OPEN ACCESS Freely available online

PLOS ONE

Vitamin D Prevents Endothelial Progenitor Cell Dysfunction Induced by Sera from Women with Preeclampsia or Conditioned Media from Hypoxic Placenta

Lars Brodowski[1☯], Jennifer Burlakov[1☯], Ashley C. Myerski[2], Constantin S. von Kaisenberg[1], Magdalena Grundmann[1], Carl A. Hubel[2]*, Frauke von Versen-Höynck[1]*

1 Department of Obstetrics and Gynecology, Hannover Medical School, Hannover, Germany, **2** Magee- Womens Research Institute and Foundation, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America

Abstract

Context: Placenta-derived circulating factors contribute to the maternal endothelial dysfunction underlying preeclampsia. Endothelial colony forming cells (ECFC), a sub-population of endothelial progenitor cells (EPCs), are thought to be involved in vasculogenesis and endothelial repair. Low vitamin D concentrations are associated with an increased risk for preeclampsia.

Objective: We hypothesized that the function of human fetal ECFCs in culture would be suppressed by exposure to preeclampsia-related factors–preeclampsia serum or hypoxic placental conditioned medium– in a fashion reversed by vitamin D.

Design, Setting, Patients: ECFCs were isolated from cord blood of uncomplicated pregnancies and expanded in culture. Uncomplicated pregnancy villous placenta in explant culture were exposed to either 2% (hypoxic), 8% (normoxic) or 21% (hyperoxic) O_2 for 48 h, after which the conditioned media (CM) was collected.

Outcome Measures: ECFC tubule formation (Matrigel assay) and migration were examined in the presence of either maternal serum from preeclampsia cases or uncomplicated pregnancy controls, or pooled CM, in the presence or absence of 1,25$(OH)_2$ vitamin D_3.

Results: 1,25$(OH)_2$ vitamin D_3 reversed the adverse effects of preeclampsia serum or CM from hypoxic placenta on ECFCs capillary-tube formation and migration. Silencing of VDR expression by VDR siRNA, VDR blockade, or VEGF pathway blockade reduced ECFC functional abilities. Effects of VDR or VEGF blockade were partially prevented by vitamin D.

Conclusion: Vitamin D promotes the capillary-like tubule formation and migration of ECFCs in culture, minimizing the negative effects of exposure to preeclampsia-related factors. Further evaluation of the role of vitamin D in ECFC regulation and preeclampsia is warranted.

Citation: Brodowski L, Burlakov J, Myerski AC, von Kaisenberg CS, Grundmann M, et al. (2014) Vitamin D Prevents Endothelial Progenitor Cell Dysfunction Induced by Sera from Women with Preeclampsia or Conditioned Media from Hypoxic Placenta. PLoS ONE 9(6): e98527. doi:10.1371/journal.pone.0098527

Editor: Anne Croy, Queen's University, Canada

Received March 14, 2014; **Accepted** May 1, 2014; **Published** June 2, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data are included within the manuscript.

Funding: The study was supported by National Institutes of Health P01HD030367 (CAH), UL1RR024153 and UL1TR000005 (University of Pittsburgh Clinical and Translational Science Institute), and a HiLF grant by Hannover Medical School. LB was supported by the Boehringer Ingelheim Fonds travel grant program. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: chubel@mwri.magee.edu (CAH); vonVersen-Höynck.Frauke@mh-hannover.de (FVV)

☯ These authors contributed equally to this work.

Introduction

Preeclampsia remains one of the most common causes of maternal and fetal morbidity and mortality in the developed world [1–3]. Although the pathogenesis of preeclampsia is still not fully understood, a multi-stage model is generally accepted. The utero-placental syndrome with impaired placental development in the first stage of the disease causes generalized maternal endothelial dysfunction as a main clinical feature of preeclampsia in the second stage [4]. An array of placenta-derived factors are candidate contributors to endothelial dysfunction in preeclampsia [5–7].

Endothelial progenitor cells (EPCs) are believed to play an important role in vascular homeostasis and in the repair of injured endothelium and neovascularization [8]. EPCs participate in both

wound healing and angiogenesis. Decreased cell numbers of hematopoietic EPCs in the maternal circulation have been described as a potential sign of impaired endothelial repair capacity in preeclampsia [9,10]. The late outgrowth sub-population of EPCs, also referred to as "endothelial colony forming cells" (ECFCs), have true endothelial-like characteristics, unlike the hematopoietic EPCs studied in the context of preeclampsia previously [11]. ECFCs are highly proliferative and migrate to sites of vessel formation, possessing the ability to differentiate into mature endothelial cells, to participate in vessel repair and to form *de novo* endothelium [12]. Recent data suggest that fetal ECFCs posses the ability to cross the placenta and participate in *de novo* maternal vessel formation in the pregnant uterus [13].

Vitamin D deficiency may be a risk factor for developing preeclampsia [14–18]. However, the underlying mechanisms are unclear. Our previous data suggest a VEGF dependent effect of vitamin D on ECFC proliferation and angiogenesis capability [19]. Given that the nature of the endothelial cell dysfunction and the role of ECFCs in preeclampsia are not entirely clear, we undertook this study in order to explore the effects of potentially relevant factors, i.e. serum from preeclamptic women or conditioned medium from placental villous explants exposed to hypoxic (2% O_2) and hyperoxic (21%) oxygen tension, on ECFC function. In addition, we aimed to investigate whether the addition of 1,25$(OH)_2$ vitamin D_3 to the culture media can prevent ECFC dysfunction under these conditions.

Materials and Methods

This collaborative study was performed at Magee-Womens Research Institute, Pittsburgh, PA and at the Department of Obstetrics and Gynecology, Hannover Medical School, Germany. The University of Pittsburgh Institutional Review Board and the Ethical Committee at Hannover Medical School approved the study and informed written consent was obtained from each woman.

Patient Blood Sample Collection

Twelve healthy women with uncomplicated, normotensive pregnancies (controls) and 12 women with preeclampsia provided pre-delivery maternal blood samples for our study, 6 of each group being primiparous and 6 multiparous. All had singleton pregnancies. Clinical and demographic data describing these pregnant subjects, all of whom delivered at Magee-Womens Hospital, are presented in Table 1. Patients were matched for gestational age at the time of blood sampling, body mass index (BMI) and race. Patients with preeclampsia had gestational hypertension and proteinuria beginning after 20 weeks of pregnancy with resolution of clinical symptoms postpartum. Gestational hypertension was recognized as an absolute blood pressure $\geq$140 mmHg systolic and/or $\geq$90 mmHg diastolic after 20 weeks of gestation. Proteinuria was defined as $\geq$300 mg per 24-h urine collection, $\geq$2+ protein on voided urine sample, $\geq$1+ protein on catheterized urine specimen, or a protein-creatinine ratio of $\geq$0.3. Women with uncomplicated pregnancy were normotensive and without proteinuria throughout gestation, and delivered healthy babies. All patients were non-smokers by self-report, and were without clinical history of preexisting renal, vascular, or metabolic disease.

The maternal peripheral venous blood was withdrawn into sterile collection tubes. The blood samples were incubated at room temperature for 30 min and then centrifuged for 20 min at 2,000×g at RT. The serum was stored at −80°C for later use. Once all samples were acquired, four separate pools of serum were created (combining n = 6 patient samples/pool), namely primiparous uncomplicated pregnancy, multiparous uncomplicated pregnancy, primiparous preeclampsia (PE) and multiparous PE pools. Both the individual serum samples and the serum pools were used for the ECFC functional assays.

Culture of Placental Villous Explants and Preparation of Conditioned Medium

The clinical and demographic data for women who provided both placentas and umbilical cord blood for this study are presented in Table 2. Placental villous explant preparation and culture was carried out according to published protocols with some modifications at Hannover Medical School [20]. Briefly, placentas from 16 uncomplicated, healthy pregnancies delivered by vaginal or Cesarean section were obtained within 10 min of delivery. All of these patients but two had singleton pregnancies. Biopsies were excised from the maternal side of the placenta, after removal of the decidua, midline between the central and lateral part of the placental edge. The tissue was immediately transported to the laboratory in ice-cold phosphate buffered saline (PBS) containing 2% penicillin/streptomycin. After rinsing the placental pieces in PBS to wash out blood, large vessels and decidua were removed by blunt dissection. Placental villous explants (1–2 mg each in size) were dissected and used for experiments under different oxygen conditions. An average of 50 mg of finely dissected villous tissue was placed into each well of a 12-well plate containing 1.5 ml of Medium 199 (Sigma-Aldrich, St- Louis, MO, USA) supplemented with 2% Fetal Bovine Serum (FBS, Biochrom, Berlin, Deutschland) and 1% penicillin–streptomycin (Biochrom, Berlin, Deutschland). The plates were incubated under controlled oxygen conditions (2% O_2, 8% O_2 and 21% O_2) in three separate incubator chambers (Xvivo, Biospherix Inc., USA) at 37°C, 5% CO_2 for 48 h. The CM was centrifuged (3,200 rpm, 4°C, 5 min) and the cell-free supernatants were frozen at −80°C for later use. For the experiments the CM were pooled according the oxygen conditions and stored in aliquots at −80°C. As control, M199 medium supplemented with 2% FBS, 1% penicillin–streptomycin (non-conditioned medium, NCM) was employed in the same ratio as conditioned medium (CM).

ECFC Cell Isolation and Culture

Umbilical cord blood was collected into sterile Vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) immediately after delivery. Within 2 h of collection, the blood was centrifuged at 400 g for 10 min at room temperature, and the plasma was removed and replaced with plasma replacement buffer (PBS containing EDTA [7.4 g in 1 l, Sigma Aldrich, Steinheim, Germany] and 1% penicillin/streptomycin). The umbilical cord blood/plasma replacement buffer mixture was then doubled in volume by addition of an equal amount of isolation buffer (PBS, 2% FBS, 1% penicillin/streptomycin). This blood suspension was layered on Ficoll Plus (GE Healthcare, Buckinghamshire, England) and subjected to density gradient centrifugation (400 g, 40 min) with brake in the off position. The mononuclear cell layer was collected and 50 million cells per well seeded into collagen-1-coated 6-well plates (BD Biosciences, Heidelberg, Germany) using Endothelial Basal Medium 2 (EBM-2, Lonza, Walkersville, MD, USA), supplemented with EGM-2 Single Quot Kit (Lonza) in supplier-recommended concentrations of human recombinant epidermal growth factor, fibroblast growth factor, VEGF, ascorbic acid, hydrocortisone, recombinant insulin-like growth factor) containing 10% FBS and 1% penicillin/streptomycin. After 10–21 days (range) of cultivation ECFCs appeared as adherent single layers of cobblestone shaped, late outgrowth cells that formed colonies (>50 cells). Individual colonies were harvested using

Table 1. Clinical and demographic data of patients who provided maternal blood samples.

Variable	Uncomplicated pregnancy (n = 12)	Preeclampsia (n = 12)	P value
Maternal age (y)	25.9±7.0	29.2±8.3	NS
Gestational age at time of blood sampling (wks)	36.5±4.2	37.4±2.3	NS
Gestational age at delivery (wks)	36.6±4.1	37.5±2.3	NS
Multiparous- n (%)	6 (50%)	6 (50%)	NS
Maternal pre-pregnancy BMI (kg/m^2)	25.5±5.3	26.5±5.8	NS
Gestational SBP, pre-delivery (mm Hg)	120±13	151±17	<0.001
Gestational SBP before 20 week gestation (mm Hg)*	118±8	107±9	<0.01
Gestational DBP, pre-delivery (mm Hg)	71±7	91±9	<0.001
Gestational DBP before 20 week gestation (mm Hg)*	71±5	69±9	NS
Birth weight (g)	2877±930	2648±689	NS
Birth weight percentile	52.4±23.6	33.6±30.7	NS
Birth weight percentile <10th- n (%)	0 (0%)	2 (17%)	NS
Caesarean delivery- n (%)	3 (25%)	4 (33%)	NS
Labor at the time of blood sampling- n (%)	8 (67%)	6 (50%)	NS
Maternal Race, Black – n (%)	4 (33%)	4 (33%)	NS
Baby gender, male- n (%)	7 (58%)	3 (25%)	NS

BMI, body mass index; DBP, SBP, diastolic and systolic blood pressure (average of last three measurements). Data are given as mean ± SD or number (percentage). *Early BP values were not available for 3 preeclampsia patients; all patients were normotensive postpartum.
doi:10.1371/journal.pone.0098527.t001

cloning rings and replated separately in tissue culture flasks. ECFCs were used at 80–90% confluence and between passages 4–7 in experiments.

Phenotyping of ECFCs

The endothelial phenotype of the isolated blood cells was confirmed by flow cytometry using VEGFR-2, CD31, CD34, CD133 and CD45 as well as appropriate isotype controls, and by using fluorescein isothiocyanate-labeled Ulex europaeus agglutinin I (lectin; Sigma-Aldrich, Steinheim, Germany) for cell surface staining, and acetylated low-density lipoprotein (Dil-Ac-LDL; Biomedical Technologies, Stroughton, MA) to confirm cellular uptake of Dil-Ac-LDL, as described previously in detail [19].

RNA Interference

RNA interference experiments were performed with siRNA for VDR (Silencer Validated siRNA VDR, Life Technologies, AM51331) and scrambled siRNA (Silencer Negative Control No. 1 siRNA, Life Technologies, Carlsbad, USA) using Dharmafect 1 (Dharmacon, Lafayette, CO, USA), according to the manufacturer's instructions. Briefly, cells were plated and then transfected at 80–90% confluence with 50 nM siRNA for 24 h. The efficiency of siRNA transfection was tested using fluorescein-conjugated control siRNA.

Table 2. Clinical and demographic data for the uncomplicated pregnant women who provided placental samples for villous explant culture (data are mean +/− SD).

Variable	
n	16
Maternal Age (y)	33±3.3
Multiparous – n (%)	9 (56%)
Maternal prepregnancy BMI (kg/m^2)	27.1±6
Gestational SBP, pre-delivery (mmHg)	119.9±13.5
Gestational DBP, pre-delivery (mmHg)	68.6±11.3
Birth Weight (g)	3437.5±735
Gestational Age (weeks)	38.8±1.3
Caesarean delivery- n (%)	9 (56%)
Labor with delivery –n (%)	7 (44%)
Smoking- n (%)	0 (0%)

doi:10.1371/journal.pone.0098527.t002

In vitro Angiogenesis Assay

We used an in vitro angiogenesis assay (endothelial tubule formation in Matrigel) as a test of ECFC function. 17,000 ECFCs/well were seeded into 96 well plates, each well pre-coated with 50 µl growth factor- reduced Matrigel (BD Biosciences, Bedford, MA). The ECFCs in Matrigel assay were exposed to pooled sera from normal pregnancy or preeclampsia patients (5% v/v concentration in EBM-2). We also tested the effect of the individual (non-pooled) serum samples (uncomplicated control versus preeclampsia; n = 12 samples each) at 5% v/v. The experiments were performed in the presence and absence of 10 nM $1,25(OH)_2$ vitamin D_3 [21,22]. In separate experiments, conditioned media (CM) or non-conditioned media (NCM) were added at 25% v/v concentration in EBM-2. After 14 h of incubation, digital images were obtained at 2.5x magnification. The total length of tubules per visual field (per well) was determined with Image J software. All experiments were done in triplicate wells from which values were averaged (n = number of experiments).

Cell Migration Assay

To analyze ECFC migratory ability, ECFCs were pre-treated with EBM-2+2% v/v FBS, with or without 10 nM $1,25(OH)_2$ vitamin D_3 for 24 h. The confluent ECFC monolayers were scratched using a sterile P200 pipette tip to produce a lane free of cells as described before [23]. Pooled sera from normal or preeclamptic patients was added at 5% v/v concentration. CM or NCM were added at 50% v/v concentration. The sera and media experiments were performed in the presence or absence of 10 nM $1,25(OH)_2$ vitamin D_3. Light microscopic images were obtained immediately after the scratch (T_0) and at the end of the experiment (18 h for CM; 8 h for serum). Migration into the scratch wound was analysed using Image J software and calculated as percentage of wound closure (percentage of original area that became occupied by cells by migration into the wound area). All experiments were done in quadruplicate wells from which values were averaged.

VDR Silencing and VEGF Pathway Inhibition

The VDR was blocked with the VDR antagonist pyridoxal-5-phosphate (0.5 mM, Cell Signaling/New England Biolabs, Frankfurt am Main, Germany) or down-regulated by transfection with validated VDR siRNA. We and others have shown that vitamin D has a stimulatory effect on the VEGF pathway, which is important for angiogenesis and repair of vessels [19,24]. Therefore, we also inhibited the VEGF pathway by SU5416 (0.5 µM, Sigma Aldrich, Steinheim, Germany).

Vitamin D_3 Measurement in Patient Serum

25(OH) Vitamin D concentrations in the individual maternal serum samples were determined using a LIAISON 25(OH) Vitamin D TOTAL Assay (DiaSorin Inc., USA), as per the manufacturers recommendations, at Hannover Medical School.

Statistical Analysis

Statistical analysis was performed after testing for normality distribution by Kolmogorov-Smirnov-test. One-way analysis of variance, Kruskal-Wallis test, unpaired t-test, Mann-Whitney U or Wilcoxon-signed rank test were used as appropriate (Prism 4 software package, GraphPad Software Inc., La Jolla, CA). Demographic data are expressed as means and standard deviation (SD) and experimental data as means and standard error (SEM), with $P<0.05$ considered as statistically significant.

Results

Patient Demographics

Maternal age, pre-pregnancy body-mass index, race and parity were not statistically different between the preeclampsia cases and uncomplicated pregnancy controls who provided serum samples (Table 1). By definition, women with preeclampsia had significantly higher systolic and diastolic blood pressures at delivery compared to controls. The gestational age at the time of venipuncture was not significantly different between the groups. Table 2 shows the characteristics of the uncomplicated pregnancy patients that contributed their placentas for villous explant culture. Two of 16 were twin pregnancies.

In vitro Angiogenesis

Figures 1 A–B show the effects of serum from preeclampsia patients compared to serum from uncomplicated controls on capillary-tube formation by ECFCs, in which data from the primiparous serum pool and the multiparous serum pool are combined for each pregnancy outcome group. Here, tubule lengths are expressed as percent relative to the value (set at 100%) obtained in the presence of uncomplicated pregnancy pooled sera (control without supplemental vitamin D). Tubule formation was significantly less in the presence of preeclampsia serum (84±3%; $P<0.001$; n = 15 experiments) compared to uncomplicated pregnancy serum (Figure 1A). As shown in Figure S1, tubule assemblage was significantly impaired by either the primiparous preeclampsia pooled sera compared to primiparous control pooled sera (90±2.5%; P = 0.01; n = 6 experiments), or multiparous preeclampsia pooled sera compared to multiparous control pooled sera (79±4.5%; P = 0.01; n = 9 experiments).

We tested, the impact of $1,25(OH)_2$ vitamin D_3 on tubule formation when in the simultaneous presence of pooled control or pooled preeclampsia sera. Vitamin D at 1 nM and 10 nM significantly increased total tubule length in the preeclamptic and control serum-treated groups (Figure 1A and Figure S1). As shown in Figure 1A, in which data from primiparous and multiparous sera are combined, 1 nM and 10 nM $1,25(OH)_2$ vitamin D_3 stimulated tubule formation in the presence of both control sera (1 nM: 114±4%; P = 0.01; n = 15; 10 nM: 114±4%; P = 0.01; n = 12) and preeclampsia sera (1 nM: 124±4%; P = 0.01; n = 15; 10 nM: 123±4%; $P<0.001$; n = 12) such that the effects of preeclampsia vs. control sera were no longer significantly different ($p>0.05$).

We tested the effect of the individual (non-pooled) serum samples (uncomplicated control versus preeclampsia; n = 12 samples each) using one ECFC cell line derived from a control pregnancy and found comparable results. With control and preeclampsia serum samples tested as tandem pairs and matched for BMI, gestational and maternal age, 11 out of 12 serum samples from preeclamptic women lead to reduced tubule lengths compared to the control serum. In summary, serum of preeclamptic patients impaired angiogenetic capacity compared to control serum (87±4%; P = 0.01; n = 12). Relative to tubule formation in the presence of control pregnancy serum alone (100%), $1,25(OH)_2$ vitamin D_3 increased tubule formation (1 nM: 106±4%; P = 0.02; n = 12; 10 nM: 110±3%; P = 0.01; n = 12). Relative to tubule formation in the presence of preeclampsia serum alone (100%), $1,25(OH)_2$ vitamin D_3 likewise increased tubule formation (1 nM: 109±4%; P = 0.03; n = 12; 10 nM: 114±4%; P = 0.01; n = 12). In the presence of vitamin D (10 nM), preeclampsia vs. control patient sera no longer had significantly different effects ($P>0.05$).

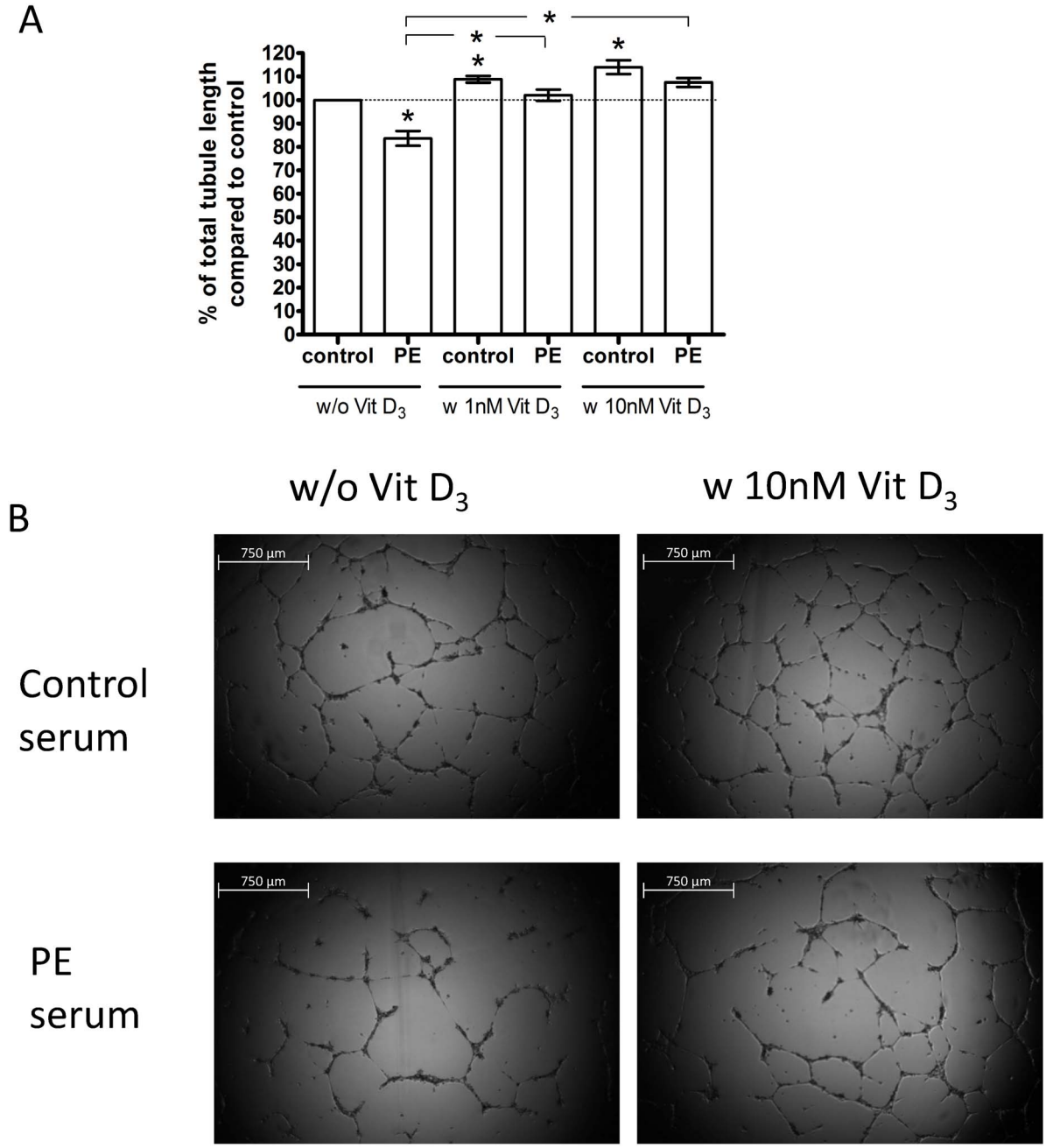

Figure 1. Effect of uncomplicated pregnancy (control) sera and preeclampsia (PE) sera, and 1,25(OH)$_2$ vitamin D$_3$, on capillary-tube formation by ECFCs in a Matrigel assay. ECFCs were cultured in endothelial basal medium (EBM) +5% v/v sera. Capillary-tube formation (average total tubule length per microscopic field) was analyzed after 14 h by visual microscopy at 2.5 magnification (A). Data are expressed as percentage of the control in the absence of vitamin D. Results represent mean values of total tubule length ± SEM of at least 6 independent experiments; *$P<0.05$ vs. control; Horizontal bars with asterisk (−*−): $P<0.05$, preeclampsia sera without vitamin D vs. preeclampsia sera with vitamin D. (B) Representative photomicrographs of ECFCs after incubation in Matrigel with EBM+5% v/v patient sera. Scale bar represents 750 µm.
doi:10.1371/journal.pone.0098527.g001

Figures 2 A–C display the results of treatment of ECFCs with placental villous explant CM (from cultures under 2%, 8% and 21% O_2) compared to NCM. The effect of 1,25(OH)$_2$ vitamin D$_3$ is illustrated within the figures. ECFCs from uncomplicated pregnancies were used, and total tubule length was determined in the *in vitro* Matrigel angiogenesis model. There was no difference in tubule formation between the 2% O_2, 8% O_2 and 21% O_2 CM (Figure 2 A). However, a reduction of angiogenesis with CM from 2% O_2 villous explant culture was observed compared to NCM (81±7.4%; P = 0.03, n = 9). Vitamin D did not exhibit a significant effect under these conditions.

Migration

As shown in Figure 3 A–C we found a reduction in migration of ECFCs when in the presence of primiparous (90±3%; P = 0.02; n = 6 experiments) or multiparous (92±6%; P = 0.03; n = 12 experiments) preeclampsia pooled sera compared to the corresponding control pooled sera. Combining data from primi- and

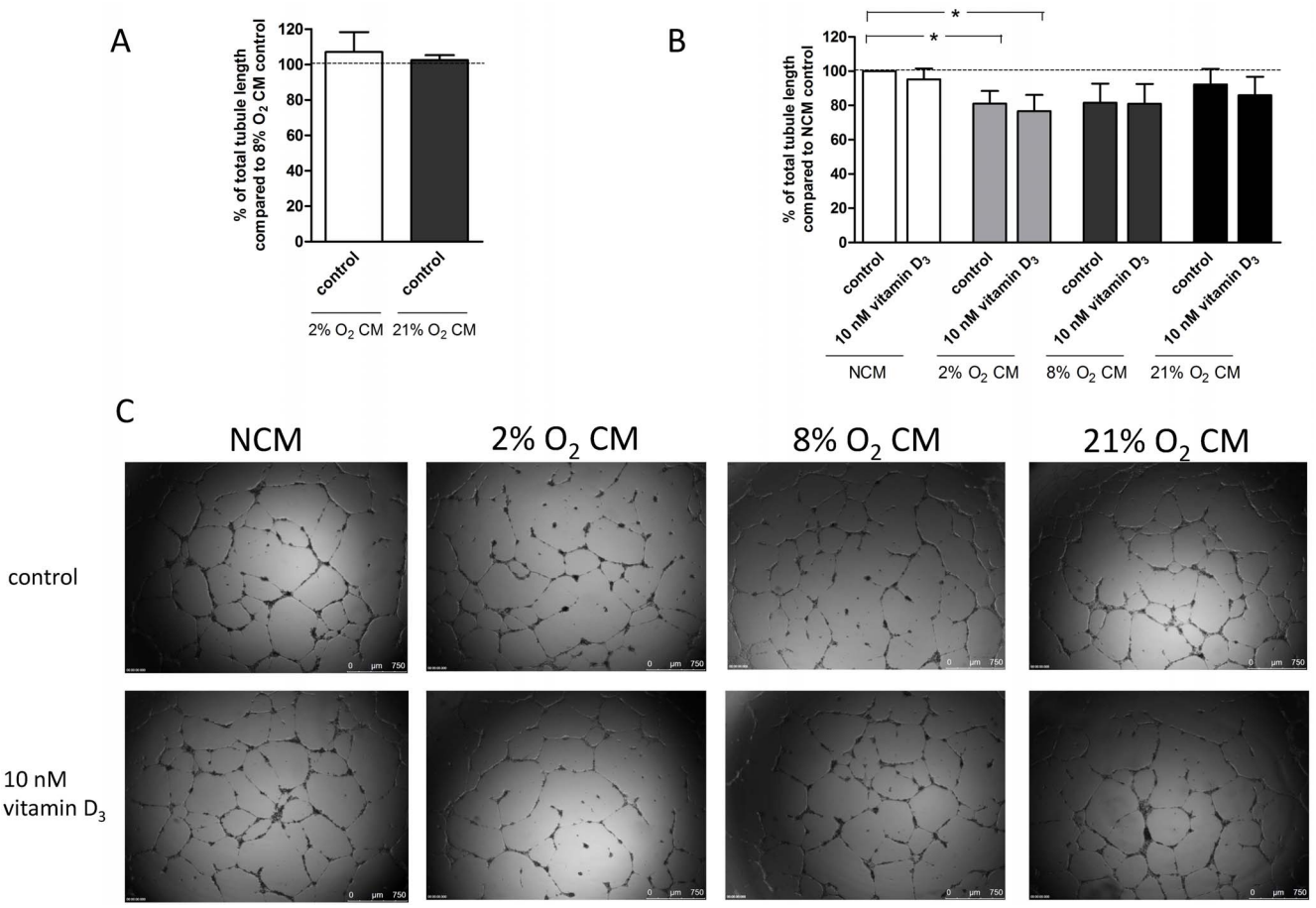

Figure 2. Effect of villous explant conditioned medium (2% O_2, 8% O_2, 21% O_2 CM) and 1,25(OH)$_2$ vitamin D_3 on capillary-tube formation in a Matrigel assay. ECFCs were cultured in endothelial basal medium (EBM) +25% CM (A–C). Capillary-tube formation (average total tubule length per microscopic field) was analyzed after 14 h by visual microscopy at 2.5 magnification. Data are expressed as percentage of control (2A: 8% O_2 CM+0 nM vitamin D_3; 2B: NCM+0 nM vitamin D_3). Results represent mean values of total tubule length ± SEM of at least 9 independent experiments; *$P<0.05$ vs. control, C: Representative photomicrographs of ECFCs after plating on Matrigel cultured in EBM+25% v/v CM. Scale bar represents 750 µm.
doi:10.1371/journal.pone.0098527.g002

multiparous patient samples, the negative effect of preeclampsia pooled sera on ECFC migration remained significant (92±4%; P = 0.01; n = 18).

As shown in Figure 3, the simultaneous addition of 1,25(OH)$_2$ vitamin D_3 increased the migrative abilities of ECFCs into the scratch wound during incubation with pooled primiparous (1 nM: 114±10%; P = 0.03; n = 6; 10 nM: 115±6%; P = 0.053; n = 6) or multiparous (1 nM: 120±5%; P = 0.01; n = 12; 10 nM: 114±4%; P = 0.01; n = 9) preeclampsia pooled sera (100%). We found a stimulating effect of 10 nM 1,25(OH)$_2$ vitamin D_3 in the presence of pooled sera from primiparous controls (1 nM: 109±5%; P = 0.11; n = 6; 10 nM: 117±10%; P = 0.03; n = 6) and stimulating effect of both 1 nM and 10 nM 1,25(OH)$_2$ vitamin D_3 in the presence of pooled sera from multiparous controls (1 nM: 120±4%; P<0.001; n = 12; 10 nM: 120±6%; P = 0.03; n = 9). When combining data from primi- and multiparous patient pools, 1,25(OH)$_2$ vitamin D_3 enhanced ECFC migration both in the presence of control sera (1 nM: 116±3%; p<0.001; n = 18; 10 nM: 119±5%; P = 0.01; n = 15) and preeclampsia sera (1 nM: 118±5%; P = 0.01; n = 18; 10 nM: 115±3%; P<0.001; n = 15), such that the differential effects of case vs. control sera remained significant. ECFC migration in the presence of preeclampsia sera + vitamin D was not different from uncomplicated pregnancy sera alone.

Placental villous explant CM derived from 2% O_2 incubations (86.1±7.2%, P = 0.02, n = 9) and 21% O_2 incubations (86±4.1%, P = 0.02, n = 10) significantly reduced ECFC migration, when compared to CM from 8% O_2 (Figure 4 A). Compared to NCM, explant CM from 2% O_2 (74±7%; P = 0.005) and 21% O_2 (71±7.1%, P = 0.003, n = 10), but not 8% O_2, impaired ECFC migration (Figure 4 B). Compared to absence of vitamin D, 1,25(OH)$_2$ vitamin D_3 improved endothelial cell migration in 2% O_2 CM (110±4.4%, P = 0.046) and 21% O_2 CM (124±8.7%, P = 0.02), but not significantly in 8% O_2, (113±7.9%, P = 0.15) (n = 10). 1,25(OH)$_2$ vitamin D_3 normalized the negative effects of 2% O_2 CM (95±5.5%, P = 0.36) and 21% O_2 CM (108±9.9%, P = 0.46) when compared to 8% O_2 CM (data not shown). ECFCs treated with 2%, 8% or 21% CM and vitamin D were also not statistically different compared to NCM (P>0.05), (Figures 4 B and C).

VDR Blocking and Inhibition of VEGF Pathway

The effects of VDR and VEGF inhibitors (P5P and SU4516, respectively) on ECFC tubule formation were tested in the presence of pooled control sera (Figure 5 A) or pooled

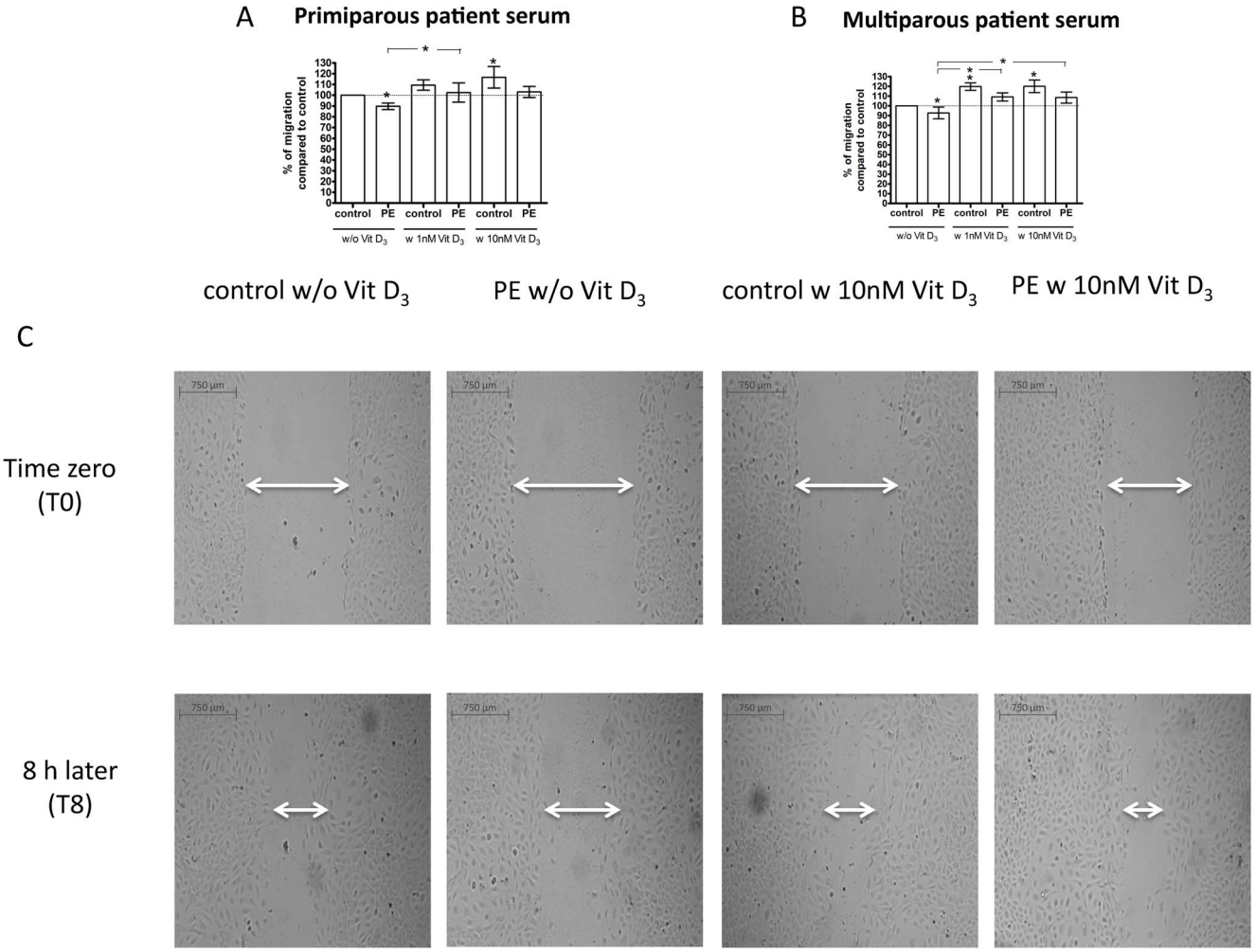

Figure 3. Effect of uncomplicated pregnancy (control) sera and preeclampsia (PE) sera from primiparous (A) and multiparous (B) women, and 1,25(OH)$_2$ vitamin D$_3$, on ECFC migration. ECFCs of uncomplicated pregnancies were cultured in endothelial basal medium (EBM) +5% v/v patient sera and treated with or without 1 nM or 10 nM 1,25(OH)$_2$ vitamin D$_3$. The migration of ECFCs into the scratch wound was assessed. ECFC migration was reduced in the presence of preeclampsia sera compared to control sera of primiparous (A) and multiparous (B) women. Vitamin D restored or improved migrative capacity. Results represent mean ± SEM percent wound filling of at least 6 independent experiments, *P<0.05 vs. control; Horizontal bars with asterisk (−*−): P<0.05, preeclampsia sera without vitamin D vs. preeclampsia sera with vitamin D. C: Representative images of ECFC monolayers with scratch wounds at 0 h (a, c) and 8 h (b, d) of incubation. Scale bar represents 750 µm.
doi:10.1371/journal.pone.0098527.g003

preeclampsia sera (Figure 5 B). P5P inhibited tubule formation (percent relative to control sera alone: 71±8.9%; P = 0.045; n = 4; percent relative to preeclampsia sera alone: 74±6.8%; P = 0.02; n = 5). SU5416 similarly inhibited tubule formation (percent relative to control sera alone: 36±13.5%; P = 0.02; n = 4; percent relative to preeclampsia sera alone: 48±11.9%; P = 0.02; n = 4). Silencing of the VDR also impaired tubule formation (percent relative to control sera alone: 59±7.2%; P = 0.01; n = 4; percent relative to preeclampsia sera alone: 78±2.6%; P = 0.01; n = 4), (data compared to non-silenced control ECFCs). Non-targeting siRNA transfected ECFCs were used as an internal control. Total ECFC tubule lengths were not affected by the non-targeting siRNA (percent relative to control sera alone: 98±1.8%; P = 0.27; n = 4; percent relative to preeclampsia sera alone: 104±4.8%; P = 0.5; n = 4). Vitamin D significantly increased tubule formation in the presence of uncomplicated pregnancy (Figure 5 A) or preeclampsia sera (Figure 5 B) (control = sera without VDR or VEGF inhibitors). Vitamin D significantly reversed the negative effects of VDR blockade (P5P) on tubule formation, and non-significantly attenuated the negative effects of VEGF blockade (SU5416), but had no effect after VDR silencing (Figure 5 A and B).

The impact of VDR or VEGF pathway inhibition on ECFC migration was tested in the presence of 2% O_2 incubation-derived villous explant culture CM (Figure 5 C). A significant reduction of scratch wound closure was observed in the presence of P5P (78±4.9%; P = 0.004, n = 7) and SU5416 (87±5.3%; P = 0.04, n = 8). Vitamin D had a rescuing effect on ECFC migration after blocking the VDR (P5P+VD: 111±4.4%; P = 0.049, n = 7), but not the VEGF pathway (SU5416+ VD: 104±4.6%; P = 0.46, n = 8).

Maternal Vitamin D Status

None of the patients had normal (replete) vitamin D levels (> 30 ng/ml [25]). More women with preeclampsia (PE: 7/12, NP: 3/12) showed severe vitamin D deficiency (<10 ng/ml), although not significantly so (P = 0.21). There was no significant relationship

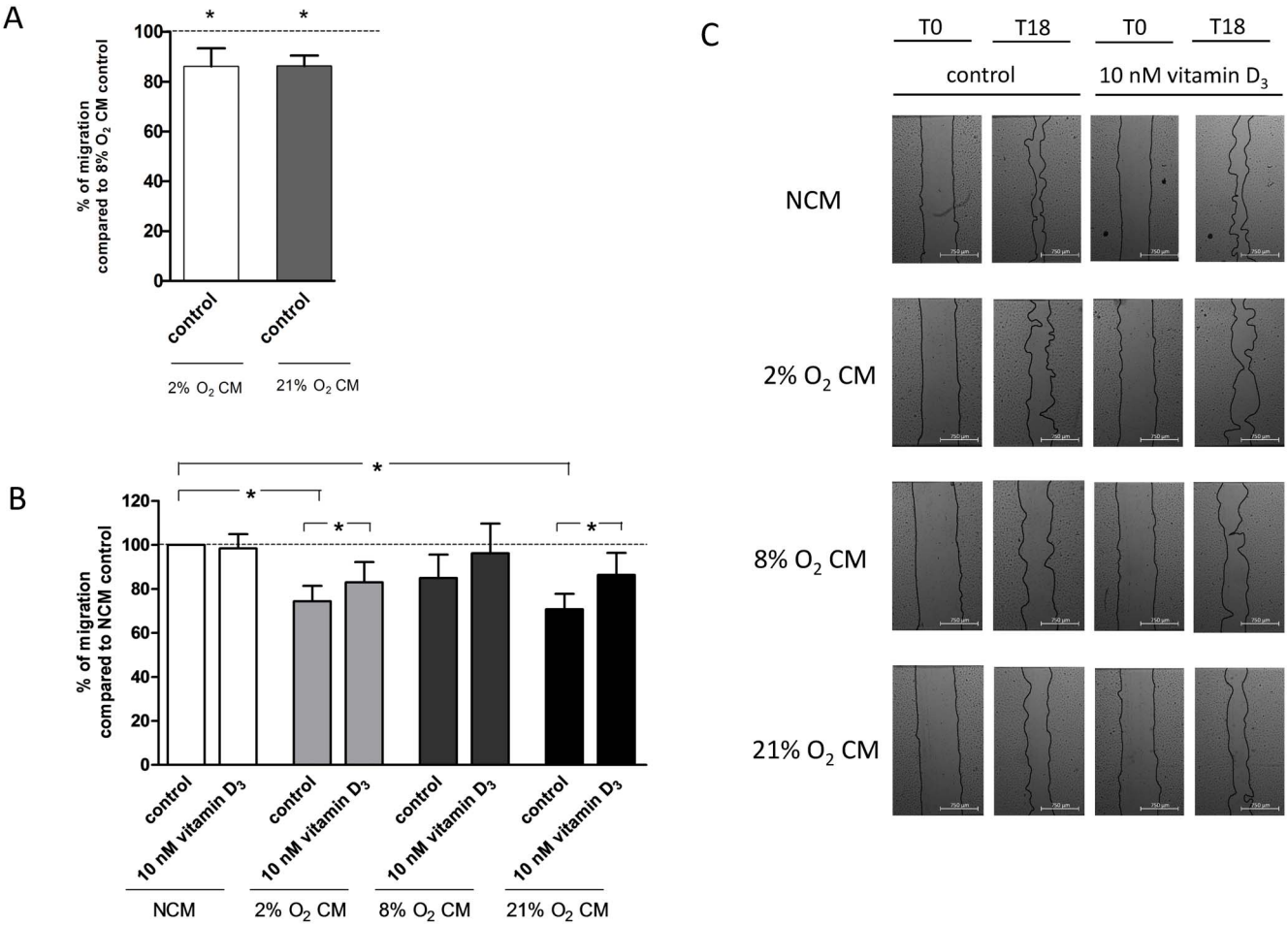

Figure 4. Effect of villous explant conditioned medium (2% O_2, 8% O_2, 21% O_2 CM) and 1,25(OH)$_2$ vitamin D_3 on ECFC migration. ECFCs of uncomplicated pregnancies were cultured in endothelial basal medium (EBM) +50% v/v CM and treated without (vehicle control) or with 10 nM 1,25(OH)$_2$ vitamin D_3. The migration of ECFCs into the scratch wound was assessed. ECFC migration was lower in 2% O_2 and 21% O_2 villous explant CM compared to 8% O_2 CM (A) and NCM (B). Vitamin D restored migrative capacity. Results represent mean ± SEM percent wound filling, N = 10, *$P<0.05$ vs. control (0 nM vitamin D). C: Representative images of ECFC monolayers with scratch wounds at 0 h and 18 h of incubation. Scale bar represents 750 µm.
doi:10.1371/journal.pone.0098527.g004

between maternal serum vitamin D concentration and fetal ECFC response to the patient serum in cell culture (P = 0.45).

Discussion

One major finding of this study is that ECFCs exhibited markedly decreased tubule formation and migration *in vitro* in response to serum from women with preeclampsia compared to serum from women with uncomplicated pregnancies. Both pooled serum and individual patient serum samples from women with preeclampsia exhibited these inhibitory effects. A second major finding is that suppression of ECFC migration was also observed in response to conditioned media collected from placental villous explants cultured under aberrant oxygen conditions [2% O_2 ("hypoxic") or 21% ("hyperoxic")] when compared to either 8% O_2 ("normoxic") or non-conditioned media. These inhibitory effects on ECFC behaviors were substantially reversed by exogenous administration of vitamin D in the physiologic range. VDR signaling antagonism (pyridoxal-5-phosphate) reduced ECFC tubule formation and migration in a fashion partially reversed by exogenous vitamin D, likely related to competition for binding to the vitamin D receptor (VDR).

During the first weeks of normal pregnancy, the placental environment is hypoxic (~2% O_2), but the oxygen level rises up to 6–8% O_2 around 12 weeks of gestation, which is considered physiologic during the second and third trimester of pregnancy [26]. At term, 6–8% O_2 is thought to represent "normoxia" and 1–2% O_2 "hypoxia" for villous tissue [27]. In this study 2% O_2 was used as hypoxic, 8% O_2 as normoxic and 21% O_2 as "hyperoxic" conditions as in previous studies [28,29]. We speculate that our findings of reduced ECFC functional capacities during incubation with both low (2%) and high (21%) O_2 might relate to increased generation of anti-angiogenic/pathogenic factors under these conditions compared to 8% O_2 explant culture conditions. Both hypoxia, especially with fluctuations in oxygen concentration, and hyperoxia can result in placental oxidative stress with release of inflammatory cytokines and/or anti-angiogenic factors [30]. In preeclampsia, low or fluctuating oxygen levels might persist due to impaired utero-placental blood flow and lead to disturbances in the placenta. The associated increased release of placenta-derived factors is believed to contribute to the disturbed maternal endothelial homeostasis [1,31]. The nature/effects of the circulating placental factors remain intensively investigated [31].

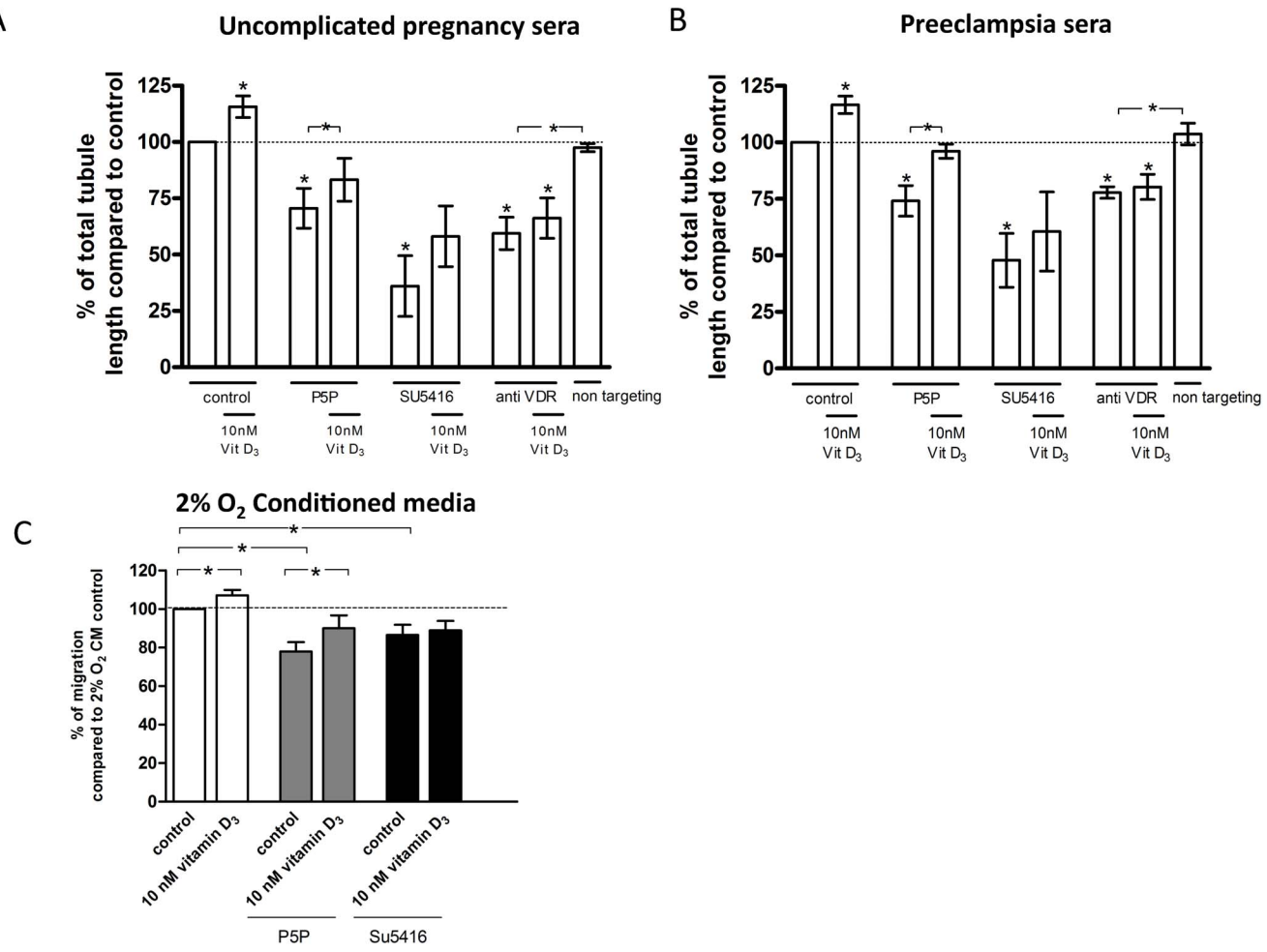

Figure 5. Effect of 1,25(OH)$_2$ vitamin D$_3$ and the inhibitors pyridoxal-5-phosphate, SU5416 and vitamin D receptor (VDR) small interfering (si)RNA on ECFC capillary-tube formation or migration. ECFCs were incubated with uncomplicated pregnancy sera (A) or preeclampsia sera (B) or 2% O_2 villous explant conditioned medium (C), and in the presence or absence (control) of 0.5 mM pyridoxal-5-phophate, 0.5 μM SU5416, VDR siRNA, or non-targeting (scrambled) siRNA. The effects of each of these exposures were additionally examined both in the absence (vehicle) or presence of 10 nM 1,25(OH)$_2$ vitamin D$_3$. Total tubule lengths (A, B) or migration (C) were determined and expressed as percent relative to control. Results represent mean ± SEM of at least 4 independent experiments. *$P<0.05$ compared to control. Other significant differences ($P<0.05$) are indicated by the horizontal bars.
doi:10.1371/journal.pone.0098527.g005

Previously, metabolic footprinting of placental villous explant conditioned culture media identified differences as a function of normoxic, hypoxic and hyperoxic incubation conditions [32]. Uncomplicated pregnancy villous tissue incubated under hypoxic conditions, and preeclampsia villous tissue incubated under normoxic conditions produced very similar metabolic footprints [32]. Others have reported that endothelial cell proliferation was reduced by plasma, but not serum, from preeclamptic women [33]. This is in contrast to our study were we show a significant negative effect of preeclampsia serum on ECFCs in vitro.

Vitamin D deficiency has been linked to an approximate 5-fold increased risk for the development of preeclampsia [14–16]. The vitamin D receptor (VDR) is expressed in human placenta, endothelial cells and (we have shown) in cord ECFCs [19,34,35]. Potentially relevant to preeclampsia, vitamin D regulates key target genes associated with implantation, trophoblast invasion and anti-inflammatory responses in maternal decidua and fetal trophoblast [36–38]. Vitamin D regulates angiogenesis through direct effects on VEGF gene transcription [19,24,39]. Our published data show a stimulating and VEGF-dependent effect of physiologic concentrations of vitamin D on fetal (cord blood) ECFC function [19]. We now demonstrate a stimulating effect of vitamin D on ECFC migration and capillary-tube formation, overcoming adverse effects of serum from preeclamptic women. Vitamin D also enhanced ECFC function after treatment with hypoxic or hyperoxic villous CM. These effects were dependent on VDR activation as indicated by silencing and blocking the receptor. Here we also confirmed that blocking of the VEGF pathway impairs ECFC function. However, vitamin D did not substantially restore the inhibiting effect of the VEGF pathway inhibitor SU5416 in the presence of patient sera or villous CM.

Vitamin D was able to rescue the angiogenic deficits caused by preeclampsia sera (Figure 1), but not those caused by 2% oxygen conditioned media (Figure 2). Chronic hypoxia might not exactly model the effects of fluctuations (hypoxia-reoxygenation) thought to frequently occur with preeclampsia [30]. The milieu in maternal serum may be more complex, with levels of maternally derived factors differing by pregnancy outcome group. Alternatively, concentrations of anti-angiogenic factors may be higher in placenta CM compared to maternal serum. The magnitude of

change (increase) in tubule formation effected by vitamin D was greater when in the presence of preeclampsia sera compared to uncomplicated pregnancy sera, such that tubule formation became equalized (Figure 1). This differential response to vitamin D according to sera was not observed with migration. The reason for this non-uniformity of response remains unclear.

Preeclampsia is a risk factor for cardiovascular events in the mother and offspring later in life [31,40]. Potentially germane to this, early life vitamin D deficiency in a rat model was associated with endothelial dysfunction and elevated blood pressure in the offspring [41], and a study in mice found vitamin D deficiency in pregnancy to lead to maternal hypertension and altered placental and fetal development [42]. These *in vivo* data are consistent with the hypothesis that vitamin D insufficiency predisposes pregnant women and their offspring to disturbed endothelial homeostasis.

ECFCs, a sub-population of EPCs, posses the unique ability for vasculogenesis – the *de novo* formation of blood vessels from progenitor cells. The few studies of fetal ECFCs and pregnancy complications describe a reduction in fetal ECFC numbers and colony formation in diabetic pregnancies or pregnancies with growth restricted infants [43,44]. Most interestingly recent data point to the ability of human fetal ECFCs, but not human fetal endothelial cells, to transmigrate to the maternal bloodstream and then home to locations of maternal uterine vasculogenesis [13]. Our data suggest that fetal ECFCs respond to pathogenic stimuli by impairment of functional activities and that these can be reversed by vitamin D. A logical future step would be to accumulate conditioned media from gestational age-matched preeclampsia and uncomplicated pregnancy villous placenta for comparison. The extent to which villous fragments in explant culture accurately retain their *in vivo* phenotype is uncertain. However, it was previously reported that, compared to conditioned media from uncomplicated, term pregnancy villous explants, conditioned media from preeclampsia placental villous explants suppress *in vitro* tubule formation and migration of human umbilical vein endothelial cells (HUVEC) in culture [45]. These effects appeared to be attributable to increased secretion of the soluble receptor, soluble vascular endothelial growth factor receptor-1 (sVEGFR-1, also known as sFLT1), by preeclampsia explants, consistent with the elevated placental secretion of sFLT1 in *vivo* [5].

Several reports indicate that $1,25(OH)_2$ vitamin D either decreases or has no effect on endothelial cell proliferation or angiogenesis *in vivo* or *in vitro* [46,47]. The divergent results might reflect heterogeneity among endothelial cell subtypes. ECFCs reportedly differ from mature human umbilical vein endothelial cells (HUVEC) or human umbilical artery endothelial cells in the expression of differentiation-related surface markers, proliferation rates, or telomerase activities [48]. However the reason for the apparent distinct proangiogenic effects of vitamin D on ECFCs is presently unclear. To speculate, vitamin D might upregulate the release of factors by progenitor cells that, in turn, stimulate angiogenic behaviors in autocrine fashion. Hematopoietic endothelial progenitor cells, a more prevalent circulating cell type compared to ECFCs, express functional vitamin D receptors. Conditioned media from vitamin D treated hematopoietic endothelial progenitors increased tubule networks of human aortic endothelial cells in Matrigel in vitro, whereas vitamin D alone did not [49].

To our knowledge this is the first study to investigate the effect of placenta-derived factors on ECFC function and to explore a putative positive role of vitamin D for restoring endothelial function in this context. Effective preventive or therapeutic strategies for preeclampsia do not exist to date. It is plausible that ensuring vitamin D sufficiency before and during pregnancy will reduce endothelial dysfunction and disease development in mother and offspring.

Supporting Information

Figure S1 Effect of uncomplicated pregnancy (control) pooled sera and preeclampsia (PE) pooled sera from primiparous (A) and multiparous (B) women, and $1,25(OH)_2$ vitamin D_3, on capillary-tube formation by ECFCs in a Matrigel assay. ECFCs were cultured in endothelial basal medium (EBM) +5% v/v sera. Capillary-tube formation (average total tubule length per microscopic field) was analyzed after 14 h by visual microscopy at 25x magnification. Data are expressed as percentage of the control in the absence of vitamin D. Results represent mean values of total tubule length ± SEM of at least 6 independent experiments; $*P<0.05$ vs. control; Horizontal bars with asterisk (−*−): $P<0.05$, preeclampsia serum without vitamin D vs. preeclampsia serum with vitamin D.
(TIFF)

Acknowledgments

We thank the Clinical Data Core and staff of Magee-Womens Hospital and the Division of Obstetrics at Hannover Medical School for invaluable assistance.

Author Contributions

Conceived and designed the experiments: LB JB MG FVV CAH. Performed the experiments: LB JB. Analyzed the data: LB JB ACM FVV CAH. Contributed reagents/materials/analysis tools: FVV CAH CSVK. Contributed to the writing of the manuscript: LB JB ACM MG CSVK FVV CAH.

References

1. Ilekis JV, Reddy UM, Roberts JM (2007) Preeclampsia–a pressing problem: an executive summary of a National Institute of Child Health and Human Development workshop. Reprod Sci 14: 508–523.
2. Wang Y, Gu Y, Zhang Y, Lewis DF (2004) Evidence of endothelial dysfunction in preeclampsia: decreased endothelial nitric oxide synthase expression is associated with increased cell permeability in endothelial cells from preeclampsia. Am J Obstet Gynecol 190: 817–824.
3. Roberts JM (2000) Preeclampsia: what we know and what we do not know. Semin Perinatol 24: 24–28.
4. Redman CW, Sargent IL (2005) Latest advances in understanding preeclampsia. Science 308: 1592–1594.
5. Maynard SE, Min JY, Merchan J, Lim KH, Li J, et al. (2003) Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest 111: 649–658.
6. Dechend R, Homuth V, Wallukat G, Kreuzer J, Park JK, et al. (2000) AT(1) receptor agonistic antibodies from preeclamptic patients cause vascular cells to express tissue factor. Circulation 101: 2382–2387.
7. Cockell AP, Learmont JG, Smarason AK, Redman CW, Sargent IL, et al. (1997) Human placental syncytiotrophoblast microvillous membranes impair maternal vascular endothelial function. Br J Obstet Gynaecol 104: 235–240.
8. Asahara T, Isner JM (2002) Endothelial progenitor cells for vascular regeneration. J Hematother Stem Cell Res 11: 171–178.
9. Luppi P, Powers RW, Verma V, Edmunds L, Plymire D, et al. (2010) Maternal circulating CD34+VEGFR-2+ and CD133+VEGFR-2+ progenitor cells increase during normal pregnancy but are reduced in women with preeclampsia. Reprod Sci 17: 643–652.
10. Lin C, Rajakumar A, Plymire DA, Verma V, Markovic N, et al. (2009) Maternal endothelial progenitor colony-forming units with macrophage characteristics are reduced in preeclampsia. Am J Hypertens 22: 1014–1019.

11. Sieveking DP, Buckle A, Celermajer DS, Ng MK (2008) Strikingly different angiogenic properties of endothelial progenitor cell subpopulations: insights from a novel human angiogenesis assay. J Am Coll Cardiol 51: 660–668.
12. Sipos PI, Crocker IP, Hubel CA, Baker PN (2009) Endothelial Progenitor Cells: Their Potential in the Placental Vasculature and Related Complications. Placenta.
13. Sipos PI, Rens W, Schlecht H, Fan X, Wareing M, et al. (2013) Uterine vasculature remodeling in human pregnancy involves functional macrochimerism by endothelial colony forming cells of fetal origin. Stem Cells 31: 1363–1370.
14. Tabesh M, Salehi-Abargouei A, Esmaillzadeh A (2013) Maternal vitamin D status and risk of pre-eclampsia: a systematic review and meta-analysis. J Clin Endocrinol Metab 98: 3165–3173.
15. Bodnar LM, Catov JM, Simhan HN, Holick MF, Powers RW, et al. (2007) Maternal vitamin D deficiency increases the risk of preeclampsia. J Clin Endocrinol Metab 92: 3517–3522.
16. Robinson CJ, Wagner CL, Hollis BW, Baatz JE, Johnson DD (2011) Maternal vitamin D and fetal growth in early-onset severe preeclampsia. Am J Obstet Gynecol 204: 556 e551–554.
17. Haugen M, Brantsaeter AL, Trogstad L, Alexander J, Roth C, et al. (2009) Vitamin D supplementation and reduced risk of preeclampsia in nulliparous women. Epidemiology 20: 720–726.
18. Hypponen E, Hartikainen AL, Sovio U, Jarvelin MR, Pouta A (2007) Does vitamin D supplementation in infancy reduce the risk of pre-eclampsia? Eur J Clin Nutr 61: 1136–1139.
19. Grundmann M, Haidar M, Placzko S, Niendorf R, Darashchonak N, et al. (2012) Vitamin D improves the angiogenic properties of endothelial progenitor cells. Am J Physiol Cell Physiol 303: C954–962.
20. von Versen-Hoynck F, Rajakumar A, Bainbridge SA, Gallaher MJ, Roberts JM, et al. (2009) Human placental adenosine receptor expression is elevated in preeclampsia and hypoxia increases expression of the A2A receptor. Placenta 30: 434–442.
21. Diaz L, Noyola-Martinez N, Barrera D, Hernandez G, Avila E, et al. (2009) Calcitriol inhibits TNF-alpha-induced inflammatory cytokines in human trophoblasts. J Reprod Immunol 81: 17–24.
22. Halhali A, Villa AR, Madrazo E, Soria MC, Mercado E, et al. (2004) Longitudinal changes in maternal serum 1,25-dihydroxyvitamin D and insulin like growth factor I levels in pregnant women who developed preeclampsia: comparison with normotensive pregnant women. J Steroid Biochem Mol Biol 89–90: 553–556.
23. Bainbridge SA, Roberts JM, von Versen-Hoynck F, Koch J, Edmunds L, et al. (2009) Uric acid attenuates trophoblast invasion and integration into endothelial cell monolayers. Am J Physiol Cell Physiol 297: C440–450.
24. Cardus A, Panizo S, Encinas M, Dolcet X, Gallego C, et al. (2009) 1,25-dihydroxyvitamin D3 regulates VEGF production through a vitamin D response element in the VEGF promoter. Atherosclerosis 204: 85–89.
25. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, et al. (2011) The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. J Clin Endocrinol Metab 96: 53–58.
26. Pringle KG, Kind KL, Sferruzzi-Perri AN, Thompson JG, Roberts CT (2010) Beyond oxygen: complex regulation and activity of hypoxia inducible factors in pregnancy. Hum Reprod Update 16: 415–431.
27. Miller RK, Genbacev O, Turner MA, Aplin JD, Caniggia I, et al. (2005) Human placental explants in culture: approaches and assessments. Placenta 26: 439–448.
28. Heazell AE, Lacey HA, Jones CJ, Huppertz B, Baker PN, et al. (2008) Effects of oxygen on cell turnover and expression of regulators of apoptosis in human placental trophoblast. Placenta 29: 175–186.
29. Heazell AE, Moll SJ, Jones CJ, Baker PN, Crocker IP (2007) Formation of syncytial knots is increased by hyperoxia, hypoxia and reactive oxygen species. Placenta 28 Suppl A: S33–40.
30. Burton GJ (2009) Oxygen, the Janus gas; its effects on human placental development and function. J Anat 215: 27–35.
31. Chen CW, Jaffe IZ, Karumanchi SA (2014) Pre-eclampsia and cardiovascular disease. Cardiovasc Res 101: 579–586.
32. Dunn WB, Brown M, Worton SA, Crocker IP, Broadhurst D, et al. (2009) Changes in the metabolic footprint of placental explant-conditioned culture medium identifies metabolic disturbances related to hypoxia and pre-eclampsia. Placenta 30: 974–980.
33. Smarason AK, Sargent IL, Redman CW (1996) Endothelial cell proliferation is suppressed by plasma but not serum from women with preeclampsia. Am J Obstet Gynecol 174: 787–793.
34. Pospechova K, Rozehnal V, Stejskalova L, Vrzal R, Pospisilova N, et al. (2009) Expression and activity of vitamin D receptor in the human placenta and in choriocarcinoma BeWo and JEG-3 cell lines. Mol Cell Endocrinol 299: 178–187.
35. Merke J, Milde P, Lewicka S, Hugel U, Klaus G, et al. (1989) Identification and regulation of 1,25-dihydroxyvitamin D3 receptor activity and biosynthesis of 1,25-dihydroxyvitamin D3. Studies in cultured bovine aortic endothelial cells and human dermal capillaries. J Clin Invest 83: 1903–1915.
36. Evans KN, Bulmer JN, Kilby MD, Hewison M (2004) Vitamin D and placental-decidual function. J Soc Gynecol Investig 11: 263–271.
37. Evans KN, Nguyen L, Chan J, Innes BA, Bulmer JN, et al. (2006) Effects of 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 on cytokine production by human decidual cells. Biol Reprod 75: 816–822.
38. Barrera D, Noyola-Martinez N, Avila E, Halhali A, Larrea F, et al. (2011) Calcitriol inhibits interleukin-10 expression in cultured human trophoblasts under normal and inflammatory conditions. Cytokine 57: 316–321.
39. Cardus A, Parisi E, Gallego C, Aldea M, Fernandez E, et al. (2006) 1,25-Dihydroxyvitamin D3 stimulates vascular smooth muscle cell proliferation through a VEGF-mediated pathway. Kidney Int 69: 1377–1384.
40. Davis EF, Lazdam M, Lewandowski AJ, Worton SA, Kelly B, et al. (2012) Cardiovascular risk factors in children and young adults born to preeclamptic pregnancies: a systematic review. Pediatrics 129: e1552–1561.
41. Tare M, Emmett SJ, Coleman HA, Skordilis C, Eyles DW, et al. (2011) Vitamin D insufficiency is associated with impaired vascular endothelial and smooth muscle function and hypertension in young rats. J Physiol 589: 4777–4786.
42. Liu NQ, Ouyang Y, Bulut Y, Lagishetty V, Chan SY, et al. (2013) Dietary vitamin D restriction in pregnant female mice is associated with maternal hypertension and altered placental and fetal development. Endocrinology 154: 2270–2280.
43. Ingram DA, Lien IZ, Mead LE, Estes M, Prater DN, et al. (2008) In vitro hyperglycemia or a diabetic intrauterine environment reduces neonatal endothelial colony-forming cell numbers and function. Diabetes 57: 724–731.
44. Sipos PI, Bourque SL, Hubel CA, Baker PN, Sibley CP, et al. (2013) Endothelial colony-forming cells derived from pregnancies complicated by intrauterine growth restriction are fewer and have reduced vasculogenic capacity. J Clin Endocrinol Metab 98: 4953–4960.
45. Ahmad S, Ahmed A (2004) Elevated placental soluble vascular endothelial growth factor receptor-1 inhibits angiogenesis in preeclampsia. Circ Res 95: 884–891.
46. Mantell DJ, Owens PE, Bundred NJ, Mawer EB, Canfield AE (2000) 1 alpha,25-dihydroxyvitamin D(3) inhibits angiogenesis in vitro and in vivo. Circ Res 87: 214–220.
47. Chung I, Han G, Seshadri M, Gillard BM, Yu WD, et al. (2009) Role of vitamin D receptor in the antiproliferative effects of calcitriol in tumor-derived endothelial cells and tumor angiogenesis in vivo. Cancer Res 69: 967–975.
48. Egorova AD, DeRuiter MC, de Boer HC, van de Pas S, Gittenberger-de Groot AC, et al. (2012) Endothelial colony-forming cells show a mature transcriptional response to shear stress. In Vitro Cell Dev Biol Anim 48: 21–29.
49. Reynolds J, Ray D, O'Neill T, Alexander MY, Bruce I (2014) Role of vitamin D in endothelial repair mechanisms in systemic lupus erythematosus. The Lancet 383: S89.