Characterization of an animal model of pregnancy-induced vitamin D deficiency due to metabolic gene dysregulation

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Vitamin D deficiency has been associated with pregnancy complications such as preeclampsia, gestational diabetes, and recurrent miscarriage. Therefore, we hypothesized differences in vitamin D status between healthy [Sprague-Dawley (SD) and Lewis (LW)] and complicated [Brown Norway (BN)] rat pregnancies. In SD, LW, and BN rats, we analyzed the maternal plasma levels of the vitamin D metabolites 25-OH-D and 1,25-(OH)2-D at prepregnancy, pregnancy, and postpartum. Analysis of the active metabolite 1,25-(OH)2-D showed a twofold increase in pregnant SD and LW rats but a nearly 10-fold decrease in pregnant BN rats compared with nonpregnant controls. BN rats had a pregnancy-dependent upregulation of CYP24a1 expression, a key enzyme that inactivates vitamin D metabolites. In contrast, the maternal renal expression of CYP24a1 in SD and LW rats remained constant throughout pregnancy. Analysis of the vitamin D receptor (VDR) indicated that LD and SD but not BN rats experience a pregnancy-induced 10-fold decrease in maternal renal VDR protein levels. Further analysis of bisulfite-converted and genomic DNA indicated that the observed differences in maternal renal regulation of CYP24a1 during pregnancy and lactation are not due to differences in CYP24a1 promoter methylation or single-nucleotide polymorphisms. Finally, supplementation with 1,25-(OH)2-D significantly improved the reproductive phenotype of BN rats by increasing litter size and maternal-fetal weight outcomes. We conclude that BN rats represent a novel animal model of pregnancy-specific vitamin D deficiency that is linked to pregnancy complications. Vitamin D deficiency in BN rats correlates with maternal renal CYP24a1 upregulation followed by CYP27b1 upregulation.

calcitriol; CYP24a1; metabolism; kidney; placenta

THE SECOSTEROID VITAMIN D has well-established classic effects on bone metabolism and mineral homeostasis (7, 37). Vitamin D3 (cholecalciferol) is generated from 7-dehydrocholesterol by a photolytic (UVB) conversion in the skin epidermis and from dietary animal sources, and vitamin D2 (ergocalciferol) is obtained by dietary plant sources. Vitamin D is endogenously activated by mitochondrial 25-hydroxylase enzymes that synthesize 25-OH-D, followed by a second hydroxylation at the 1α position that yields the active metabolite of vitamin D: 1α,25-(OH)2-D (calcitriol). This step is catalyzed by a rate-limiting enzyme, 25(OH)-1α-hydroxylase (CYP27b1), that is expressed abundantly in the renal cortex. In the blood, vitamin D metabolites are transported bound to albumin and the vitamin D binding protein (VDBP) (13, 26).

At the molecular level, vitamin D mediates its biological effects by binding and activating the vitamin D receptor (VDR) (19, 21). Active VDR, heterodimerized with the retinoid X receptor, regulates gene expression by targeting gene promoters containing vitamin D response elements. This leads to activation or repression of transcription, resulting in gene expression changes (19, 21). A classic gene regulated in this manner is the vitamin D-inactivating enzyme CYP24A1 (24-hydroxylase). Most cells that express VDR also express CYP24A1, providing a unique local feedback control mechanism to regulate the vitamin D effects (13, 19, 21). In healthy nonpregnant mammals, the circulating levels of 1α,25-(OH)2-D are tightly regulated; low levels of 1α,25-(OH)2-D stimulate the release of parathyroid hormone (PTH) that upregulates renal CYP27b1 expression, and high levels of 1α,25-(OH)2-D stimulate VDR that upregulates CYP24A1 expression.

Vitamin D metabolism changes significantly during mammalian pregnancy (27, 40). Maternal plasma levels of ionized calcium and the abundant precursor 25-(OH)-D do not change significantly during normal pregnancy. In contrast, circulating levels of active 1α,25-(OH)2-D increase severalfold from early pregnancy and remain high during the entire length of pregnancy (27, 42, 55). In rodent pregnancy models, studies suggest that increased circulating 1α,25-(OH)2-D levels are caused by upregulation of renal CYP27b1 expression (17, 18, 43). In normal human placenta and decidua from the first and second trimesters, expression of CYP27b1 and VDR is increased by more than 10-fold compared with endometrium, whereas the contrary is true for placental CYP24a1 expression (10). A recent study has demonstrated that CYP24a1 expression in the human placenta is downregulated by hypermethylation of its promoter (37). Increased maternal 1α,25-(OH)2-D levels during pregnancy are not a consequence of increased PTH, which remains low during human and rodent pregnancy (24, 38). Thus, the mechanisms that lead to higher plasma levels of 1α,25-(OH)2-D during normal pregnancy have not been fully elucidated.

On a global basis, vitamin D deficiency during pregnancy is highly prevalent (23, 44, 50). Recent clinical studies have demonstrated that vitamin D deficiency is associated with human pregnancy complications such as preeclampsia, gestational diabetes, and recurrent miscarriage (5, 14, 48, 52, 53). In preeclampsia, maternal circulating levels of the precursor 25-OH-D are similar or slightly lower than normal; however, the active metabolite 1α,25-(OH)2-D is decreased significantly compared with women with healthy pregnancies (47). Placenta-
tal CYP27b1 mRNA expression is higher, and that of VDR and CYP24a1 is lower in pregnancies complicated by preeclampsia (11). Furthermore, CYP27b1 activity in preeclamptic placentas is just one-tenth of the activity in normal placentas (9). Altogether, although multiple studies suggest an important role of vitamin D in mammalian pregnancy, our knowledge of the physiological and pathophysiological factors that determine vitamin D status during gestation remains limited.

Our laboratory has been investigating the Brown Norway (BN) rat, which is an inbred rat strain characterized by fetal loss and small litter sizes (16, 25). Placental insufficiency in the BN rat is associated with decreased trophoblast invasion into the uterine decidua compared with other rat strains (16, 25). Therefore, we studied the vitamin D status and metabolism of BN rats compared with other rat strains. We have found that BN rat pregnancy is characterized by vitamin D deficiency due to metabolic gene dysregulation. In addition, supplementation with calcitriol led to improved pregnancy outcomes. Thus, the BN rat represents a novel animal model of metabolic dysregulation of vitamin D that can be used to further investigate the role of this important hormone in mammalian pregnancy.

**MATERIALS AND METHODS**

Animals. Three-month-old outbred Sprague-Dawley (SD) rats, BN inbred rats from barrier P07, and Lewis (LW) inbred rats from barrier H49 were obtained from Charles River Laboratories (Wilmington, MA). SD rats were chosen as the control strain due to multiple reports on their reproductive phenotype, including vitamin D status (18, 25). LW rats were chosen as an inbred control rat strain due to their reproductive and immune phenotype (12, 25). All rats were housed at the Loma Linda University Animal Research Facility under conditions of 14 h of fluorescent light, 10 h of darkness, ambient temperature of 20°C, and relative humidity of 30–60%. We obtained SD females from a single colony to decrease genetic variability. All rats were maintained in a temperature of 20°C, and relative humidity of 30 – 60%. We obtained SD females from a single colony to decrease genetic variability. All rats were housed in a temperature of 20°C, and relative humidity of 30 – 60%. We obtained SD females from a single colony to decrease genetic variability.

Breeding and tissue collection. Rats were bred by overnight monogamous pairing of a female with a male, as described previously (16). The following day was considered day 0 of pregnancy (P0). Pregnancy was confirmed by examining vaginal plugs on day 0 and weight gain by day 10 of pregnancy (P10). Female rats were euthanized by CO2 asphyxiation at various stages, including nonpregnancy (NP), pregnancy days 8, 13, 17, and 21 (P8, P13, P17, and P21, respectively), postpartum lactation day 10 (PP10), and postweaning day 1 (PW1). Pups were weaned on day 21 of life. After euthanization, blood was quickly collected directly from the heart using heparin blood tubes, followed by separation of plasma at 3,000 × 5-min centrifugation and storage at −80°C. Tissues were harvested, weighed, snap-frozen in liquid nitrogen, and stored at −80°C for later analysis.

Vitamin D supplementation. Cholecalciferol supplementation was achieved by feeding rats with customized diets before breeding and throughout gestation. Rat chow diets were designed by Harlan Laboratories, (Indianapolis, IN) and consisted of a control purified diet suitable for reproduction containing 1,000 IU cholecalciferol/kg diet (TD 02403, control diet), or 3,000 IU cholecalciferol/kg diet (TD 09134, cholecalciferol-supplemented diet). Calcitriol supplementation was achieved by implanting on P9 a subcutaneous miniosmotic pump (model 2004; Alzet, Cupertino, CA) that administers 6 μg/day of either vehicle (50% ethanol, 50% propylene glycol), 0.33 ng/μl (2 ng/day), or 1.33 ng/μl (8 ng/day) calcitriol.

**ELISA analysis of vitamin D metabolites, calcium, VDBP, and PTH.** Plasma levels of 25-OH-D and 1α,25-(OH)2-D were analyzed using commercially available EIA kits that determine total (bound and free) levels of vitamin D metabolites (Immunodiagnostic Systems, Scottsdale, AZ). The sensitivity of the 25-OH-D kit is 5 nM, the intra- and interassay variability is 5.3–8.7% coefficient of variation (CV), and it is specific for 25-OH-D and 24,25-(OH)2-D. The sensitivity of the 1α,25-(OH)2-D EIA kit is 6 pM, the intra- and interassay variability is estimated at 10 and 20% CV, and it is highly specific for 1α,25-OH-D. Maternal plasma levels of total calcium were assayed using a colorimetric kit (Abcam, San Francisco, CA), immunoreactive PTH was assayed using an ELISA kit (Immunotopics, San Clemente, CA), and VDBP levels were determined using an ELISA kit (Genway Biotech, San Diego, CA), following each respective manufacturer’s instruction manuals. Normal plasma values for adult humans and rodents range from 75 to 200 nM 25-OH-D, 50 to 195 pM 1α,25-(OH)2-D, 11 to 55 pg/ml intact PTH, and 300 to 550 mg/l VDBP. Normal total serum calcium levels range from 8.5 to 11.8 mg/dl.

**QUANTITATIVE REAL-TIME PCR.** RNA isolation, RT reaction, and real-time PCR were performed as described previously (16, 35). Exon-spanning primers were obtained from the literature and confirmed by standardized efficiency testing. Samples were analyzed on the Roche LightCycler 1.5 (Roche, Indianapolis, IN), using the Quantitect SYBR green kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. The following primers were used: rat CYP24 forward 5’-CAACACCTGGAAAGCCTATCG-3’, reverse 5’-CGCT- GGCATCTCTGTCCCTT-3’, rat CYP27b1 forward, 5’-ACCCATT- GCATCTCTTCC-3’, reverse 5’-GATTGAGTCCTCTCAGGT3’; rat VDR forward 5’-AGGAAACCGGCAGACT-3’, reverse 5’-CT- GTACCTCTCATGTC-3’, rat β-actin forward 5’- GGCCACG- CTGAAAAGATGA-3’, reverse 5’-GACCAGAGCATACG- ACA-3’. Quantitative analysis was performed with the aid of standard curves using two different control plasmids, as described previously (35). Data is reported as fg mRNA divided by ng β-actin mRNA. Means ± SE were obtained for each gestational and rat strain group.

**SDS-PAGE and immunoblotting.** From each group, snap-frozen placentas, kidney cortex, heart apex, and liver samples were homogenized in RIPA buffer (20 mM Tris·HCl, pH = 7.4, 0.05% SDS, 0.5% deoxycholate, 1% NP-40, 1 mM EDTA, and 20% glycerol) containing fresh Halt protease and phosphatase inhibitors (ThermoFisher, Pittsburgh, PA), sonicated for 3 min at low frequency, and centrifuged at 10,000 rpm × 10 min at 4°C. Protein lysates were then analyzed by Western immune blotting, as described previously (16, 35). Briefly, samples were separated on 4%–15% SDS–PAGE and transferred to polyvinylidene fluoride membranes. Membranes were blocked in 5% nonfat dried milk in 0.05% Tris-buffered saline (TBST) for 1 h and then probed in primary antibody overnight at 4°C. The following antibodies were used: monoclonal anti-VDR, rabbit polyclonal anti-CYP24a1 and anti-CYP27b1 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution; monoclonal anti-β-tubulin and anti-HSP90 (Cell Signaling Technology, Danvers, MA) and monoclonal anti-β-actin (Ambion, Austin, TX) at 1:5,000 dilution. All the antibodies were diluted in blocking buffer containing 5% nonfat dry milk in TBST. After three 10-min washes with TBST, the membranes were incubated with corresponding secondary antibodies that were diluted at 1:2,000. Bound antibodies were visualized using the SuperSignal West-Femto substrate (Pierce ThermoFisher, Pittsburgh, PA). Digital images were captured using the Alpha Innotech ChemiImager Imaging System with a high-resolution charge-coupled device camera and quantified using the Alpha Innotech ChemiImager 4400 software (Cell Biosciences, Santa Clara, CA). Relative VDR, CYP24a1, and CYP27b1 protein expression were calculated with respect to β-actin (or HSP90 for liver and heart samples), and fold changes in protein expression were determined. To estimate differences in protein levels between kidney and placenta, equal protein loading was confirmed by analyze-
ing the protein levels of both β-actin and β-tubulin. To compare band densitometries of the same protein between different gels, a standard sample (non-pregnant BN kidney) was used in every gel.

**Genomic DNA and methylation analysis.** The NCBI databank sequence NC_005102.3 of the BN rat strain, chromosome 3, was utilized to study the rat CYP24a1 promoter, as described previously (28). Genomic DNA was isolated from NP and P17 maternal kidneys of BN, SD, and LW rats, using the Wizard genomic DNA isolation kit (Promega ThermoFisher, Pittsburgh, PA). To determine methylation status of the CYP24a1 promoter, DNA samples were treated with bisulfite using the epTect Bisulfite kit (Qiagen) according to the manufacturer’s instructions. Converted DNA was amplified using primers directed to bisulfite modified genomic DNA: CYP24BisF1, 5'-TAGTTGTTAGGGGAGGAGGG-3'; CYP24BisR1, 5'- AACAACCTCTTTTACCCAT-3'; CYP24BisF2, 5'- TGGTAGTTTAGGGTAGAAATTAG-3'; CYP24BisR2, 5'- AAACAAAACACCTCTTTTAAAC-3'. Amplification conditions were as follows: 94°C for 2 min, 55°C for 30 s, 72°C for 1 min × 40 cycles, and 72°C for 5 min. Resulting amplicons were cloned into TOPO TA cloning vector (Invitrogen, Carlsbad, CA) for automated fluorescent sequencing, as described (15). Data were analyzed using CLC software, and clones showing <80% conversion or identified as clonal were not included in further analysis. The methylation levels for each amplicon were determined using the formula CpG methylated sites/total CpG sites × 100 for each clone. In addition, the 5'-untranslated region and promoter of CYP24a1 were analyzed by automated fluorescent sequencing to examine genetic variants among the three rat strains. Both plus and minus DNA strands were sequenced, aligned, and analyzed by CLC Sequence Viewer 6 software (CLCBio, Cambridge, MA). Determination of transcription factor binding sites, including the vitamin D response elements, was performed using MattInspector software (Genomatix, Munich, Germany).

**Statistical analysis.** All data are presented as means ± SE. We used one-way ANOVA and Bonferroni post hoc analysis using SPSS 16.0 (IBM, Armonk, NY) to determine significant differences between rat strains or gestational ages. Statistical significance was determined as \( P < 0.05 \).

**RESULTS**

**Vitamin D status in BN, SD, and LW rat pregnancies.** We investigated the maternal circulating levels of vitamin D metabolites in BN rats compared with the commonly studied SD outbred rat strain and the LW inbred rat strain, both of which manifest healthy reproductive phenotypes. In nonpregnant animals, the plasma levels of 25-OH-D, as determined by ELISA, were 85 ± 12 (BN), 73 ± 8 (LW), and 75 ± 6 nM (SD). Pregnancy was associated with a significant decrease in the maternal levels of 25-OH-D at P17 and P21 compared with the nonpregnant levels in all three rat strains (Fig. 1A).

ELISA determined a significant increase in the active metabolite 1α,25-(OH)₂-D in both LW and SD rats during pregnancy starting at P13 and throughout the rest of gestation and lactation (Fig. 1B). In contrast, BN rats demonstrated a significant decrease in maternal levels of active vitamin D starting at P13, with the lowest levels shown at P17 (31 ± 11 pM) and slowly recovering toward normal levels throughout lactation (Fig. 1B). At PW1, the maternal levels of active vitamin D returned to prepregnant levels (Fig. 1B).

The changes in maternal circulating levels of vitamin D metabolites were confirmed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). First, LC-
MS/MS confirmed that all three rat strains experience a decline in the levels of 25-OH-D3 by P17. Second, LC-MS/MS confirmed that pregnant SD and LW rats have increased levels, in contrast with pregnant BN rats, which have significantly decreased levels of 1α,25-(OH)2-D3 by P17 compared with nonpregnant levels (data not shown). We also observed significant differences between LC-MS/MS and ELISA methods. Plasma 25-OH-D3 levels measured by LC-MS/MS were significantly higher for all nonpregnant rats than those obtained by ELISA. In contrast, plasma 1α,25-(OH)2-D3 levels measured by LC-MS/MS were significantly lower in nonpregnant SD and LW than those measured by ELISA (data not shown).

We then studied maternal circulating levels of total calcium, VDBP, and immunoreactive PTH. Maternal plasma calcium levels remained constant throughout pregnancy and were not significantly different among the three rat strains (Fig. 1C). Maternal circulating VDBP levels increased significantly throughout pregnancy and lactation and did not significantly differ among the three rat strains (Fig. 1D). Finally, maternal PTH levels increased significantly in BN pregnancy compared with LW and SD pregnancies (Fig. 1E). Overall, the BN rat reproductive phenotype is characterized by significant vitamin D deficiency that correlates with increased PTH.

Expression of CYP27b1. The maternal kidney has been proposed to be the main regulator of vitamin D homeostasis throughout pregnancy, followed by the placenta (17, 18). In the maternal kidney, CYP27b1 mRNA expression was significantly increased in late pregnancies of BN and SD rats compared with nonpregnancy (Fig. 2A). Similarly, the levels of CYP27b1 protein were also increased significantly in late pregnancies of all three rat strains compared with NP levels, with pregnant BN rats showing the highest expression at P21 (Fig. 2C). In the placenta, CYP27b1 mRNA expression from midpregnancy (P13) to late pregnancy (P21) was constant and was not significantly different among the three rat strains (Fig. 2B). Placental expression of CYP27b1 protein, however, in-
creased significantly from P13 to P21 in all three rat strains and was significantly higher in BN placentas compared with SD and LW placentas (Fig. 2D).

We also studied the kidney/placenta ratios of CYP27b1 mRNA and protein expression. At P13, the mRNA ratios ranged from 0.4 to 0.7 for the three rat strains, indicating that the placenta had higher levels of CYP27b1 mRNA than maternal kidney (Fig. 2E). This ratio was increased significantly in LW rats at P17 and P21 (Fig. 2E). Similarly, at P13, the kidney/placenta protein ratios were 0.3 to 0.4 for the three rat strains, indicating that P13 placentas have significantly higher levels of CYP27b1 protein than maternal kidneys (Fig. 2F). This ratio was increased significantly in all three rat strains at P21, with ratios of 0.7 for SD and LW rats and 1.9 for BN rats (Fig. 2E). The largest increase in protein ratios (7.7-fold of P13 ratio at P21) was observed in BN rats.

Expression of CYP24a1. We next studied the expression profile of the vitamin D-inactivating enzyme CYP24a1. In the maternal kidneys, there were no differences in the levels of CYP24a1 mRNA and protein in nonpregnant animals among the three rat strains (Fig. 3, A and C). However, pregnancy increased CYP24a1 mRNA and protein abundance significantly in BN rats, peaking at P17 and returning to nonpregnant levels at PP10 (Fig. 3, A and C). In contrast, pregnancy decreased CYP24a1 expression significantly in LW and SD rats (Fig. 3, A and C). In the placenta, CYP24a1 mRNA abundance was significantly higher in P13 BN placentas compared with SD and LW rats (Fig. 3B). In addition, CYP24a1 mRNA and protein levels decreased significantly at later stages (P17 and P21) in all three rat strains (Fig. 3, B and D). Nevertheless, BN placentas had significantly higher expression of CYP24a1 protein than SD and LW placentas (Fig. 3D).

Fig. 3. Maternal renal CYP24a1 expression is upregulated in BN pregnancy. CYP24a1 mRNA and protein expression were determined by real-time PCR and immunoblotting, as described in MATERIALS AND METHODS. A and B: bar graphs of relative CYP24a1 mRNA levels (fg CYP24a1/ng β-actin mRNA) in maternal kidney (A) and placentas (B). C and D: bar graphs of relative CYP24A1 protein levels (CYP24A1/β-actin) and representative immunoblots in maternal kidney (C) and placentas (D). E and F: CYP24a1 kidney/placenta ratios for mRNA (G) and protein (H). Data represent the fold of NP values and shown as means ± SD (n ≥ 3/group). *P < 0.05 vs. SD rat values; †P < 0.05 vs. NP values; ‡P < 0.05 vs. P13 values.
We further analyzed CYP24a1 expression ratios for kidney/placenta. At P13, the kidney/placenta CYP24a1 mRNA ratio was 4 for SD rats, 8 for LW rats, and 12 for BN rats (Fig. 3E). Importantly, this ratio increased significantly in all three rat strains, with the highest on P17 (36 ± 9 for SD, 21 ± 6 for LW, and 242 ± 75 for BN rats, P < 0.05; Fig. 3E). At the protein level, P13 kidney/placenta ratios were 0.5 to 0.7 for all three rat strains. This ratio increased significantly only in BN rats on P17 (3.5-fold ratio of P13) and P21 (5.2-fold ratio of P13) (Fig. 3F).

CYP24a1 promoter methylation and genetic variations. CYP24a1 expression can be regulated by promoter methylation (37). Therefore, we analyzed the methylation status of the CYP24a1 promoter in the kidney. Analysis of proximal promoter segments −215/+132 and −658/−393 showed no significant differences in CpGs’s methylation (<1%) in P17 rats (3.5-fold ratio of P13) (Fig. 3F).

We also sequenced a ∼1,280-bp segment of the CYP24a1 promoter (Fig. 4). The sequences in BN rats were identical to those in the NCBI BN rat genome database. We found one single nucleotide polymorphism (SNP) in SD rats at position −1,115 (C/T) and 1 SNP in LW rats at −86 (G/A). Analysis of transcription factor binding sites, using TFSearch and Genomatix softwares, revealed binding sites for myeloid zinc finger, GATA-binding factors, nuclear factor erythroid derived 2, NF-kB member REL, heat shock factor 2, and VDR in all three rat strains (Fig. 4).

VDR expression. CYP24a1 expression is highly regulated by the transcription factor VDR (13, 21, 26). To examine alternative mechanisms of CYP24a1 regulation in pregnancy, we then analyzed the VDR expression throughout gestation and at lactation day 10. In the maternal kidney, BN rats showed lower VDR mRNA levels compared with SD and LW rats (Fig. 5, A and C). In all three rat strains, pregnancy downregulated renal VDR mRNA levels, with no significant differences among the three rat strains (Fig. 5A). Pregnancy decreased renal VDR protein levels significantly in SD and LW rats but not in BN rats (Fig. 5C). The effect was stronger at P21; VDR protein levels decreased ninefold in SD kidneys and 15-fold in LW kidneys (P < 0.05; Fig. 5C). In the placenta, there were few significant changes in VDR mRNA levels between P13, P17, and P21 placentas, with the exception of a significant increase in VDR mRNA expression in LW placentas at P21 (Fig. 5B). At the protein level, there was a significant increase in P21 VDR protein expression in all three rat strains compared with P13 placental values (Fig. 5D), and SD rats had lower VDR protein levels than and LW rats at P21.

Analysis of VDR kidney/placenta ratios revealed that kidney levels of VDR mRNA were always significantly higher than those of placenta at all times tested (Fig. 5E). In addition, the rat strains differ significantly in the kidney/placenta ratios of VDR mRNA at P13 (83 ± 15 for SD, 144 ± 15 for LW, and 190 ± 44 for BN; Fig. 5E). This ratio decreased significantly in SD and LW rats at later pregnancy stages but not in BN rats (Fig. 4E). At the protein level, kidney/placenta ratios on P13 were 0.4 ± 0.1 for SD rats, 0.2 ± 0.03 for LW rats, and 1.0 ± 0.1 for BN rats (P < 0.05 vs. SD/LW ratios), and they did not change significantly at later pregnancy stages (Fig. 5F).

To provide comparative analysis of VDR expression, we also determined VDR protein levels in heart apex and liver of nonpregnant and P21 pregnant rats. BN rats showed a significant decrease in heart and liver VDR protein expression at P21 compared with NP values (Fig. 6). Furthermore, analysis of heart and liver CYP27b1 protein expression revealed a similar decrease in heart and liver expression in BN pregnant compared with BN nonpregnant rats (Fig. 6). In contrast, pregnant SD and LW rats had comparable heart and liver VDR, CYP24a1, and CYP27b1 protein levels compared with nonpregnant (Fig. 6).

Supplementation with calcitriol, but not cholecalciferol, improves pregnancy outcomes in BN rats. To determine the effects of vitamin D supplementation on pregnancy outcomes, we designed dietary approaches to improve vitamin D status in the pregnant BN rat, and the results are shown in Table 1. BN rats fed a customized diet with three times the levels of cholecalciferol from preconception to pregnancy showed increased maternal plasma levels of 25-OH-D from 74 to 118 nM, with smaller increases in the levels of the active metabolite 1α,25-(OH)_2-D from 38 to 44 PM (Table 1). In contrast, SD rats fed the cholecalciferol-supplemented diet showed a significant increase in maternal plasma levels of both 25-OH-D and 1α,25-(OH)_2-D. Importantly, this ratio increased significantly in all three rat strains, with the highest on P17 (36 ± 9 for SD, 21 ± 6 for LW, and 242 ± 75 for BN rats, P < 0.05; Fig. 3E). At the protein level, P13 kidney/placenta ratios were 0.5 to 0.7 for all three rat strains. This ratio increased significantly only in BN rats on P17 (3.5-fold ratio of P13) and P21 (5.2-fold ratio of P13) (Fig. 3F).

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1α,25-(OH)₂-D (Table 1). Cholecalciferol-supplemented diet, however, did not alter maternal serum calcium, maternal body weight gain, litter size, or fetal or placental weight in either BN or SD rats compared with control diet (Table 1).

We next attempted to improve vitamin D status in BN rats by chronically administrating a small daily dose of the active vitamin D metabolite calcitriol via subcutaneous miniosmotic pumps (Table 2). Daily doses of 2 and 8 ng/day significantly and dose-dependently increased the levels of maternal circulating 1α,25-(OH)₂-D from 15.5 (solvent treated) to 135 (2 ng/day) and 485 pM (8 ng/day) (Table 2). Active vitamin D levels of solvent-treated rats were significantly lower than nonsurgically intervened BN rats at P17 (16 vs. 38 pM; Tables 1 and 2). Calcitriol at a dose of 8 ng/day induced significant toxicity, manifested as maternal hypercalcemia and weight loss, and decreased fetal and placental weights compared with solvent-treated rats but prevented fetal loss, as evidenced by an increase in litter size (Table 2). In addition, calcitriol dose-dependently decreased the levels of plasma PTH, decreased the maternal renal CYP27b1 mRNA levels, and increased VDR and CYP24a1 mRNA levels (Table 2). Calcitriol at a lower dose of 2 ng/day increased the circulating levels of active vitamin D 10-fold, did not affect maternal serum calcium levels, and was able to improve pregnancy outcomes by increasing litter size, preventing fetal resorption, and increasing maternal, fetal, and placental weights (Table 2). The reproductive outcomes of low-dose calcitriol-treated BN rats, although improved, did not reach the optimal levels observed in SD pregnancies (Tables 1 and 2).

DISCUSSION

The present study has demonstrated the following novel findings: 1) BN rat pregnancy is characterized by functional vitamin D deficiency, as defined by 25-OH-D levels <75 nM and 1α,25-(OH)₂-D levels lower than 50 pM; 2) vitamin D deficiency in BN pregnancy is associated with maternal renal and placental upregulation of CYP24a1 and CYP27b1, in-
increases in plasma PTH, and maternal heart and liver downregulation of CYP27b1 and VDR; and 3) vitamin D supplementation in the BN rat significantly improves pregnancy outcomes. In addition, pregnant SD and LW rats that are characterized by healthy reproductive outcomes show 1) maternal functional vitamin D sufficiency characterized by high (>150 pM) plasma levels of 1α,25-(OH)2-D but low (<75 nM) plasma levels of 25-OH-D, 2) maternal renal CYP24a1 downregulation that is not dependent on genetic (polymorphisms) or epigenetic (promoter methylation) factors, and 3) maternal renal VDR downregulation that is stronger at the protein level than at the mRNA level, suggesting posttranscriptional mechanisms of regulation.

With the aid of different rat strains, we have discovered nonclassical mechanisms of pregnancy-specific regulation of vitamin D status. In human pregnancy, the levels of 25-OH-D remain constant or slightly decreased, whereas the levels of the active metabolite 1α,25-(OH)2-D double or triple throughout gestation and lactation (27, 43). Similarly, analysis of the active metabolite 1α,25-(OH)2-D revealed that both LW and SD rats have sufficient circulating levels at nonpregnancy, with a nearly twofold increase in nonpregnancy values in late gestation. In contrast, BN rats, despite having normal levels of 1α,25-(OH)2-D at nonpregnancy, manifested a significant fivefold decrease in nonpregnancy values throughout gestation. Plasma metabolite values obtained with LC-MS/MS were significantly different for both 25-OH-D and 1α,25-(OH)2-D in nonpregnant animals, which further confirmed previous reports on the differences among methods (49, 51). Nevertheless, data obtained with LC-MS/MS confirmed vitamin D deficiency in pregnant BN and vitamin D sufficiency in SD/LW rats, as shown by pregnancy-specific changes in 1α,25-(OH)2-D.
expression did not prevent decreases in 1,25-(OH)2-D levels increases in PTH and maternal/placental increases in CYP27b1 fourfold the levels of nonpregnancy values. Interestingly, in PTH were increased significantly in BN rat pregnancy to nearly pregnancy are characterized by increased PTH. The levels of CYP27b1 upregulation in late gestation. This was not

In all of our rat strains, we observed significant increases in maternal renal upregulation of CYP27b1 (17, 43), although this increased active vitamin D levels in mammalian pregnancy is the maternal renal upregulation of CYP27b1 (17, 43), although this theory remains unproven. Furthermore, it has been suggested that the placenta activates vitamin D precursors into calcitriol, thereby providing this molecule to the fetoplacental unit as well as contributing calcitriol for the maternal system (10, 56). In all of our rat strains, we observed significant increases in CYP27b1 expression; however, BN rat pregnancy showed the highest CYP27b1 upregulation in late gestation. This was not surprising considering that PTH is an important physiological stimulant of renal CYP27b1 expression (13, 36), and BN rat pregnancies are characterized by increased PTH. The levels of PTH were increased significantly in BN rat pregnancy to nearly fourfold the levels of nonpregnancy values. Interestingly, increases in PTH and maternal/placental increases in CYP27b1 expression did not prevent decreases in 1,25-(OH)2-D levels in pregnant BN but it likely contributes to recovery of vitamin D sufficiency during the lactation and weaning stages. We speculate that pregnancy-specific regulation of extrarenal CYP27b1 may contribute to vitamin D sufficiency/deficiency. In support of this theory, we observed significant CYP27b1 downregulation in pregnant BN heart and liver tissue that was not observed in SD and LW rats. Our data indicate that rat pregnancy is characterized by classical regulation of vitamin D through PTH and PTH-mediated upregulation of maternal renal CYP27b1. In addition, our data suggest that extrarenal regulation of CYP27b1 is PTH independent and can potentially contribute to vitamin D sufficiency.

In nonpregnant mammals, increases in vitamin D metabolites, particularly calcitriol, are well known to upregulate the renal expression of VDR and CYP24a1 (4, 29, 41, 46). This feedback mechanism prevents vitamin D toxicity and is present in every cell that contains VDR (19, 21). In contrast, our studies clearly demonstrate uncoupling of renal VDR/CYP24a1 expression and 1,25-(OH)2-D plasma levels during pregnancy. High levels of 1α,25-(OH)2-D2 are associated with downregulation of VDR and CYP24a1 in pregnant SD/LW rats, and low levels of 1α,25-(OH)2-D2 are associated with

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**Table 1. Effect of cholecalciferol supplementation on maternal vitamin D status and pregnancy outcomes**

<table>
<thead>
<tr>
<th>Outcomes on Pregnancy Day 17</th>
<th>Brown Norway</th>
<th>Sprague-Dawley</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control diet</td>
<td>VD3 diet</td>
</tr>
<tr>
<td></td>
<td>Control diet</td>
<td>VD3 diet</td>
</tr>
<tr>
<td>25-(OH)-D, nM</td>
<td>74 ± 8</td>
<td>118 ± 13*</td>
</tr>
<tr>
<td>1α, 25-(OH)2-D, pM</td>
<td>38 ± 6</td>
<td>44 ± 8</td>
</tr>
<tr>
<td>Serum calcium, nM</td>
<td>10 ± 0.6</td>
<td>14 ± 0.5</td>
</tr>
<tr>
<td>Maternal weight gain, g</td>
<td>38 ± 5</td>
<td>35 ± 7</td>
</tr>
<tr>
<td>Litter size (n)</td>
<td>3.5 ± 2</td>
<td>3.0 ± 2.5</td>
</tr>
<tr>
<td>Fetal resorptions (n)</td>
<td>2.2 ± 1.5</td>
<td>2.3 ± 1</td>
</tr>
<tr>
<td>Fetal weight, g</td>
<td>0.76 ± 0.06</td>
<td>0.81 ± 0.06</td>
</tr>
<tr>
<td>Placental weight, g</td>
<td>0.32 ± 0.03</td>
<td>0.30 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5. VD3, vitamin D3. Control customized diet contained 1,000 IU VD3/kg diet, and VD3-supplemented diet contained 3,000 IU VD3/kg diet. *P < 0.05 control vs. VD3 diet; †P < 0.05, Brown Norway vs. Sprague-Dawley rats.

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**Table 2. Effect of calcitriol supplementation on Brown Norway rat maternal vitamin D status and pregnancy outcomes**

<table>
<thead>
<tr>
<th>Outcomes on Pregnancy Day 17</th>
<th>Control (n = 4)</th>
<th>Calcitriol, 2 ng/day (n = 4)</th>
<th>Calcitriol, 8 ng/day (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma 25-(OH)-D, nM</td>
<td>65 ± 6</td>
<td>51 ± 10*</td>
<td>27 ± 7*</td>
</tr>
<tr>
<td>Plasma 1α, 25-(OH)2-D, pM</td>
<td>16 ± 3</td>
<td>135 ± 25*</td>
<td>485 ± 98*</td>
</tr>
<tr>
<td>Serum calcium, mg/dl</td>
<td>11.2 ± 0.4</td>
<td>11.9 ± 0.6</td>
<td>13.7 ± 0.4*</td>
</tr>
<tr>
<td>Plasma PTH</td>
<td>159 ± 31</td>
<td>12 ± 3*</td>
<td>2 ± 1*</td>
</tr>
<tr>
<td>Maternal weight gain, g</td>
<td>33 ± 3</td>
<td>59 ± 6*</td>
<td>−12 ± 18*</td>
</tr>
<tr>
<td>Litter size (n)</td>
<td>3.0 ± 1.0</td>
<td>7.0 ± 1.5*</td>
<td>6.0 ± 1.5*</td>
</tr>
<tr>
<td>Fetal resorptions (n)</td>
<td>1.5 ± 0.8</td>
<td>0 ± 0*</td>
<td>0 ± 0*</td>
</tr>
<tr>
<td>Fetal weight, g</td>
<td>0.758 ± 0.06</td>
<td>1.06 ± 0.22*</td>
<td>0.52 ± 0.32*</td>
</tr>
<tr>
<td>Placental weight, g</td>
<td>0.31 ± 0.01</td>
<td>0.37 ± 0.04*</td>
<td>0.27 ± 0.11</td>
</tr>
<tr>
<td>Renal 27bl mRNA</td>
<td>1 ± 0.3</td>
<td>0.25 ± 0.1*</td>
<td>0.08 ± 0.03*</td>
</tr>
<tr>
<td>Renal 24al mRNA</td>
<td>41 ± 5</td>
<td>82 ± 26*</td>
<td>109 ± 25*</td>
</tr>
<tr>
<td>Renal VDR mRNA</td>
<td>76 ± 27</td>
<td>97 ± 20</td>
<td>120 ± 21*</td>
</tr>
</tbody>
</table>

Values are means ± SE. PTH, parathyroid hormone; VDR, vitamin D receptor. *P < 0.05 vs. control; †fetuses were not viable.
CYP24a1 upregulation in pregnant BN rats. This uncoupling has also been reported in the process of aged small mammals that have high renal VDR/CYP24a1 expression and low 1α,25-(OH)₂-D plasma levels (1). The mechanisms that lead to VDR/CYP24a1 upregulation during aging and VDR/CYP24a1 downregulation in healthy pregnancy have not been elucidated. We did not observe significant renal CYP24a1 promoter methylation or SNPs that could account for differences in CYP24a1 regulation among the three rat strains. Therefore, we propose that upstream factors that are pregnancy specific (metabolic, immune derived, hormonal factors) determine the expression of VDR and CYP24a1 in pregnancy and vitamin D status during pregnancy (Fig. 7). In support of this hypothesis, a report has shown that hypoxia upregulates CYP24a1 in human placental ex vivo cultures (33).

Our studies also revealed that, in healthy pregnancies, downregulation of VDR protein levels was significantly stronger than downregulation of VDR mRNA. This observation was further corroborated by the kidney/placenta ratios; VDR/mRNA ratios ranged from 25 to 220 compared with VDR protein ratios of only 0.5 to 1.2, indicating that maternal kidneys have more copies of VDR mRNA than placentas but similar copies of translated proteins. Because VDR is a nuclear hormone, and other nuclear hormones are highly regulated by proteasomal degradation (22, 35), we speculate that healthy pregnancy is characterized by increased renal proteasomal degradation of VDR, which then limits the ability of calcitriol to upregulate the CYP24a1 transcription. Altogether, maternal renal VDR downregulation seems to be a pregnancy-specific physiological adaptation to prevent maternal renal catabolism of calcitriol. This in turn leads to increases in circulating levels of 1α,25-(OH)₂-D that are available to other maternal organs as well as the fetoplacental unit.

Vitamin D supplementation studies revealed that pregnancy complications in BN rats can be partially rescued by increasing the circulating levels of 1α,25-(OH)₂-D. Our data support the hypothesis that vitamin D sufficiency is necessary to ensure healthy pregnancy outcomes, as suggested by the clinical association of vitamin D deficiency with pregnancy complications (5, 14, 52, 53) and offspring childhood diseases (9a, 32, 54). We speculate that maternal vitamin D deficiency could be a contributing factor in the susceptibility of BN rats for pregnancy disorders (16, 25) as well as postnatal allergic-induced asthma (12). In addition, we propose that BN rats can be a useful model to investigate the molecular and physiological roles of vitamin D during mammalian pregnancy. Recent studies have suggested that vitamin D effects in mammalian pregnancy include the regulation of the immune, endocrine, and cardiovascular systems (2, 3, 30, 31, 39) warranting further research.

Finally, our studies revealed that dietary supplementation with cholecalciferol failed to increase 1α,25-(OH)₂-D levels in BN rats, which confirms the important metabolic dysregulation of BN pregnancy. We speculate that a similar scenario could occur in some pregnant women that have significant metabolic dysregulation due to underlying physiological factors, disease, or genetics and might not be able to maintain adequate levels of 1α,25-(OH)₂-D even when supplemented with vitamin D precursors. Our studies suggest possible explanations as to why recent clinical studies on vitamin D supplementation throughout pregnancy have not clearly identified a beneficial role of vitamin D in the prevention of pregnancy complications (6, 8, 20, 45). Therefore, further studies are required to elucidate novel biomarkers of vitamin D sufficiency and deficiency and to understand upstream mechanisms that regulate maternal vitamin D metabolism during pregnancy and lactation.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
R.G. and E.M.-G. conceived and designed the research; R.G., L.Z., A.B.B., D.J.B., B.O., and E.M.-G. performed the experiments; R.G., L.Z., A.B.B., D.J.B., B.O., and E.M.-G. analyzed the data; R.G., L.Z., A.B.B., D.J.B., L.D.L., B.O., and E.M.-G. edited and revised the manuscript; E.M.-G. approved the final version of the manuscript.

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