

## Decreased expression of pigment epithelium derived factor (PEDF), an inhibitor of angiogenesis, in placentas of unexplained stillbirths

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### **SUMMARY**

Normal placental vascular development depends upon the complex interactions between angiogenic inducers and inhibitors within the placenta. Alterations within the placental microenvironment can promote an imbalance in angiogenic mediators which may be associated with adverse perinatal outcomes. The purpose of this study was to investigate the placentas of infants with unexplained stillbirth as compared to live-born infants and to determine whether alterations in angiogenic inducer vascular endothelial growth factor (VEGF) or inhibitor pigment epithelium-derived factor (PEDF) are associated with altered angiogenesis, vascular remodeling and stillbirth. Placentas of 22 unexplained stillbirths and 44 age-matched live-born controls were scored for microvascular density (MVD), vasculopathy and microvascular permeability. A subset was scored for expression of angiogenic inducer VEGF and inhibitor pigment epithelium-derived factor. Stillborn placentas demonstrated higher MVD than controls (mean±SD:

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116.6±46.3 v. 60.8±13.5, respectively,  $p < 0.001$ ). Vasculopathy was present in 10/22 (45%) stillbirths compared to 0/44 (0%) controls ( $p < 0.001$ ); increased vascular permeability was present in 15/22 (68%) cases and 5/44 (11%) controls ( $p < 0.001$ ). PEDF expression was significantly lower in stillborn placentas (1.7±0.3) than live-born controls (3.6±0.8,  $p < 0.01$ ) while VEGF expression was similar (3.3±0.7 v. 3.7±0.4, respectively,  $p > 0.05$ ). In conclusion, we found that unexplained stillbirth is associated with loss of angiogenic inhibitor PEDF, vasculopathy and heightened angiogenesis in the placenta. *Reproductive Biology* 2008, 8, 2:107-120.

**Key words:** stillbirth, angiogenesis, placenta, PEDF

## INTRODUCTION

Normal placental vascular development depends upon the complex interactions between angiogenic inducers and inhibitors within the placental microenvironment [2]. Placental development requires abundant and precise growth of new blood vessels (vasculogenesis) early in pregnancy followed by elaboration of these blood vessels (angiogenesis) as pregnancy progresses. Early in development, endothelial cells are stimulated by growth factors such as vascular endothelial growth factor (VEGF; [4, 19]), a potent inducer of angiogenesis [8]. Both placental [3, 14] and maternal serum [16] levels of VEGF are high during this early period of gestation. However, once the placenta reaches a critical mass, angiogenic inducers plateau to stabilize vessel growth in normal placentation [8, 20]. The factors responsible for inducing vascular quiescence at this stage are unclear. Here, we present evidence to suggest that pigment epithelium-derived factor (PEDF), a potent inhibitor of angiogenesis [6] is highly expressed in the vasculature and trophoblasts of placentas obtained from women with normal pregnancies.

Pigment epithelium-derived factor, a multifunctional 50 kDa secreted glycoprotein is known to be expressed in the placenta [21], however, its functional role in placental angiogenesis has not yet been reported. PEDF is one of the most potent angiogenic inhibitors identified to date

[6]. In several tissue beds, environmental stimuli including hypoxia and inflammation, result in the up-regulation of VEGF and down-regulation of PEDF [7, 22, 26, 27], thus altering the angiogenic balance to favor a pro-angiogenic microenvironment. Although VEGF is essential to establish a rich vascular network in early placental development [12], sustained VEGF elevation could have deleterious consequences later in gestation. For instance, VEGF, a potent mitogen to vascular smooth muscle cells, induces myointimal hyperplasia in epicardial vessels [10] in the setting of Kawasaki's disease. In the epicardial vessels, this alteration results in arterial narrowing (vasculopathy), consequently, compromising blood flow and promoting tissue hypoxia. Prolonged upregulation of VEGF could promote the same pathologic process in placental vessels. In addition, capillary induction by angiogenic stimuli can result in poorly formed vascular junctions leading to leaky vasculature [23] and PEDF, an anti-permeability factor, has been shown to overcome VEGF-induced permeability [15, 25]. The counterbalance of endogenous angiogenic inhibitors, like PEDF, is essential to maintain both vascular quiescence and to ensure normal vascular integrity.

As in other tissues bed, alterations within the placental microenvironment can promote an imbalance in angiogenic mediators. We hypothesize that prolonged up-regulation of VEGF or down-regulation of PEDF may alter the placental vasculature and contribute to adverse perinatal outcomes, such as stillbirth. The purpose of this study was to investigate the placentas of infants with unexplained stillbirth as compared to live-born infants and to determine whether alterations in angiogenic inducer VEGF or inhibitor PEDF are associated with vascular remodeling and adverse fetal outcome.

## **MATERIALS AND METHODS**

### **Selection of cases and controls**

We reviewed records from all deliveries of singleton stillborn infants  $\geq 24$  weeks estimated gestational age at Northwestern Memorial Hospital from

January 1, 1998 to December 31, 2002. Cases with congenital anomalies (determined either by autopsy or gross inspection), congenital infection (confirmed by autopsy or culture findings), aneuploidy or evidence of significant maternal-fetal hemorrhage (>than 10 fetal cells per 5000 maternal red blood cells (RBC; [17]) were excluded. All remaining cases were considered to represent unexplained stillbirth.

Controls included placentas matched by gestational age ( $\pm 2$  weeks) in a 2:1 ratio to cases. Gestational age in each group was calculated by reported menstrual dates and/or ultrasound dating parameters. To compare placentas of normal pregnancies to those of stillbirths, only placentas from mothers without underlying medical conditions, hypertensive disease of pregnancy or prolonged rupture of membranes (greater than 24 hours) who delivered a normally grown live-born infant (between the tenth and ninetieth birth-weight percentile for estimated gestational age; [1]) were considered eligible. Placentas of infants with congenital anomalies, congenital infection or aneuploidy were excluded. The study was approved by Northwestern University's Institutional Review Board.

### **Immunohistochemical studies**

A pathologist (SC), blinded to clinical information, examined all archival histopathologic specimens. Formalin-fixed placentas had undergone routine pathological examination which included gross inspection of the placenta with sectioning at the site of the cord insertion and grossly abnormal areas in addition to gross and histologic examination of normal and abnormal-appearing portions of the umbilical cord. Representative sections were submitted for this study. Specimens had previously been formalin fixed, paraffin embedded and hematoxylin and eosin (H&E) stained. Additional paraffin-embedded tissue sections were deparaffinated, rehydrated, and washed with PBS according to standard procedure. Immunohistochemical staining was performed using primary antibodies directed against von Willebrand Factor (VWF; DAKO, Denmark, 1:200 dilution), PEDF (produced and purified as previously described, 1:75 dilution; [6]) and VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, 1:100 dilution) using

an Avidin-Biotin-Peroxidase protocol and Vectastain elite kit (Vector Laboratories, Inc., Burlingame, CA) as previously described [7]. Negative controls for each antibody were used to confirm antigen specificity. Microvascular density (MVD), a hallmark of angiogenesis, was defined as the number of VWF positive endothelial-lined vessel per high power field (HPF, 40 $\times$ ) averaged over five non-overlapping fields. Heightened angiogenesis was defined as MVD  $\geq$ 100 vessels/HPF. Small and medium-sized arteries were graded on a 1-5 vasculopathy scale and averaged over 5 non-overlapping fields as originally described by Heath and Edwards [13]. Briefly, a score of 1 was defined as normal, 2 as medial hyperplasia, 3 as medial and intimal hyperplasia, 4 as luminal stenosis  $>$ 50% and 5 as plexogenic lesions with recanalization. Vasculopathy was defined as a score  $\geq$ 4. Microvascular permeability was measured by RBC extravasation which was considered to be present if visualized in  $\geq$ 4 of 5 non-overlapping fields. For VEGF and PEDF expression, immunolocalization studies were performed in a subset of samples (6 cases and 6 controls), each with an estimated gestational age  $\geq$ 32 weeks, a gestational age at which the placental vasculature is presumed to be quiescent. The intensity of the stain in the endothelium and trophoblasts was graded on a 1 - 4 scale (1=no staining, 2=focal, mild staining, 3=moderate staining, 4=diffuse, intense staining) and averaged over 5 non-overlapping high power fields (HPF, 40 $\times$ ). Additional histopathologic factors analyzed include significant infarction ( $>$ 15% placental area) and the presence of organizing or occlusive thrombi.

### **Statistical analysis**

Spearman rank correlation, Mann-Whitney U, Student t-test, and Chi-square were used for statistical analyses (Minitab 13, Minitab Inc., State College, PA). As underlying maternal medical conditions and preeclampsia were included in the cases but not the controls, additional analyses were performed in which these cases were excluded. For all analyses, a p value of  $<$ 0.05 was considered significant.

## RESULTS

Ninety-nine cases of singleton intrauterine fetal demise (IUFD) occurred at  $\geq 24$  weeks estimated gestational age at our institution between January 1, 1998 and December 31, 2002 and all medical records were reviewed (fig. 1). Of the 57 cases of unexplained stillbirth, 22 had placental tissue available

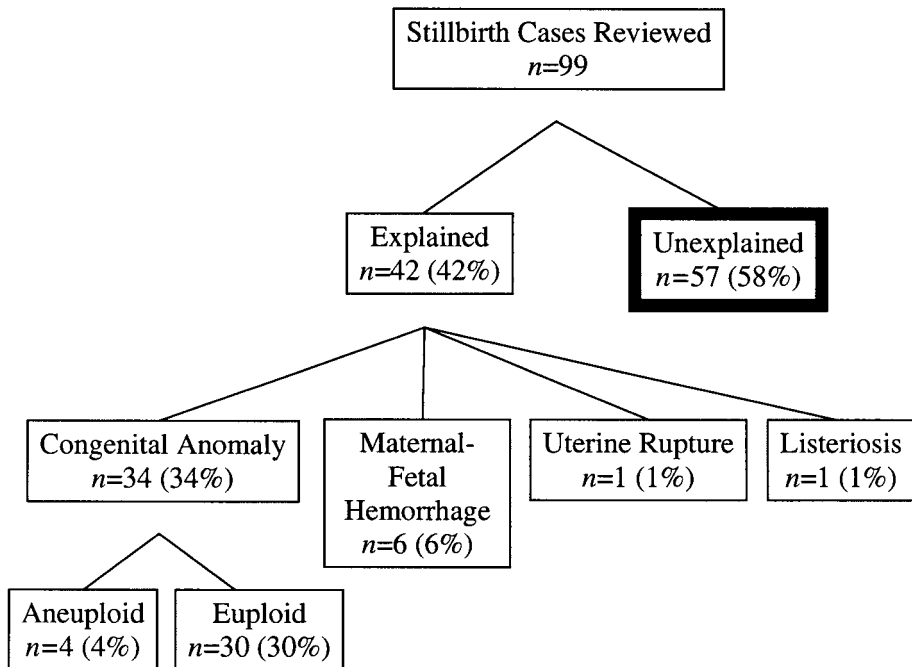


Figure 1. Selection of cases.

for analysis and these comprise the cases in this study set. The controls were matched by gestational age and maternal demographic data are presented in Table 1. Of note, 7 (31.8%) of the 22 women with unexplained stillbirth had underlying medical disease or preeclampsia and 4 (18.2%) of the fetuses were growth restricted as compared to none in the control group.

Placentas of stillborn infants manifested a significantly higher MVD (mean $\pm$ SD 116.6 $\pm$ 46.3, fig. 2g, h) as compared to live-born controls

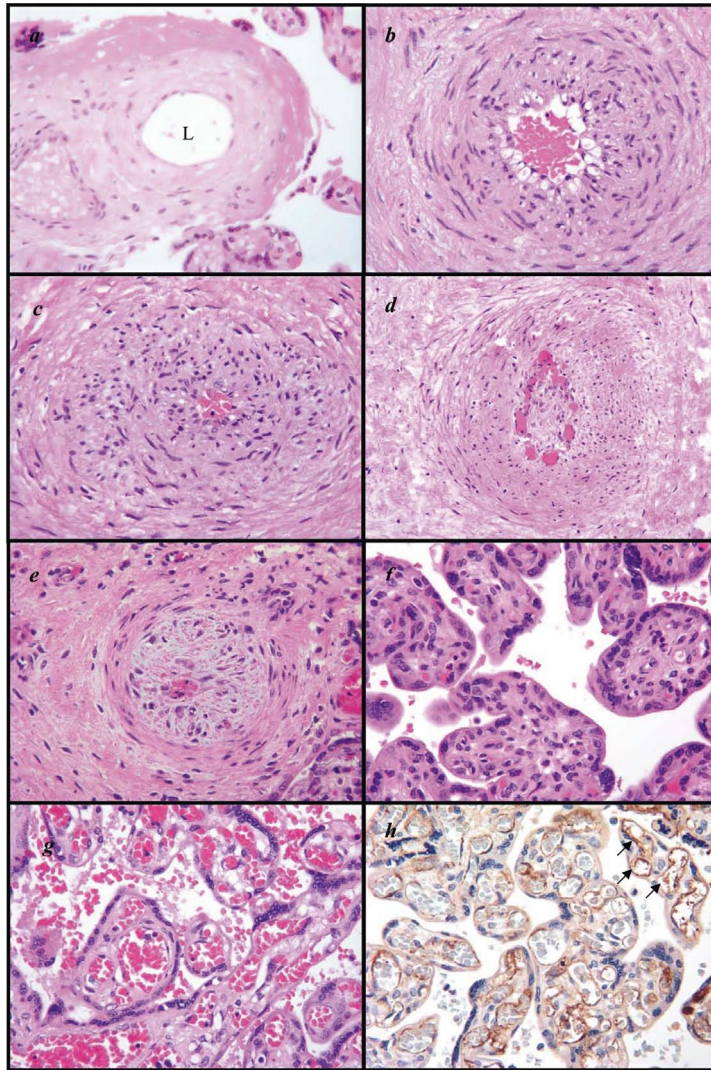
Table 1. Maternal and obstetric characteristics

	Stillbirth n=22	Controls n=44
Maternal age (years, mean+SD)	30.5+6.9	30.3+7.4
Nulliparity	6 (27.0)	21 (47.7)
Chronic hypertension	3 (13.6)	0 (0)
RA/SLE	4 (18.2)	0 (0)
Pregestational diabetes	1 (0.05)	0 (0)
Preeclampsia	5 (23)	0 (0)
Substance abuse	1 (0.05)	0 (0)

All data presented as n (%), unless otherwise indicated. RA/SLE indicates rheumatoid arthritis or systemic lupus erythematosus. Seven of the 22 (31.8%) women with stillbirth had underlying medical conditions or preeclampsia. Three of these 7 women (42.8%) carried multiple diagnoses.

(60.8±13.5,  $p<.001$ ; fig. 2f). Furthermore, 14/22 (63%) of the stillbirth placentas demonstrated heightened angiogenesis ( $\geq 100$  vessels/HPF) as compared to 0/44 (0%) of live-born placentas. The placentas of stillborn infants demonstrated marked vascular remodeling with significantly higher vasculopathy scores [median 3.5, quartile 1 (Q1):3.0, quartile 3 (Q3): 4.5] as compared to live-born placentas (1.4, Q1:1.2, Q3:1.6,  $p<0.001$ ; fig. 2a-e). Vasculopathy was apparent in 10/22 (45%) of placentas of stillbirths as compared to 0/22 (0%) of the normal placentas ( $p<0.001$ ). Finally, alterations in vascular permeability as evidenced by increased RBC extravasation occurred frequently in placentas of stillborn infants [15/22 (68%)] as compared to live-born controls [5/44 (11%),  $p<0.001$ ]. These and additional histologic findings are summarized in Table 2.

To control for the potential impact of underlying maternal medical conditions or preeclampsia on placental vascular development, we excluded these patients and repeated the analyses (tab. 3). In this subset of patients, organizing or occlusive thrombi were no longer significantly more common in the placentas of stillbirths [2/15 (13%)] as compared to controls [0/44 (0%),  $p=0.06$ ]. All other results were not substantively altered. In the total group, MVD and vasculopathy scores were highly correlated (Spearman's rho 0.63,  $p<0.001$ ) as were MVD and RBC extravasation (Spearman's rho 0.71,  $p<0.001$ ).



*Figure 2.* Vasculopathy scores and elevated microvascular density (MVD). a/ Normal vessel (magnification 40 $\times$ , score=1). L denotes vessel lumen. b/ Vessel demonstrating medial hyperplasia (40 $\times$ , score=2). c/ Vessel demonstrating medial and intimal hyperplasia (40 $\times$ , score=3). d/ Vessel demonstrating >50% luminal stenosis (40 $\times$ , score=4). e/ Vessel demonstrating plexogenic lesions with recanalization (40 $\times$ , score=5). f/ Hematoxylin and eosin (H&E) stain demonstrating MVD in the placenta of a live-born infant (40 $\times$ ). g/ H&E stain demonstrating heightened MVD in the placenta of a stillborn infant. h/ Endothelial cell positivity highlighted with von Willebrand factor (VWF) antigen confirms increased MVD (40 $\times$ ) in the placenta of a stillborn infant. Arrows indicate endothelial cell VWF positivity within terminal villi.



*Table 2.* Comparison between stillborn cases and gestational age-matched normally grown live-born controls

	Stillbirth n=22	Controls n=44	p-value
Gestational age (weeks, mean+SD)	33.5+ 5.8	34.0+4.8	NS
MVD (mean+SD)	116.6+ 46.3	60.8+13.5	<0.001
Increased angiogenesis	14 (63)	0 (0)	<0.001
Vasculopathy score (median, Q1,Q3)	3.5 (3.0, 4.5)	1.4 (1.2, 1.6)	<0.001
RBC extravasation	15 (68)	5 (11)	<0.001
Infarction	6 (22)	0 (0)	<0.001
Thrombi	3 (14)	0 (0)	<0.05
Vasculopathy	10 (45)	0 (0)	<0.001

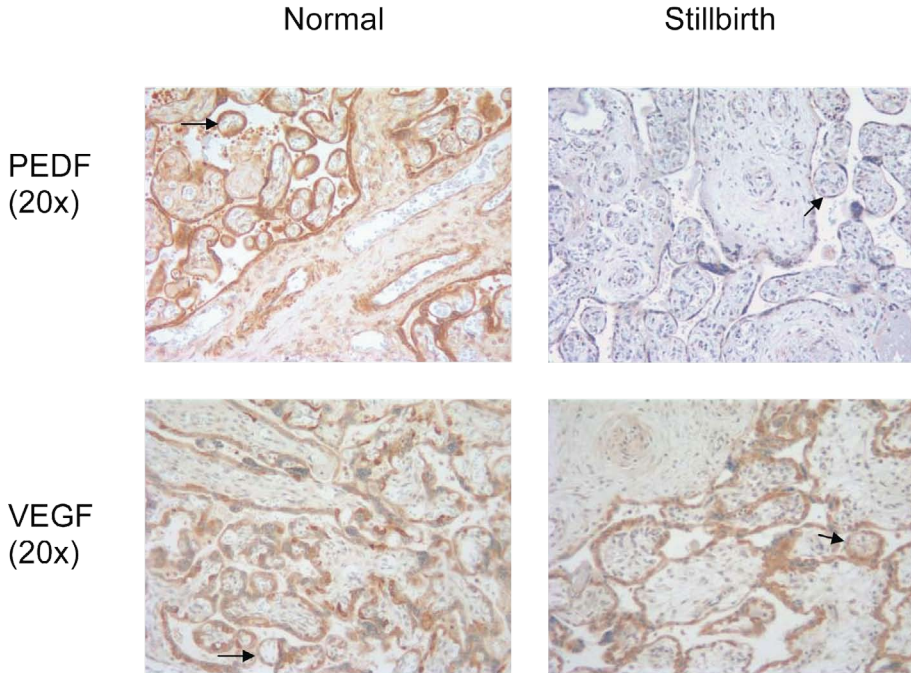
All data presented as n (%) unless otherwise specified. Increased angiogenesis is defined as MVD>100 vessels/HPF. NS=non-significant, MVD=microvascular density, RBC=red blood cell, Q1=quartile 1 cut point, Q3=quartile 3 cut point

*Table 3.* Comparison between stillborn cases without underlying maternal disease or preeclampsia and gestational age-matched normally grown live-born controls

	Stillbirth n=15	Controls n=44	p-value
Gestational age (weeks, mean+SD)	34.9+4.5	34.0+4.8	NS
MVD (mean+SD)	110.0+48.9	60.8+13.5	<0.01
Increased angiogenesis	8 (53)	0 (0)	<0.001
Vasculopathy score (median, Q1,Q3)	4.0 (3.0, 4.5)	1.4 (1.2, 1.6)	<0.001
RBC extravasation	10 (67)	5 (11)	<0.001
Infarction	4 (27)	0 (0)	<0.001
Thrombi	2 (13)	0 (0)	0.06
Vasculopathy	8 (53)	0 (0)	<0.001

All data presented as n (%) unless otherwise specified. Increased angiogenesis is defined as MVD>100 vessels/HPF. NS=non-significant, MVD=microvascular density, RBC=red blood cell, Q1=quartile 1 cut point, Q3=quartile 3 cut point

Placentas of stillborn infants demonstrated significantly lower PEDF expression (fig. 3) in trophoblasts ( $1.7 \pm 0.3$ ) and endothelial cells ( $1.6 \pm 0.4$ ) than in placentas of live-born infants ( $3.6 \pm 0.8$ ,  $p < 0.01$  and  $2.3 \pm 0.4$ ,  $p < 0.01$ , respectively). Surprisingly, VEGF expression was not significantly different in the stillbirth placentas as compared to the controls in either the trophoblasts ( $3.7 \pm 0.4$  vs.  $3.3 \pm 0.7$ , respectively,  $p > 0.05$ ) or the endothelium



*Figure 3.* VEGF and PEDF immunolocalization in placentas of stillborn infants and live-born controls. PEDF expression in trophoblasts, indicated by the intense brown stain (score=4), is notably higher in the placentas of live-born controls as compared to the placentas of stillborn infants (score=1) in which the blue hematoxylin counterstain is most apparent. VEGF expression in trophoblasts, indicated by the brown stain, is similar in placentas of stillborn infants (score=3) as compared to live-born controls (score=3). Arrows point to a representative terminal villus in each panel.

( $2.1 \pm 1$  vs.  $2.2 \pm 0.5$ , respectively,  $p > 0.05$ ; fig. 3). The ratio of VEGF to PEDF expression in the trophoblasts was significantly higher in cases ( $1.9 \pm 0.5$ ) as compared to controls ( $1.1 \pm 0.5$ ,  $p < 0.05$ ). When data were stratified by degree of angiogenesis, PEDF expression was significantly lower in the setting of elevated angiogenesis ( $1.7 \pm 0.2$ ) as compared to lower angiogenesis ( $3.3 \pm 1.1$ ,  $p < 0.01$ ) whereas VEGF expression did not significantly differ ( $3.0 \pm 0.8$  vs.  $3.7 \pm 0.4$ , respectively,  $p > 0.05$ ).

## DISCUSSION

Our study is the first to demonstrate that normal third trimester placentas express a high level of angiogenesis inhibitor PEDF in both trophoblasts and endothelial cells. Even in this small study, loss of PEDF in the placenta highly correlated with stillbirth and increased angiogenesis. We hypothesized that an alteration in the balance of angiogenic mediators which favored VEGF could result in aberrant placental microvasculature and vascular remodeling as demonstrated here. Surprisingly, though, alterations in VEGF expression were not evident in the placentas of stillborn infants. Rather, our findings suggest that PEDF may be a signal for regulating vascular proliferation or quiescence later in development. Loss of PEDF is correlated with these pathologic consequences, suggesting that PEDF may play an important role in regulating normal placental vasculature.

In addition, placentas of infants with unexplained stillbirth demonstrate a marked vasculopathy which was not observed in the placentas of normally-grown live-born infants. These arterial changes are comparable to those described within the vasculature of stem villi in fetal thrombotic vasculopathy (FTV; [18]). Interestingly, we do not appreciate areas of extensive avascular villi as is commonly seen in FTV, instead, we note marked neoangiogenesis. Arterial changes similar to those found in our study had previously been described in growth-restricted infants with abnormal Doppler velocimetry of umbilical artery blood flow [9]. As these abnormal Doppler findings are a marker for fetal hypoxemia [24], the authors suggest that these arterial changes may reflect chronic intrauterine hypoxia. Taken together, it is plausible that intrauterine hypoxia is a potential mechanism by which endothelial and trophoblast expression of PEDF could be down-regulated. However, other pathologic processes including inflammation, endothelial activation, thrombosis or lipid disturbances may contribute to the process of vascular remodeling.

Our findings suggest that alterations in the placental vascular bed may compromise placental function and be associated with adverse fetal outcomes. However, our retrospective study is limited in that we are unable to assess causality. Similarly, our study is limited in that we

are not adequately able to assess the exact timing of either fetal death or the development of the observed pathologic changes. There is some controversy in the literature as to whether some or all of these alterations could have occurred as a result of post-mortem changes. Although luminal abnormalities involving total or partial occlusion of the arteries due to stasis in stem villi can be apparent after 2 days of demise [11], remodeling of the arterial wall that we have described here requires active migration of vascular smooth muscle cells. Vascular remodeling is typically a flow-dependent process [5] making it unlikely that these particular findings occurred after the fetal demise. In addition, a similar vascular phenotype has been reported in the setting of live-born infants who experienced other adverse perinatal outcomes [9, 18] demonstrating that the findings, in these cases, occurred prior to fetal demise.

Our study introduces a new functional mediator in placental vasculature and provides a framework in which to examine placental vascular development in normal and complicated pregnancies. We utilized placental microvascular density to characterize the angiogenic environment within the placental bed and we provide evidence to suggest that these alterations in our study are not associated with increased VEGF expression. Instead, we demonstrated that PEDF, an endogenous inhibitor of angiogenesis, is expressed in normal placentas and its loss is associated with stillbirth and heightened angiogenesis. Subsequent studies which investigate the interactions between angiogenic inhibitor PEDF and inducer VEGF under altered environmental conditions such as intrauterine hypoxia may further elucidate potential mechanisms involved in perinatal disease processes such as intrauterine fetal demise.

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