Original Article

Reproductive Sciences
2015, Vol. 22(3) 358-366

DOI: 10.1177/1933719114542020
rs.sagepub.com
SAGE

Gestational Diabetes Mellitus Upregulates Vitamin D Receptor in Extravillous Trophoblasts and Fetoplacental Endothelial Cells

Julia Knabl, MD[1], Rebecca Hüttenbrenner[1], Stefan Hutter, MD[1], Maria Günthner-Biller, MD[1], Christina Riedel, MSc[1,2], Ursula Hiden, PhD[1,3], Franz Kainer, MD[1], Gernot Desoye, PhD[1,3], and Udo Jeschke, PhD[1]

Abstract

Objective: Gestational diabetes mellitus (GDM) is often accompanied by low maternal vitamin D, that is, calcitriol ($1,25[OH]_2$ vitamin D3), levels. Here, we tested the hypothesis that the placental vitamin D receptor (VDR) is regulated by calcitriol and altered in GDM with distinct changes in different placental cell types. Specifically, we aimed to localize VDR in human term placentas from normal and GDM pregnancies, to quantify its cellular expression and to study in vitro its regulation by its physiological agonist calcitriol. **Study Design:** Placental tissue slides of 80 patients (40 with GDM/40 controls) were double stained for VDR and human leukocyte antigen G to identify extravillous trophoblasts (EVTs). Staining intensity was semiquantified. Quantitative real time-polymerase chain reaction and Western blotting measured VDR messenger RNA (mRNA) and protein in decidual tissue. The trophoblast cell line BeWo was used to study in vitro VDR regulation by calcitriol (0.01, 0.1, and 1 nmol/mL). **Results:** Vitamin D receptor protein and mRNA levels are upregulated ($P < .05$) in EVT (1.8-fold) as well as in placental endothelium (5.8-fold) of patients with GDM. Expression of VDR is regulated by calcitriol in a bimodal manner: high doses (0.1 and 1 nmol/mL) caused downregulation, whereas the low dose (0.01 nmol/mL) resulted in VDR upregulation. **Conclusion:** Vitamin D receptor is upregulated in EVT and endothelium of GDM placentas. This could be due to low maternal vitamin D levels in patients with GDM because in vitro low calcitriol doses upregulate VDR in trophoblast cells.

Keywords

gestational diabetes mellitus, vitamin D receptor, nuclear receptor, trophoblasts

Introduction

Vitamin D through its vitamin D receptor (VDR) is classically known to regulate bone metabolism and mineral homeostasis. Beyond its classical targets such as bone, gut, and kidney, VDRs are found in other tissues such as the immune system, pancreatic B cells, and the placenta, that is, trophoblasts. In these target tissues, nonclassical actions of the vitamin D endocrine system include control of cellular proliferation and differentiation, regulation of hormone secretion, and modulation of immune responses.[1,2] Low maternal vitamin D concentrations are associated with insulin resistance in pregnancy[3] and increase the risk of gestational diabetes mellitus (GDM).[4] The underlying causal factors for this association have not yet been identified.

Gestational diabetes mellitus is defined as glucose intolerance, with its onset or first recognition during pregnancy, affecting 3% to 8% of all pregnancies.[5] It is clinically diagnosed later in pregnancy when women fail to maintain normoglycemia, because of a pronounced peripheral resistance to insulin.[6]

The VDR belongs to the family of nuclear steroid hormone receptors. Vitamin D receptor binds its major ligand $1,25(OH)_2$ vitamin D3 (calcitriol) and recruits another receptor of this

[1] Department of Obstetrics and Gynecology, Ludwig-Maximilians-Universität, Munich, Germany
[2] Institut für Soziale Pädiatrie und Jugendmedizin, Ludwig-Maximilians-Universität München, Munich, Germany
[3] Department of Obstetrics and Gynecology, Medical University Graz, Graz, Austria

Corresponding Author:
Udo Jeschke, Department of Gynaecology and Obstetrics, Campus Innenstadt, Ludwig-Maximilian-University Munich, Maistrasse 11, D-80337 Munich, Germany.
Email: udo.jeschke@med.uni-muenchen.de

group, for example, retinoid X receptor α to form a heterodimer. This complex can further interact with the VDR response elements of targeted genes. Vitamin D receptor in its classical function—like in kidney and bone—is homologous upregulated by vitamin D. This regulation is absent in reproductive organs and other tissues, which are not involved in calcium homeostasis.[7] Especially in trophoblasts, VDR regulation by vitamin D has not yet been demonstrated. A recent study did not find significant changes in VDR expression in the human placenta in GDM compared to control pregnancies.[8] This study focussed on the villous trophoblast as the first placental target for vitamin D in the maternal circulation. However, the placenta is a multicellular tissue and it cannot be ruled out that other placental cells can be affected by GDM. Possible expression changes in the extravillous trophoblast (EVT) of GDM placentas are still unknown, a recent study of our group could identify a change in other nuclear receptors in GDM EVT.[9] Vitamin D promotes immune responses in the maternal decidua and fetal trophoblasts.[10-12] As EVT is the site of implantation and immune modulation[13] and GDM alters human placental macro- and microvascular reactivity as a result of endothelial dysfunction,[14-16] we focussed on specific changes in these 2 sites of the diabetic fetomaternal interface.

Therefore, we hypothesized that GDM is associated with a change in VDR expression in placental endothelial cells and the EVT, 2 other important placental compartments. To this end, we investigated the expression pattern and the regulation of the nuclear receptor VDR in normal and diabetic placentas with a particular focus on these compartments.

Materials and Methods

The study was approved by the ethics committee of the Ludwig-Maximilian-University (LMU) Munich, Germany, and written informed consent was obtained from the patients. Placental tissues were obtained from 80 women. All women underwent an oral glucose tolerance test[17] at weeks 24 to 28 of gestation. According to the criteria of the German Diabetes Society (capillary whole blood; fasting glucose >90 mg/dL, 1 hour >180 mg/dL, and 2 hours > 155 mg/dL; GDM was defined with 2 values above this limits), 40 women were diagnosed with GDM. Immediately after delivery, the samples were taken from the central part of the placenta. In addition, a cotyledon structure showing signs of sufficient blood supply was chosen. Calcified, necrotic, and visually ischemic areas were excluded from collection. Pieces ($2 \times 2 \times 2$ cm^3) were dissected, containing decidua, extravillous, and villous trophoblasts as well as amniotic epithelial cells. Each sample was further separated in decidual and villous tissue, frozen separately, and stored at -80°C. A second set of placental tissue was fixed in 4% buffered formalin for 24 hours and thereafter embedded in paraffin. The patients were monitored at least once a week at the Diabetes Center of the Department of Internal Medicine LMU Munich. All patients in the GDM group were treated with insulin and showed a mean hemoglobin A1c of 5.8%. An expert in diabetes therapy monitored the blood glucose protocols once a week. A total of 75% of the patients had been under good glucose control according to their mean blood glucose. Demographic and clinical data of the study population are summarized in Table 1. Perinatal and clinical data of the study group were published recently.[9]

Table 1. Clinical Details of the Patients With Gestational Diabetes Mellitus (GDM) and of the Normal Control Group (Mean ± SD).[a]

	GDM	Controls	
Maternal age, years	32.82 ± 4.56	31.15 ± 6.10	ns
BMI (prepregnancy)	28.1 ± 6.96	23.4 ± 6.21	$P < .0001$[b]
Median gravidity (min, max)	2 (1, 6)	2 (1, 7)	ns
Median parity (min, max)	1 (1, 4)	1 (1, 3)	ns
Gestational age at delivery	39.85 ± 1.29	39.78 ± 1.35	ns
Birth weight, g	3611.4 ± 536	3316.9 ± 502	$P = .006$[b]
Umbilical artery pH	7.30 ± 0.08	7.29 ± 0.09	ns
Median 5 min APGAR (min, max)	10 (8, 10)	10 (8, 10)	ns
% vaginal delivery	67	80	ns
% underwent labor	85	85	ns
Mean HbA1c%	5.8	nd	

Abbreviations: BMI, body mass index; GDM, gestational diabetes mellitus; HbA1c, hemoglobin A1c; nd, not done in the control group; ns, not significant.
[a]The Mann-Whitney *U* test was applied to compare clinical outcome data of the 2 groups.
[b]*P* values which are statistically significant.

Double Immunofluorescence Staining

Vitamin D receptor-expressing cells in the decidua of placental tissue were characterized by examining cryosections (thickness: 8 μm). Slides were fixed with acetone and blocked by Ultra V Block (Lab Vision, Fremont, California). The slides were incubated overnight at 4°C with primary antibodies against VDR and human leukocyte antigen G (HLA-G) as a marker for EVT cells; goat-antirabbit Cy 3 secondary antibody, which will appear red, was used for the VDR and goat-antimouse Cy 2 secondary antibody, which will appear green, for HLA-G. Finally, the slides were embedded in mounting buffer containing 4′,6-diamino-2-phenylindole and examined with a Zeiss Axiophot photomicroscope (Jena, Germany).

Immunohistochemistry

The sections with a thickness of 2 to 3 μm were deparaffinized, the antigen was unmasked by cooking in a pressure cooker with citrate buffer (pH = 6.0), and the sections were blocked with human AB serum. Slides were incubated with the primary VDR antibody for 16 hours at 4°C. The Vectastain Elite Mouse immunoglobulin G (IgG) ABC-Kit (Vector Laboratories, Burlingame) was used for visualization. The sections were stained with VIP Substrate Kit (Vector Laboratories) and counterstained by using methyl green (Vector Laboratories). Sections were dehydrated and coverslipped

with Shandon Consul Mount Medium (Thermo Fisher Scientific, Waltham, Massachusetts). For the negative control, the primary antibody was replaced by species-specific isotype control antibody. The signal was quantified using the semiquantitative immunoreactive score (IRS)[18] analyzed by 2 examiners until consensus was reached. The IRS was calculated by multiplying optical staining intensity (graded as 0 = none, 1 = weak, 2 = moderate, and 3 = strong staining) and the percentage of stained cells (0 = no staining, 1 = $\leq$10% of the cells, 2 = 11%-50% of the cells, 3 = 51%-80% of the cells, and 4 = $\geq$81% of the cells).

Western Blot Analysis

A total of 1 mL lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich, St Louis, Missouri) and radioimmunoprecipitation assay buffer (Sigma-Aldrich), in a dilution 1:100, was added to the decidual placental tissue, and then the tissue was homogenized using a Sonifier ultrasonic cell disrupter (Branson Ultrasonics S.A., Geneve, Switzerland). The tissue homogenates were centrifuged at 1000 rpm for 5 minutes, and the protein concentration in the supernatant was determined via Bradford assay as described recently.[19] A protein amount of 2 µg of β-actin and 20 µg of VDR was used for Western blotting. The lysate proteins were first separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, Massachusetts). Between each step, the membrane was washed in casein solution from the Vectastain ABC-AmP mouse-IgG-Kit for Western blot detection (Vector Laboratories). The membrane was blocked in casein solution for 1 hour and then incubated with the primary antibodies, the antihuman VDR rabbit polyclonal antibody (Abcam), diluted 1:400 in casein, and the β-actin mouse monoclonal antibody (Sigma), diluted 1:1000 in casein, for 16 hour at 4°C. Afterward, the membrane was incubated with the biotinylated antirabbit IgG/antimouse IgG secondary antibody for 45 minutes and the ABC reagent for 20 minutes, both from the Vectastain ABC-AmP rabbit/mouse IgG-Kit (Vector Laboratories). The blots were developed with the 5-bromo-4-chloro-3'-indolyphosphate/nitro-blue tetrazolium (BCIP/NBT)-chromogen substrate solution. Western blots were detected with the Bio-Rad Universal Hood II (Bio-Rad Laboratories, Hercules, California) and quantified using the Bio-Rad Quantity One Software (Bio-Rad Laboratories). The blot was repeated 3 times.

RNA Isolation, Processing, and Real-Time Polymerase Chain Reaction

Total RNA was extracted from the decidual tissue using the Rneasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Quantification and evaluation of the purity of the isolated RNA were carried out with a NanoPhotometer (Implen, Munich, Germany). A ratio of absorbance at 260/280 nm $\sim$2.0 was accepted as pure. The reverse transcription was performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Weiterstadt, Germany) according to the protocol of the manufacturer in a mastercycler gradient (Eppendorf, Hamburg, Germany) for 10 minutes at 25°C, 2 hours at 37°C, 5 seconds at 85°C, and at 4°C on hold. The real-time polymerase chain reactions (PCRs) were accomplished in optical 96-well reaction microtiter plates covered with optical caps in a volume of 20 µL, containing 1 µL TaqMan Gene Expression Assay 20 × (Hs01045840_m1 for VDR; Applied Biosystems), 10 µL TaqMan Univeral PCR Master Mix 2 × (Applied Biosystems), 8 µL H_2O (diethylpyrocarbonate-treated deionized water; Sigma, Taufkirchen, Germany), and 1 µL template. For thermal cycling, a ABI PRISM 7500 Fast (Applied Biosystems) was used for 20 seconds at 95°C, followed by 40 cycles of amplification for 3 seconds at 95°C and 30 seconds at 60°C. Quantification was carried out by the $2^{-\Delta\Delta CT}$ method using β-actin (Hs99999903_m1) as housekeeping gene, since it revealed a stable expression in placenta and decidua after normal and GDM pregnancies.

Cell Culture and Cell Stimulation

As a trophoblast model, the chorioncarcinoma cell line BeWo was obtained from the European Collection of cell cultures (Salisbury, United Kingdom, passage numbers 22 and 23, polyclonal). Cells were cultured in Dulbecco modified eagle medium (DMEM; Biochrom, Germany, 3.7 g/L $NaHCO_3$, 4.5 g/L D-glucose, 1.028 g/L stable glutamine, and Na-pyruvate) supplemented with 10% heat-inactivated fetal calf serum (FCS) without antibiotics and antimycotics. The BeWo cells were grown on sterile 12 multiwell slides at a density of 1 million cells/mL DMEM. For the stimulation with human calcitriol, the cells were cultured with DMEM containing 10% FCS for 4 hours and with fresh DMEM without FCS for the remaining hours. After 24 hours, cells were stimulated with 0.01, 0.1, or 1 nmol/mL human calcitriol (Sigma-Aldrich) for 4 hours for messenger RNA (mRNA) quantification by PCR and for 48 hours for immunocytochemical analysis, respectively. Control cells were incubated without stimulants. For quantification of total RNA, the NucleoSpin RNAII Kit (Macherey-Nagel, Düren, Germany) was used according to the manufacturer's protocol. For each dose of calcitriol, 3 independent experiments were performed.

Immunocytochemistry

First, the slides were fixed in ice-cooled methanol for 20 minutes. Afterward, the cells were permeabilized by 0.2% NP40 (Sigma-Aldrich) for 10 minutes. Between each step, the slides were washed with PBS (Biochrom). After blocking with power block (diluted 1:10 in aqua dest) for 3 minutes, the slides were incubated with the VDR antibody, diluted 1:1000 in PBS, for 16 hours at 4°C. The Vectastain Elite Mouse IgG ABC-Kit (Vector Laboratories, Burlingame) was used for visualization. Finally, the slides were stained with 3-amino-9-ethylcarbazole plus (AEC plus; Dako, Glostrup, Denmark) for 10 minutes,

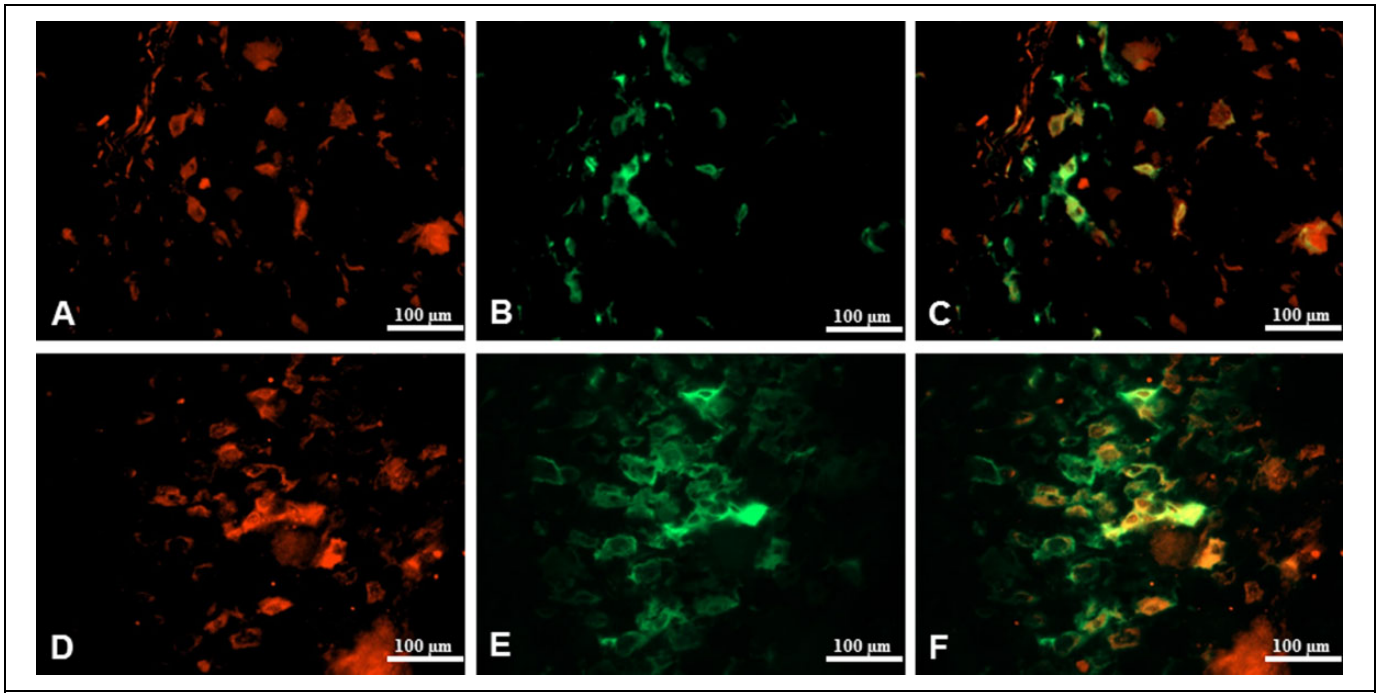

Figure 1. Double-immunofluorescence staining of EVT for VDR and HLA-G. Vitamin D receptor is marked with the Cy-3-labeled secondary antibody and stained red. It is expressed in EVT of GDM placentas (A) and normal controls (D). The HLA-G as a specific marker for the EVT that is stained with the Cy-2-labeled secondary antibody giving a green signal. The HLA-G is expressed in EVT of GDM positive placentas (B) and normal controls (E). Triple filter excitation shows a simultaneous expression of VDR and HLA-G in EVT of GDM placentas (C) and normal controls (F). The blue staining of the nucleus results from the 4′,6-diamino-2-phenylindole (DAPI) mounting buffer. All images were taken at a magnification of 40×. EVT indicates extravillous trophoblast; GDM, gestational diabetes mellitus; HLA-G, human leukocyte antigen G; VDR, vitamin D receptor.

counterstained with hemalaun for 30 seconds, washed in tap water, and coverslipped.

Immunocytochemistry evaluation was performed by the intensity of positively stained cells. The examination was semiquantitative, including the possibilities of no staining (negative), weak (singular), moderate (2-fold), and strong (3-fold) staining intensity and expressed as percentage (%). Only cells with intensity +2 or +3 were included for further analysis.

Statistical Analysis

Statistical analysis was performed using the nonparametrical Mann-Whitney *U* signed rank tests and the *t* test for comparison of the means. Univariate and multivariate linear regression models were used to analyze the associations of clinical outcome data with IRS. The models were adjusted for potential (maternal age, parity, and umbilical artery pH) and known (body mass index [BMI], fetal gender, and gestational age) confounders.[20] Statistical significance was assumed at P values <.05.

Results

Localization of Placental VDR Expression

Vitamin D receptor was localized in the nucleus of EVT, the villous syncytiotrophoblast (SCT), and endothelium in placentas from both GDM and non-GDM pregnancies. Double staining using antibodies against HLA-G, a specific marker for most EVTs, and VDR, the EVT cells were identified as VDR-expressing cells in the decidua of placental tissue (Figure 1).

Expression of VDR is Upregulated in GDM Trophoblasts and Villous Endothelium

The VDR expression in GDM placentas was modestly upregulated in the SCT (IRS 7 vs 3, $P < .05$) and more profoundly in the EVT (IRS 8 vs 2, $P = .001$). In addition, we identified a strong upregulation of VDR also in the villous endothelial capillaries in GDM (IRS 8 vs 1, $P = .001$) as compared to normal pregnancies (Figure 2).

To confirm the immunohistochemistry results, we performed Western blotting for VDR in decidual tissue containing EVT in patients with GDM and normal controls (Figure 3).[21] Decidua from patients with GDM showed stronger VDR levels than decidua from normal controls (1.7-fold, $P = .001$). Vitamin D receptor protein levels were normalized to β-actin.

Vitamin D Receptor Expression

The mRNA expression of VDR was increased 2.3-fold in the decidual tissue containing EVT in patients with GDM as

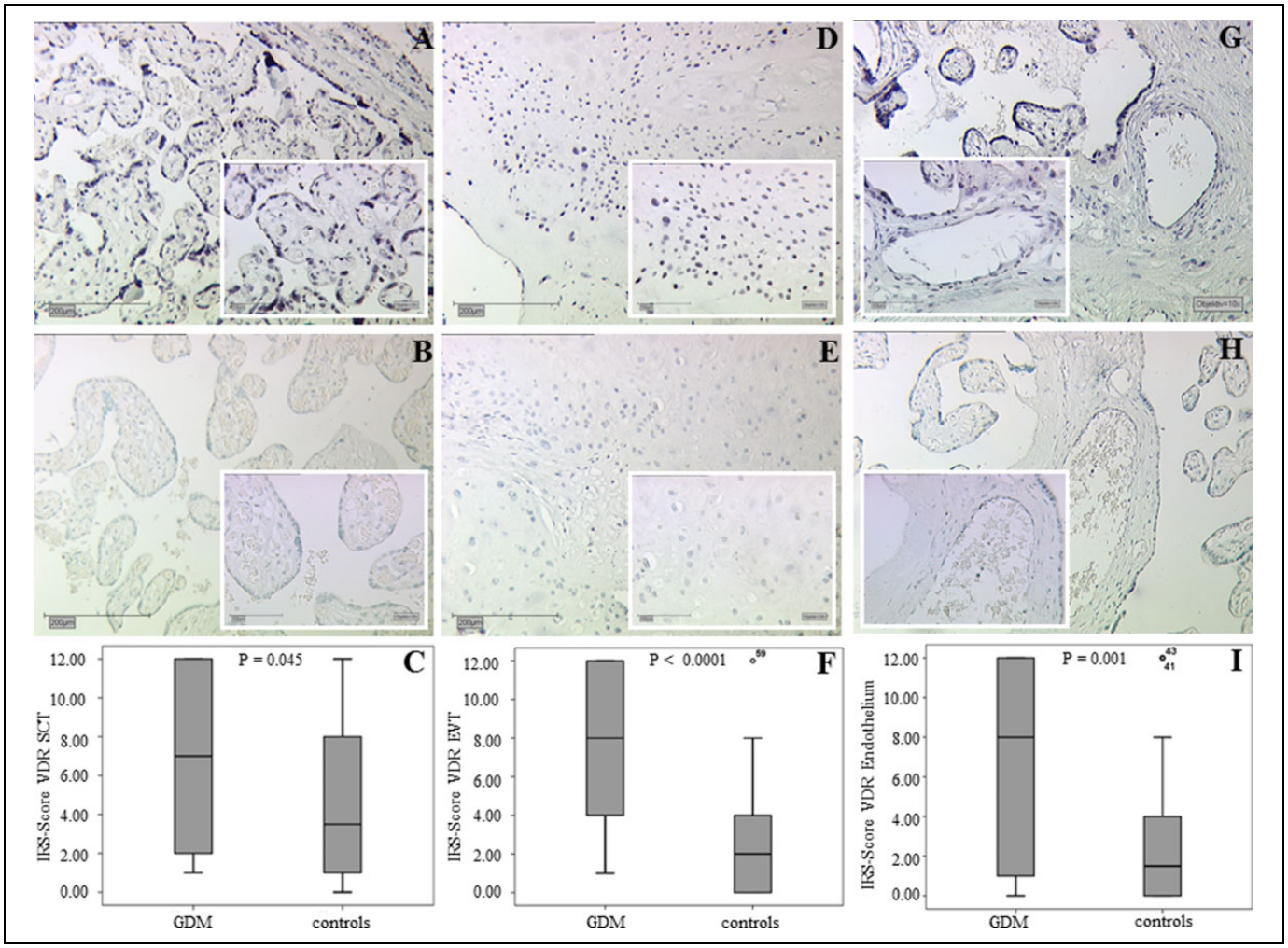

Figure 2. Representative immunohistochemical staining for VDR expression in normal and GDM placentas. A positive staining was detected in the SCT (A and B), in the EVT (D and E), and in the endothelium (G and H) GDM and control placentas, respectively. Pictured were taken at a magnification of 10× and 25×. The quantification of VDR expression analysis in GDM and control placentas for SCT (C), EVT (F), and endothelium (I) is shown in the boxplots. The boxes represent the range between the 25th and 75th percentiles with a horizontal line at the median. The bars delineate the 5th and the 95th percentiles. The circle indicates values more than 1.5 box lengths from the 75th percentile. Numbers at circle indicate sample number. The VDR expression was significantly higher in GDM placentas (SCT: $P = .045$, EVT: $P < .001$, and endothelium: $P = .001$). EVT indicates extravillous trophoblast; GDM, gestational diabetes mellitus; SCT, syncytiotrophoblast; VDR, vitamin D receptor.

compared to control participants ($P = .0001$; Figure 3). As our study groups differed in BMI and birth weight (see Table 1), we analyzed the statistical associations of these potential confounders with VDR expression. Birth weight, but not BMI, was associated with VDR. However, the GDM effect on VDR staining intensity was still significant for EVT (1.8-fold, $P = .01$) and fetal endothelium (5.8-fold, $P = .0001$) after adjusting for birth weight.

The linear regression analyses showed a significant positive association of birth weight with VDR staining in EVT and fetal endothelium but not with SCT. These associations disappeared after adjusting for confounders for birth weight.[20] Therefore, the confounder analysis demonstrated no correlation of birth weight and VDR expression.

Vitamin D Receptor Expression in BeWo Cells

Human calcitriol showed a bimodal dose-dependent effect on VDR protein and mRNA levels determined by immunocytochemistry and quantitative PCR. Concentrations of 0.1 and 1 nmol/mL induced a significant ($P = .008$, 100% of moderately and strongly stained cells vs 10%; $P = .009$, 90% of moderately and strongly stained cells vs 40%, respectively) reduction in VDR protein. In contrast, stimulation with 0.01 nmol/mL calcitriol induced a significant ($P = .007$) increase in VDR protein levels (0% of moderately and strongly stained cells vs 90%; Figure 4).

These results were also confirmed on mRNA level: stimulation of BeWo cells with 0.1 and 1 nmol/mL human calcitriol

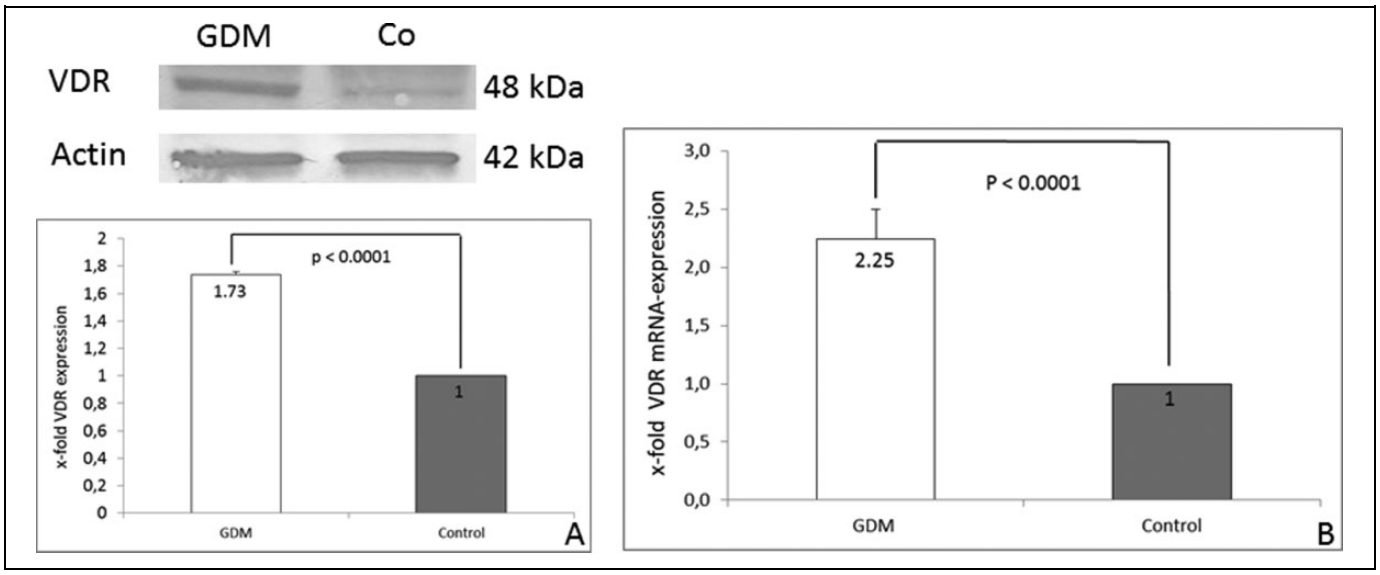

Figure 3. Quantification of VDR protein and mRNA in EVT from normal and GDM placentas. A, Western blotting for VDR in decidual tissue containing EVT in patients with GDM and normal controls generated a major band in the molecular mass range of 48 kDa. The GDM decidua showed a stronger staining intensity for VDR than the normal controls (1.73-fold, $P < .001$). B, Quantitative real-time polymerase chain reaction (RT-PCR) showed a significant upregulation of VDR mRNA expression in GDM-positive EVT compared to normal controls (2.25-fold, $P < .001$). EVT indicates extravillous trophoblast; GDM, gestational diabetes mellitus; mRNA, messenger RNA; VDR, vitamin D receptor.

significantly ($P = .035$, $P < .0001$) reduced VDR-mRNA expression by 70% and 50%, respectively. In contrast, 0.01 nmol/mL human calcitriol induced a significant ($P = .07$) increase in VDR-mRNA expression by 100% (Figure 4).

Discussion

To the best of our knowledge, this is the first study showing increased levels of VDR in EVTs and fetoplacental endothelium associated with maternal GDM. Our results are in contrast to an earlier study, which failed to find significant changes in VDR expression in villous placental tissue of patients with GDM. However, the study of Cho et al[8] did not analyze decidual tissue, EVT, or the placental endothelium. Therefore, we focused here on these compartments. Furthermore, our study included more patients with GDM (40 GDM placentas), which may have enabled us to identify even a modest VDR upregulation in the villous trophoblast in addition to the strong upregulation of VDR in villous endothelium of GDM placentas.

Surprisingly, the VDR was stronger upregulated in GDM in the extravillous than in villous trophoblasts. This finding by semiquantitative immunohistochemistry was also confirmed at the protein and mRNA level. Extravillous trophoblast represents the site of trophoblast invasion. Therefore, we performed in vitro experiments with the trophoblast tumor cell line BeWo—a model for invasive trophoblasts.[22,23] These experiments revealed that high doses (0.1 and 1 nmol/mL) of the natural VDR ligand calcitriol decreased, whereas a low dose (0.01 nmol/mL) increased VDR mRNA expression.

Although there is no consensus on optimal levels of vitamin D in humans, vitamin D deficiency is mostly defined as a 25(OH) vitamin D3 level of less than 20 ng/mL.[24] Vitamin D serum levels range between 82 and 110 pg/mL, which corresponds to 0.2 and 2.6 nmol/L.[25] The placenta produces calcitriol with tissue concentrations above serum levels.[26] Therefore, we decided to use these higher concentrations for our experiments. Interestingly, calcitriol in different concentrations resulted in opposing effects with VDR down- and upregulation, respectively. Although we had no information about sun exposition or dietary cholecalciferol intake, multiple studies have shown that maternal serum vitamin D concentrations are lower in patients with GDM than in non-GDM controls.[3,27]

Because vitamin D deficiency is a known risk factor for glucose intolerance[28] and vitamin D levels are reduced in diabetic pregnancies,[3] low maternal vitamin D levels are one possible reason for enhanced VDR expression.

Vitamin D protects endothelial tissue against renovascular dysfunction.[29] Thus, upregulation of VDR found in the fetoplacental endothelium could be a sign of vitamin D deficiency even in the fetal circulation in GDM: cord blood vitamin D concentration in GDM pregnancies has not yet been published, but maternal vitamin D concentration usually correlates with infant cord serum[30] and was reported to be lower in type 1 diabetic pregnancies.[31] In addition, an involvement of vitamin D and endothelial dysfunctions seems likely as vitamin D induces NO production in endothelial cells,[32] and endothelial dysfunction in GDM is accompanied by increased NO production.[33] The question, whether the fetus is also affected by these changes found in the fetoplacental endothelium, thus remains.

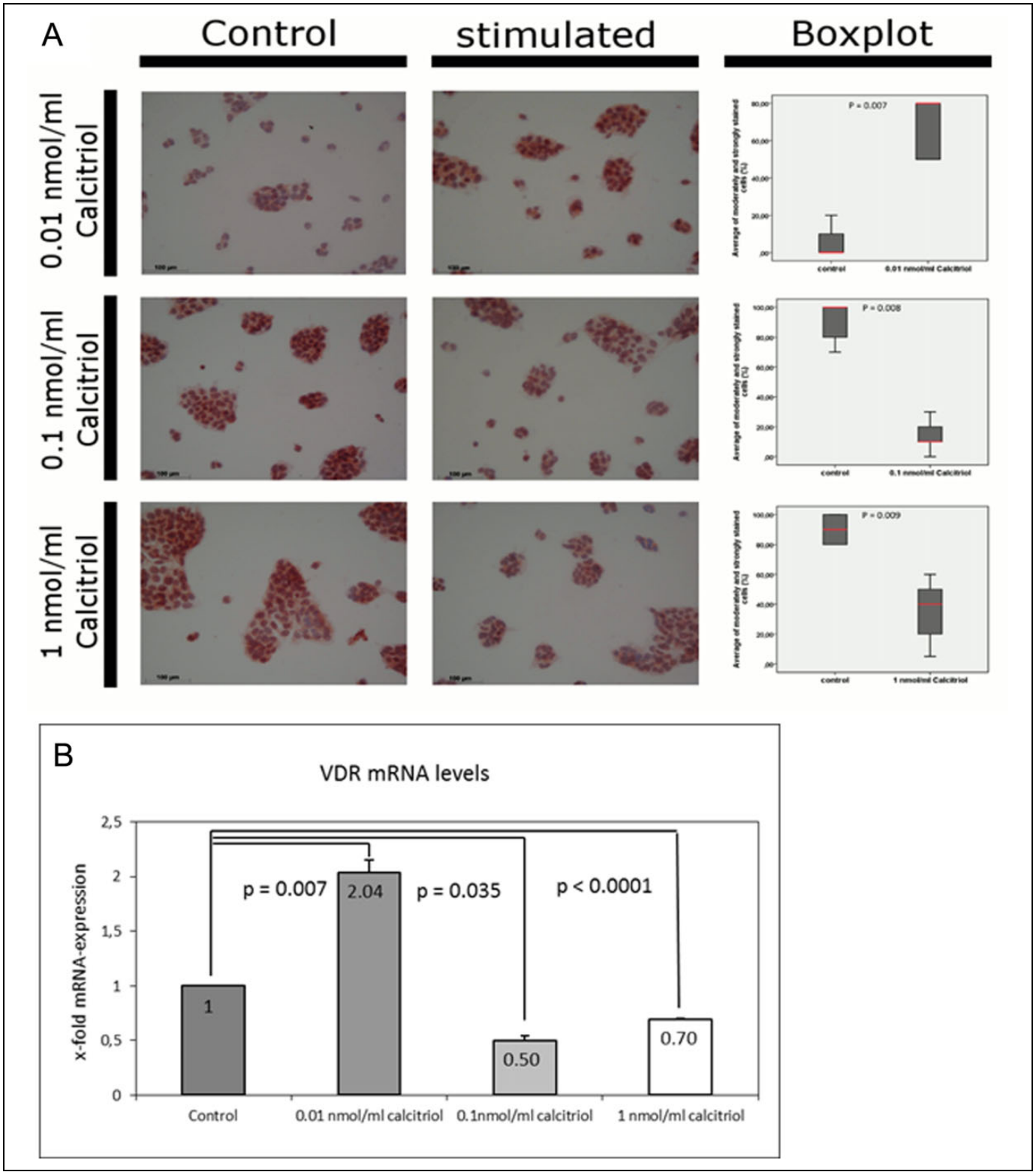

Figure 4. Regulation of VDR expression by calcitriol. A, Immunocytochemical analysis revealed that concentrations of 0.1 and 1 nmol/mL human calcitriol reduced ($P = .008$ and $P = .009$), whereas stimulation with 0.01 nmol/mL calcitriol increased VDR protein levels ($P = .007$). Images were taken at a magnification of 20×. The boxes represent the range between the 25th and 75th percentiles with a horizontal line at the median. The bars delineate the 5th and the 95th percentiles. B, Quantitative real-time polymerase chain reaction (RT-PCR) showed a downregulation of VDR mRNA expression after incubation with 0.1 nmol/mL (0.5-fold, $P = .035$) and 1 nmol/mL (0.7-fold, $P < .0001$) human calcitriol for 2 hours. Stimulation with 0.01 nmol/mL human calcitriol for 2 hours increased ($P = .007$) VDR mRNA expression (2.04-fold, $P = .007$). mRNA indicates messenger RNA; VDR, vitamin D receptor.

Above all, we could confirm the hypothesis that enhanced VDR expression is a result of low vitamin D levels by cell stimulation experiments. As the regulation of VDR expression in its nonclassical function is tissue specific[7] and no data are available for trophoblasts, this is the first report demonstrating downregulation of VDR in trophoblast cells by physiological concentrations of its natural ligand calcitriol. For our in vitro experiments, we utilized the choriocarcinoma cell line BeWo, which shows the characteristics of invasive trophoblasts.[22,23,34,35]

An important finding is the identification of EVTs as VDR expressing cells in the decidua of the fetal–maternal interface. These EVTs showed the strongest VDR upregulation of all placental compartments studied in GDM. Extravillous trophoblasts invade the maternal uterus and form the maternofetal interface. In this way, EVTs prevent allo-recognition and killing of fetal cells by maternal natural killer cells, cytotoxic T cells, and macrophages.[36] Increasing evidence suggests that GDM is a proinflammatory state similarly to preexisting diabetes.[37] Recent studies demonstrate that vitamin D promotes immune responses in maternal decidua and fetal trophoblasts[10-12] and regulates placental inflammation.[11] Therefore, we speculate that VDR upregulation might be a compensatory mechanism to low vitamin D levels. Vitamin D receptor on trophoblasts controls cellular proliferation and differentiation.[38] Such actions of VDR are likely to be changed under conditions of low maternal calcitriol status like GDM.

Collectively, our data demonstrate upregulation of the VDR system in trophoblasts and endothelium of pregnancies affected by GDM. Confounder analysis showed that this effect is not due to fetal birth weight or BMI of the mother.

Acknowledgments

The authors thank Simone Hofmann, Susanne Kunze, and Christina Kuhn for excellent technical support.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received the following financial support for the research, authorship, and/or publication of this article: The study was supported by the Medical Faculty of the Ludwig-Maximilians-Universität München.

References

1. Verstuyf A, Carmeliet G, Bouillon R, Mathieu C. Vitamin D: a pleiotropic hormone. *Kidney Int*. 2010;78(2):140-145.
2. Evans KN, Bulmer JN, Kilby MD, Hewison M. Vitamin D and placental–decidual function. *J Soc Gynecol Investig*. 2004;11(5):263-271.
3. Soheilykhah S, Mojibian M, Rashidi M, Rahimi-Saghand S, Jafari F. Maternal vitamin D status in gestational diabetes mellitus. *Nutr Clin Pract*. 2010;25(5):524-527.
4. Zhang C, Qiu C, Hu FB, et al. Maternal plasma 25-hydroxyvitamin D concentrations and the risk for gestational diabetes mellitus. *PLoS One*. 2008;3(11):e3753.
5. Metzger BE, Coustan DR. Summary and recommendations of the Fourth international workshop-conference on Gestational diabetes mellitus. The organizing committee. *Diabetes Care*. 1998;21(suppl 2):B161-B167.
6. Butte NF. Carbohydrate and lipid metabolism in pregnancy: normal compared with gestational diabetes mellitus. *Am J Clin Nutr*. 2000;71(5 suppl):1256S-1261S.
7. Gensure RC, Antrobus SD, Fox J, Okwueze M, Talton SY, Walters MR. Homologous up-regulation of vitamin D receptors is tissue specific in the rat. *J Bone Miner Res*. 1998;13(3):454-463.
8. Cho GJ, Hong SC, Oh MJ, Kim HJ. Vitamin D deficiency in gestational diabetes mellitus and the role of the placenta. *Am J Obstet Gynecol*. 2013;209(6):560. e1-e8.
9. Knabl J, Huttenbrenner R, Hutter S, et al. Peroxisome proliferator-activated receptor-gamma (PPARgamma) is down regulated in trophoblast cells of gestational diabetes mellitus (GDM) and in trophoblast tumour cells BeWo in vitro after stimulation with PPARgamma agonists. *J Perinat Med*. 2014;42(2):179-187.
10. Liu N, Kaplan AT, Low J, et al. Vitamin D induces innate antibacterial responses in human trophoblasts via an intracrine pathway. *Biol Reprod*. 2009;80(3):398-406.
11. Liu NQ, Kaplan AT, Lagishetty V, et al. Vitamin D and the regulation of placental inflammation. *J Immunol*. 2011;186(10):5968-5974.
12. Evans KN, Nguyen L, Chan J, et al. Effects of 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 on cytokine production by human decidual cells. *Biol Reprod*. 2006;75(6):816-822.
13. Warning JC, McCracken SA, Morris JM. A balancing act: mechanisms by which the fetus avoids rejection by the maternal immune system. *Reproduction*. 2011;141(6):715-724.
14. Pardo F, Arroyo P, Salomon C, et al. Role of equilibrative adenosine transporters and adenosine receptors as modulators of the human placental endothelium in gestational diabetes mellitus. *Placenta*. 2013;34(12):1121-1127.
15. Hiden U, Lassance L, Tabrizi NG, et al. Fetal insulin and IGF-II contribute to gestational diabetes mellitus (GDM)-associated up-regulation of membrane-type matrix metalloproteinase 1 (MT1-MMP) in the human feto-placental endothelium. *J Clin Endocrinol Metab*. 2012;97(10):3613-3621.
16. Wadsack C, Desoye G, Hiden U. The feto-placental endothelium in pregnancy pathologies. *Wien Med Wochenschr*. 2012;162(9-10):220-224.
17. Carpenter MW, Coustan DR. Criteria for screening tests for gestational diabetes. *Am J Obstet Gynecol*. 1982;144(7):768-773.
18. Remmele W, Stegner HE. Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue [in German]. *Der Pathologe*. 1987;8(3):138-140.
19. Heublein S, Vrekoussis T, Kuhn C, et al. Inducers of G-protein coupled estrogen receptor (GPER) in endometriosis: potential implications for macrophages and follicle maturation. *J Reprod Immunol*. 2013;97(1):95-103.
20. Cogswell ME, Yip R. The influence of fetal and maternal factors on the distribution of birthweight. *Semin Perinatol*. 1995;19(3):222-240.

21. Battaglia S, Maguire O, Thorne JL, et al. Elevated NCOR1 disrupts PPARalpha/gamma signaling in prostate cancer and forms a targetable epigenetic lesion. *Carcinogenesis*. 2010;31(9):1650-1660.
22. Hirano T, Higuchi T, Katsuragawa H, et al. CD9 is involved in invasion of human trophoblast-like choriocarcinoma cell line, BeWo cells. *Mol Hum Reprod*. 1999;5(2):168-174.
23. Grummer R, Hohn HP, Mareel MM, Denker HW. Adhesion and invasion of three human choriocarcinoma cell lines into human endometrium in a three-dimensional organ culture system. *Placenta*. 1994;15(4):411-429.
24. Holick MF. Vitamin D deficiency. *N Engl J Med*. 2007;357(3): 266-281.
25. Seely EW, Brown EM, DeMaggio DM, Weldon DK, Graves SW. A prospective study of calciotropic hormones in pregnancy and post partum: reciprocal changes in serum intact parathyroid hormone and 1,25-dihydroxyvitamin D. *Am J Obstet Gynecol*. 1997;176(1 pt 1):214-247.
26. Zerwekh JE, Breslau NA. Human placental production of 1 alpha,25-dihydroxyvitamin D3: biochemical characterization and production in normal subjects and patients with pseudohypoparathyroidism. *J Clin Endocrinol Metab*. 1986;62(1): 192-196.
27. Lau SL, Gunton JE, Athayde NP, Byth K, Cheung NW. Serum 25-hydroxyvitamin D and glycated haemoglobin levels in women with gestational diabetes mellitus. *Med J Aust*. 2011; 194(7):334-337.
28. Rudnicki PM, Molsted-Pedersen L. Effect of 1,25-dihydroxy cholecalciferol on glucose metabolism in gestational diabetes mellitus. *Diabetologia*. 1997;40(1):40-44.
29. Dong J, Wong SL, Lau CW, et al. Calcitriol protects renovascular function in hypertension by down-regulating angiotensin II type 1 receptors and reducing oxidative stress. *Eur Heart J*. 2012;33(23):2980-2990.
30. Markestad T, Aksnes L, Ulstein M, Aarskog D. 25-Hydroxyvitamin D and 1,25-dihydroxyvitamin D of D2 and D3 origin in maternal and umbilical cord serum after vitamin D2 supplementation in human pregnancy. *Am J Clin Nutr*. 1984;40(5):1057-1063.
31. Bennett SE, McPeake J, McCance DR, et al. Maternal vitamin D status in type 1 diabetic pregnancy: impact on neonatal vitamin D status and association with maternal glycaemic control. *PLoS One*. 2013;8(9):e74068.
32. Molinari C, Uberti F, Grossini E, et al. 1alpha,25-dihydroxy cholecalciferol induces nitric oxide production in cultured endothelial cells. *Cell Physiol Biochem*. 2011;27(6):661-668.
33. Guzman-Gutierrez E, Arroyo P, Salsoso R, et al. Role of insulin and adenosine in the human placenta microvascular and macrovascular endothelial cell dysfunction in gestational diabetes mellitus. *Microcirculation*. 2014;21(1):26-37.
34. Orendi K, Gauster M, Moser G, Meiri H, Huppertz B. The choriocarcinoma cell line BeWo: syncytial fusion and expression of syncytium-specific proteins. *Reproduction*. 2010;140(5):759-766.
35. Wadsack C, Hrzenjak A, Hammer A, et al. Trophoblast-like human choriocarcinoma cells serve as a suitable in vitro model for selective cholesteryl ester uptake from high density lipoproteins. *Eur J Biochem*. 2003;270(3):451-462.
36. Kumpel BM, Manoussaka MS. Placental immunology and maternal alloimmune responses. *Vox Sang*. 2012;102(1):2-12.
37. Radaelli T, Varastehpour A, Catalano P, Hauguel-de Mouzon S. Gestational diabetes induces placental genes for chronic stress and inflammatory pathways. *Diabetes*. 2003;52(12):2951-2958.
38. Shin JS, Choi MY, Longtine MS, Nelson DM. Vitamin D effects on pregnancy and the placenta. *Placenta*. 2010;31(12):1027-1034.