Human placental villous stromal extracellular matrix regulates fetoplacental angiogenesis in severe fetal growth restriction

Shuhan Ji, Diane Gumina, Kathryn McPeak, Radu Moldovan, Miriam D. Post, Emily J. Su

Division of Reproductive Sciences, Department of Obstetrics and Gynecology, University of Colorado School of Medicine, Aurora, CO, U.S.A.

Department of Pharmacology, University of Colorado School of Medicine, Aurora, CO, U.S.A.

Department of Pathology, University of Colorado School of Medicine, Aurora, CO, U.S.A.

Division of Maternal-Fetal Medicine/Division of Reproductive Sciences, Department of Obstetrics and Gynecology, University of Colorado School of Medicine, Aurora, CO, U.S.A.

Abstract

Pregnancies complicated by severe, early-onset fetal growth restriction with abnormal Doppler velocimetry (FGRadv) have a sparse villous vascular tree secondary to impaired angiogenesis. As endothelial cell (EC) and stromal matrix interactions are key regulators of angiogenesis, we investigated the role of placental stromal villous matrix on fetoplacental EC angiogenesis. We have developed a novel model of generating placental fibroblast (FB) cell-derived matrices (CDMs), allowing us to interrogate placenta-specific human EC and stromal matrix interactions and their effects on fetoplacental angiogenesis. We found that as compared with control ECs plated on control matrix, FGRadv ECs plated on FGRadv matrix exhibited severe migrational defects, as measured by velocity, directionality, accumulated distance, and Euclidean distance in conjunction with less proliferation. However, control ECs, when interacting with FGRadv CDM, also demonstrated significant impairment in proliferation and migratory properties. Conversely several angiogenic attributes were rescued in FGRadv ECs subjected to control matrix, demonstrating the importance of placental villous stromal matrix and EC-stromal matrix interactions in regulation of fetoplacental angiogenesis.

Introduction

Fetal growth restriction (FGR), which occurs when a fetus is unable to meet his or her inherent growth potential, is clinically defined as an estimated fetal weight (EFW) of less
than the tenth percentile for gestational age [1]. A particular subset of these growth-restricted fetuses is categorized as early-onset FGR, defined as EFW or abdominal circumference measurement less than the third percentile for gestational age and/or the presence of absent or reversed umbilical artery end-diastolic velocities diagnosed prior to 32 weeks of gestation [2]. These fetuses exhibit a phenotype on the severe end of the spectrum, with substantially higher risks of adverse perinatal and long-term outcome, including stillbirth, neonatal death, neurodevelopmental delay, and chronic lung disease when compared with either gestational age-matched, appropriately grown fetuses or those with less severe, late-onset FGR that typically deliver at or near term gestation [3–8].

From a pathophysiologic standpoint, early-onset FGR most often arises secondarily to aberrant placental function. An underdeveloped fetoplacental vasculature, clinically evident by Doppler ultrasonography as absent or reversed umbilical artery end-diastolic velocities, results in increased fetal cardiac afterload. At the same time, reduced villous surface area impairs fetal oxygen extraction from the maternal intervillous space [9]. This combination of fetal hypoxia and elevated cardiac afterload results in myocardial impairment and ultimately, death in the absence of delivery.

Currently, other than delivery, no preventative or therapeutic measures exist for severe, early-onset FGR. Clinical management paradigms attempt to time delivery where gestational age is maximized while minimizing prolonged exposure of a fetus to an aberrant in utero environment [1]. However, while data from clinical trials continue to demonstrate that delivery can prevent stillbirth in early-onset FGR, survivors exhibit a higher incidence of neonatal death, trading in utero demise for postnatal mortality [10]. Furthermore, the incidence of neuroimpairment at school-age was similar in survivors irrespective of whether delivery occurred immediately after identification of severe umbilical artery blood flow abnormalities (absent or reversed end-diastolic velocities) or with expectant management in an attempt to gain more maturity [11,12]. Similarly, in a more recent trial comparing various antenatal surveillance tools to guide timing of delivery in early-onset FGR, there was no difference in the proportion of infants surviving without neurodevelopmental delay among the three groups [13]. Even interventional trials, where women with severe, early-onset FGR were administered sildenafil, a phosphodiesterase type 5 inhibitor that has the potential to improve uteroplacental perfusion, showed no differences in prolongation of pregnancy, fetal growth velocity, or overall pregnancy outcome as compared with placebo [14,15]. Taken together, these clinical data underscore the need to better understand mechanisms of severe FGR if efficacious prevention or therapy is to be developed.

While there are other less severe criteria that can result in diagnosis of early-onset FGR, our laboratory is most interested in these FGR cases that are further complicated by abnormal (absent or reversed) umbilical artery Doppler velocimetry (FGRadv), as these are the pregnancies at highest risk for adverse perinatal and long-term outcome. As compared with normal pregnancies where ongoing fetoplacental angiogenesis allows for a proper decrement in fetoplacental vascular resistance as gestation progresses, placentas from FGRadv pregnancies typically exhibit at least a one-third reduction in villous vascular volume as a result of impaired angiogenesis in the latter half of pregnancy [9,16–20].
We have previously found that human, fetoplacental endothelial cells (ECs) primarily isolated from FGRadv placentas demonstrate compromised cell migration and angiogenesis as compared with ECs from placentas of gestationally age-matched, appropriately grown fetuses [19,21–23]. While these data suggest that there are inherent defects within FGRadv ECs that impair their angiogenic potential, this human cell model overlooks other important factors that regulate angiogenesis. Specifically, one critical element is the microenvironment of the fetoplacental vasculature. While the microenvironment has been well-established as a critical regulator of angiogenesis in a variety of tissues, the placental villous stroma and its effects on the fetoplacental vasculature is understudied [24,25]. Existing data demonstrate that FGR placentas exhibit significantly diminished villous stromal volume within intermediate and terminal villi, the latter being the presumed site of ongoing angiogenesis in pregnancy [26,27]. These findings suggest that at least some degree of alteration within the placental microenvironment is present in FGR.

On a cellular level, villous stroma is composed primarily of pericytes, Hofbauer cells, and fibroblasts (FBs), with FBs acting as the main cell type driving extracellular matrix (ECM) production [16,28]. We have developed a method of isolating villous stromal FBs with which we generate placental FB-specific, cell-derived matrices (CDMs). Primarily isolated fetoplacental ECs from either FGRadv or control human placentas are plated on either FGRadv or control CDM, allowing us to interrogate how EC and villous stromal matrix interactions impact fetoplacental angiogenesis. We present evidence that while intrinsic qualities in FGRadv ECs contribute to impaired angiogenesis, EC–ECM signaling significantly influences fetoplacental EC proliferation and migration.

Methods

Subjects

After approval by the Colorado Multiple Institutional Review Board (COMIRB), subjects from two separate groups were identified: (1) Singleton pregnancies complicated by severe, early-onset FGR with an EFW of less than the 10th percentile for gestational age AND absent or reversed end-diastolic umbilical artery velocities (FGRadv), and (2) Singleton pregnancies that resulted in full-term, appropriately grown fetuses delivered via cesarean section in the absence of labor (control).

Gestational age was confirmed using criteria defined by the American College of Obstetricians and Gynecologists (ACOG), the American Institute of Ultrasound in Medicine (AIUM), and the Society for Maternal-Fetal Medicine (SMFM) [29]. Additionally, actual birth weight was used to confirm growth restriction in the FGRadv cohort and appropriate growth in the control group. Exclusion criteria for both groups included fetal anomaly, fetal aneuploidy, fetal or maternal infection, diabetes, history of thrombosis, or antiphospholipid antibody syndrome. Additionally, any concomitant medical comorbidities that could result in uteroplacental insufficiency were also considered exclusion criteria for the control cohort. Eligible subjects were then approached, and informed consent was obtained.
**Primary fetoplacental EC isolation and culture**

Human fetoplacental villous ECs were isolated/cultured from placentas of both groups as previously described with minor modifications [30,31]. After isolation, ECs were cultured in Endothelial Growth Media-Microvascular (EGM-MV) medium supplemented with 5% fetal bovine serum, bovine brain extract with heparin, epidermal growth factor, hydrocortisone, and gentamicin/amphotericin B (Lonza) at 37°C in a humidified incubator with 5% CO₂. On the basis of previous data, only primary cells up to fifth passage were used in experiments to avoid changes in phenotype [30].

**Primary placental villous stromal FB isolation and culture**

Within 60 min after delivery, 30 g of chorionic villi were dissected after removal of the basal and chorionic plates. Samples were washed twice with 100 ml of Hank’s balanced salt solution (HBSS; Sigma) in the presence of gentamicin (Gibco) and once with 50 ml RPMI-1640 (Gibco) with 2% FBS and 1% antibiotic/antimycotic solution (C-RPMI-1640). Samples were minced using a disposable scalpel in a Petri dish. Vessels and calcifications that were visible to the naked eye were discarded, and the remaining pieces of chorionic villi were no more than 1 mm³ in size. Minced samples were then digested in 150 ml C-RPMI-1640 with 0.28% Collagenase D (Roche), 0.25% Dispase II (Sigma), 0.002% DNase I (Sigma) for 2 h in a shaking incubator at 37°C. A discontinuous Percoll (Sigma) gradient of ten layers (10, 20, 30, 35, 40, 45, 50, 55, 60, and 70%) was prepared during the digestion period in two 30-ml glass centrifuge tubes. After digestion, the cell suspension was filtered through four layers of sterile gauze (Fisher Scientific) followed by 70-μm cell strainers (Falcon) and centrifuged at 300×g for 10 min at 22°C. The cell pellet was then resuspended and washed with C-RPMI-1640 and centrifuged again at 300×g for 10 min at 22°C. The cell pellet was then resuspended in 6 ml C-RPMI-1640, with 3 ml of the suspension carefully layered on each Percoll gradient. This was then centrifuged at 3060 rpm for 20 min at 22°C without brakes. Percoll layers 3–5 (located between 30 and 40%) containing cells were then transferred to a fresh tube, washed with 30 ml C-RPMI-1640, and centrifuged again at 500×g for 10 min at 22°C. This cell pellet was then collected and resuspended in 20 ml of Endothelial Growth Media-2 (EGM-2; Lonza) and centrifuged at 1500 rpm for 5 min at 22°C.

Cells were then counted and seeded at a density of 1 × 10⁷ cells per dish on to 10-cm dishes pre-coated with Attachment Factor solution (Cell Applications). Cells were cultured for an additional 10 days, with media changes occurring every other day. Cells were then trypsinized, which effectively eliminates trophoblast cells. Cells within this suspension then underwent selection with CD31 Dynabeads (Invitrogen), thereby removing microvascular ECs, and then plated once again. The remaining FB cells were then cultured in full Fibroblast Growth Media-2 (FGM-2; Lonza) supplemented with 2% fetal bovine serum, insulin, fibroblast growth factor, and gentamicin/amphotericin B. Cellular morphology of FBs, which are distinct from trophoblast and ECs, were confirmed via bright-field microscopy, and cell purity was assessed via immunofluorescence and flow cytometry.
**Cellular immunofluorescence**

Fetoplacental ECs and villous stromal FBs were separately plated on glass coverslips lining 24-well plates. At 90–100% confluence, cells were fixed in 4% formaldehyde (Tousimis). After thorough washing with PBS, cells were blocked with 2% BSA (Sigma) for 1 h at room temperature followed by incubation in primary antibody at 4°C with slow shaking overnight. Cells were then washed and then incubated with secondary antibody conjugated with AlexaFluor 488 (Invitrogen) for 1 h at room temperature. The glass coverslips were mounted to glass slides using Lab Vision™ PermaFluor™ Aqueous Mounting Medium (Thermo Scientific). Cells were then visualized using the Olympus FV1000 confocal fluorescence microscope. Primary antibodies: anti-CD31 (Sigma P8590; 1:200), anti-vimentin (Sigma V6630; 1:100), vWF (Sigma F3250; 1:400), anti-α-smooth muscle antibody (Sigma A2547; 1:400), anti-chondroitin proteoglycan sulfate 4 (Abcam ab129051; 1:100). Negative controls with anti-mouse and anti-rabbit secondary antibodies alone were performed on both cell types with no fluorescence visualized.

**Flow cytometry**

Fetoplacental ECs and villous stromal FBs were analyzed by flow cytometry. Briefly, cells were detached with 0.05% trypsin, centrifuged at 1500 rpm for 3 min, and resuspended in PBS. Cells were then counted, and 1 × 10⁶ cells were then blocked with 0.1% BSA for 30 min at 4°C. Cells were then incubated with fluorophore-conjugated antibodies or isotype IgG controls for 1 h at 4°C. DAPI staining was utilized to determine cell viability. All samples were subjected to FACSCanto II/Celesta flow cytometer (BD) and analyzed with FlowJo v10 software. The analysis of each marker was compared with isotype-stained cells, with viability cells gated with DAPI. Antibodies: FITC-conjugated anti-CD31 (BD Biosciences 555445, 1:200), AlexaFluor 488-conjugated anti-CD45 (BioLegend 304019, 1:200), AlexaFluor 488-conjugated anti-CD105 (BioLegend 323209; 1:200), APC-conjugated anti-CD90 (BioLegend 328113; 1:200), and APC-conjugated anti-CD146 (BioLegend 361015, 1:200). Isotype controls: FITC-IgG (BioLegend 400108; 1:200), Alexa-Fluor-IgG (BioLegend 400132; 1:200), APC-IgG (BioLegend 400120; 1:200).

**Generation of villous stromal FB-derived conditioned media**

Villous stromal FBs isolated from both control and FGRadv placentas were grown in 6-cm dishes with full FGM-2 medium until 50–70% confluence. After 2 h of starvation, basal FGM-2 without FBS or supplements was added to the cells. After FBs were cultured for 24 h, the medium was then collected and centrifuged at 1500 rpm for 5 min. After discarding the cellular debris, the supernatant was then collected and stored at −80°C until ready for use. ECs were ultimately treated in media made up of 50% CM and 50% full EGM-MV after determination that this was the highest conditioned media (CM):media ratio that did not result in large amounts of cell death.

**Wound scratch assays**

Fetoplacental EC migration in response to CM was measured in four groups: (1) Control ECs cultured in control CM, (2) Control ECs cultured in FGRadv CM, (3) FGRadv ECs cultured in control CM, and (4) FGRadv ECs cultured in FGRadv CM. Briefly, equal
numbers of ECs were seeded in 12-well plates and cultured in full EGM-MV media until 100% confluent. Thereafter, cells were starved for 2 h in basal media without FBS or supplements and then scratched with P200 pipette tip. Basal medium was removed and ECs were then treated with the CM treatment (50% CM/50% full EGM-MV) as noted in the four groups above. ECs were immediately then imaged with Incucyte® S3 Live-Cell Analysis System (Essen BioScience) for real-time imaging. Time-lapsed, lime-cell images were taken at 0, 3, 6, 9, 12, 15, 17, 21, and 24 h for each sample. Images were then analyzed with ImageJ software (https://imagej.nih.gov/ij/). The degree of wound healing, which correlates to EC migration, was assessed by measuring the remaining cell-free (non-closure) area and then converted into percent closure.

Generation of placental villous stromal FB CDMs

CDMs were generated from control and FGRadv stromal FBs as described in Nature Protocols with minor modifications [32]. Briefly, 24-well cell culture plates with or without sterile glass coverslips underwent coating with 0.2% (w/v) gelatin (BD) for 1 h at 37°C. Wells were then washed twice with PBS and then cross-linked with 1% (v/v) glutaraldehyde (Sigma) at room temperature for 30 min. Following a PBS wash, wells were then treated with glycine (1 M, VWR) at room temperature for 20 min to quench any remaining glutaraldehyde. Thereafter, wells were washed and then incubated in full FGM-2 for 1 h at 37°C.

After preparation of gelatin-coated plates or coverslips, FBs isolated from control or FGRadv placentas were seeded in full FGM-2 at a density of 2 x 10^5 cells per well with the goal of confluence within 12–16 h. After reaching 100% confluence, the medium was replaced with full FGM-2 supplemented with 50 μg/ml freshly prepared ascorbic acid. This medium was replaced daily with freshly prepared ascorbic acid daily for 5–7 days. Once a uniform matrix was visualized via bright-field microscopy, FBs were washed with PBS. FBs were then decellularized from the CDM by a pre-warmed extraction buffer supplemented with 2% (v/v) ammonium hydroxide (Sigma) and 0.5% (v/v) Triton-X (Fisher Scientific) in 1× PBS. FBs were observed under bright-field microscopy during this process in order to optimize the length of cell extraction, which typically occurred within 5 min of treatment for both control and FGRadv FBs. After decellularization, the extraction buffer was immediately removed via gentle pipetting to avoid disrupting the CDM scaffold. CDMs were then washed twice with PBS, and residual cellular DNA was then removed by additional incubation with 10 μg/ml DNase I (Roche) at 37°C for 30 min. The DNase I solution was then removed by gentle pipetting, and CDMs were washed twice with PBS once again. Bright-field microscopy was used to confirm matrix integrity and uniformity of matrix deposition, and CDMs were utilized for subsequent experiments thereafter.

CDM immunofluorescence

After confirmation by bright-field microscopy of a uniform and intact matrix, CDMs generated on glass coverslips were fixed for immunofluorescence with 10% formaldehyde (Tousimis) at room temperature for 20 min. CDMs were then washed three times with PBS, blocked with 30% (v/v) horse serum (Gibco) at room temperature for 1 h. Thereafter, CDMs were incubated in primary antibody at room temperature for 2 h and washed three times with
PBS, followed by incubation with secondary antibody conjugated with Alexa Fluor 488 (Invitrogen) for 1 h at room temperature. Glass coverslips were then mounted on to glass slides once again using Lab Vision™ PermaFluor™ Aqueous Mounting Medium, and the Olympus FV1000 confocal fluorescent microscope was utilized once again to visualize CDMs. Primary antibodies: anti-collagen I (Novus NB600-408; 1:200); anti-fibronectin (Sigma F3648; 1:400). Negative controls with secondary antibody alone were performed for CDM with no fluorescence visualized.

**Endothelial proliferation on individual ECM substrates**

Twenty-four-well cell culture plates were coated with collagen I (PureCol® EZ gel, Advanced BioMatrix, 100 μg/ml), fibronectin (Gibco, 100 μg/ml), or as negative controls, BSA (100 μg/ml) or nude plastic. Equal numbers of control ECs ($1 \times 10^4$ cells/well) were then plated on these individual substrates or control CDM (see next section for CDM methodology). Time-lapsed, live-cell imaging was then performed with IncuCyte, with images captured at 0, 1, 2, 3, 4, and 5 days. Images were then analyzed with ImageJ software, with the proliferation rate of ECs assessed by measuring the actual cell number at each time point.

**Endothelial proliferation on CDM**

In addition to the above experiment where control ECs were grown on control CDM and compared with EC proliferation on key, individual ECM substrates, control and FGRadv EC proliferation on control and FGRadv CDM was also assessed. Specifically, four groups were compared: (1) Control ECs plated on control CDM, (2) Control ECs plated on FGRadv CDM, (3) FGRadv ECs plated on control CDM, and (4) FGRadv ECs plated on FGRadv CDM. After CDM generation by control and FGRadv FBs in 24-well tissue culture plates, control and FGRadv ECs were plated in equal numbers ($1 \times 10^4$ cells/well) on their respective CDM. Time-lapsed, live-cell imaging was performed once again with IncuCyte, with images captured at 0, 1, 2, 3, 4, and 5 days. EC proliferation rate was then calculated by cell count at each time point as captured via ImageJ.

**EC directional migration on CDM**

With the same four groups as noted above, equal numbers of ECs ($5 \times 10^3$ cells/well) were seeded on the respective CDMs and immediately placed in the IncuCyte live-cell analysis system. Time-lapsed images were captured every 20 min for 0–24 h each sample. For each experimental group, centers of mass, which represent the average of all single cell endpoints accounting for the x- and y-trajectory of cell movement, were generated after analyzing at least 180 cell tracks per group in order to qualitatively evaluate overall EC migration. Thereafter, single-cell migration was analyzed with the ImageJ tracking tool, with directionality, velocity, accumulated distance, and Euclidean distance assessed via the Ibidi chemotaxis tool (http://ibidi.com/manual-images-analysis/171-chemotaxis-and-migration-tool.html). Fifteen cells per well, with each experiment performed in triplicate in four separate subjects, led to 180 cells being tracked per experimental group. Cells that were dividing, colliding, or undergoing cell death were excluded in the analyses.
Bright-field microscopy assessment of CDM stability

After decellularization, CDM was maintained in sterile PBS and pen/strep at 4°C, and images of CDM deposition were captured with the 10x objective on days 0, 7, and 14.

Protein isolation and Western blotting

Protein extraction from human fetoplacental ECs was performed using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher, U.S.A.) with addition of phosphatase and protease inhibitors (Cell Signaling Technology). Protein concentrations were determined by colorimetric bicinchoninic acid protein assay (Thermo Fisher), and equal concentrations of total protein were loaded in each well. Samples were subjected to a commercially available, capillary-based immunoassay (ProteinSimple; Bio-Techne, San Jose, CA) with the following antibodies: Caspase-3 (Cell Signaling 9662s; 1:500), cleaved caspase-3 (Cell Signaling 9661s, 1:10), collagen I (Novus NBP1-30054; 1:25), and fibronectin (Thermo MA5-11981; 1:2000). Relative protein expression for each protein of interest was normalized to total protein as per ProteinSimple methodology.

Statistical analysis

Clinical characteristics between control and FGRadv subjects were compared using paired t tests after confirmation of normal distribution of datasets by the Shapiro–Wilk test for normality. All experiments were performed on at least four representative subject samples, with each experiment repeated in triplicate. Representative images are shown from a single subject, with analysis of numerical and graphical representation accounting for all subjects. Numerical data are reported as means of the replicates performed within all the subjects, with error bars representing the standard error of the mean (SEM). Normal distribution of experimental datasets was also confirmed with the Shapiro–Wilk test, and statistical analysis for comparison of groups (more than two) was performed with one-way ANOVA with Tukey’s post-hoc comparison if the overall one-way ANOVA was statistically significant. For comparison of two groups, a Student’s t test was utilized. A value of $P<0.05$ was considered significant.

Results

Clinical characteristics of control and FGRadv subjects

Clinical characteristics for subjects are presented in Table 1. With the exception of gestational age, all continuous variables were normally distributed. As anticipated, there were significant differences in gestational age at delivery, neonatal birthweight, and placental weight. Birthweight percentiles ranged from 33 to 58 percent for control subjects. All FGRadv subjects had birthweights between the 1st and 7th percentile and exhibited either persistent absent or reversed end-diastolic umbilical artery Doppler velocimetry.

Primarily isolated fetoplacental ECs and villous stromal FBs express distinguishing cell-specific markers

Flow cytometry demonstrated that primarily isolated fetoplacental ECs express EC-specific cell surface markers, including platelet EC adhesion molecule (PECAM, CD31), endoglin...
(CD105), and melanoma cell adhesion molecule (MCAM, CD146). These cells were negative for Thy-1 cell surface antigen (CD90), and FB cell surface protein [33,34], and protein tyrosine phosphatase receptor type C (PTPRC, CD45), a monocyte cell surface marker [35]. In contrast, primarily isolated villous stromal FBs were positive for CD90, but negative for CD31, CD105, CD146, and CD 45 (Supplementary Figure S1A). Immunofluorescence for intracellular proteins also confirmed purity of isolated cells. While both ECs and FBs stain positive for vimentin, von Willebrand factor (vWF) was exclusively expressed in fetoplacental ECs, while α-smooth muscle actin (α-SMA) was solely expressed in villous stromal FBs (Supplementary Figure S1B).

**Fetoplacental EC migration is not differentially regulated by secreted factors from control or FGRadv FBs**

We first wanted to investigate whether secreted factors from placental-specific, villous stromal FBs differentially regulate fetoplacental angiogenesis in both control and FGRadv ECs. To do this, we generated CM from either control FBs or FGRadv FBs. Control ECs were then subjected to either control FB- or FGRadv FB-derived CM, and similarly FGRadv ECs were also exposed to both forms of CM. Wound scratch assays were then performed under live-cell imaging. As anticipated, FGRadv ECs, in general, continued to demonstrate significantly impaired migration as compared with control ECs, regardless of which type of CM the cells received (Figure 1; black versus red lines). However, although there was a statistically significant difference among all four groups via ANOVA at each time point, post-hoc comparisons at the 24-h endpoint showed that percent closure was comparable when comparing control ECs cultured in control CM to control ECs cultured in FGRadv CM (Figure 1B, black solid vs black dashed line; Figure 1C, black font). At all timepoints including the 24-h endpoint, there were statistically non-significant trends toward delayed wound closure in FGRadv ECs cultured in FGRadv CM as compared with those grown in control CM (Figure 1B, red solid vs red dashed line; Figure 1C, red font). Together, these data suggest that while there is likely some biologic effect of CM on EC migratory capacity, placental stromal FB-derived soluble factors alone do not significantly regulate EC migration.

**CDMs are able to be generated from villous stromal FBs**

While soluble growth factors are present within stromal tissue, their availability are regulated by several factors. One of the most important of these is ECM, the primary, non-cellular constituent of stroma. ECM is able to directly sequester growth factors, impacting how they are recognized by cells. Furthermore, the specific composition of proteins and glycosaminoglycans that make up the intermeshing network of ECM also regulate cellular function, indicating the complexity of stromal matrix beyond that of just soluble growth factors.

In order to interrogate the role of EC and villous stromal interactions on fetoplacental angiogenesis, we first generated CDMs with our primarily isolated placental villous stromal FBs. Confluent FBs were cultured and treated with ascorbic acid to promote matrix cross-linking for 5–8 days. After uniform deposition of matrix, FBs, which were stained with chondroitin sulfate proteoglycan 4 (CSPG4), a transmembrane protein expressed by FBs that
is not secreted in the absence of stimulation [36], remained present overlying the matrix (Figure 2A). After the CDM was decellularized, FBs were no longer evident, although there was some nuclear debris as evidenced by DAPI staining (Figure 2A).

To further characterize villous stromal FB CDM, fibronectin was chosen as a candidate ECM that has previously been shown to be highly expressed in placental stroma [37]. While some fibronectin was evident via immunofluorescence in pre-decellularization CDM, the staining distribution was most suggestive of intracellular expression within the FBs, confirming the persistence of a confluent FB layer that is capable of secreting fibronectin (Figure 2B). In contrast, after decellularization, a uniform matrix was evident as shown by fibronectin staining (Figure 2B). Furthermore, bright-field imaging demonstrated the stability of villous stromal FB CDM for up to 14 days post-decellularization (Figure 2C).

We then sought to determine whether fetoplacental ECs would adhere to and proliferate on villous stromal FB CDM, and ECs were plated on pre- and post-decellularization CDM. Live-cell imaging of ECs stained with a lipophilic membrane stain (diI) was used to assess ECs for 72 h. Similar to ECs plated on tissue culture-treated plates, ECs adhered and continued proliferating on post-decellularization CDM for at least 72 h. In contrast, EC adherence and proliferation were significantly impaired on pre-decellularization CDM after ~24 h in culture, demonstrating that decellularization of CDM is required for proper EC adherence (Supplementary Figure S2).

**EC proliferation is most robust when plated on CDMs**

We then wanted to determine whether CDMs had relevant physiologic effects on adherence and proliferation as compared with standard tissue culture plates. To do this, we plated control ECs on routine tissue culture dishes and bovine serum albumin as an additional negative control. Based upon existing data suggesting strong expression of fibronectin and collagen I within the ECM of the human placenta [37], we also grew cells on these two commercially available ECM proteins and on control CDM. After time-lapsed, live-cell imaging for 5 days, cellular proliferation was significantly enhanced when ECs were grown on CDM as compared with the other conditions for the first 3 days (Figure 3). This robust proliferation persisted through days 4 and 5, although there was also enhanced proliferation on those days when cells were grown on collagen I.

**Control and FGRadv EC proliferation is mediated by CDM**

After confirming the ability of ECs to adhere to and proliferate on post-decellularization CDM, which will be referred to as just CDM from here on out, our next question was whether EC–matrix interactions affected cellular proliferation. In order to better delineate the individual contributions of ECs and CDM, four groups, as depicted in Figure 4, were compared: (1) Control ECs plated on control CDM, (2) Control ECs plated on FGRadv CDM, (3) FGRadv ECs plated on control CDM, and (4) FGRadv ECs plated on FGRadv CDM. Live-cell imaging was performed for 5 days, with time-lapsed imaging demonstrating significant differences between groups on all 5 days (Figure 5A and Supplementary Figure S3). As anticipated, FGRadv ECs plated on FGRadv CDM exhibited significantly impaired proliferation at days 4 and 5 (Figure 5B). There was a non-significant trend toward
improvement in FGRadv EC proliferation when subjected to control CDM as compared with FGRadv ECs plated on FGRadv CDM. Notably, however, there was a statistically significant impairment in control EC proliferation when exposed to FGRadv CDM on all 5 days as compared with control ECs plated on control CDM (Figure 5B), which was distinctly different than control ECs subjected to FGRadv FB CM (Figure 1B). Furthermore, among all four groups, proliferation was most deficient in control ECs plated on FGRadv CDM, establishing that villous stromal matrix plays a critical role in EC proliferation (Figure 5B). As shown in Supplementary Figure S4, these findings were not a result of differences in apoptosis among groups.

**Placental CDM significantly regulates fetoplacental EC migration**

To better understand how stromal matrix regulates angiogenesis beyond just proliferation, we investigated the effect of EC–CDM interactions on EC directional migration in the same four groups as noted above. EC directional migration was assessed with live-cell imaging for 24 h at 20-min time intervals. Figure 6A shows representative spider plots for at least 180 cells for each of the four conditions. The centers of mass for these spider plots, which represent the average of all single cell endpoints accounting for the x- and y-trajectory of cell movement, demonstrate that CDM has a much stronger influence on EC directional migration, with impaired migration in both control and FGRadv ECs plated on FGRadv CDM (Figure 6A). Taken another way, FGRadv CDM compromises control EC migration, whereas FGRadv EC migration appears partially rescued in the presence of control CDM. These results were further confirmed by absolute quantification of accumulated distance (total distance that a cell travels) and Euclidean distance (straight-line distance between the start and endpoint of a cell) (Figure 6B), both of which show attenuated migration of control ECs plated on FGRadv CDM and enhanced migration of FGRadv ECs grown on control CDM. Similarly, directionality, which is a measure of whether a cell travels in a straight and organized line or moves in a disordered and scrambled fashion, was significantly impaired in both types of ECs plated on FGRadv CDM and improved in FGRadv ECs plated on control CDM. Analysis of cellular velocity also showed comparable findings. In contrast, there were no significant differences in all four measures of migration when control ECs plated on control CDM were compared with FGRadv ECs plated on control CDM. Together, these data suggest that not only does CDM regulate several functional aspects of EC migration but that control CDM is able to rescue the inherent defects seen in FGRadv ECs.

**FGRadv CDM exhibit differential expression of key ECM proteins**

As our data suggested that CDM itself has a substantially greater impact on EC proliferation and migration as compared with secreted factors from FB-generated CM, we sought to determine whether there was differential expression of key ECM proteins between control and FGRadv CDM. Based upon existing data suggesting strong expression of fibronectin and collagen I within the ECM of the human placenta [37], we performed immunofluorescence of control and FGRadv CDM for these two important ECM proteins. Imaging of fluorescent intensity demonstrated a significant reduction in both collagen I and fibronectin in FGRadv CDM, with striking differences in structural organization (Figure 7). To determine whether matrix was uniformly deposited by both control and FGRadv FBs,
however, we stained both control and FGRadv CDM with thrombospondin I, a matrix protein that has been shown to be expressed in similar levels within placental stromal cells of normal and growth-restricted pregnancies [38]. Both immunofluorescence and gray-scale imaging of thrombospondin demonstrate a similar appearance of the matrix and confirm that the differences seen in collagen I and fibronectin are not the result of a scant matrix in FGRadv CDM (Figure 7). As shown in Supplementary Figure S5, these findings are not the result of inherent EC expression of matrix proteins.

**Discussion**

No preventative or therapeutic strategies for severe, early-onset FGR have proven effective, and thus, understanding the mechanisms that underlie its pathophysiology is essential if clinical outcomes are to be improved. In normal pregnancies, fetoplacental angiogenesis is ongoing throughout gestation. Angiogenesis further accelerates, starting at approximately 25 weeks of pregnancy, resulting in exponential growth of the fetoplacental vasculature until term gestation [39,40]. This degree of vascular development is necessary for the progressive decrease in fetoplacental vascular resistance that occurs in normal pregnancies as gestation continues [17,41]. In contrast, placentas from FGRadv pregnancies exhibit impaired development of the villous vasculature, with fewer small arterial vessels and abnormally slender, elongated, and poorly branched villous vessels [42–44]. More specifically, there is a reduction in density of small diameter vessels that reside primarily within terminal villi of FGRadv placentas [42–44], suggesting that the angiogenic defects accompanying FGRadv are the result of impaired angiogenesis within the distal segments of placental villi.

Within terminal villi, fetoplacental capillaries or sinusoids are surrounded by placental villous stroma, which is composed primarily of FBs, myofibroblasts, macrophages (Hofbauer cells), and ECM, the majority of which is synthesized and deposited by villous FBs [16,37,45–47]. To our knowledge, the placenta villous stroma has been much less extensively studied than other components within the human placenta. Existing data demonstrate that placental villous stromal cells express approximately 100 ECM genes, as determined from data mining of an mRNA database [37]. From a pathophysiologic standpoint, pregnancies complicated by FGR exhibit significantly less stromal volume in comparison to healthy control and even preeclamptic placentas [26,27]. However, the mechanistic role of villous stroma on placental development, including angiogenesis, is not known. It is clear from other literature, though, especially that of tumor biology, that tissue microenvironment, including stroma, is critical in regulation of tumor angiogenesis [24,48].

Tissue microenvironment is made up of several dynamic cellular and non-cellular components, including FBs, immune cells, and ECM, all of which are essential for both normal physiologic and aberrant pathophysiologic processes. ECM is a key component of the tissue microenvironment. Beyond its classic role of providing structural support for cells and tissues, ECM also serves as an active participant in biochemical and biophysical signaling to neighboring cells. Through its function as a storage compartment for both stimulators and inhibitors of angiogenesis along with its role in cellular adhesion through cell surface receptors such as integrins, various signaling cascades are activated. This cell–matrix bi-directional interaction ultimately influences many facets of cell behavior including...
proliferation and migration, both of which are cellular functions that are critical in regulation of angiogenesis [24,48–50].

We were surprised to find that CM generated from control or FGRadv FBs had no consistent, statistically significant effect on either control or FGRadv EC migration. Technically, at 12 h, there was statistically impaired wound closure of control ECs cultured in FGRadv CM as compared with those grown in control CM. This finding resolved by 18 h. Within FGRadv ECs exposed to FGRadv CM, there were statistically non-significant trends towards decreased wound closure at all time points when cultured in FGRadv CM as compared with control CM. Ultimately, it is certainly possible that with an increased sample size, we would have detected consistent, statistically significant differences with diminished migration in ECs when exposed to FGRadv CM. These findings, however, were compelling enough to demonstrate that further investigation of EC–stromal interactions and stromal effects on fetoplacental angiogenesis were warranted. As availability of soluble factors would be further regulated by ECM, we opted to move forward and evaluate the global role of stroma on placental ECs by generating placental FB-specific CDM.

Within our model of primary EC and FB isolation from human placentas, villous FB-derived CDM appreciably influenced EC proliferation and migration without any effect on apoptosis. Specific to proliferation, there was a trend toward enhanced FGRadv EC proliferation when subjected to control CDM as compared with FGRadv CDM. Most strikingly, however, control EC proliferation was significantly diminished when plated on FGRadv CDM as compared with cells grown on control CDM. Furthermore, the degree of impaired proliferation was even worse than that of FGRadv ECs regardless of the type of CDM to which they were exposed, illustrating the ability of placental stromal matrix to communicate to fetoplacental ECs and dictate their ability to grow and divide.

We also found that placental FB-derived CDM significantly affects EC migration. FGRadv ECs plated on FGRadv CDM exhibited disordered migration, as anticipated, in comparison with control ECs grown on control CDM. However, not only was there a statistically significant increase in EC migration when FGRadv ECs were exposed to control CDM rather than FGRadv CDM, but all migrational parameters, including velocity, accumulated distance, directionality, and Euclidean distance were essentially completely rescued in FGRadv ECs plated on control CDM. Along those same lines, control ECs grown on FGRadv CDM exhibited significant impairment in all four migrational metrics to a level similar to that of FGRadv ECs plated on FGRadv CDM. Together, these data further confirm that not only do ECs from FGRadv placentas exhibit inherent angiogenic defects but that villous stromal matrix also plays a substantial role in both normal and pathological fetoplacental angiogenesis.

Given the significant contribution of stromal matrix on EC proliferative and migratory properties, we questioned whether there were specific differences in compositional make-up between control and FGRadv CDM. We chose to first investigate two key candidate ECM proteins, fibronectin and collagen I, and we found that there was less expression of both in FGRadv CDM. However, although bright-field microscopy showed uniform deposition of both control and FGRadv matrices, the topographic distribution of both fibronectin and
collagen I within FGRadv CDM suggested the potential for structural differences in matrix deposition between these two groups. To test this, we chose to query thrombospondin-1. While thrombospondin-1 has been shown to be expressed at similar levels within placental stromal cells of both normal and growth-restricted pregnancies [38], it has also been shown to carry significant anti-angiogenic properties in non-placental tissue [51–53]. Thus, we chose this particular matrix protein, hypothesizing that this ECM protein would be seen in either similar or even greater amounts in FGRadv CDM. We found no difference in thrombospondin-1 expression between control and FGRadv CDM, with immunofluorescence and gray-scale imaging demonstrating similar topographic deposition of thrombospondin-1. These findings further support that global CDM deposition is similar between control and FGRadv subjects while also suggesting that lower expression levels and aberrant topographical deposition of fibronectin and collagen I in FGRadv CDM may be key drivers of impaired EC proliferation and migration.

While our data demonstrate overt differences in CDM composition between control and FGRadv matrices, there are certainly other potential mechanistic explanations that could further explain our findings. First, although villous stromal CDM appears to play a more dominant role in EC proliferation and migration, we have previously shown that inherent defects exist within FGRadv ECs that affect their angiogenic potential. We found that low expression of the aryl hydrocarbon receptor nuclear translocator (ARNT) transcription factor in FGRadv ECs resulted in down-regulation of vascular endothelial growth factor A (VEGFA) expression and EC migration and tube formation [19,22]. However, administration of VEGFA or rescue of ARNT expression in FGRadv ECs only partially improved their migratory properties [23], suggesting that there are other mechanisms that underlie the intrinsic deficiencies in FGRadv ECs. More specific to EC–ECM bi-directional interactions, others have shown that ARNT expression also regulates expression of key EC integrins such as αv, β1, and β3 [54,55]. Additionally, a decrease in FGRadv EC VEGFA expression as a result of lower ARNT could further impair integrin activation, further suggesting that differences in integrin expression and activation between control and FGRadv ECs may also mechanistically contribute to our findings. Second, ECs themselves also secrete various matrix metalloproteinases, which degrade various protein substrates within ECM, rendering matrix ligands more or less accessible to cell surface receptors such as integrins [56]. Third, ECs themselves also contribute to production of ECM, which serves as yet an additional mechanism that could regulate EC–ECM bi-directional signaling [57–59].

Strengths of the present study include the use of primarily isolated ECs and FBs isolated from human specimens that exhibit a very specific and key phenotype of FGR to generate a clinically relevant model of fetoplacental EC and villous stromal matrix interactions. A limitation of the present study, however, is that the placental FB-derived model of CDM may not fully recapitulate the exact environment of villous stroma. That being said, EC–ECM interactions, which are well-established to regulate angiogenesis in other tissues, have not yet been studied within the human placenta, and our findings contribute fundamental yet critical knowledge surrounding fetoplacental angiogenesis. Another limitation of the present study is that control ECs were isolated from placentas of full-term, uncomplicated pregnancies, as angiogenesis is not static throughout gestation [40]. However, utilizing placentas from appropriately grown, gestational age-matched fetuses that delivered preterm
for some other etiology other than FGR also leads to other potential confounders. For example, there is increasing data to suggest that placental pathology related to placental insufficiency, a key factor in FGRadv pregnancies, may also result in many cases of spontaneous preterm birth [60,61], and this could have led to dilution of findings if preterm control specimens were utilized. Furthermore, even taking into account the limitations of control specimens from full-term pregnancies, our findings strongly suggest that CDM derived from these specimens rescues FGRadv EC migrational defects, making this a potential avenue for development of future interventions in FGRadv pregnancies.

In summary, our findings demonstrate the importance of bi-directional interactions between fetoplacental EC and villous stromal matrix in regulation of placental angiogenesis. Specifically, we show the key role that ECM plays in regulation of EC angiogenic properties, with significant EC impairment of control ECs exposed to FGRadv CDM and rescuing of EC migration in FGRadv ECs plated on control CDM. Continued research in this area will be essential to better understand mechanisms underlying both normal and pathologic angiogenesis. In turn, this knowledge will be critical if preventative and/or therapeutic strategies for FGRadv are to be developed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

The data that support the findings of the present study are available from the corresponding author upon reasonable request.

Abbreviations

- **ARNT**: aryl hydrocarbon receptor nuclear translocator
- **CDM**: cell-derived matrix
- **CM**: conditioned media
- **EC**: endothelial cell
- **ECM**: extracellular matrix
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Clinical perspectives

- Placentas from pregnancies complicated by severe, early-onset FGR (FGRadv) have a sparse villous vascular tree secondary to impaired angiogenesis, resulting in detrimental pathophysiologic consequences to the fetus and high risk for adverse perinatal outcome. Although stromal microenvironment has been demonstrated to be a critical regulator of angiogenesis in other tissues, this has never been studied within the human placenta.

- Fetoplacental vessels within the villous vascular tree are surrounded by villous stroma. We have developed a novel method of isolating human placental villous stromal FBs and generating placental-specific, FB-derived matrices. The present study demonstrates that normal fetoplacental ECs grown on matrix generated from FBs of FGRadv placenta exhibit deficient proliferation and migration. In contrast, matrix derived from FBs of normal placenta rescue FGRadv EC proliferation and migration.

- Our results are the first to demonstrate the role of human placental villous stromal matrix on regulation of fetoplacental EC angiogenic properties, opening an additional avenue of investigation into methods by which to improve outcomes in FGRadv pregnancies.
Figure 1. Fetoplacental EC migration is not significantly regulated by secreted factors from control and FGRadv FBs

(A) Representative images of wound scratch assays at 6-h time intervals for a total of 24 h with control and FGRadv ECs subjected to CM generated from control or FGRadv FBs. A blue-colored mask was applied to ECs by ImageJ. (B) There were statistically significant differences between groups (n=4/group) at 6-h (F(3, 44) = 24.89, P<0.0001), 12-h (F(3, 44) = 42.17, P<0.0001), 18-h (F(3, 44) = 50.48, P<0.0001), and 24-h (F(3, 44) = 32.70, P<0.0001). Post-hoc comparisons also demonstrated that when grown in control CM, significant differences persisted between control EC and FGRadv EC migration (solid black vs solid red line: 6-h P<0.001, 12-h P<0.0001, 18-h P<0.0001, 24-h P<0.001). This difference was seen once again between control and FGRadv EC cohorts when cultured in FGRadv CM (dashed black vs dashed red line: 6-h P<0.0001, 12-h P<0.0001, 18-h P<0.0001, 24-h P<0.0001). (**** P<0.0001). (C) The table summarizes post-hoc comparisons of the effect of CM on wound closure. Post-hoc P-values are listed comparing control ECs grown in control CM as compared with those cultured in FGRadv CM (black font; solid black vs dashed black line in (B)). P-values are also listed comparing FGRadv ECs cultured in control CM as compared with those grown in control CM (red font; solid red vs dashed red line in (B)).
Figure 2. Placental villous stromal FBs are able to generate uniform and stable CDMs

(A) Representative immunofluorescence images of CSPG4, a protein expressed within FBs that is not secreted in the absence of stimulation, show the persistence of FBs on a 7-day-old CDM prior to decellularization. After decellularization, FBs were no longer evident, although there was still some cellular debris as shown by DAPI staining. (B) Fibronectin, an ECM protein known to be highly expressed in placental villous stroma, demonstrates uniform deposition of CDM post-decellularization in these representative images. (C) Representative bright-field images show the persistence of stable, uniformly deposited placental FB-derived CDM up to 14 days after decellularization when generated from either control or FGRadv FBs. A blue-colored mask was applied to CDM by ImageJ.
Figure 3. EC proliferation is most robust when grown on CDM

(A) Representative images from days 0, 1, 3, and 5 of proliferation of control ECs plated on various ECM substrates or control CDMs with negative controls of BSA and nude tissue culture plastic. A blue-colored mask was applied to ECs by ImageJ. (B) Statistically significant differences existed among groups on day 1 (F(4, 55) = 102.90, P<0.01), day 2 (F(4, 55) = 52.92, P<0.01), day 3 (F(4, 55) = 33.44, P<0.001), day 4 (F(4, 55) = 67.21, P<0.001), and day 5 (F(4, 55) = 396.80, P<0.0001). Importantly, there were significant post-hoc differences between EC proliferation when grown on CDM as compared with nude plastic or BSA as early as day 1, which persisted through all 5 days (P<0.0001). There was also significantly enhanced proliferation of ECs when plated on fibronectin or collagen I in contrast with proliferation when grown on nude plastic or BSA starting at day 4 (P<0.05) that persisted into day 5 (P<0.001). (*P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001).
Figure 4.
Experimental design to elucidate the individual and synergistic effects of fetoplacental ECs and placental stromal matrix on EC proliferation and migration.
Figure 5. EC proliferation is mediated by placental FB CDMs

(A) Representative images of time-lapsed, live-cell imaging of control and FGRadv EC proliferation on control and FGRadv FB CDMs over a 5-day time period. A blue-colored mask was applied to ECs by ImageJ. (B) Statistically significant differences between groups (n=4/group) were present at day 1 (F(3, 44) = 3.65, P<0.05), day 2 (F(3, 44) = 3.34, P<0.05), day 3 (F(3, 44) = 3.21, P<0.05), day 4 (F(3, 44) = 7.19, P<0.001), and day 5 (F(3, 44) = 12.49, P<0.0001). While post-hoc analysis only showed significant differences between control ECs/control CDM and FGRadv ECs/FGRadv CDM on days 4 (P<0.05) and 5 (P<0.0001), proliferation significantly differed between control ECs/control CDM and control ECs/FGRadv CDM on all 5 days (P<0.05 for days 1, 2, 3; P<0.001 for days 4, 5), demonstrating that FGRadv CDM significantly impairs control EC proliferation. There was also a trend toward a significant difference between FGRadv ECs/FGRadv CDM and FGRadv ECs/Control CDM (P=0.14), suggesting that control matrix may partially rescue FGRadv EC proliferation. (*P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001).
Figure 6. Migrational properties of ECs are significantly regulated by placental FB CDMs

(A) Spider plots depict the centers of mass for all four experimental groups, with each plot containing at least 180 representative cell tracks per condition. The centers of mass for both control and FGRadv ECs plated on control CDM are qualitatively larger than ECs subjected to FGRadv CDM, illustrating the robust migratory capacity of both control and FGRadv ECs when interacting with control matrix. In contrast, although EC migration is deficient as expected for FGRadv ECs plated on FGRadv CDM, control EC migration demonstrates a similar qualitative level of impairment when subjected to FGRadv CDM. (B) There were statistically significant differences among all four experimental groups with regard to accumulated distance (F(3, 177) = 60.33, P<0.0001), Euclidean distance (F(3, 177) = 26.23; P<0.0001), directionality (F(3, 177) = 15.61, P<0.0001), and velocity (3, 177) = 60.33, P<0.0001). For each EC migration parameter, statistically significant differences persisted between control ECs plated on control CDM as compared with FGRadv ECs plated on FGRadv CDM (P<0.0001), but each parameter was also significantly impaired when comparing control ECs plated on control CDM and control ECs plated on FGRadv CDM. Similarly, there were significant differences in all parameters when FGRadv ECs plated on FGRadv CDM were compared with FGRadv ECs plated on control CDM. In contrast, there were no significant differences in all four measures of migration when control ECs plated on control CDM were compared with FGRadv ECs plated on control CDM. Most importantly, graphical representation of each EC migration parameter demonstrates that spider plot/centers of mass findings were affected most by Euclidean distance and directionality, suggesting that the impaired migration seen in ECs subjected to FGRadv CDM stems from disordered and scrambled EC motility. (**P<0.01, ***P<0.001, ****P<0.0001).
Figure 7. FGRadv CDM exhibit differential expression of key ECM proteins

Immunofluorescence analysis demonstrates differential expression of collagen I and fibronectin, two key ECM proteins, with significantly lower expression of collagen I ($P<0.0001$) and fibronectin ($P<0.0001$) in FGRadv. While imaging of these two ECM proteins suggests the possibility of suboptimal matrix deposition in FGRadv, immunofluorescence with thrombospondin-1 demonstrates similar uniformity of the matrix between control and FGRadv CDM. Together, this suggests that although matrix deposition is consistent in both control and FGRadv CDM, differential expression of certain key ECM proteins may be one mechanism that contributes to aberrant EC proliferation and migration. (****$P<0.0001$).
Table 1
Clinical characteristics (data presented as mean ± SEM or as median with IQR)

|                                | Control (n=4) | FGRadv (n=4) |
|--------------------------------|---------------|--------------|
| Maternal age (years)           | 31.75 ± 2.69  | 33.25 ± 4.09 |
| Gestational age at delivery (weeks)† | 39.07 (39.00–39.68) | 30.72 (28.75–33.43) |
| Doppler velocimetry (absent/reversed) | 0/0          | 2/2          |
| Neonatal birthweight (g) (percentile range)* | 3314 ± 119.8 (33–58%) | 800 ± 40.2 (1–7%) |
| Placental weight (g)³         | 578 ± 27.5    | 193.5 ± 17.9 |
| Neonatal sex (M/F)            | 2/2           | 2/2          |
| Route of delivery (vaginal/C-section) | 0/4          | 0/4          |

* P<0.0001 by unpaired, two-tailed t test.

† P<0.05 by Mann–Whitney test.