Expressions of vitamin D metabolic components VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR in placentas from normal and preeclamptic pregnancies

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Vitamin D insufficiency/deficiency (3, 7, 29), the information on placental vitamin D metabolism and function during pregnancy is lacking. To determine whether altered vitamin D metabolic system is present in preeclamptic placentas, we examined vitamin D binding protein (VDBP), 25-hydroxylase (CYP2R1), 1α-hydroxylase (CYP27B1), 24-hydroxylase (CYP24A1), and vitamin D receptor (VDR) expressions in placentas from normotensive and preeclamptic pregnancies. By immunostaining we found that in normal placentas VDBP, CYP24A1, and VDR expressions are localized mainly in trophoblasts, whereas CYP2R1 and CYP27B1 expressions are localized mainly in villous core fetal vessel endothelium. Protein expressions of CYP2R1 and VDR are reduced, but CYP27B1 and CYP24A1 expressions are elevated, in preeclamptic compared with normotensive placentas. Because increased oxidative stress is an underlying pathophysiology in placental trophoblasts in preeclampsia, we further determined whether oxidative stress contributes to altered vitamin D metabolic system in placental trophoblasts. Trophoblasts isolated from normal-term placentas were treated with hypoxic-inducing agent CoCl2, and protein expressions of VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR were determined. We found that hypoxia-induced downregulation of VDBP, CYP2R1, and VDR and upregulation of CYP27B1 and CYP24A1 expressions were consistent with that seen in preeclamptic placentas. CuZnSOD expression was also downregulated in trophoblasts treated with CoCl2. These results provide direct evidence of disrupted vitamin D metabolic homeostasis in the preeclamptic placenta and suggest that increased oxidative stress could be a causative factor of altered vitamin D metabolism in preeclamptic placentas.
MATERIALS AND METHODS

Chemicals and reagents. Antibodies for CYP2R1 (S1968), CYP24A1 (S1171 for Western blotting), and catalase were from Epitomics (Burlingame, CA), antibodies for CYP27B1 (H-90), CYP24A1 (H-87 for immunostaining), V (D-6), and CuZn superoxide dismutase (CuZn-SOD; N-19) were purchased from Santa Cruz Biotechnology (San Diego, CA), and Dulbecco’s modified Eagle’s medium (DMEM), Percoll, and protease inhibitors were from Sigma (St. Louis, MO). All other chemicals were from Sigma unless otherwise noted.

Tissue collections. Third-trimester placentas from normotensive and preeclamptic pregnant women were collected immediately after delivery from the main hospital of Louisiana State University Health Sciences Center-Shreveport, Shreveport, LA. First- and second-trimester placentas were collected from selective pregnancy terminations at the Department of Obstetrics and Gynecology, the First Hospital of Harbin Medical University, China. Tissue collection was approved by both institutions. Normal pregnancy is defined as maternal blood pressure <140/90 mmHg without obstetrical and medical complications. Preeclampsia is defined as blood pressure >140/90 mmHg with at least two separate readings with proteinuria (>1+) at dipstick or >300 mg protein/24-h urine. A total of 34 placentas were used in this study, with 17 from normal-term pregnancies, 11 from preeclampsia, three from first-trimester pregnancies, and three from second-trimester pregnancies. None of the patients had signs of infection, nor were they smokers. None of the patients was complicated with diabetes, but two patients in the preeclamptic group had chronic hypertension. Freshly obtained placental tissues were processed immediately for either fixation, snap-freezing, or trophoblast isolation.

Immunohistochemistry. Placental tissue pieces were fixed with 10% formalin and embedded in paraffin. Expressions of VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR were examined by immunostaining of paraffin-embedded tissue sections. A standard immunohistochemistry staining procedure was performed. Briefly, a series of defaturation was done with xylene and ethanol alcohol. Antigen retrieval was performed by boiling tissue slides in citric buffer, and hydrogen peroxide was used to quench the endogenous peroxidase activity. After blocking with goat serum, tissue sections were incubated with the primary antibody at 4°C overnight and then biotinylate-conjugated peroxidase was used to quench the endogenous peroxidase activity. After blocking with goat serum, tissue sections were incubated with the primary antibody at 4°C overnight and then biotinylate-conjugated secondary antibody at room temperature for 3 h. Stained slides were counterstained with Gill’s formulation hematoxylin. Tissue sections stained with isotype IgG or without primary antibody served as negative controls. Slides stained with the same antibody were all stained at the same time. Stained slides were then reviewed under microscope, and images were captured with PictureFrame computer software (Uptronics, Sunnyvale, CA) and recorded to a microscope-linked PC computer.

Trophoblast isolation and culture. Trophoblasts were isolated from normal-term placentas (n = 3) by trypsin digestion (0.125% trypsin solution containing 100 μmol of DNase I and 5 mmol of MgCl2) in DMEM at 37°C for 90 min. Isolated trophoblasts were further purified by Percoll gradient centrifugation, and contaminated red blood cells were eliminated by incubation of isolated trophoblast cells with red blood cell lysis buffer, as described previously (37). Isolated trophoblasts (5 × 10⁵ cells/well) were then seeded into six wells/plate and incubated with DMEM containing 5% fetal bovine serum and antibiotics. Culture medium was changed after overnight incubation. To induce trophoblast oxidative stress, cells were then treated with different concentrations of CoCl2 at 0, 100, 250, and 500 μM for 48 h. CoCl2 is a hypoxic mimetic agent that has been widely used as an oxidative stress stimulator. At the end of the experiment, total cellular protein was extracted by ice-cold protein lysis buffer containing 50 mM Tris, 0.5% NP-40, and 0.5% Triton X-100 with protease inhibitors of phenylmethylsulfonyl fluoride, dithiothreitol, leupeptin, and aprotinin. Samples were stored at −70°C until assay.

Protein expression by Western blot. Expressions for VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR were examined by Western blot in snap-frozen placental tissues and in isolated trophoblasts after culture. For placental tissue expression, total tissue protein was extracted from snap-frozen tissue. For trophoblast expression, cellular protein was extracted after cells were treated with CoCl2, as stated above. For Western blot, briefly, an aliquot of total cellular protein (15 μg of each sample) was subjected to electrophoresis and then transferred to nitrocellulose membranes. The membranes were probed with a primary antibody and then secondary antibody. The bound antibodies were visualized with an enhanced chemiluminescence detection Kit (Amersham, Arlington Heights, IL). Nitrocellulose membranes were stripped and blocked before they were probed with different primary antibodies. Protein expressions for CuZnSOD and catalase were also determined. Band densities were scanned and analyzed by National Institutes of Health Image 1.16 software. Relative density for each of the target molecules was normalized by β-actin expression.

RESULTS

Expression of VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR in normal and preeclamptic placentas. To determine whether altered vitamin D metabolism components are present in preeclamptic placentas, placental tissue protein expressions of VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR were determined by immunostaining and by Western blot. Immunostaining data show compartmental localization/distribution of VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR within villous tissue. Western blot results represent the total amount of protein in the placenta tissue. A total of 22 placentas were used in this experiment. Immunostaining was done in tissue sections from 12 placentas (6 from normal and 6 from preeclamptic pregnancies). For each antibody staining, all slides were stained at the same time, and consistent results were obtained. Western blot was done using total tissue protein extracts from snap-frozen tissue homogenates (5 from normal and 5 from preeclamptic pregnancies). The patient demographic information was summarized in Table 1. Figure 1 shows immunostaining and Western blot results for VDBP,

Table 1. Demographic characteristics for normal and preeclamptic pregnant women

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Preeclampsia</th>
<th>P Value</th>
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<tr>
<td>Maternal age, yr</td>
<td>24 ± 7</td>
<td>24 ± 4</td>
<td>0.844</td>
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<tr>
<td>Racial status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age, wk</td>
<td>37° ± 2°</td>
<td>32° ± 4°</td>
<td>0.0007</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td></td>
<td></td>
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<tr>
<td>Systolic</td>
<td>125 ± 16</td>
<td>165 ± 13</td>
<td>0.0001</td>
</tr>
<tr>
<td>Diastolic</td>
<td>69 ± 14</td>
<td>98 ± 11</td>
<td>0.0004</td>
</tr>
<tr>
<td>Gravidity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pimigravid</td>
<td>5</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Multigravid</td>
<td>6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Mode of delivery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Caesarean section</td>
<td>7</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Placenta weight, g</td>
<td>557 ± 109</td>
<td>326 ± 127</td>
<td>0.0022</td>
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Data are presented as means ± SD; n = 11 in each group.
CYP2R1, CYP27B1, CYP24A1, and VDR expressions in normal and preeclamptic placental tissues. For immunostaining, VDBP and VDR are strongly expressed in syncytiotrophoblasts in normal placentas (Fig. 1, A–E), but markedly reduced in syncytiotrophoblasts in preeclamptic placentas (Fig. 1, F–J). In normal placentas, CYP2R1 is expressed in syncytiotrophoblasts, stromal cells, and villous endothelial cells. CYP2R1 expression is much reduced in preeclamptic placentas (Fig. 1, B and G). CYP27B1 is strongly expressed in villous fetal vessel endothelium in normal placenta (Fig. 1, C). In contrast, CYP27B1 is strongly expressed in trophoblasts and less detected in villous fetal vessel endothelium in preeclamptic placenta (Fig. 1, D). CYP24A1 is expressed in trophoblasts in normal placentas (Fig. 1, A), and its expression is increased in placentas from preeclampsia (Fig. 1, I).

The abundance of total protein expressions for VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR determined by Western blot is shown in Fig. 1, bottom. The bar graphs show the relative density of VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR protein expressions after normalization with β-actin expression. *P < 0.05 and **P < 0.01; PE vs. normal. ST, syncytiotrophoblasts; V, villous fetal vessels.

VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR expressions in normal and preeclamptic placental tissues. For immunostaining, VDBP and VDR are strongly expressed in syncytiotrophoblasts in normal placentas (Fig. 1, top, A and E), but markedly reduced in syncytiotrophoblasts in preeclamptic placentas (Fig. 1, top, F and J). In normal placentas, CYP2R1 is expressed in syncytiotrophoblasts, stromal cells, and villous endothelial cells. CYP2R1 expression is much reduced in preeclamptic placentas (Fig. 1, top, B and G). CYP27B1 is strongly expressed in villous fetal vessel endothelium in normal placenta (Fig. 1, top, C). In contrast, CYP27B1 is strongly expressed in trophoblasts and less detected in villous fetal vessel endothelium in preeclamptic placenta (Fig. 1, top, H). CYP24A1 is expressed in trophoblasts in normal placentas (Fig. 1, top, D), and its expression is increased in placentas from preeclampsia (Fig. 1, top, I).

The abundance of total protein expressions for VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR determined by Western blot is shown in Fig. 1, bottom. The bar graphs represent the relative density of VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR expressions after adjustment by β-actin expression. Consistent with immunostaining data, total tissue protein expressions of CYP2R1 and VDR are reduced and total tissue protein expressions of CYP27B1 and CYP24A1 increased in preeclamptic placental tissues compared with normal placental tissues. Although immunostaining of VDBP was reduced in preeclamptic placentas, its total protein expression was not significantly different between normal and preeclamptic placentas by Western blot.

Expressions of VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR in placentas at different gestational ages. Because most placentas in the preeclamptic group were delivered before term (<37 wk), we then determined whether the altered protein expressions between normal and preeclamptic placentas were due to the differences in gestational age. We examined VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR expressions by immunostaining in nine human placentas: three from 8–10 wk (1st trimester), three from 16–18 wk (2nd trimester), and three from 37–38 wk (term) of pregnancy. For each antibody staining, all slides were stained at the same time, and consistent results were obtained. Representative immunostainings of VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR expressions at different gestational ages are shown in Fig. 2. Slides stained with isotype IgG antibody or slides stained only with a secondary antibody showed negative staining (data not shown).

Interestingly, differential compartmental localization of
CYP2R1 and CYP27B1 expressions was noticed in the human placenta at different gestational ages. CYP2R1 is strongly expressed in cytotrophoblasts in the first-trimester placentas and also strongly expressed in villous core fetal vessel endothelium and stromal cells in the term placentas. CYP27B1 is expressed in both cyto- and syncytiotrophoblasts in the first-trimester placentas, and its expression in trophoblasts is reduced in the second trimester and further reduced in the term placentas. In contrast, CYP27B1 is positive in the villous core fetal vessel endothelium in the second-trimester placentas and strongly expressed in the fetal vessel endothelium in the term placentas. Bar, 50 μm.

Oxidative stress induced alteration of VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR expressions in placental trophoblasts. Trophoblasts were isolated from normal-term placentas (n = 3) and treated with CoCl2 at different concentrations (0, 100, 250, and 500 μM) for 48 h. We choose CoCl2 because CoCl2 is a hypoxic mimetic agent that has been widely used to induce oxidative stress in various tissue and cell culture studies, including trophoblasts (9, 15, 17, 18). At the end of incubation, total cellular protein was collected. Protein expressions for VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR were determined by Western blot. As shown in Fig. 3, VDBP, CYP2R1, and VDR expressions were significantly downregulated, whereas CYP27B1 expression was significantly upregulated, in trophoblasts treated with CoCl2 compared with the untreated cells. CYP24A1 expression was slightly, but not statistically, increased. The bar graphs represent the relative densities of VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR expressions after normalization by β-actin expression in cultured trophoblasts.

Oxidative stress altered expressions of CuZnSOD and catalase in placental trophoblasts. To confirm increased oxidative stress in trophoblasts treated with CoCl2, we determined expressions of antioxidant CuZnSOD and catalase in cells treated with CoCl2. Our results showed that CuZnSOD expression was reduced but that catalase expression was increased in tropho-
blasts treated with CoCl2 compared with the untreated control cells. Reduced CuZnSOD expression and increased catalase expression were also dose-dependently associated with increased CoCl2 concentrations in the culture (Fig. 4).

**DISCUSSION**

Altered mRNA expressions for CYP27B1, CYP24A1, or VDR were previously reported to be either increased or decreased in preeclamptic placentas compared with normal placentas (11, 13). In our study, we determined the distributions and expressions of major vitamin D metabolic components, including VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR, by immunostaining and by Western blot analysis in placental tissues from normal and preeclamptic pregnancies. By immunostaining, we found that in normal-term placentas VDBP and VDR are expressed mainly in trophoblasts and that CYP27B1 is expressed predominantly in villous vessel endothelium. VDBP, CYP2R1, and VDR expressions were reduced and CYP27B1 and CYP24A1 expressions increased in preeclamptic placentas compared with normal placentas. These results provide direct evidence of disrupted vitamin D metabolic homeostasis in preeclamptic placenta.

VDBP is a multifunctional protein in the plasma. Studies have shown that VDBP carries ~88% of the 25(OH)D3 and 85% of the 1,25(OH)2D3 in the circulation (6, 33). It binds to and transports bioactive vitamin D to target organs and cells. In the present study, we found that VDBP is expressed in both cyto- and syncytiotrophoblasts of normal placentas throughout pregnancy. Reduced trophoblast expression of VDBP in preeclamptic placentas was detected by immunostaining but not by Western blot of total protein in snap-frozen tissues. This discrepancy could be due to blood trapped in the snap-frozen tissue, which contributes to the total amount of VDBP detected by Western blot. Although the exact function of VDBP in placental trophoblasts is not known, if VDBP is a key carrier/binding protein for 25(OH)D3 and 1,25(OH)2D3 then it is expected that trophoblast VDBP could be a cellular reservoir of bioactive vitamin D at the maternal-fetal interface during pregnancy. Reduced VDBP expression in trophoblasts could be an indicator of reduced cellular VDBP activity or decreased 25(OH)D3 and 1,25(OH)2D3 availability within trophoblasts in preeclamptic placentas. Low-density lipoprotein-related protein 2 (also known as megalin) might also play a role. It mediates endocytosis of VDBP (30). Whether deregulation of megalin is present in placental trophoblasts in preeclampsia needs further investigation.

Two major vitamin D metabolizing enzymes, CYP2R1 and CYP27B1, were examined. CYP2R1 converts calciferol to 25(OH)D3 (calcidiol), which is further converted to 1,25(OH)2D3 (calcitriol) by CYP27B1. We found different patterns of CYP2R1 and CYP27B1 expressions between the first-trimester and the term placentas, i.e., the dominant expression of CYP2R1 and CYP27B1 in trophoblasts in the first-trimester placenta that switches to the dominant expression of CYP2R1 and CYP27B1 in the villous fetal vessel endothelium in the term placenta. Although in the present study we did not measure 1,25(OH)2D3 production by placental trophoblasts or endothelial cells, the presence of CYP2R1 together with
CYP27B1 in trophoblasts and then in the villous core fetal vessel endothelium undoubtedly suggests that trophoblasts might be a source of maternal 1,25(OH)\(_2\)D\(_3\) during pregnancy and that the fetal vessel endothelium could be a source of fetal 1,25(OH)\(_2\)D\(_3\) towards term.

Protein expression for CYP2R1 is significantly reduced and protein expression for CYP27B1 significantly elevated in preeclamptic placenta compared with normal placenta. In normal-term placentas, CYP27B1 is weakly expressed in trophoblasts but strongly expressed in villous vessel endothelium, whereas CYP2B1 is expressed mainly in placental trophoblasts and barely detectable in villous vessel endothelium in preeclamptic placentas. CYP2B1 is the key enzyme to convert 1,25(OH)\(_2\)D\(_3\) from 25(OH)D\(_3\). Although the exact mechanism of deregulation of trophoblast expressions of CYP2R1 and CYP27B1 seen in preeclamptic placentas is not known, data obtained from our cell culture study suggest that hypoxia/oxidative stress downregulates CYP2R1 expression, whereas increased CYP27B1 expression could be a compensatory response due to increased oxidative stress. The predominant expression of CYP2B1 in villous vessel endothelium in normal placenta suggests that villous vessel endothelium could be a source of fetal 1,25(OH)\(_2\)D\(_3\) during pregnancy, and reduced CYP2B1 expression in villous endothelium may explain in part the low cord blood levels of 1,25(OH)\(_2\)D\(_3\) found in women with preeclampsia (16). CYP24A1 is a major vitamin D catabolizing enzyme. It degrades 25(OH)D\(_3\) and 1,25(OH)\(_2\)D\(_3\) to 24,25(OH)\(_2\)D\(_3\) and 1,24,25(OH)\(_3\)D\(_3\). We found that CYP24A1 was weakly expressed in placental trophoblasts throughout pregnancy in normal placentas, but its expression was markedly increased in preeclamptic placentas. Increased CYP24A1 expression suggests the possibility of increased degradation of bioactive vitamin D in placental trophoblasts in preeclampsia. Increased CYP24A1 expression may also be due to reduced DNA methyltransferase expression observed in placental trophoblasts in preeclampsia (data not shown), since specific methylation of CYP24A1, but not CYP2B1 and VDR, is found in placental trophoblasts and promoter methylation decreases CYP24A1 activity (26).

VDR is strongly expressed in trophoblasts of normal placentas, and its expression was reduced significantly in preeclamptic placentas. The presence of VDR together with CYP2R1, CYP2B1, and CYP24A1 in trophoblasts clearly indicates the presence of the vitamin D metabolic system and autocrine regulatory signaling in placental trophoblasts. It is well known that the biological function of 1,25(OH)\(_2\)D\(_3\) is mediated through binding to its receptor on cells. Most if not all effects of 1,25(OH)\(_2\)D\(_3\) are mediated by VDR, acting primarily by regulating the expression and downstream signaling (10, 23, 24, 40). Reduced trophoblast expression of VDR could result in altered downstream vitamin D signaling in preeclamptic placentas. Reduced VDR expression may also be due to the lack of 1,25(OH)\(_2\)D\(_3\) within the cells since the level of VDR expression is dependent on the ligand’s availability. Increased trophoblast CYP24A1 expression and the possibility of increased degradation of 1,25(OH)\(_2\)D\(_3\) further support the concept of disrupted vitamin D metabolic homeostasis in preeclamptic placenta.

The reason for altered VDBP, CYP2R1, CYP2B1, CYP24A1, and VDR expressions in preeclamptic placentas is not clear, although the association of promoter methylation with CYP24A1, but not CYP2B1 and VDR, gene expression has been reported in placental trophoblasts (26). Since increased oxidative stress is an underlying pathophysiology in the preeclamptic placenta, we have tested specifically whether hypoxia/oxidative stress contributes to the altered vitamin D metabolic components in preeclamptic placentas. Trophoblast cells were isolated from normal-term placentas. Trophoblast oxidative stress was induced by treating the cells with different doses of CoCl\(_2\). CoCl\(_2\) is a hypoxic mimic agent that has been widely used to induce oxidative stress in various in vitro cell and tissue culture studies, including trophoblasts (9, 18). We found that not only VDBP and VDR but also CYP2R1 expressions were dose-dependently reduced in cells treated with CoCl\(_2\). In contrast, CYP27B1 expression was dose-dependently increased in cells treated with CoCl\(_2\). CYP24A1 expression was slightly increased in cells treated with CoCl\(_2\). The phenomena of hypoxia mimic-induced downregulation of VDBP, CYP2R1, and VDR expressions and upregulation of CYP27B1 expression are consistent with the findings of reduced VDBP, CYP2R1, and VDR expressions and increased CYP27B1 expression seen in trophoblasts of preeclamptic placentas. Trophoblast CYP24A1 expression was slightly, but not significantly, upregulated when treated with CoCl\(_2\). This could be due to the differences in the duration and the degree of hypoxia-induced oxidative stress in cultured trophoblasts. Nonetheless, these results provide convincing evidence that...
oxidative stress could deregulate the vitamin D metabolic system in placental trophoblasts. Oxidative stress could be a causative factor leading to compromised vitamin D homeostasis in placental trophoblasts in preeclampsia.

Previous published works have shown numerous biological effects of vitamin D3 in the placenta (31). Vitamin D is involved in the regulation of placental lactogen expression (34), human chorionic gonadotropin secretion (4), and calcium transport (21) in the placenta. The presence of CYP2R1 and CYP27B1 supports the role of trophoblast production of bioactive vitamin D and its role in controlling local immune tolerance and innate and adaptive immunity at the maternal-fetal interface during pregnancy. Although it is expected that proper vitamin D metabolism plays critical roles in trophoblast function and placental development during pregnancy, the consequences of altered placental vitamin D metabolic homeostasis in preeclampsia are not known. Moreover, it is not clear whether reduced VDR expression is associated with defective placentation in preeclampsia complicated pregnancies and premature births. This study was supported in part by grants from the National Institutes of Health (RO1 NICHD (HD-36822) and RO1 NHLBI (HL-65997)) to Y. Wang.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

R.M., J.S., L.J.G., and Y.W. did the conception and design of the research; R.M., X.G., S.Z., and Y.W. performed the experiments; R.M., Y.G., J.S., and Y.W. analyzed the data; R.M., Y.G. and Y.W. prepared the figures. R.M., J.S., L.J.G., and Y.W. approved the final version of the manuscript. R.M., Y.G., S.Z., J.S., and Y.W. did the conception and design of the research; R.M. and Y.W. prepared the figures; R.M., S.Z., and Y.W. analyzed the data; R.M., Y.G., S.Z., and Y.W. interpreted the results of the experiments; R.M., J.S., and Y.W. approved the final version of the manuscript.

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