Vitamin D stimulates miR-26b-5p to inhibit placental COX-2 expression in preeclampsia

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Vitamin D insufficiency or deficiency during pregnancy has been associated with an increased risk of preeclampsia. Increased placental cyclooxygenase-2 (COX-2) activity was proposed to contribute to the inflammatory response in preeclampsia. This study was to investigate if vitamin D can benefit preeclampsia by inhibiting placental COX-2 expression. Placenta tissues were obtained from 40 pregnant women (23 normotensive and 17 preeclampsia). miR-26b-5p expression was assessed by quantitative PCR. Vitamin D receptor (VDR) expression and COX-2 expression were determined by immunostaining and Western blot. HTR-8/SVneo trophoblastic cells were cultured in vitro to test anti-inflammatory effects of vitamin D in placental trophoblasts treated with oxidative stress inducer CoCl2. 1,25(OH)2D3 was used as bioactive vitamin D. Our results showed that reduced VDR and miR-26b-5p expression, but increased COX-2 expression, was observed in the placentas from women with preeclampsia compared to those from normotensive pregnant women. Transient overexpression of miR-26b-5p attenuated the upregulation of COX-2 expression and prostaglandin E2 (PGE2) production induced by CoCl2 in placental trophoblasts. 1,25(OH)2D3 treatment inhibited CoCl2-induced upregulation of COX-2 in placental trophoblasts. Moreover, miR-26b-5p expression were significantly upregulated in cells treated with 1,25(OH)2D3, but not in cells transfected with VDR siRNA. Conclusively, downregulation of VDR and miR-26b-5p expression was associated with upregulation of COX-2 expression in the placentas from women with preeclampsia. 1,25(OH)2D3 could promote miR-26b-5p expression which in turn inhibited COX-2 expression and PGE2 formation in placental trophoblasts. The finding of anti-inflammatory property by vitamin D through promotion of VDR/miR-26b-5p expression provides significant evidence that downregulation of vitamin D/VDR signaling could contribute to increased inflammatory response in preeclampsia.

Preeclampsia is a pregnancy-specific disorder characterized by new-onset hypertension, which occurs most often after week 20 of gestation and frequently near term1. It has been estimated that preeclampsia affects 5–8% of all pregnant women and remains the leading cause of maternal and prenatal morbidity and mortality in the world2. Moreover, preeclampsia increases the risk of cardiovascular disease later in life3. Several underlying mechanisms have been proposed in preeclampsia including immune maladaptation, uteroplacental ischemia, increased trophoblast deportation, and imbalances between angiogenic and antiangiogenic factors1,2. Although the exact cause of preeclampsia is still unclear, an excessive systemic inflammatory response is well accepted as a hallmark of this pregnancy-related disorder4. Plasma levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) have been shown to be elevated in preeclampsia. The finding of significantly higher IL-6/IL-10 ratio in women who had preeclampsia twenty years ago compared with healthy pregnancies, supports the notion of long-lasting increase in the inflammatory status in women who had preeclampsia5.

Preeclampsia is a placentally induced disorder of pregnancy6. According to this theory, the exaggerated maternal inflammatory response in preeclampsia is attributed to the hypoxic placenta which is associated with abnormal production of cytokines7, debris8, and prostaglandins9, such as thromboxane (TXA2) and prostacyclin (PGI2), by placental trophoblast cells. TXA2 is a potent vasoconstrictor, while PGI2 is a vasodilator. A decreased ratio of PGI2:TXA2 production is a characteristic of trophoblast dysfunction in preeclampsia8. One of the rate-limiting steps in prostaglandin synthesis is cyclooxygenase (COX) activity. Studies have shown that placental...

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COX-2 expression is significantly higher in preeclampsia than in normal pregnancy. Increased COX-2 expression is a marker of increased inflammatory response in preeclampsia. COX-2 is an inducible enzyme and can be induced by hypoxia and oxidative stress. When COX-2 enzyme is activated, excess prostaglandin E$_2$ (PGE$_2$) is released to involve in the oxidative stress-induced inflammation response. Therefore, inhibition of COX-2/ PGE$_2$ signaling might be beneficial in this disease.

As post-transcriptional regulators, microRNAs (miRNAs) have been found to regulate almost every aspect of cell function. Emerging evidence has accumulated in recent decades and indicates an important role of miRNAs in preeclampsia. In many cases, miRNAs are found to control fundamental processes that are directly involved in preeclampsia, such as angiogenesis, trophoblast proliferation and invasion and immune tolerance. miR-26b is highly expressed in the placenta, ovary, breast, liver and heart. Studies have shown that miR-26b participates in the development and progression of many tumor cells by targeting COX-2. miR-26b is also reported to be involved in preterm delivery by suppressing COX-2 in the human placenta. However, the placental miR-26b/ COX-2 axis has never been studied in preeclampsia.

Vitamin D deficiency or insufficiency during pregnancy has been considered a risk factor for preeclampsia. Vitamin D exerts anti-inflammatory effects in placental trophoblasts by suppressing TNF-α production and COX-2 expression. We previously reported that vitamin D exerted anti-oxidative activity through inhibition of inflammatory microparticle shedding from placental trophoblasts. A recent study suggested that vitamin D could stimulate the expression of multiple miRNAs through VDR to inhibit pro-labor gene expression in the human placenta. To further study the beneficial effects of vitamin D on placental trophoblasts, we examined VDR, miR-26b-5p, and COX-2 expression in placentas from normotensive and preeclamptic pregnant women in this study. Using HTR-8/SVneo cells as an in vitro testing trophoblast model, we investigated whether vitamin D could stimulate miR-26b-5p to inhibit oxidative stress-induced COX-2 expression and PGE$_2$ release in placental trophoblasts.

### Clinical characteristics.

The clinical characteristics of the study subjects are presented in Table 1. Note that the maternal body mass index (BMI) was significantly higher in women with preeclampsia than normotensive pregnancies ($p < 0.01$). The maternal gestational age at delivery was significantly shorter ($p < 0.01$) and the frequency of cesarean section was markedly higher in preeclamptic pregnant women than women with normal pregnancies. In addition, the infant birth weight was significantly lower in women with preeclampsia ($p < 0.01$).

### Reduced placental VDR and miR-26b-5p expression is associated with increased COX-2 expression in preeclampsia.

We first determined if aberrant VDR expression and miR-26b-5p expression are present in the placentas of women with preeclampsia. VDR expression was examined in placental tissue by immunohistochemistry staining and in primary isolated placental trophoblasts by Western blot. miR-26b-5p expression in the placenta was determined by quantitative PCR. Representative images of VDR and miR-26b-5p expression in the placentas from normotensive and preeclamptic women are shown in Fig. 1. Our results clearly showed that VDR expression was markedly reduced in the placenta (Fig. 1A) and primary isolated placental trophoblasts (Fig. 1B) from women with preeclampsia. Interestingly, placental miR-26b-5p expression was also significantly downregulated in preeclampsia (Fig. 1C).

COX-2 is a target of miR-26b-5p. Therefore, we examined placental COX-2 expression. In contrast to VDR and miR-26b-5p expression, COX-2 expression was obviously increased in the placentas from preeclamptic pregnant women (Fig. 1A), suggesting that upregulation of placental COX-2 expression is associated with down-regulation of VDR and miR-26b-5p expression in women with preeclampsia.

### Transient overexpression of miR-26b-5p inhibits increased COX-2 expression and PGE$_2$ production induced by CoCl$_2$ in placental trophoblasts.

To determine if reduced miR-26b-5p expression contributes to the activated COX-2/prostaglandins system that are relevant to preeclampsia, we examined the role of miR-26b-5p in the oxidative stress-induced inflammatory response. Transient overexpression of miR-26b-5p was induced by transfection of miR-26b-5p mimics into HTR-8/SVneo trophoblastic cells. As shown in

| Variables | Normal (n=23) | PE (n=17) | P value |
|-----------|---------------|-----------|---------|
| Maternal age, year | 29 ± 0.47 | 30.47 ± 0.61 | > 0.05 |
| Body mass index, kg/m$^2$ | 21.37 ± 0.56 | 25.68 ± 0.93 | 0.0002 |
| Gestational age, week | 39.81 ± 0.17 | 36.62 ± 0.65 | < 0.0001 |
| Systolic pressure, mmHg | 118.5 ± 2.42 | 163 ± 3.76 | < 0.0001 |
| Diastolic pressure, mmHg | 77.17 ± 1.62 | 104 ± 2.89 | < 0.0001 |
| Infant birth weight, g | 3572 ± 42.15 | 2802 ± 159 | < 0.0001 |
| Infant gender: % female | 43.47 | 47.06 | ND |
| Mode of delivery: cesarean % | 4.35 | 76.47 | ND |

Table 1. Clinical characteristics of normal and preeclamptic pregnancies. Data are expressed as mean ± SE. ND not determined.
Fig. 2A,B, COX-2 expression was significantly increased in trophoblasts treated with CoCl₂. PGE₂ level was also markedly elevated when the cells were treated with CoCl₂ (Fig. 2C). However, the CoCl₂-induced increase of COX-2 expression and PGE₂ formation could be clearly attenuated when the cells were transfected with miR-26b mimics (Fig. 2B,C), that is, overexpression of miR-26b-5p suppresses the increased COX-2/PGE₂ signaling induced by CoCl₂ in placental trophoblasts.

To further determine the role of miR-26b-5p in the placental vasculature in preeclampsia, PGI₂ production by placental trophoblasts was also examined by ELISA. In contrast to PGE₂, PGI₂ release was significantly reduced in cells treated with CoCl₂, and overexpression of miR-26b-5p could partially prevent the CoCl₂-induced decrease in PGI₂ production by placental trophoblasts (Fig. 2E). Because arachidonic acid (AA) is firstly converted to prostaglandin H₂ (PGH₂) when COX-2 is induced, and PGH₂ is then further transformed into PGI₂ by downstream enzyme prostacyclin synthase (PGIS). We next determined if the CoCl₂-induced decrease of PGIS in placental trophoblasts. As we expected, the expression of PGIS was downregulated in the cells cultured with CoCl₂ compared to the untreated cells, and this CoCl₂-induced decrease of PGIS was reversed when the cells were transfected with miR26b mimics (Fig. 2D).

Vitamin D attenuates the oxidative stress-induced upregulation of COX-2 expression in placental trophoblasts. To further test if vitamin D exerts anti-inflammatory properties through inhibition of COX-2 in placental trophoblasts, we examined COX-2 expression in placental trophoblasts treated with CoCl₂ in the presence or absence of 1,25(OH)₂D₃. As shown in Fig. 3A, COX-2 expression was dose-dependently increased in the trophoblasts treated with different concentrations of CoCl₂. Interestingly, in contrast to cells treated with CoCl₂, COX-2 expression was dose-dependently decreased in the trophoblasts treated with 1,25(OH)₂D₃ (Fig. 3B). Importantly, our results showed that the CoCl₂-induced increase in COX-2 expression was markedly attenuated in cells treated with 1,25(OH)₂D₃ compared to those without 1,25(OH)₂D₃ (Fig. 4A,B). COX-2 expression was also examined by immunofluorescence staining. Consistent with the Western blot data, 1,25(OH)₂D₃ could inhibit increased COX-2 expression induced by CoCl₂ (Fig. 4C). These data are similar to what we found in cells transfected with miR-26b-5p mimics, as shown in Fig. 2.

Vitamin D promotes miR-26b-5p expression via VDR in placental trophoblasts. To determine if vitamin D exerts any biological effects on miR-26b-5p expression in placental trophoblasts, we assessed miR-26b-5p expression in HTR-8/SVneo cells treated with 1,25(OH)₂D₃. Surprisingly, we found that 1,25(OH)₂D₃ could significantly stimulate miR-26b-5p expression in placental trophoblasts. As shown in Fig. 5A, the 1,25(OH)₂D₃-induced upregulation of miR-26b-5p expression was in a dose-dependent manner. We next determined the effects of vitamin D on the expression of miR-26b-5p under oxidative stress. Our data showed that CoCl₂-induced decrease in miR-26b-5p expression could be clearly reversed in the cells treated with 1,25(OH)₂D₃ compared to those not treated with 1,25(OH)₂D₃ (Fig. 5B).

To further study the specificity of 1,25(OH)₂D₃-induced miR-26b-5p expression in placental trophoblasts, VDR siRNA was transfected into HTR-8/SVneo cells followed by treatment with 1,25(OH)₂D₃. Intriguingly, we found that miR-26b-5p expression was significantly increased in the control cells treated with 1,25(OH)₂D₃, but not in the VDR-siRNA-transfected cells.
but not in the cells transfected with VDR siRNA (Fig. 3C). This result indicates that the 1,25(OH)2D3-induced upregulation of miR-26b-5p expression in placental trophoblasts is mediated through VDR.

**Vitamin D inhibits COX-2 expression via the VDR-miR-26b-5p pathway in placental trophoblasts.** Lastly, we determined if vitamin D downregulates COX-2 through the VDR-miR-26b-5p pathway. VDR siRNA or miR-26b inhibitors was transfected into HTR-8/SVneo cells in the presence of 1,25(OH)2D3. As shown in Figs. 1, 6, 25(OH)2D3 treatment alone led to a markedly decrease in COX-2 expression in placental trophoblasts; however, such a 1,25(OH)2D3-induced decrease in COX-2 was significantly prevented in the cells transfected with VDR siRNA or miR-26b inhibitors.

**Discussion.** In the present study, we had important findings that placental expression of VDR and miR-26b-5p was markedly reduced in women with preeclampsia compared to normotensive pregnant women. On the contrary, placental COX-2 expression was notably increased in the placenta from women with preeclampsia. COX-2 is the inducible isoenzyme of COX, upregulated by various inflammatory stimuli and cytokines. COX-2 and COX-2-derived prostaglandins play essential role in chronic inflammation and cancer. Increased COX-2 expression is associated with elevated levels of inflammatory cytokines in women with preeclampsia. Our findings of decreased VDR and miR-26b-5p expression related to increased COX-2 expression in the placenta suggest that reduced VDR expression and miR-26b-5p expression are connected to increased placental inflammatory response in preeclampsia. We previously found that trophoblasts from preeclamptic placentas produced more inflammatory cytokines, including TNF-α, sTNFR1, IL-6, and IL-8, than those from normal placentas. These data support the notion that increased inflammatory cytokine production is associated with upregulated COX-2 expression in placentas from preeclamptic women. Collecting with the current finding, we believe that downregulated placental VDR and miR-26b-5p expression contributes to the increased inflammatory response in preeclampsia.

Our study further found that CoCl2-induced upregulation of COX-2 expression was significantly reversed in cells transfected with miR-26b mimics, which demonstrated that miR-26b exerted anti-inflammatory activity in
the context of oxidative stress by targeting COX-2 in placental trophoblasts. We also assessed the specificity of the anti-inflammatory effects of miR-26b on COX-2 expression by transfection of miR-26b inhibitors into placental trophoblasts. Our findings showed that COX-2 expression was significantly increased in cells transfected with miR-26b-5p inhibitors compared with untreated cells. These data demonstrate that miR-26b can specifically suppress oxidative stress induced inflammatory response by targeting COX-2 in placental trophoblasts.

In addition, the effector molecular of inflammation PGE2 and the vasodilator PGI2 concentrations were measured using ELISA assay in this study. PGE2 is known as a pro-inflammatory molecular that is able to act on four kinds of receptor subtypes to elicit disparate actions26. The findings of CoCl2-induced increase in COX-2 expression and PGE2 synthase was clearly suppressed by overexpression of miR26b-5p in placental trophoblast, suggesting miR-26b can alleviate the oxidative stress-induced inflammation injury by inhibiting COX-2/PGE2 signaling in placental trophoblast. In contrast to PGE2, PGI2 release is reduced when the cells were cultured with CoCl2, and this CoCl2-induced reduction of PGI2 was partially prevented by overexpression of miR26b. PGI2 is well known to counteract the vasoconstriction and platelet aggregation effects of TXA2. It was reported that reduced PGI2 production, but not increased TXA2 production, occurs before onset of clinical signs of preeclampsia27, suggesting that elevating PGI2 production is a crucial part of the strategy to balance the abnormal vasodilator-vasoconstrictor ratio present in preeclampsia. Therefore, the finding of prevention of miR-26b against CoCl2-induced decrease of PGI2 synthesis indicates that miR-26b may benefit the placental vasculature by promoting trophoblastic vasodilators synthesis in preeclampsia.

It is notable that CoCl2 exerts opposite effects on PGE2 and PGI2 synthesis in the present study. It has been suggested that hypoxia may exert different actions on COX-2 and its downstream PGIS expression. For example, Mercedes Camacho et al. reported that 1% O2-induced hypoxia upregulated inflammation-stimulated expression of COX-2 and PGIS and PGI2 release in human vascular smooth muscle cells and endothelial cells28. On the contrary, using CoCl2 to induce hypoxia, Wang et al.29 and Li et al.30 found that CoCl2-induced hypoxia upregulated COX-2 but inhibited PGIS expression in macrophage co-cultured human cardiac microvascular endothelial cells. Our data showed that CoCl2 clearly downregulated PGIS expression in placental trophoblasts, which is associated with the decrease of PGI2 release induced by CoCl2. Since the placental PGI2 release was significant lower in preeclampsia, we investigated the alterations of placental PGIS expression in normal and preeclamptic pregnancies using IHC. We found that the PGIS expression was reduced in the placentas from preeclamptic pregnancy compared to those from normal pregnancy (Supplementary Fig. 1). These data support the notion that decrease of PGI2 release from placentas is due to the decrease of PGIS expression induced by placental hypoxia in preeclampsia.

Several studies have shown that vitamin D insufficiency/deficiency during pregnancy is a risk factor for preeclampsia, while vitamin D supplementation can reduce the risk of this disease31. The biological activity of vitamin D is regulated through its receptor, VDR32. In the present study, we found that placental VDR expression was reduced in women with preeclampsia. 1,25(OH)2D3 treatment could significantly decrease COX-2 expression in placental trophoblast, and such a decrease was significantly reversed when VDR was knocked down by siRNA. In

**Figure 3.** Dose-effects of vitamin D and CoCl2 on COX-2 expression in placental trophoblasts. (A) Protein expression for COX-2 in HTR-8/SVneo cells treated with different concentrations of CoCl2. The bar graphs show relative protein expression after normalization against β-actin from four independent experiments. CoCl2 induced a dose-dependent increase in COX-2 expression. ***P < 0.001: 250 μM vs control. (B) Protein expression of COX-2 in HTR-8/SVneo cells treated with different concentrations of 1,25(OH)2D3. In contrast to CoCl2, 1,25(OH)2D3 causes a dose-dependent decrease in COX-2 expression in placental trophoblasts. The bar graphs show relative protein expression after normalization to β-actin from three independent experiments. **P < 0.01: treated vs control cells.
addition, we also found that the CoCl$_2$-induced upregulation of COX-2 expression was markedly suppressed by 1,25(OH)$_2$D$_3$. Similar effects were seen in the cells transfected with miR-26b mimics. These data indicate that vitamin D/VDR signaling exerts anti-inflammatory and antioxidative stress properties by suppressing COX-2 activity in placental trophoblasts.

There are only a few studies that have investigated the association between vitamin D and miRNAs in pregnancy. For example, Enquobahrie et al. found that 10 miRNAs in peripheral blood, including miR-93, miR-573, miR-589 and miR-574-5p, were downregulated in women with low vitamin D levels compared with those with high vitamin D levels, suggesting that maternal vitamin D concentrations in early pregnancy are associated with maternal post transcription gene regulation. Zhou et al. reported that vitamin D could promote cell migration and invasion by downregulating miR-21 expression in human placental trophoblast cells. We previously found that VDR and miR-126 expression was reduced in maternal systemic endothelial cells in preeclampsia, and that, vitamin D could inhibit TNF-α-induced vascular cell adhesion molecule (VCAM) expression/production in endothelial cells by promoting miR-126 expression. In the current study, we demonstrated that vitamin D could increase miR-26b-5p expression in placental trophoblasts in a dose-dependent manner. Using VDR siRNA, we also found that VDR knockdown not only blocked 1,25(OH)$_2$D$_3$ stimulated VDR expression, but also suppressed 1,25(OH)$_2$D$_3$-induced upregulation of miR-26b-5p expression. These data suggest that 1,25(OH)$_2$D$_3$ stimulated VDR could promote the upregulation of miR-26b-5p in placental trophoblasts. The upregulation of miR-26b-5p expression stimulated by vitamin D/VDR signaling may be a novel mechanism of the anti-inflammatory activity of vitamin D in preeclampsia. Figure 7 shows a diagram that connects vitamin D, VDR, miR-26b gene, and their anti-inflammatory effects on COX-2/PGE$_2$ signaling in placental trophoblasts.

In the present study, the HTR-8/SVneo cells are used to study placental trophoblast functions. The HTR-8/SVneo cells are derived from first trimester extravillous trophoblast and immortalized by transfection with simian virus 40 (SV40). Since the HTR-8/SVneo cells are widely used to as a model of extravillous trophoblasts, to strengthen our findings, villous trophoblast cell models like BeWo, JEG-3 chorioncarcinoma cell line or primary isolated placental trophoblasts may be required to further investigate the trophoblast functions.
Figure 5. Vitamin D promotes miR-26b-5p expression in placental trophoblasts. (A) 1,25(OH)₂D₃ stimulates miR-26b-5p expression in HTR-8/SVneo cells. The bar graphs show relative miR-26b-5p expression after normalization to U6 expression in each sample from seven independent experiments. *P < 0.05: 100 nM of 1,25(OH)₂D₃ treated vs control. (B) miR-26b-5p expression in HTR-8/SVneo cells treated with CoCl₂ in the presence or absence of 1,25(OH)₂D₃, showing that 1,25(OH)₂D₃ could prevent the CoCl₂-induced decrease in miR-26b-5p expression in placental trophoblasts. The bar graphs show relative miR-26b-5p expression after normalization to U6 expression in each sample from eleven independent experiments. **P < 0.01: 1,25(OH)₂D₃ treated vs control. ***P < 0.01: CoCl₂ alone vs control. ****P < 0.001: 1,25(OH)₂D₃ + CoCl₂ vs CoCl₂ alone. (C) Inhibition of VDR expression prevents 1,25(OH)₂D₃-induced increase in miR-26b-5p expression in placental trophoblasts. VDR siRNA was transfected into HTR-8/SVneo cells. The bar graphs show relative miR-26b-5p expression after normalization to U6 in each sample from four independent experiments. *P < 0.05: 1,25(OH)₂D₃ treated vs control. **P < 0.01: VDR siRNA and 1,25(OH)₂D₃ + VDR siRNA vs 1,25(OH)₂D₃ treated alone, respectively.

Figure 6. Vitamin D promotes miR-26b-5p expression to inhibit COX-2 in placental trophoblasts. (A) Protein expression of COX-2 in HTR-8/SVneo cells treated with VDR siRNA or miR-26b inhibitors for 48 h followed by the addition of 1,25(OH)₂D₃ at a final concentration of 100 nM. The bar graph shows relative COX-2 expression after normalized by β-actin in each sample from three independent experiments. *P < 0.05: 1,25(OH)₂D₃ + miR-26b inhibitor vs 1,25(OH)₂D₃ alone. **P < 0.01: 1,25(OH)₂D₃ treated vs control. #P < 0.05: 1,25(OH)₂D₃ + VDR siRNA vs 1,25(OH)₂D₃ alone. (B) VDR protein expression in the experiment described in A. The bar graph shows relative VDR expression after normalization to β-actin in each sample from three independent experiments. *P < 0.05: 1,25(OH)₂D₃ treated vs control. **P < 0.01: 1,25(OH)₂D₃ + VDR siRNA vs 1,25(OH)₂D₃ alone.
In summary, this study has revealed a novel mechanism by which vitamin D downregulates COX-2 /PGE₂ signaling and may reduce the risk of preeclampsia. Our findings also provide significant insights into the medical benefits of vitamin D/VDR signaling during pregnancy.

Methods

Patients and sample collection. Placentas were collected immediately after delivery at the Second Affiliated Hospital of Harbin Medical University. A total of 40 placentas were used in the study, 23 from normal and 17 from preeclamptic pregnancies. Diagnostic criteria for study participants were used as previously described²¹. Normal pregnancy was defined as pregnancy with a blood pressure < 140/90 mmHg, and without proteinuria or obstetrical and medical complications²¹. Diagnosis of preeclampsia was defined as follows: a sustained systolic blood pressure of ≥ 140 mmHg or a sustained diastolic blood pressure of ≥ 90 mmHg on two separate readings; a proteinuria measurement of 1+ or more on a dipstick; or ≥ 300 mg of protein in a 24-h urine specimen²¹. Smokers were excluded. The demographic data including maternal age, body mass index, gestational age at delivery, blood pressure, and infant birth weight, are summarized in Table 1.

Study approval. Placenta collection was approved by the Ethical Committee for the Use of Human Samples of Harbin Medical University (# 82001577). All the participants signed a written informed consent for study enrollment. All the experiments were performed in accordance with the relevant guidelines and regulations of ethics committee of Harbin Medical University.

Trophoblast isolation. Placental trophoblasts were isolated by trypsin digestion, further purified by Percoll gradient centrifugation and cultured in six-well plates (5 × 10⁶ cells per well) in Dulbecco's modified Eagle medium supplemented with fetal bovine serum and antibiotics. The trophoblasts were maintained at 37 °C and 5% CO₂ for at least 24 h to spontaneously differentiate into syncytiotrophoblasts prior to further analysis.

Cell culture and treatment. The immortalized human trophoblast cell line HTR-8/SVneo (BeNa Culture Collection, Beijing, China) was cultured in DMEM/F12 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. The trophoblasts were maintained at 37 °C and 5% CO₂ for at least 24 h to spontaneously differentiate into syncytiotrophoblasts prior to further analysis.

Immunohistochemical staining. A standard immunohistochemistry staining procedure was performed as previously described. After blocking, placental tissue sections were incubated with primary antibodies specific for hum VDR (Affinity Biosciences, Jiangsu, China), PGIS (Affinity Biosciences, Jiangsu, China) or COX-2 (Bimake, Shanghai, China) overnight at 4 °C. The corresponding biotinylated secondary antibodies and ABC staining system was subsequently used according to the manufacturer's instructions. Slides stained with the same antibody were all processed at the same time. The stained slides were reviewed under a microscope and images were captured by a digital scanning microscopy imaging system (PreciPoint, Germany).
Western blot analysis. Placental tissue and trophoblast protein expression of VDR, COX-2 and PGIS was examined by Western blot. An aliquot of 10 μg of total protein was subjected to electrophoresis and then transferred to a polyvinylidene fluoride membrane. After blocking, the membranes were probed with primary antibodies against VDR, COX-2, or PGIS followed by the corresponding secondary antibodies (Bimake, Shanghai, China). The bound antibody was visualized with an enhanced chemiluminescent detection kit (Yeasen, Shanghai, China). The bands for VDR, COX-2 and PGIS were detected at 48KD, 69KD and 57KD, respectively. The band density was analyzed by ImageJ software (National Institutes of Health, USA). β-actin expression was used as the loading control for each sample.

miR-26b mimic and VDR siRNA transfection. miR-26b overexpression was achieved by transfection of miR-26b mimic, and VDR downregulation was achieved by transfection of VDR siRNA into HTR-8/SVneo cells using the Lipofectamine 2000 transfection reagent. miR-26b mimic and VDR siRNA were purchased from GenePharma and Sangon Biotech (Shanghai, China), respectively. Transfection was performed when the cells reached 60–70% confluence. Total RNA was extracted with TRIzol reagent approximately 48 h after transfection, and miR-26b-5p expression was determined by quantitative PCR. Total cellular protein was collected to determine COX-2 expression. The medium was also collected to determine PGE2 and PGI1 production.

Quantitative polymerase chain reaction (qPCR). Total RNA was extracted from placental tissue or HTR-8/SVneo cells with TRIzol reagent. cDNA was synthesized using the Mir-X miRNA First Strand Synthesis Kit (Takara, Japan) following the manufacturer’s instructions. miR-26b-5p expression was determined by qPCR. The qPCR was performed in 20 μL solutions using the SYBR Premix Ex Taq II Kit (Takara, Japan). The expression of U6 snRNA was determined and served as the endogenous control for the expression of miRNA-26b-5p. The relative expression values were calculated by the ΔΔCT method of relative quantitation using an Applied Biosystems 7500 Real-Time PCR System. The primer for miR-26b-5p was 5′-UUCAAGUAAUUCAGGAAUGGU-3′, which was synthesized by Sangon Biotech (Shanghai, China).

ELISA assay. PGE2 and PGI1 concentrations were measured using enzyme-linked immunosorbent assay (ELISA). The ELISA kits for the detection of human PGE2 and PGI1 were purchased from Elabscience Biotechnology (Wuhan, China). The sensitivity of the ELISA kits for detection of PGE2 and PGI1 was 18.75 pg/mL. The assay was carried out according to the manufacturer’s instructions. Both PGE2 and PGI1 standards were serially diluted, with ranges of 3.9 to 500 pg/mL. An aliquot of 50 μL of each sample was assayed in duplicate. After reaction, the plates were read at 450 nm by an autolamplate reader (Molecular Devices, USA). The Within-assay variations were <8% for all the assays.

Data presentation and statistics. Data are presented as mean ± SEM. Statistical analysis was performed with unpaired t-test to compare two groups. Ordinary one-way ANOVA followed by Tukey’s post hoc test was performed to compare multiple groups using GraphPad Prism 8 software. A probability level <0.05 was considered statistically significant.

Data availability The datasets are available from the corresponding author on reasonable request.

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Author contributions
H.Z. and J.X. conceived and designed the experiments; Y.C. and X.J. performed the experiments; Y.C. and Y.H. analyzed the data; J.W. and C.L. contributed reagents/materials/analysis tools; X.Y. collected the placentas and clinical information of study subjects; Y.C. and J.X. wrote the paper. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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