 9