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Overexpression of the aryl hydrocarbon receptor nuclear translocator (ARNT) partially rescues fetoplacental angiogenesis in severe fetal growth restriction

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Abstract

Pregnancies complicated by severe fetal growth restriction with abnormal umbilical artery Doppler velocimetry (FGRadv) are at substantial risk for adverse perinatal and long-term outcomes. Impaired angiogenesis of the placental vasculature in these pregnancies results in a sparse, poorly branched vascular tree, which structurally contributes to the abnormally elevated fetoplacental vascular resistance that is clinically manifested by absent or reversed umbilical artery Doppler indices. Previous studies have shown that aryl hydrocarbon receptor nuclear translocator (ARNT) is a key mediator of proper placental angiogenesis, and within placental endothelial cells (ECs) from human FGRadv pregnancies, low expression of ARNT leads to decreased vascular endothelial growth factor A (VEGFA) expression and deficient tube formation. Thus, the aim of this study was to determine the effect of VEGFA administration or ARNT overexpression on angiogenic potential of FGRadv ECs. ECs were isolated and cultured from FGRadv or gestational age-matched control placentas and subjected to either vehicle vs VEGFA treatment or transduction with adenoviral-CMV (ad-CMV) vs adenoviral-ARNT (ad-ARNT) constructs. They were then assessed via wound scratch and tube formation assays. We found that VEGFA administration nominally improved FGRadv EC migration ($p<0.01$) and tube formation ($p<0.05$). ARNT overexpression led to significantly enhanced ARNT expression in FGRadv ECs ($p<0.01$), to a level similar to control ECs. Despite this, FGRadv EC migration ($p<0.05$) and tube formation ($p<0.05$) were still only partially rescued. This suggests that although ARNT does play a role in

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Authors' contributions

All three authors designed experiments. SJ and HX performed experiments. All three authors analyzed data and interpreted results. SJ and EJS wrote the manuscript.

Declarations of interest

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fetoplacental EC migration, other factors in addition to ARNT are likely also important in placental angiogenesis.

Introduction

Fetal growth restriction (FGR) occurs when a fetus' individual growth potential is unable to be met, and of all the various etiologies, placental insufficiency is the most common pathology associated with FGR (1, 2). The diagnosis of FGR, in general, increases risks for adverse perinatal outcome, neurodevelopmental delay, and adulthood diseases such as cardiovascular disease, diabetes, and obesity (2–6). These outcomes increasingly worsen as the degree of FGR worsens, and in the most severe cases, where there is early-onset FGR in conjunction with fetal Doppler abnormalities, the prognosis is oftentimes dismal, with a high risk for morbidity and mortality (7–11).

Of the various antenatal surveillance tools available to monitor the fetus, only umbilical artery and ductus venosus Dopplers have been shown to potentially improve outcomes in fetuses with FGR (2, 12, 13). Abnormally elevated impedance in the umbilical artery suggests the presence of underlying placental insufficiency in FGR. Furthermore, when absent or reversed end-diastolic velocities are present in umbilical arteries, there is a substantial increase in risk for adverse outcome – specifically, either perinatal mortality or a significantly preterm delivery with the attendant complications of prematurity that are further exacerbated by FGR (2, 14–16). Together, these data establish the importance of the fetoplacental circulation and vasculature in fetal growth and pregnancy outcome.

In uncomplicated pregnancies, the fetoplacental circulation is established by approximately two weeks after conception and continues to expand first via vasculogenesis followed by angiogenesis (17, 18). Notably, angiogenesis dramatically accelerates starting at approximately 25 weeks of gestation and continues until 40 weeks (19, 20). This gives rise to a vascular bed that has been estimated to be approximately 550 km in length and 15 m² in surface area at term gestation (21). In turn, this translates to a normal, progressive decrease in fetoplacental vascular resistance as pregnancy progresses that is reflected by increasing end-diastolic velocities within the umbilical artery (22–24). In contrast, placentas from FGR pregnancies with aberrantly high vascular resistance as clinically represented by abnormal Doppler velocimetry (FGR_{adv} = FGR with abnormal Doppler velocimetry) exhibit a sparse, poorly branched vascular tree that arises, at least in part, secondary to abnormal angiogenesis in the latter half of pregnancy (25–28).

We have previously found that aryl hydrocarbon receptor nuclear translocator (ARNT) is expressed in lower quantities within fetoplacental endothelial cells (ECs) primarily isolated from FGR_{adv} placentas in comparison to ECs from gestational age-matched, appropriately grown fetuses (29). ARNT, a member of the basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) family of transcription factors, is essential for proper placental vascularization and embryonic development. Transgenic ARNT-deficient mice are embryonically lethal secondary to severe deficiencies in yolk sac and placental labyrinthine vascularization (30–32). More specific to the endothelium, conditional EC knock-out of ARNT resulted in about 40% embryonic lethality, with no identifiable blood within the umbilical cord or placentas

on gross inspection of these demised embryos (33). Among live embryos, EC-specific disruption of ARNT showed a trend toward smaller embryos than littermate controls that led to statistically significant neonatal growth restriction (33). Collectively, these findings demonstrate that ARNT is critical for proper placental vascularization and fetal growth.

Within our model of primarily isolated fetoplacental ECs, we have previously found that FGRadv ECs demonstrate deficient angiogenic potential with impaired tube formation secondary to diminished EC motility (29). This was mediated, at least in part, by lower ARNT expression, whereby ARNT ablation in normal ECs also led to fewer angiogenic branch points and total tube length. Furthermore, knock-out of ARNT in these same ECs also led to decreased binding of ARNT heterodimers to key response elements within the vascular endothelial growth factor A (VEGFA) proximal promoter, resulting in decreased VEGFA transcription and expression (29). Thus, we hypothesized that administration of VEGFA or rescue of ARNT expression in FGRadv ECs would result in improvement of FGRadv EC migration, and our objective was to determine the effect of VEGFA treatment or ARNT overexpression on angiogenic potential of FGRadv ECs.

Methodology

Subjects

After approval by the institutional review board at Northwestern University and the Colorado Multiple Institutional Review Board (COMIRB), subjects from three separate groups were identified: [1] Singleton pregnancies complicated by FGR and delivered prematurely (gestational age range 24-34 weeks), as defined by an estimated fetal weight of less than the 10th percentile for gestational age AND absent or reversed end-diastolic umbilical artery velocities (FGRadv); [2] Singleton pregnancies with appropriately grown fetuses between the 10th and 90th percentile for gestational age that were gestational age-matched (within 10 days) and sex-matched to FGRadv subjects that were delivered prematurely secondary to spontaneous preterm birth etiologies (controls) ; and [3] Singleton pregnancies that resulted in full-term, appropriately grown fetuses delivered via cesarean section in the absence of labor (term controls). Cells from this third group were utilized primarily for methodologic establishment.

All subjects, regardless of their categorization, were required to meet solid dating criteria as 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) (34). Furthermore, actual birth weight was used to confirm growth restriction status in group 1 and appropriate growth in groups 2 and 3. Specific to group 2 (control subjects), patients with concomitant medical comorbidities such as preeclampsia, hypertension, or other processes that could result in uteroplacental insufficiency were ineligible. This was done in an effort to minimize the likelihood of impaired placental angiogenesis (even in the setting of appropriate growth) in these preterm control subjects. Additionally, cells from group 2 were only utilized as preterm control cells if placental pathology did not demonstrate any evidence of placental insufficiency, and any control subjects who were found to have histologic evidence of maternal or fetal undervascular perfusion were excluded. Exclusion criteria for all groups consisted of fetal anomaly, fetal aneuploidy, fetal

or maternal infection, diabetes, history of thrombosis, or antiphospholipid antibody syndrome. Appropriate subjects were approached, and informed consent was obtained.

Endothelial cell isolation and culture

Human fetoplacental villous ECs were isolated/cultured within one hour after delivery as previously described with minor modifications (35, 36). After isolation, ECs were cultured in media supplemented with 5% fetal bovine serum, bovine brain extract with heparin, epidermal growth factor, hydrocortisone, and gentamicin/amphotericin B (Lonza, USA) at 37° C (95% humidity, 5% CO₂). As our previous findings showed that EC migrational defects in FGRadv were independent of oxygen concentration, all experiments were performed at ambient oxygen levels (29). Consistent with previous data, primarily isolated ECs demonstrated nearly 100 percent purity, and ECs were cultured up to the fifth passage to avoid changes in phenotype, with persistently low ARNT expression in FGRadv ECs despite passage number (data not shown) (35, 37). For ECs undergoing VEGFA administration, ECs were cultured with vehicle or VEGFA 60 ng/mL (R&D, USA), with this specific concentration chosen secondary to findings from previous studies (38).

ARNT overexpression

For transient overexpression of ARNT, we utilized the adenoviral-ARNT (ad-ARNT) construct commercially available from Vector Biolabs (USA). This recombinant human adenovirus type 5 lacking the E1 and E3 genes essential for viral replication expresses ARNT under the control of a CMV promoter. The same adenoviral-CMV backbone without the ARNT insert was utilized as a control (ad-CMV), as was the same adenoviral vector that expresses EGFP (ad-GFP). The titer of each construct was determined with Adeno-XTM qPCR Titration Kit (Takara Bio, USA) as per manufacturer protocol, and additional virus was purified as needed using the Adeno-X Maxi Purification Kit (Takara Bio) and further titrated.

To determine the optimal quantity of viral particles per cell for infection, ECs were seeded in 12-well plates at a density of 1×10^5 cells/well and cultured in full media $\times$ 24 hours. Upon reaching approximately 80% confluence, 0, 50, 100, 150, and 200 ad-GFP viral particles/cell were added for 6 hours. Culture media was then changed, and cells were incubated for an additional 48 hours in full media. Optimal multiplicity of infection (MOI) was then determined by microscopy taking into account both GFP transduction efficiency and cytopathic effect. After establishing the optimal MOI of 100, ECs were plated and grown to approximately 70-80% confluence and then subjected to either ad-CMV or ad-ARNT for 6 hours. Again, culture media was then changed, and cells were incubated for an additional 48 hours in full media prior to undergoing downstream experiments.

Protein isolation and western blotting

Protein extraction from human fetoplacental ECs was performed using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher, USA) 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 SDS-PAGE and

transferred onto polyvinylidene difluoride membranes. Membranes were probed using antibodies against ARNT (1:1000; BD Biosciences, USA), hypoxia inducible factor 1-alpha (HIF1A, 1:1000; BD Biosciences), VEGFA (1:500; Proteintech, USA), and beta-actin (1:5000; Sigma-Aldrich, USA). Anti-rabbit and anti-mouse IgG conjugated to horseradish peroxidase (Cell Signaling Technology, USA) were used as secondary antibodies (ARNT 1:3000, HIF1a 1:3000, VEGFA 1:3000, actin 1:10,000). Lastly, immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Millipore Sigma, USA) and quantified/normalized via densitometric analysis.

Wound scratch assays

Equal numbers of ECs were cultured in full media until 100% confluent (~24 hours) and then scratched with a P200 pipette tip. ECs were then further subjected to vehicle or VEGFA treatment in full media. For overexpression studies, after transduction with ad-CMV or ad-ARNT vectors, equal numbers of ECs were cultured in full media until 100% confluent and then scratched with a P200 pipette tip. Full media was chosen as this was not only required to maintain EC viability with prolonged imaging but also felt to be more representative of in vivo conditions than starvation. Furthermore, the phenotype of impaired motility in FGRadv ECs was maintained despite exposure to full media.

Immediately after scratching, cells were then imaged with Incucyte ZOOM® (Essen BioScience, USA) for real-time imaging of cells in culture. Time-lapsed, live images were taken at 0, 4, 8, 12, 16, 20, and 24 hours for each sample. Images were then analyzed with ImageJ software (<https://imagej.nih.gov/ij/>). The degree of wound healing, which correlates with EC migration, was assessed by measuring the remaining cell-free (non-closure) area and then converted to percent closure.

Tube formation assays

ECs were subjected to vehicle versus VEGFA 60 ng/mL or ad-CMV versus ad-ARNT transduction and then subjected to Geltrex™ reduced factor basement membrane matrix (Thermo Fisher), which was added in a volume of 50 µL/well to a 96-well plate and allowed to polymerize at 37° C for 30 minutes. After polymerization, ECs were stained with calcein AM and plated on the matrix in equal numbers (1.5×10^4 cells/well in 200 µL of media) (39). Tube formation was observed under an inverted microscope (Zeiss Axiovert 40 CFL) after 24 hours. Images were captured with a Zeiss AxioCam camera attached to the microscope. The tube formation was quantified by measuring the long axis of the individual cells on the matrix using the ImageJ Angiogenesis Analyzer. Mean values of branch points and total length in each sample were used to numerically represent tube formation.

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 cellular experiments were performed on 4–5 matched pairs of subjects, with each repeated in triplicate, using cells between the second and fourth passage. Representative images of experiments are from one or two subjects, with analysis of numerical results 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 SEM. Normal distribution of experimental datasets was also confirmed with the Shapiro-Wilk test, and thus, statistical analysis for comparison of groups was performed using one-way ANOVA with Tukey adjustment for multiple comparisons if the overall one-way ANOVA was statistically significant. A value of $p < 0.05$ was considered significant.

Results

Subject characteristics

Selected clinical information for subjects are presented in Table 1. Each control subject was gestational age-matched (± 10 days) to FGRadv cases with an attempt to match for neonatal sex whenever possible. Despite matching for gestational age, neonatal birth weight ($p < 0.05$) and placental weights ($p < 0.05$) were significantly lower in the FGRadv cohort in comparison to gestational age-matched controls.

ARNT overexpression in FGRadv fetoplacental ECs results in up-regulation of VEGFA expression.

To first determine the optimal MOI that yielded the highest transduction efficiency and lowest cytopathic effect, we first subjected term control fetoplacental ECs to various concentrations of viral particles per cell (ranging from 0 to 200 ad-GFP viral particles/cell). We found that 100 MOI was optimal, with high ad-GFP transduction efficiency with minimal cytopathic effects (Figure 1A).

We then subjected both control and FGRadv ECs to ad-ARNT constructs, utilizing ad-CMV empty backbone vectors as a control. Similar to our previous findings, ARNT expression after ad-CMV transduction was significantly lower in FGRadv ECs in comparison to control ECs ($p < 0.001$, Figure 1B, 1C). Infection with ad-ARNT resulted in significantly increased ARNT protein expression only in FGRadv ECs ($p < 0.01$), although the degree of expression was still less than basal levels in control ECs with ad-CMV transduction (Figure 1B, 1C). We also investigated the effect of ARNT overexpression on one of its heterodimeric partners, HIF1A, which together with ARNT initiates VEGFA transcription. As anticipated, there was no effect of ARNT overexpression on HIF1A protein levels. However, ARNT overexpression led to a significant increase in VEGFA protein expression ($p < 0.05$), suggesting that transduction of this ad-ARNT vector is biologically functional (Figure 1B, 1C).

Additional VEGFA administration to FGRadv ECs only partially improves EC migration.

Although ARNT overexpression resulted in up-regulation of VEGFA protein expression, this was not to the level of basal VEGFA expression in control ECs. Thus, we next wanted to determine whether administration of additional VEGFA would improve FGRadv EC migrational defects that were previously noted. We first performed wound scratch assays, which continued to confirm deficient EC migration in FGRadv ECs in comparison to control ECs ($p < 0.001$, Figure 2). Control ECs demonstrated nearly 100 percent closure both under vehicle and VEGFA treatment at 24 hours. In contrast, VEGFA administration in FGRadv

ECs resulted in a small, but statistically significant improvement in wound closure ($p<0.01$, Figure 2).

Similarly, we utilized tube formation assays as an additional method of assessing angiogenic potential. Although the one-way ANOVA demonstrated statistical significance when looking at branch points overall ($p<0.05$), we did not find any statistically significant difference between groups with post-hoc analysis (Figure 3A, 3B). There was a trend toward fewer branch points in FGRadv ECs under vehicle treatment in comparison to control ECs ($p=0.053$), which is unlike previous data demonstrating a statistically significant difference (29). There did continue to be a statistically significant decrease in total length between control and FGRadv ECs treated with vehicle ($p<0.001$, Figure 3A, 3C). Additionally, VEGFA treatment of FGRadv ECs resulted in a slight but significant increase in total tube length ($p<0.05$), suggesting that additional factors beyond just VEGFA are needed to ameliorate EC migrational defects seen in FGRadv ECs (Figure 3).

Rescuing of ARNT expression in FGRadv ECs also does not completely restore EC angiogenic potential.

VEGFA was an enticing candidate to initially study both because of its known pro-angiogenic effects and because it is a downstream target gene of ARNT. However, ARNT regulates other downstream genes beyond just VEGFA, and thus, we hypothesized that rescuing ARNT expression in FGRadv ECs would improve angiogenic defects beyond that of just VEGFA administration.

To test this hypothesis, we subjected control and FGRadv ECs to wound scratch assays after transduction with either ad-CMV or ad-ARNT at 100 MOI. Similar to control ECs exposed to either vehicle or VEGFA, we found that at 24 hours, there was essentially 100 percent wound closure in control ECs regardless of the adenoviral construct to which these ECs were exposed (Figure 4). However, FGRadv ECs infected with ad-CMV controls demonstrated a mean closure of approximately 57 percent. This was significantly increased with ARNT overexpression, resulting in an average of 79 percent closure ($p<0.01$, Figure 4).

These findings also corresponded to that of tube formation assays after ad-CMV or ad-ARNT transduction. Specifically, the impairment in both branch points and total tube length seen in FGRadv ECs was significantly improved with ARNT overexpression from a statistical perspective ($p<0.05$, Figure 5). However, the degree of phenotypic rescue was modest and did not reach basal levels of tube formation seen in control ECs (Figure 5).

Discussion

Currently, the only treatment for pregnancies complicated by FGRadv is to time delivery such that gestational age is maximized while fetal exposure to the compromised uterine environment is minimized (2, 13). Of all the various ultrasound modalities, Doppler assessments of the fetoplacental circulation in FGR are the most correlative with perinatal outcome and oftentimes help guide timing of delivery, highlighting the important influence of the fetoplacental vasculature on fetal growth and pregnancy outcome (2, 4, 40–42). Yet, clinical evidence repeatedly demonstrates that even with the additional information gained

by fetal Dopplers, these infants remain at high risk for perinatal morbidity and mortality (2, 5, 8, 10, 11, 43, 44). Thus, significant improvements in FGRadv clinical outcomes are unlikely unless we can first better understand mechanisms underlying the development of abnormal fetoplacental blood flow.

From a structural perspective, FGRadv placentas have a sparsely branched, abnormally elongated distal villous vasculature, contributing to aberrantly high fetoplacental vascular impedance (25, 27, 28, 45–47). Previous animal models have shown that ARNT is important for proper placental vascular development and fetal growth (30–33). Within a model of primarily isolated fetoplacental ECs, we have also found that FGRadv ECs express lower levels of ARNT, resulting in less VEGFA transcription and EC migration/angiogenesis (29, 48). Thus, in this study, we sought to determine whether administration of VEGFA or rescue of ARNT expression in human FGRadv ECs would improve migrational defects present in ECs primarily isolated from FGRadv placentas. We found that overall, the impairment in FGRadv EC migration was only partially recovered after either VEGFA treatment or ARNT overexpression, suggesting that other factors in addition to ARNT also mediate fetoplacental EC migration and angiogenesis.

Specifically, we found that VEGFA treatment of FGRadv ECs resulted in a statistically significant increase in FGRadv EC wound closure and total tube length when compared to FGRadv ECs subjected to vehicle. The magnitude of improvement from a physiologically relevant perspective, however, was marginal. One potential explanation for this is that pooled results of FGRadv ECs subjected to wound scratch assays with vehicle treatment demonstrated nearly two-fold less closure than FGRadv ECs that underwent basal ad-CMV transduction. Thus, it is possible that VEGFA had to “overcome” a batch of FGRadv ECs that were substantially impaired during the wound scratch assay. However, ECs were utilized from the same eight subjects for both VEGFA administration and ARNT overexpression experiments, and a similar degree of baseline impairment of tube formation was seen in FGRadv ECs subjected to either vehicle treatment or ad-CMV transduction. Furthermore, these tube formation assays also demonstrated only minor improvement with VEGFA treatment, with no statistically significant increase in number of branch points and a statistically significant but physiologically marginal enhancement in total tube length.

On the one hand, we had initially anticipated that administration of additional VEGFA, especially at supraphysiologic dosages, would significantly enhance FGRadv EC migration for two main reasons. First, this would restore a key growth factor that was found to be deficient in FGRadv ECs mediated in part by ARNT (29). Second, others have shown that VEGFA expression is reduced in syncytiotrophoblast (STB) in FGRadv pregnancies, and it is possible that VEGFA is deficient within the fetoplacental vasculature secondary to *both* EC and STB derangements (27, 49). On the other hand, it was ultimately not surprising to us that VEGFA treatment alone did not fully rescue FGRadv EC migratory defects because ARNT has other downstream targets other than VEGFA. Moreover, others have found that ablation of key mediators of cardiovascular function, such as the G proteins $G_{\alpha q}$ or $G_{\alpha 11}$, inhibit VEGFA-induced human umbilical vein EC (HUVEC) proliferation and migration, suggesting that even if VEGFA is present in adequate quantities, other pathways are also important in mediating EC migration and angiogenesis (50–52). Likewise, it would also be

extremely unlikely for an organ as complex as the human placenta to be regulated by just one angiogenic growth factor.

Given the marginal improvement in FGRadv EC migration with VEGFA administration, the next obvious step was to investigate whether rescuing of ARNT expression in these ECs could better improve FGRadv EC angiogenic potential. Based upon densitometric analysis of our western blots of a total of 10 subjects, ad-ARNT transduction in FGRadv ECs resulted in an increase in ARNT protein expression to approximately 80 percent of basal expression in control ECs subjected to ad-CMV constructs, and the overall difference in expression between these two groups on post-hoc analysis was technically not statistically significant ($p=0.14$). Despite similar expression levels of ARNT between these two groups, rescuing of ARNT in FGRadv ECs led to improvements in wound scratch closure and tube formation that were only moderate and of similar magnitude to that of VEGFA treatment. These findings suggested to us that other downstream targets of ARNT besides VEGFA may not necessarily play a significant role in fetoplacental angiogenesis, and there are likely other mediators beyond ARNT that are also critically important.

Strengths of our study include the use of cells obtained from human specimens that demonstrate a very specific and severe phenotype of placental insufficiency. Several limitations, though, exist. One potential limitation is that ECs isolated from placentas of gestational age-matched, appropriately grown fetuses are not true “controls” in that they delivered preterm secondary to some other underlying pathology. However, specimens obtained from entirely normal pregnancies that deliver at term gestation are also not ideal controls in that angiogenesis may not be static throughout gestation. Furthermore, placental vascular resistance normally decreases with advancing gestational age, and we felt that this could be a more substantial confounder in assessing mechanisms of fetoplacental angiogenesis. Thus, in an attempt to obtain control ECs from uncompromised fetoplacental vessels, we only utilized ECs from control placentas after formal pathologic analysis ruled out any evidence of placental insufficiency. Another potential concern is that the isolation and culture process of ECs may inadvertently alter its phenotype in unrecognized ways, although we have previously found that lower ARNT expression is seen in both in vivo immunohistochemical and in vitro data (29). Furthermore, this current model precludes investigation of other potentially important interactions such as those that occur between EC-trophoblast and/or EC-villous stroma. Finally, a potential conceptual concern is that isolation of ECs from placentas at the time of delivery is not necessarily reflective of real-time, mechanistic events. Animal models of FGR, including those in rodents and sheep, are informative but differ in many different respects to a human placenta. From a feasibility standpoint, obtaining human placental tissue via procedures such as chorionic villus sampling (CVS) also has several inherent limitations. First, the amount of tissue is inadequate to isolate the number of cells needed to pursue mechanistic experiments. Second, CVS is not routine in FGRadv and in general, is also being utilized less frequently as other non-invasive methods of evaluating the fetus are being developed (53). Third, many CVS specimens will be abnormal at baseline, as they are being done secondary to sonographic findings. Finally, even if the CVS specimen is ultimately found to be normal, it would be unlikely to represent a placenta that will go on to manifest the FGRadv phenotype given the rarity of CVS procedures and the overall low frequency of FGRadv pregnancies.

From a translational perspective, the fetoplacental vasculature, although not the only important mediator of fetal health and disease, is an attractive potential clinical target for several reasons. First, flow/velocity abnormalities are readily detectable clinically via Doppler ultrasound. Second, fetal Doppler aberrations are highly predictive of adverse perinatal outcome. Third, and perhaps most importantly, placental angiogenesis is on-going throughout all of gestation, making it possible that this vasculature could be targeted in both a preventative and therapeutic fashion. Although we found only partial salvage of FGRadv EC migration with administration of additional VEGFA or rescuing of ARNT expression, it is possible that even a fractional enhancement in placental angiogenesis could potentially translate into some improvement in perinatal outcome for these severely compromised pregnancies. However, our data also suggests that there are other mediators beyond ARNT. Future studies, including bioinformatic approaches to identify other potential pathways that are contributing to impaired EC migration despite restoration of ARNT, will help to elucidate additional mechanisms underlying impaired fetoplacental angiogenesis. This is critical if substantial improvements in FGRadv pregnancy outcomes are to be made.

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Abbreviations list

ARNT:	Aryl hydrocarbon receptor nuclear translocator
Ad-:	Adenoviral
EC:	Endothelial cell
FGR:	Fetal growth restriction
FGRadv:	Fetal growth restriction with abnormal umbilical artery Doppler velocimetry
VEGFA:	Vascular endothelial growth factor A

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Clinical Perspectives

- Abnormally elevated vascular resistance within the fetoplacental circulation of pregnancies complicated by fetal growth restriction (FGRadv) is highly related to adverse perinatal outcome, and one primary cause of this is impaired placental angiogenesis. Prior studies have demonstrated that the aryl hydrocarbon receptor nuclear translocator (ARNT) is essential for proper placental vascular development.
- This study shows that although human FGRadv endothelial cells (ECs) express lower quantities of ARNT, resulting in impaired EC migration, rescuing of ARNT expression in these cells partially improves FGRadv EC angiogenic defects.
- Our results suggest that ARNT is an important transcription factor in mediating proper placental vascular development and that augmenting its expression in FGRadv placentas could improve fetoplacental angiogenesis and perinatal outcome. However, other mechanisms beyond ARNT likely also contribute to impaired placental angiogenesis in FGRadv pregnancies, and further investigation is warranted.

Figure 1. FGRadv ECs transduced with ad-ARNT express ARNT in similar quantities to control ECs.

(A) An ad-GFP construct demonstrates high transduction efficiency with minimal cytopathic effects at 100 MOI in fetoplacental ECs. (B) Representative western blots of two control (Subject 1 – S1, Subject 2 – S2) and two gestational age-matched ECs from pregnancies complicated by FGRadv (Subject 3 – S3 matched to S1, Subject 4 – S4 matched to S2). (C) Densitometric analysis of western blot data for ten gestational age-matched subjects (N=5 controls, N=5 FGRadv) demonstrates statistically significant overexpression of ARNT in FGRadv ECs with ad-ARNT transduction ($p<0.01$), which results in a partial but significant increase in vascular endothelial growth factor A (VEGFA) expression. Overall $p<0.0001$ for ARNT and VEGFA using one-way ANOVA with post-hoc comparisons shown in the graphs.

Figure 2. VEGFA administration in FGRadv ECs incompletely rescues EC migration.

(A) Representative images of wound scratch assays from ECs isolated from control and FGRadv placentas in the presence or absence of VEGFA treatment. (B) Graphical representation of wound scratch assays for eight subjects (N=4 controls, N=4 FGRadv) shows slight but statistically significant improvement in FGRadv EC wound closure with VEGFA treatment in comparison to vehicle ($p<0.01$). Overall $p<0.0001$ using one-way ANOVA with post-hoc comparisons shown in the graphs.

Figure 3. FGRadv ECs subjected to VEGFA treatment demonstrate limited improvement in tube formation.

(A) Representative images of ECs from control and FGRadv placentas ECs subjected to tube formation assays in the presence and absence of vascular endothelial growth factor A (VEGFA) treatment. Graphical representation of (B) branch points and (C) total tube length for eight subjects (N=4 controls, N=4 FGRadv) shows slight but statistically significant improvement in FGRadv EC total length with VEGFA treatment in comparison to vehicle ($p<0.05$). No significant differences were seen in branch points between any of the groups. Overall $p<0.05$ using one-way ANOVA for branch points and $p<0.0001$ for total length with post-hoc comparisons shown in the graphs.

Figure 4. Ad-ARNT transduction in FGRadv ECs partially improves EC migratory defects. (A) Representative images of wound scratch assays from ECs of control and FGRadv placentas subjected to transduction of either ad-CMV or ad-ARNT vectors. (B) Graphical representation of wound scratch assays for eight subjects (N=4 controls, N=4 FGRadv) shows slight but statistically significant improvement in FGRadv EC wound closure with ARNT overexpression in comparison to ad-CMV ($p < 0.05$). Overall $p < 0.0001$ using one-way ANOVA with post-hoc comparisons shown in the graphs.

Figure 5. Restoration of ARNT expression in FGRadv ECs partially rescues tube formation deficits.

(A) Representative images of ECs from control and FGRadv placentas subjected to tube formation assays after either ad-CMV or ad-ARNT transduction. Graphical representation of (B) branch points and (C) total tube length for eight subjects (N=4 controls, N=4 FGRadv) shows that rescue of ARNT expression in FGRadv ECs partially improves both branch points ($p<0.05$) and total tube length ($p<0.01$). Overall $p<0.01$ using one-way ANOVA for branch points and $p<0.0001$ for total length with post-hoc comparisons shown in the graphs.

Table 1.
Clinical characteristics of Control and FGAdv subjects.

Data are represented as mean $\pm$ SEM;

	Control (N=5)	FGAdv (N=5)
Maternal age (years)	32.0 $\pm$ 2.7	34.4 $\pm$ 4.0
Gestational age at delivery (weeks)	28.7 $\pm$ 1.3	27.6 $\pm$ 1.2
Neonatal birthweight (g) *	1121 $\pm$ 201	675 $\pm$ 158
Placental weight (g) *	306 $\pm$ 20	159 $\pm$ 34
Neonatal sex (M/F)	2/3	2/3
Route of delivery (vaginal/C-section)	3/2	1/4

*
p<0.01.