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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Kovacs, Christopher S., Mandy L. Woodland, Neva J. Fudge, and James K. Friel The vitamin D receptor is not required for fetal mineral homeostasis or for the regulation of placental calcium transfer in mice. Am J Physiol Endocrinol Metab 289: E133–E144, 2005. First published March 3, 2005; doi:10.1152/ajpendo.00354.2004.—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 homeostasis. Vdr null mice were profoundly hypocalcemic, conceived infrequently, and had significantly fewer viable fetuses in utero that 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 (wild type, Vdr+/+, and Vdr null fetuses), there was no alteration in serum Ca, P, or Mg, parathyroid hormone, placental Ca2+ transfer, Ca and Mg content of the fetal skeleton, and morphology and gene expression in the fetal growth plate. Vdr null fetuses did have threefold 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.

fetus; calcitriol; skeletal mineralization; parathyroid hormone-related protein; parathyroid hormone; calbindin-D9k; calcium-adenosine triphosphatase; calcium transporter 1 and 2; 1α-hydroxylase; placenta

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(OH)2D3 (57, 62), and yet circulating 1,25(OH)2D3 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(OH)2D3 in response to PTH because, in the model of calcium-sensing receptor gene (Casr) ablation, a stepwise increase in PTH levels was associated with a stepwise increase in 1,25(OH)2D3 levels (28).

Several experimental models have suggested that 1,25(OH)2D3 is required for normal fetal calcium and bone homeostasis. A 72-h infusion of a crude polyclonal antiseraum raised against 1,25(OH)2D3 decreased the ovine fetal blood calcium level (50). Bilateral nephrectomy in fetal sheep removed the main source of 1,25(OH)2D3 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(OH)2D3 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 1α-hydroxylase-deficient Hanover pig model (33) demonstrated that fetuses of homozygous 1,25(OH)2D3-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 Hanover 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 are only limited data from humans about whether vitamin D or

IT IS WELL APPRECIATED THAT VITAMIN D, and more importantly its active metabolite calcitriol or 1,25-dihydroxyvitamin D \(1,25(OH)_2D_3\), 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(OH)2D3 in regulating fetal mineral metabolism (24, 25). The receptor for 1,25(OH)2D3 (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
1,25(OH)2D3 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 deminerlization and other rachitic changes are typically not detectable until 1 or 2 mo 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 fontanella, and wide wrists; see Ref. 58).

In view of the limited and conflicting animal and human data about the role of vitamin D or 1,25(OH)2D3 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 cause 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 Vdr null females were also mated to yield pregnancies with ED 0.5 vaginal mucus plug on the morning after mating marked day (ED) 0.5. This model allowed the assessment of fetal need for 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 2 wk before 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 previously been shown to be effective in Vdr null mice (35).

**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 embryonic day (ED) 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 Pelletier Thermal Cycler (MJ Research, Cambridge, MA). The nucleotide sequences for Vdr PCR primers were as follows (forward and reverse, respectively): 5′-CTG CCC TGC TCC ACA GTC CTT-3′ and 5′-GCA GAC TCT CCC ATG TGC AAA 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 was counted. The number of viable fetuses in utero is normally higher than the litter size observed 6–24 h after birth because of early culling of the litter by the mother and neonatal deaths resulting from other causes.

**Diet.** The mice routinely received a standard chow diet that contained 1% calcium (LabDiet SP00; PMI Nutrition, Richmond, IN). To determine the effect of increased calcium intake on fetal number and
Gene expression in the cartilaginous growth plate and adjacent bone (periosteum and endosteum) was examined using the following cDNAs: pro-α1(I) chain of human type I collagen (4), pro-α1(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; see Ref. 3; gifts of B. Lanske, MGH); and murine interstitial collagenase (17) and murine 92-kDa gelatinase (type IV collagenase or matrix metalloproteinase-9; see Ref. 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 α-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).

PCR was performed using 1 µl sequence-specific primers, and 10 ng/µl random primers were added to a final concentration of 1 × RT Buffer (20 mM Tris·HCl, pH 8.4, and 5 mM KCl), 5 mM MgCl$_2$, 0.01 mM dithiothreitol, 2 U/µl RNaseOut, and 20 U/µl Superscript III RT and incubated for 10 min at 25°C followed by a 50-min incubation at 30°C. One microliter of 2 U/µl RNase H was added and incubated for 20 min at 37°C.

Real-time PCR data and analysis were obtained using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). cDNA reaction (2.0 µl), 830 pmol/µl sequence-specific primers, and SYBR Green PCR Master Mix (Applied Biosystems) were incubated at 95°C for 15 s and 60°C for 1 min for a total of 40 cycles. Nucleotide sequences for glyceraldehyde-3-phosphate dehydrogenase PCR primers (forward and reverse, respectively) were as follows: 5′-TTC TCA TCA ACC AGA AGC TCA-3′ and 5′-TCT GTG GGT TCA-3′; for parathyroid hormone-related protein (PTHrP) 5′-TCC ACA CAG CCG AAA TCA GAG CTA-3′ and 5′-TCT TG CTT CCT TCT CCA CCA-3′; and for 1α-hydroxylase 5′-TCC ACC ATT CCT CCT GCC ATT TCC-3′ and 5′-AA TAC CAC CTT CAC ACC AAT CTC-3′. PCR primer sequences for calcium transporter I (CaT1) and calcium transporter 2 (CaT2) were those that have been previously published (55).

In situ hybridization. In situ hybridization was performed on 5-µm tissue sections as described previously (34). Hybridization was performed in a humidified chamber (16 h, 55°C) with the labeled riboprobe diluted 1:20 in the hybridization solution. Sections were successively washed, RNase treated, and dehydrated in graded ethanol (EtOH) 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 in Kodak NTB-2 liquid emulsion, dried, stored in light-tight boxes, and kept at 4°C until developed (2–6 wk). The emulsion was developed using standard developer and fixer, and the sections were counterstained with hematoxylin-eosin.

All comparisons of WT with Vdr null were made between tissues obtained from within the same litter and that had been processed, embedded, and sectioned at the same time. All comparative sections were always hybridized together with the same probe and washed together 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 min in 1 ml 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, and 1 mM phenylmethylsulfonyl fluoride), and incubated on a shaker overnight at 4°C. The samples were then centrifuged at 10,000 rpm for 15 min, 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 Biolyx, Brockville, ON, Canada). Standard or sample (25 µl) was added to 200 µl of working reagent in a 96-well plate and incubated at 37°C for 30 min, and the absorbance was obtained at 570 nm using a maximal velocity 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, Canada). Sample buffer, antitoxin, and 100 µg protein were heated to 70°C for 10 min and run on a 4–12% Tris–Bis gel at 200 V for 35 min. The gels were transferred onto a polyvinylidene difluoride membrane at 30 V for 1 h. 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 revolutions/s at room temperature. Membranes were soaked for 30 s in methanol, rinsed two times with ultrapure water, and blocked for 30 min in Blocking Solution. The membranes were washed twice for 5 min in water and incubated for 1 h with either rabbit anti-PTHrP or rabbit anti-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500. After four washes with buffered saline, the blots were incubated with alkaline phosphatase-conjugated anti-rabbit IgG antibodies for 30 min. 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 5 min, blotted with filter paper, and exposed to Kodak X-Omat LS X-ray film (Amersham) for 20 s-5 min.

Histology. Sections (5 µm) were deparaffinized, rehydrated in a graded EtOH series, and transferred to distilled water. For morphological assessment of the growth plate, sections were stained with hematoxylin and eosin or 1% methyl green and 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 three times in distilled water, placed, in 2.5% sodium thiosulfate (5 min), and washed three times 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-normal bovine serum and applied at 20°C for 60 min (this step omitted from control sections). The primary antibody was detected using biotinylated goat anti-rabbit (Vector, Burlington, ON, Canada), avidin-biotin complex reagent (Vector), and diaminobenzidine-Tris-peroxidase substrate (Vector). Sections were counterstained, washed, dehydrated, and mounted.

Alizarin red S and Alcian blue preparations. Fresh fetuses from Vdr$^{–/–}$ mothers (ED 18.5) were obtained, and the skin, viscera, and adipose tissue were removed carefully. 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. After this, the fetuses were stained for 3 days in 10 ml freshly prepared staining solution at 37°C (1 vol of 0.3% Alcian blue 8GS in 70% EtOH-1 vol of 0.1% Alizarin red S in 95% EtOH-1 vol of acetic acid-17 vol of 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 (~12–48 h). They were cleared in 1% KOH containing increasing concentrations (20, 50, and 80%) of glycercine (7–10 days at each step). Finally, they were transferred to 100% glycercine for permanent storage.

Statistical analysis. Data were analyzed using SYSTAT 5.2.1 for Macintosh (SYSTAT, 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-ΔΔCt method where the target and reference are amplified in separate wells (40). Two-tailed probabilities are reported, and all data are presented as means ± SE.

RESULTS

Maternal effects of VDR 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 (Fig. 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 was 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 2 wk before mating. On the enriched diet, the frequency of confirmed pregnancy after a confirmed mating was >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 (Fig. 1B) was intermediate between that of Vdr+/− and Vdr null mothers that consumed the normal chow (Fig. 1A) and not significantly different from each other 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 g 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 vs. weight of counterpart from Vdr+/− mothers and P < 0.01 vs. 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 compared with 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 min; instead, there was a nonsignificant 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; Fig. 2). Because of far fewer available pregnancies, placental calcium transfer could be measured in only two Vdr null mothers. Again, a nonsignificant trend to increased placental calcium transfer was observed in Vdr null fetuses (169 ± 33.6 vs. 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 as follows: 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+/−, 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 (Fig. 3A). Even in the severely hypocalcemic Vdr null mothers, Vdr+/− and Vdr null fetuses maintained a normal ionized calcium level indistinguishable from that of the fetuses of Vdr+/− mothers (Fig. 3B). Because of the maternal hypocalcemia, the corresponding maternal-fetal calcium gradient was significantly (P < 0.001) increased in fetuses obtained from Vdr null mothers compared with normal (Fig. 3B).

Similar to the fetal ionized calcium levels, serum magnesium and phosphorus were unaltered among WT, Vdr+/−, 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 fetuses (Table 1).

Serum 1,25(OH)2D3 levels were unaltered in Vdr+/− compared with WT fetuses but were significantly increased in Vdr null fetuses (Fig. 4). By comparison, adult mice show a stepwise increase in 1,25(OH)2D3 from WT to Vdr+/− to Vdr null (P < 0.001), and the peak level achieved in adult Vdr null mice is almost two times that achieved in Vdr null fetuses (Fig. 4). To determine if kidney or placenta was the source of the increase in 1,25(OH)2D3, mRNA for 1α-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 (Fig. 5A) but not in placenta (Fig. 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).

Table 1. Serum magnesium, phosphorus, and PTH in fetuses obtained from Vdr+/− mothers

<table>
<thead>
<tr>
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<th>Magnesium, mmol/l</th>
<th>Phosphorus, mmol/l</th>
<th>PTH, pg/ml</th>
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<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 ± 0.12 (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>

Values are means ± SE; no. of observations in parentheses. PTH, parathyroid hormone; WT, wild type; Vdr, vitamin D receptor gene. No statistically significant differences were seen.

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 (Fig. 6, A and 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+/− mothers, which were 21.4 ± 0.7 mg in WT, 20.1 ± 0.6 mg in Vdr+/−, and 20.5 ± 0.8 mg in Vdr null (Table 2). 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+/− mothers at 12.5 ± 1.3 mg in Vdr+/− and 12.2 ± 1.3 mg in Vdr null.
null (P < 0.001 vs. counterpart). When expressed as percentage of fetal weight, the relative skeletal weights were not significantly different between Vdr null fetuses of Vdr+/− 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 of 12.6 ± 0.5 mg in Vdr+/− and 13.9 ± 0.5 mg in Vdr null fetuses (P = not significant vs. counterpart on regular diet). Thus absence of VDR within the fetus did not alter skeletal mineral content compared with Vdr+/− 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+/− and Vdr null.

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

**Table 2. Amniotic fluid calcium, magnesium, and phosphorus in fetuses obtained from Vdr+/− mothers**

<table>
<thead>
<tr>
<th></th>
<th>Calcium, mmol/l</th>
<th>Magnesium, mmol/l</th>
<th>Phosphorus, mmol/l</th>
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<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>1.36±0.04 (41)</td>
<td>1.94±0.08 (31)</td>
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<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>
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</table>

Values are means ± SE; no. of observations in parentheses. No statistically significant differences were seen.

**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; because 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). With the use of this method, the mineral present in Vdr null growth plates appeared to be distributed normally compared with WT siblings (Fig. 6, C and 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 peristeum also did not differ among WT or Vdr null fetuses (Fig. 6, C and 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 Fig. 6, E–N. The distribution pattern and intensity of expression of the mRNAs for types 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–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, calbindin-D9k and Ca2+-ATPase, by in situ hybridization. No gross abnormality

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**Fig. 4.** Fetal serum 1,25-dihydroxyvitamin D [1,25(OH)2D3]. Serum obtained on ED 17.5 and 18.5 from fetuses of Vdr+/− mothers was analyzed for 1,25(OH)2D3 level by RIA. 1,25(OH)2D3 level was increased only in Vdr null fetuses compared with WT and Vdr+/− fetuses. For comparison, mean values of 1,25(OH)2D3 in pregnant adult WT, Vdr+/− (+/−), and Vdr null (−/−) mice are represented by 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 no. of observations is indicated in parentheses.

**Fig. 5.** Real-time PCR of 1α-hydroxylase (1α-OH) mRNA. The expression of 1α-hydroxylase mRNA in WT and Vdr null kidney is shown in A, whereas the expression in placenta is shown in B. Values shown are means ± SE normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and WT. Nos. in parentheses indicate the no. of placentas studied.
of placental weight, structure, or cellular morphology was evident. By light microscopic examination, the amount and distribution of the intraplacental yolk sac was normal (Fig. 7, A and B vs. E and F). Calbindin-D9k, 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 compared with their WT siblings (Fig. 7, C vs. G). Similarly, Ca2+/H+ ATPase showed no alteration in location or intensity of expression in Vdr null placentas compared with their WT siblings (Fig. 7, D vs. 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 vs. WT placentas (data not shown).

PTHrP is known to stimulate placental calcium transfer, and, because 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 (Fig. 8, A–D). Quantitative real-time PCR on placental mRNA confirmed a statistically significant increase in PTHrP mRNA in Vdr null vs. WT placentas (Fig. 8E). Western blot of total placental protein showed no difference in PTHrP expression between WT and Vdr null.

Table 3. Absolute skeletal calcium and magnesium content of fetuses obtained from Vdr \(^{+/−}\) mothers

<table>
<thead>
<tr>
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<th>Calcium, mg/g ash</th>
<th>Magnesium, mg/g ash</th>
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<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>

Values are means ± SE; no. of observations in parentheses. No statistically significant differences were seen.
intraplacental yolk sac could not be analyzed separately from the rest of the placenta.

To further assess for possible consequences of lack of placental expression of VDR, we examined the expression of CaT1 and CaT2 (or ECaC), the two recently described apical calcium channels of which CaT1 is more abundantly expressed in the intraplacental yolk sac (arrows) in placenta (47). The expression of CaT1 was significantly increased as assessed by quantitative real-time PCR on RNA obtained from Vdr null vs. WT placentas (Fig. 9). The expression of CaT2 was 1,000-fold lower compared with 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(OH)2D3] 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 (nonsignificantly 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 minerals 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 nonsignificant drop in ionized calcium in late pregnancy (ionized calcium ~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 vs. 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 the result of 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 because of 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, compared with 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 indicate that the number of viable fetuses was significantly reduced in
by yolk sac. PTHrP immunoreactivity was consistently increased in the
with the most intense expression in the columnar cells of the intraplacental
null placentas both show PTHrP immunoreactivity throughout the placenta,
and
A
significantly increased in
mRNA normalized to GAPDH and WT. PTHrP mRNA expression was
sion at the protein level was observed in
PTHrP and actin expression in total placenta. No difference in PTHrP expres-
sions of 1,25(OH)2D3 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 Vdr ablation. In that study (51), Vdr
null mothers were mated to WT males so that all fetuses were
Vdr+/−, and those studies were controlled by pregnancies in
WT mothers mated to WT males (the Vdr null and WT female
mice were not stated to be genetically related). The authors
observed the fetal plasma calcium to be increased in Vdr+/−
fetuses compared with unrelated WT fetuses; the ionized cal-
cium was not measured. Our results indicate that the ionized
calcium, the physiologically important fraction of the plasma
calcium, was unaltered in Vdr+/− or Vdr null fetuses compared
with WT fetuses within the same pregnancies. In the same
study in Leuven-strain mice (51), fetal skeletal mineralization
was observed to be impaired in Vdr+/− fetuses of Vdr null
mothers and to be improved by maternal calcium supplemen-
tation. That study could not determine if fetal absence of VDR
contributed to the skeletal phenotype because it did not exam-
WT, Vdr+/−, and Vdr null fetuses within the same preg-
nancy. By comparing WT, Vdr+/−, and Vdr null fetuses within
the same litters, and also comparing the progeny of Vdr+/− and
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 Casr 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 it 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 measured directly in the
previously cited vitamin D deficiency models. Care (8) 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(OH)2D3. However,
due to the nature of the model (fetal nephrectomy with sub-
sequent removal of the fetus before placental perfusion was
performed), it could not be certain that the observed pheno-

Fig. 8. Parathyroid hormone-related protein (PTHrP) expression in Vdr null
placentas. A–D: immunohistochemistry for PTHrP, with sections from WT
placentas in A and B and sections from Vdr null (−/−) placentas in C and D.
A and C are nonimmune controls, whereas immunostained sections are shown
in B and D. The bilayered structure of the intraplacental yolk sac is 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. Scale bars indicate 100 μm. E: real-time PCR results for PTHrP
mRNA normalized to GAPDH and WT. PTHrP mRNA expression was
significantly increased in Vdr null vs. WT. F: representative Western blot of
PTHrP and actin expression in total placenta. No difference in PTHrP expres-
sion at the protein level was observed in Vdr null placentas vs. WT. Nos. in
parentheses indicate the no. of placentas studied.

Fig. 9. Real-time PCR of calcium transporter 1 (CaT1) mRNA. CaT1 mRNA
expression was upregulated in Vdr null placentas vs. WT. Values shown are
means ± SE normalized to GAPDH and WT. Nos. in parentheses indicate the
no. of placentas studied.

The previously cited vitamin D deficiency models in rats and
sheep had demonstrated normal fetal blood calcium and phos-
phorus 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 Vdr null model conclusively
shows that fetal loss of VDR and, thereby, the classical actions
of 1,25(OH)2D3 resulted in no effect on circulating mineral and

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type was solely the result of the effect of reduced fetal 1,25(OH)\textsubscript{2}D\textsubscript{3}. Others noted that pharmacological doses of 1,25(OH)\textsubscript{2}D\textsubscript{3} or 1\alpha-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-D\textsubscript{9k}, and Ca\textsuperscript{2+}-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-D\textsubscript{9k} 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 nonsignificantly increased), placental expression of calbindin-D\textsubscript{9k} and Ca\textsuperscript{2+}-ATPase was normal, whereas PTHrP and CaT1 mRNA was 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 nonsignificant 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 upregulation of PTHrP, which in turn upregulates placental calcium transfer.

Calbindin-D\textsubscript{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\textsubscript{9k} (29, 37). The normal expression of calbindin-D\textsubscript{9k} in Vdr null placentas indicates that it is not dependent on the VDR. This observation is consistent with previous studies in fetal rats that have noted that, although pharmacological doses of 1,25(OH)\textsubscript{2}D\textsubscript{3} will increase the placental content of calbindin-D\textsubscript{9k}, vitamin D deficiency has no quantifiable impact on the placental content of calbindin-D\textsubscript{9k} (14, 41, 61).

1\alpha-Hydroxylase is expressed in placenta and kidney during fetal development, but 1,25(OH)\textsubscript{2}D\textsubscript{3} normally circulates at low levels in the fetus. Our findings indicate that the renal 1\alpha-hydroxylase was modestly upregulated in the absence of VDR, whereas the placental 1\alpha-hydroxylase was not upregulated. The renal 1\alpha-hydroxylase is likely subject to VDR-mediated 1,25(OH)\textsubscript{2}D\textsubscript{3} feedback inhibition in the fetus just as it is in the adult, which may explain why the 1\alpha-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 compared with the mother, this may explain why Vdr null fetuses achieved only a modest increase in serum 1,25(OH)\textsubscript{2}D\textsubscript{3} level compared with 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(OH)\textsubscript{2}D\textsubscript{3} because of possible nonclassical actions of 1,25(OH)\textsubscript{2}D\textsubscript{3} that are not VDR mediated. It is conceivable that 1,25(OH)\textsubscript{2}D\textsubscript{3} is still acting on placental calcium transfer and fetal bone development through nongenomic pathways that do not require the VDR. The VDR normally forms heterodimers with one of the three retinoid X receptors [RXRs (\(\alpha\), \(\beta\), and \(\gamma\)], and it has been demonstrated that, postnatally, double-mutant mice lacking VDR and RXR\(\gamma\) have more severe impairment of growth plate development compared with 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\(\gamma\); 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, or hypophosphatemia; see Refs. 36, 60, and 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 1,25(OH)\textsubscript{2}D\textsubscript{3} 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 to more rigorously detect impaired physiology as a consequence of absence of VDR. Although 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.

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