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Decidual cells from preeclamptic pregnancies demonstrate inadequate decidualization and elevated sFlt1 production

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An abstract of

A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of
Master of Science
in Clinical Science

2019

Abstract

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Uterine stromal cell decidualization of maternal tissue is essential for implantation of and local adaptation to the fetal allograft, as well as growth and maintenance of the placenta in healthy pregnancies. Maternal defects in decidualization have recently been suggested as a possible driver of preeclampsia, a common hypertensive disorder of pregnancy. Despite being responsible for a significant global burden of maternal and infant morbidity and mortality, the cause of the disease remains unknown and treatment is limited to prompt delivery of the placenta and fetus. Preeclamptic (PE) pregnancies demonstrate shallow implantation, inadequate spiral artery remodeling, and elevated levels of the anti-angiogenic protein, soluble fms-like tyrosine kinase-1 (sFlt1). To test whether decidual stromal cells (DSCs) isolated from PE placentas exhibit inadequate re-decidualization and increased expression of sFlt1, DSCs from 7 healthy (NT-DSCs) and 6 PE (PE-DSCs) placentas were treated for 8 days (D8) with cyclic adenosine monophosphate (cAMP) to induce decidualization and levels of decidualization markers prolactin (PRL), insulin-like growth factor binding protein 1 (IGFBP1), vascular endothelial growth factor (VEGF), and sFlt1 were measured at day 0 (D0), D8, and after reversal of treatment. NT-DSCs achieved statistically significant elevations in median PRL (25.72 IQR: [5.78 - 50.04], p=0.0008) and median IGFBP1 expression (and 92.09 [1.79 - 543.10], p=0.005). PE-DSCs increased PRL and IFGBP1 expression to 6.15 [2.30 - 10.73] (p=0.18) and 8.67 [1.64 - 376.10] (p=0.04). NT-DSCs reduced sFlt1 expression at D8 to 0.25 [0.17 - 0.49] (p=0.0021) compared to 0.31 [0.25 - 0.82] (p=0.087) in PE-DSCs. These results show that, when induced to decidualize, PE-DSCs fail to increase expression of decidualization markers to levels achieved by NT-DSCs. sFlt1 expression is higher in PE-DSCs during decidualization, suggesting inadequate suppression during the crucial implantation period. These defects at the maternal fetal interface may lead to the failed spiral artery modification, decreased placental invasion of the uterus, and elevated circulating sFlt1 levels seen in PE pathology.

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ACKNOWLEDGEMENTS

I could never have completed this incredible project without Dr. Rajakumar's mentorship, guidance from MSCR program administrators and educators, or the unwavering support of my family and friends.

This thesis is based in part on a previously published journal article: Sahu MB, Deepak V, Gonzales SK, et al. Decidual cells from women with preeclampsia exhibit inadequate decidualization and reduced sFlt1 suppression. *Pregnancy Hypertension* 2019;15:64-71. doi: 10.1016/j.preghy.2018.11.003.

This work was supported by the Abraham J & Phyllis Katz Foundation. M.S. is supported in part by the National Center for Advancing Translational Sciences of the National Institutes of Health (UL1TR002378).

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INTRODUCTION

Preeclampsia (PE) is a hypertensive disease of pregnancy characterized by widespread vascular dysfunction leading to end-organ damage in the mother by late pregnancy (1, 2). There is currently no reliable means of diagnosing disease before clinical symptoms of tissue damage have appeared, at which time the only definitive treatment is prompt delivery of placenta and fetus. The etiology of preeclampsia remains unknown despite decades of research and well-characterized pathologic findings, including shallow implantation and underdeveloped spiral arteries supplying the placenta (3, 4).

In 2003, an anti-angiogenic molecule was found to be elevated in serum of pregnant women affected by preeclampsia (5). Soluble fms-like tyrosine kinase-1, or sFlt1, is produced at low levels by the placenta during healthy pregnancy and binds to and abstracts circulating vascular endothelial growth factor and placental growth factor. Though it is now accepted that elevated sFlt1 is the pathogenic molecule of pregnancy, many gaps exist in our understanding of what mechanism drives this elevation and its relationship with other pathologic features of preeclampsia (5-9).

The current study aims to better characterize the behavior of preeclamptic maternal decidual cells and their ability to modulate sFlt1 production. This *in vitro* study, using tissue samples collected from normal (NT) and preeclamptic (PE) decidua at time of delivery at Emory University Hospital Midtown, hypothesizes that decidual stromal cells (DSCs) obtained from PE placentas (PE-DSCs) will demonstrate altered decidualization potential as measured by functional molecular markers. Because the

decidualization process plays a regulatory role in sFlt1 production in the healthy endometrium, levels of sFlt1 before and after treatment were also examined.

BACKGROUND

Preeclampsia is a hypertensive disorder of pregnancy that affects 8 million pregnancies annually worldwide and is responsible for an estimated 15% of premature births in the US (1). PE typically occurs after 20 weeks of gestation with the onset of hypertension and widespread vascular dysfunction. If untreated, severe disease leads to fatal end-organ damage in the mother, including stroke, kidney failure, liver rupture, pulmonary edema, and frank eclampsia (10). The only known treatment of the condition once it has developed is the delivery of both the placenta and the fetus, resulting in higher rates of premature births and infant growth restriction (1). PE has a lasting effect on both mother and child: it is associated with a 2-fold increased risk of future cardiovascular disease (CVD) and a 5- to 12-fold increased risk of end-stage renal disease in mothers, and an increased risk of CVD in offspring (11, 12).

Though the etiology of PE is unknown, several mechanisms have been proposed, including immune response to the fetal allograft, endothelial dysfunction, and placental hypoxia (4). Until recently most models focused on the placenta as the primary causal agent in PE development (2). However, recent research has begun to implicate dysfunction before and during early pregnancy at the maternal-fetal interface (MFI). Dysfunction at the MFI has been linked to other adverse pregnancy outcomes, including placental abruption, intrauterine growth restriction, and preterm birth (13, 14). PE placentas typically demonstrate shallow implantation into the uterine myometrium and inadequate spiral artery remodeling. These characteristics have directed investigations into angiogenesis and the behavior of both migratory and receptive cells at the MFI (3).

The degree of placental invasion and implantation into the uterine wall is often reduced in PE placentas, a finding that correlates with the clinical severity of the disease (15). This process is dependent on carefully-orchestrated interactions between invading extravillous trophoblasts and maternal decidual cells at the MFI (16). Endometrial stromal cells (ESCs) of menstruating species cyclically undergo decidualization, during which they change shape and function to secrete factors promoting placental growth and embryo implantation, including insulin-like growth factor binding protein 1 (IGFBP1) and prolactin (PRL) (17-19). The initial penetration and growth of the conceptus during and in the early stages of implantation are dependent on proper reception and maintenance by the transient decidual layer (20). If implantation occurs successfully, decidualization must be sustained by factors produced by the incoming embryo.

Recent studies have begun to identify defects in the decidua of women with past or current PE pregnancies (3). Women who later developed preeclampsia were found to have lower first- and second-trimester circulating levels of IGBFP1, potentially reflecting deficient production by the decidual compartment (21). A 2009 transcriptomic analysis, seeking to determine whether hypoxia-related transcriptional changes could be characterized in first-trimester tissue obtained from women who went on to develop severe preeclampsia, instead demonstrated dysregulation of genes related to decidualization, including IGFBP1 and PRL (22). In 2017, Garrido-Gomez et al., have shown that endometrial stromal cells derived from non-pregnant women with a prior PE pregnancy failed to fully decidualize in vitro and exhibit a global depression in transcription when compared to controls, again suggesting that failed decidualization may contribute to the development of PE (23).

In healthy pregnancies, endovascular trophoblasts also invade the inner third of the uterine myometrium to promote remodeling of the spiral arteries, which serve as primary vasculature of the intervillous space (21, 24). The remodeled vessels are larger in caliber and support the increased oxygen and nutrient demands of the developing pregnancy. In preeclampsia, invasion by endovascular trophoblasts and remodeling of spiral arteries is inadequate, and hypoxic damage to the placenta occurs. As the pregnancy progresses, the damaged placenta secretes pro-inflammatory cytokines, syncytial knots, and anti-angiogenic factors into the maternal circulation, precipitating systemic microvascular derangement (15, 25).

Dysregulated production of the anti-angiogenic soluble fms-like tyrosine kinase-1 (sFlt1) is thought to contribute to the disorganized and incomplete remodeling of spiral arteries observed in PE placentas (5, 7). In healthy pregnancies, sFlt1 is produced in a controlled manner by the placenta (with minor contributions by maternal peripheral blood mononuclear cells and ESCs) to support the creation of arterial networks to supply the conceptus (7, 8). It acts by binding and inactivating pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and placental growth factor (P1GF) (5, 26). sFlt1 is found at much higher circulating levels in women with active PE; these elevated levels are associated with decreased circulating levels of free VEGF and P1GF (27). Soluble flt1 administration to pregnant rats leads to hypertension and large proteinuria; these symptoms can be ameliorated by exogenous VEGF and P1GF supplementation, suggesting that the abstraction of free VEGF and P1GF by soluble Flt1 is key to the clinical presentation of these patients (5). This altered balance between pro- and anti-

angiogenic factors causes generalized systemic endothelial dysfunction as the pregnancy progresses, culminating in end-organ damage by late pregnancy.

In preeclamptic pregnancies, serum sFlt1 levels appear to begin to rise above normal at roughly 20 weeks gestation, though a reliable blood marker to identify disease prior to symptom onset has not yet been introduced into clinical practice (5, 27-29). The timing and mechanism of excess sFlt1 production prior to its measurable elevation in maternal serum is not understood. It is not known whether sFlt1 is a driver of the disorganized and incomplete remodeling of spiral arteries that is characteristic of PE placenta, or if instead an early hypoxic insult to the preeclamptic placenta stimulates sFlt1 secretion. Experiments have shown that hypoxic cytotrophoblasts reduce sFlt1 production when exposed to aspirin, indicating that one contributor to sFlt1 elevation may occur via a COX-1 pathway (30). However, the reduced PO2 observed in first-trimester placental villi is physiologic, rather than hypoxic, suggesting that a different stressor is responsible for early-pregnancy elevations in sFlt1 and subsequent derangement of vascular growth (31). We recently found that sFlt1 production and decidualization are negatively correlated in healthy ESCs, indicating that adequate suppression of this anti-angiogenic protein at the MFI is important for successful implantation of the conceptus, and providing a potential avenue for sFlt1 elevation in the pre- and early-pregnancy period (32).

It follows that a defect in the implantation process may initiate a disruption in uterine-placental interaction, leading to poor development of the placental spiral arteries, hypoxia and interruption of nutrient delivery to the conceptus, local immune disruption, and systemic maternal derangements as seen in the clinical preeclamptic phenotype.

Understanding the effect of such a defect may bridge the gap between initiation of disease and late-stage presentation. However, the relationship between decidualization in elevated sFlt1 in preeclampsia has not yet been characterized. This forms the basis of our exploratory *in vitro* study.

METHODS

Study design

Our overarching hypothesis is that inadequate or sub-total decidualization of maternal cells plays a causal role in the development of preeclampsia. Here, our study outcome is PE and the exposure we are seeking to define in this population is ineffective decidualization. At this time our ability to evaluate decidualization is limited: we cannot currently determine decidual status at time of implantation or early pregnancy. Given this, we set up an experimental protocol to mimic and thereby evaluate the decidualization process in cells obtained from the decidua of delivered placentae. To obtain study tissue, patients were enrolled in a small case-control study. Preeclamptic patients served as cases, and normotensive patients without pathology served as controls.

Our aims are as follows:

1. To describe the baseline production of decidualization markers and sFlt1 production by preeclamptic cells and normotensive (control) cells when not stimulated to decidualize.

2. To determine whether preeclamptic cells decidualize adequately, as measured by upregulation of expression of decidualization marker genes PRL, IGFBP1, and VEGF, when exposed to decidualization treatment.

3. To determine whether preeclamptic cells downregulate sFlt1, as measured by secreted protein levels and gene expression, when exposed to decidualization treatment.

We hypothesize that treated preeclamptic cells will not upregulate production of decidualization markers PRL, IGFBP1, or VEGF, while treated normotensive controls will. Further, we hypothesize that treated preeclamptic cells will not downregulate production of sFlt1, while treated normotensive controls will.

As a proxy for decidualization potential, gene expression of key decidualization factors (PRL, IGFBP1) is used. Our aims evaluate for statistically significant fold change with decidualization treatment above baseline expression levels – in other words, an upregulation of the factors associated with decidualization. This is a relatively imperfect measure of decidualization: there is no “normal” upregulation level we can use to create a cutoff. Instead, we define successful decidualization by the aggregate response to treatment by cells obtained from phenotypically normal pregnancies. More costly methods, such as transcriptomic analysis, are more definitive measures of gene activity, but for the purposes of this exploratory study – designed to detect, but not characterize decidualization abnormalities in preeclamptic cellular behavior – we expect gene expression analysis to be effective to guide further studies.

Enrollment and sample collection

Preeclamptic (n=6) and normotensive (n=7) pregnant women were enrolled in this study through the Emory University Hospital–Midtown (Atlanta, GA) Gynecology-Obstetric clinic. This protocol was approved by the Emory Institutional Review Board and signed informed consent was obtained from all subjects under IRB00078902. PE was defined per ACOG Hypertension Guidelines (33). We included non-smoking English-speaking women above 18 years old and excluded women with a preexisting chronic condition complicating pregnancy (diabetes, pre-gestational or chronic hypertension,

congenital heart disease, autoimmune disorder, diagnosed past or present malignancy), acute complication such as non-pregnancy-related infection, known or suspected fetal anomaly, or known/suspected complication of pregnancy other than PE. Patient characteristics are summarized and shown in **Table 1**.

Samples were collected and dissected in-hospital within 30 minutes of deliver. The decidual layer of each placenta was grossly dissected away and stored in 1x PBS for transportation to the lab. There, decidual tissue was processed for stromal cell preparation as below.

Decidual stromal cell (DSC) isolation and expansion

Per published protocols, dissected tissue was enzymatically digested using 0.1% collagenase and filtered using 100–40 micron sieves (32). Filtrates were subcultured at least twice to enrich the stromal cell population, and resultant primary DSCs were trypsinized and frozen in liquid nitrogen (10% DMSO & 90% FBS). Cells were thawed and grown in DMEM/Ham's F-12 supplemented with L-glutamine containing 10% fetal bovine serum, 1.0 nM sodium pyruvate, 1% nonessential amino acid, 1% penicillin-streptomycin, and 1% amphotericin B (Hyclone). Cells were passaged at 90% confluency and treatment was initiated at passage 4. Equivalent stromal cell lineage was confirmed with vimentin staining using a previously published protocol (34).

In vitro re-decidualization and reversal

In this study we use the term "re-decidualization" to describe *in vitro* re-stimulation of stromal cells which were obtained from the placental decidua. These cells have previously undergone monthly cyclical conversions from spindle-shaped stromal

cells to cobblestone-shaped secretory decidual cells (17, 35). After isolation from the placental tissue and multiple cell culture passages in the absence of stimuli such as cyclic adenosine monophosphate (cAMP), they are de-decidualized both in form and function, but their history of prior decidualization distinguishes them from cells obtained from non-secretory endometrium (34). For re-decidualization and reversal experiments, we used both NT-DSCs (n=7) and PE-DSCs (n=6). Initially cells were seeded in 10 cm cell culture plates, allowed to reach 90% confluence and subjected to re-decidualization by 0.5 mM dibutyryl cAMP (hereafter referred to as cAMP) in decidualization medium (phenol red-free DMEM/high glucose modified (Hyclone) with 5% charcoal-stripped fetal bovine serum, 1% penicillin streptomycin, and 1% amphotericin B (34)). Cyclic AMP was used in isolation without addition of estradiol (E2) or progesterone (P4) as per previously published studies, which showed no appreciable difference in decidualization with this approach (32). Medium was changed every 4 days. Decidualization medium without cAMP was used in untreated cells used for baseline comparison. At the end of 8 days, supernatant was harvested and stored at -20°C, while cells were snap-frozen in liquid nitrogen and stored in -80°C for further analysis.

To measure effect on decidualization reversal, another subset of DSCs was simultaneously decidualized for 8 days, followed by supplementation with regular growth medium (free of cAMP) for another 8 days (RD8). At RD8, the supernatant was harvested and stored at -20°C, while pelleted cells were snap-frozen in liquid nitrogen and stored in -80°C for further analysis.

Quantitative PCR analysis for decidualization markers and sFtl1

Total RNA was isolated from all cells at passage 4 once 80% confluent, prior to exposure to decidualization; on day 8 of decidualization treatment; and on day 8 of reversal using TRIzol (Invitrogen, Carlsbad, CA) per the manufacturer's protocol. cDNA was synthesized from the isolated RNA using Superscript III Reverse Transcriptase (Invitrogen). SYBR green based quantitative PCR (qPCR) analysis was used to analyze the expression of sFlt1, PRL, IGFBP1, and VEGF using SYBR green master (primer sequences shown in **Table 2**) (32). Target gene transcript levels of each sample were normalized to human 60S ribosomal protein L17 (hRPL17) housekeeping gene and the fold change was calculated using the $2^{-\Delta\Delta C(T)}$ method (36). Each sample was run in duplicate for each gene (including housekeeping gene) and the average of both results reported. If duplicates differed from each other by ≥1, results were considered spurious and the plate was repeated.

ELISA estimation of sFlt1

Human VEGF R1/Flt1 Quantikine ELISA kits (R&D Systems, Minneapolis, MN) were used to estimate sFlt1 levels of NT-DSCs (n=6) and PE-DSCs (n=5) in culture medium at day 8 (D8) and reversal day 8 (RD8) according to manufacturer's protocol as described in prior publications (32). In a previous manuscript, we have shown that mRNA levels of PRL, IGFBP1, and VEGF follow protein levels as measured by ELISA (32). We therefore elected to measure and report only RNA levels of these markers for this exploratory investigation.

Statistical plan

As this was a pilot study without prior data on expression levels in preeclamptic decidual cells, no formal power analysis was made to determine sample size. Normally distributed data such as patient characteristics are presented as mean ± standard deviation or as proportion of total group. Skewed data is reported as median [interquartile range, 25th quartile – 75th quartile]. Clinical parameters of PE and NP patients were compared with non-parametric Mann-Whitney *U* test. Gene expression profiles and ELISA protein levels were first analyzed by Kruskal-Wallis *H* test. If a group difference was found significant by Kruskal-Wallis *H* test, paired groups were compared with subsequent Mann-Whitney *U* test. All treated samples were standardized to their own untreated baseline values. p values <0.05 were considered statistically significant. GraphPad PRISM software version 7.0 (GraphPad Software Inc., San Diego, CA) was used for analysis.

RESULTS

Clinical characteristics of study cohort

For this study, placental decidual tissue for stromal cell isolation was collected from a total of 13 women (7 NT, 6 PE). Their clinical characteristics are shown in **Table 1**. Maternal age and maternal body mass index (BMI) were comparable between mothers with PE versus NT. As expected, gestational age and blood pressure at time of delivery are significantly different in NT controls when compared to PE cases. The average gestational age at delivery of NT pregnancies is 39.8±0.6 weeks, compared to 34.0±4.0 weeks in PE pregnancies (p=0.0006). NT pregnancies had average systolic and diastolic blood pressures of 118±8 and 70±5 respectively, while PE pregnancies averaged 155±18 and 92±4 (both p=0.0012). The average neonatal weight was significantly lower in PE pregnancies compared to NT pregnancies (p=0.014), as was placental weight (p=0.048), though it is unclear if this is a result of earlier delivery, PE pathology, or some combination thereof.

Day 0 expression of sFlt1 and decidualization markers in DSCs

To examine DSC production of decidualization markers PRL, IGFBP1, and VEGF after passaging and before treatment, all cell lines were passaged three times in normal cell growth medium. RNA expression levels were then measured and compared in all untreated DSCs (**Figure 1A-B**). NT- and PE-DSCs expressed grossly higher levels of decidualization markers than previously observed in ESCs, as expected of cells originating from the maternal decidua (32). We find no significant difference in expression levels of sFlt1, IGFBP1, VEGF, or PRL between the two groups (**Figure 1C-**

D). There was considerable individual variation in all baseline gene expression values in both PE and NT-DSCs.

Decidualization of placental DSCs by cAMP

Degree of DSC decidualization was assessed at day 8 (D8) of stimulation by comparing fold change in decidualization markers PRL, VEGF, and IGFBP1 (**Figure 2A-C**). Individually, response to re-decidualization stimuli in DSCs was highly variable even within each group (**Figure 2**). Collectively, at D8 of cAMP treatment, NT-DSCs showed a significant increase in PRL mRNA levels, with a median increase of 25.72 [5.78-50.04] fold compared to its baseline untreated level (labeled 'BL', 1.0) (p=0.0008). In PE-DSCs, PRL mRNA rose to a median of 6.15 [2.30-10.73] (p=0.18). Similarly, NT-DSCs showed increased IGFBP1 mRNA levels to a median of 92.09 [1.79-543.10] (p=0.0054) fold compared to baseline (1.0). PE-DSC IGFBP1 mRNA rose a median 8.67-fold [1.64–376.10] (p=0.04). VEGF mRNA levels in NT-DSCs at D8 rose to 2.65 [1.10-5.96] (p=0.12) versus 1.66 [0.74-4.09] ($p>0.9999$) in PE-DSCs compared to respective baseline levels (1.0).

Soluble Flt1 expression in DSCs during decidualization

Previously we had demonstrated a downregulation of sFlt1 with decidualization treatment in ESCs derived from normal cycling non-pregnant women (32). Here we compared patterns of sFlt1 in PE-DSCs throughout re-decidualization to that of NT-DSCs.

Consistent with past findings in ESCs remote of pregnancies, measured sFlt1 mRNA median fold change levels in NT-DSCs at day 8 of cAMP treatment are reduced

to one quarter of D0 levels (0.25 [0.17-0.49], *p*=0.0021) (**Figure 3A**), whereas PE-DSCs did not achieve a statistically significant change from baseline (0.31 [0.25-0.82], *p*=0.087).

ELISA was used to measure levels of secreted sFlt1 protein on D8. Raw levels (pg/ml) were then normalized to baseline levels produced by untreated cells ('BL', standardized to 1.0) to obtain proportionate change (**Figure 3B**). At D8 of treatment, NT-DSCs reduced sFlt1 protein levels to a median 0.10 [0.07-0.30] (*p*=0.0017), a decrease of 0.90 or 90%. In comparison, PE-DSCs sFlt1 production was reduced to 0.19 [0.13-0.43] (*p*=0.029) fold, a decrease of 0.81 or 81%, also shown in **Figure 3B**.

Effect of removal of cAMP on decidualization markers and sFlt1 expression

Levels of decidualization markers during decidualization reversal were assessed at RD8 (**Figure 4A-C**). Reported values were produced by normalizing fold change levels in treated cells to levels produced by untreated cells (baseline, 'BL', standardized to 1.0), as was done in calculation of D8 levels. Upon reversal of decidualization, NT-DSC median fold change in expression of PRL was 1.05 [IQR: 0.48-1.54], IGFBP-1 was 0.80 [0.48-1.21], and VEGF was 0.95 [0.53-1.21]. In PE-DSCs, the fold change levels of PRL, IGFBP-1, and VEGF were 0.63 [0.31-1.13], 0.85 [0.66-1.95], and 1.18 [0.74-1.44], respectively. None of these were statistically significant changes from untreated cell levels.

By RD8, sFtl1 mRNA transcripts levels in NT-DSCs had risen to a 0.51 [0.34-0.86] fold (*p*=0.033) change from baseline, while PE-DSC expression had returned to 0.72 [0.61-1.36]. ELISA levels at RD8 were also measured and compared. When

standardized to baseline production levels, measured sFlt1 levels were 0.78 [0.63-1.09] (p=0.01) in NT-DSCs and 0.77 [0.58-1.69] (p=0.19) in PE-DSCs.

DISCUSSION

In this manuscript, we report two novel findings related to molecular events at the MFI in the development of PE. First, our results indicate that DSCs obtained from PE placentas are unable to adequately respond to decidualization stimuli *in vitro*, consistent with recent results in ESCs published by Garrido-Gomez et al. In addition, we show that when stimulated to re-decidualize these cells only partially downregulate sFlt1, the pathogenic molecule associated with PE. This contrasts with ESCs obtained from non-pregnant women and NT-DSCs, both of which significantly downregulate sFlt1 production when decidualized (23, 32). To our knowledge, this is the first report addressing the relationship between insufficient decidualization and sFlt1 production in stromal cells obtained from the decidua of PE placentas. Our findings add to the growing body of evidence implicating the maternal decidua as an early driver of the PE phenotype. We suggest that a defect in the ability of maternal stromal cells to properly decidualize is responsible for a chain of downstream effects which together lead to aberrant overexpression of sFlt1.

The notion that failed decidualization is a contributor to negative pregnancy outcomes such as PE and fetal growth restriction (FGR) has been considered for some time. In 1986, the Brosens group published evidence of inadequate physiological changes in preeclamptic maternal uterine tissue in response to trophoblastic invasion (37). Soon after, Craven et al., showed that structural changes necessary for implantation occurred in the maternal decidua prior to cellular contact with extravillous cytotrophoblasts (24). Taylor et al., related lower circulating levels of IGFBP1 to reduced trophoblastic invasion of the decidua in to-be-preeclamptic pregnancies in 1996 (21). In a 2002 review it was

suggested that partial or disrupted decidualization could initiate a series of events leading to inadequate implantation (21, 38). These and other contributions to the concept of defective decidualization have recently been summarized in a review by Conrad et al (25).

More recently, Garrido-Gomez et al., published the first experimental evidence showing that ESCs obtained from non-pregnant women with a history of PE have defects in their ability to decidualize (23). They suggested that women who are destined to develop PE have inherent errors in their response to hormonal decidualization signals. Our findings support this hypothesis. In addition to demonstrating deficient decidualization, we also show that IGFBP1 expression by PE-DSCs was 5 times lower than in NT-DSCs – a magnitude of difference which may lead to insufficient trophoblastic invasion (39, 40). This may be an artifact of multiple past dysfunctional decidualization cycles, in which abnormal responses to local decidualization stimuli over many months results in de-synchronous regulation of decidualization hormones. Our repeated passaging of the cells prior to analysis was intended to capture baseline behavior of the cells, removed from local *in vivo* stimuli. Even when passaged in culture conditions three times, PE-DSCs appear to retain a defective decidualization phenotype (32).

One model for PE development proposes that pathologically elevated sFlt1 levels seen in PE may be the result of cross-talk between placental and maternal cells. In 2007, Lockwood et al., described increased expression of sFlt1 by DSCs obtained from first-trimester pregnancies in response to thrombin (6). Placental hypoxia has been shown to result in an over-secretion of sFlt1 into maternal circulation (41). A recent study from our

lab found that placental villous explants caused co-cultured PE peripheral blood mononuclear cells (PBMCs) to overproduce sFlt1(9). These findings suggest cell-placental interactions play a key role in modulating sFlt1 production. We show here that, while NT-DSCs respond to decidualization stimulation by consistently downregulating sFtl1 production, PE-DSCs do not. Taken together, these findings may indicate that inadequate or partial decidualization of maternal DSCs leads to their overexpression of sFlt1, thereby interfering with angiogenic development in the local environment and causing hypoxic stress in the placenta. Discoordination of VEGF and sFlt1 production at this stage may lead to further dysfunction in remodeling of spiral arteries and oxygen delivery to the uteroplacental interface (42). The placenta may then in turn produce higher levels of sFtl1 which is secreted into maternal circulation, contributing to global vascular dysfunction as seen in PE.

It is important to note that our study does not use a single definitive measure of 'successful' decidualization. In general, the *in vivo* environment at time of implantation is not well characterized, and factors such as prolactin, estrogen, and IGBFP1 levels may vary widely even between clinically healthy pregnancies. Decidualization itself is highly regulated by epigenetic factors, a potential route by which conditions such as obesity and diabetes mellitus predispose women to preeclampsia (29, 43-46). Because of these constraints, our aims are somewhat recursive: we define successful decidualization by the expression characteristics observed in cells obtained from healthy pregnancies, rather than by a more objective measure with a straightforward cutoff. Certain measures, including transcriptomics, are more definitive, but these are expensive and were not thought to be feasible at this level of exploration. Nonetheless, the magnitude of

difference in response in preeclamptic cells versus control cells was considered evidence enough to pursue further research into these pathways with larger samples and more definitive methods.

Ultimately, our exploratory study is limited by our use of term tissues only, which are essential to understand behavior of diseased tissue but cannot be used to definitively characterize early causal events. This is a common constraint in the study of adverse pregnancy outcomes. While our passaging and enrichment protocol was designed to describe cellular behavior remote of pregnancy and thereby mitigate impact of gestational age on decidualization potential, it is important to note that the gestational age at time of delivery was significantly earlier in the PE mothers in this study. Future studies should also consider the impact that fetal sex, fetal outcome, differential gestational age of sample tissue, and other patient characteristics have on the behavior of maternal cells in preeclamptic pregnancies. As less invasive measures are being used developed to improve perinatal testing, availability of early-pregnancy study tissue (i.e., by chorionic villous sampling) is limited. This increases the importance of designing exploratory studies to ensure that those investigations that use early-pregnancy tissue are as targeted as possible. We believe building upon the results of this study by describing behavior of early gestational tissue in future studies will be essential to characterizing early pathology at the MFI.

Our results show that decidualization and sFlt1 production are linked and concurrently dysregulated in preeclampsia, supporting a model for disease development that is driven by early dysfunction at the MFI. These findings deserve continued investigation, as better understanding of decidual cell behavior in PE may contribute to

the discovery of a clinically useful biomarker for risk quantification, early diagnosis, and treatment to restore of a healthy phenotype.

FUNDING SOURCES:

This work was supported by the Abraham J & Phyllis Katz Foundation. M.S. is supported in part by the National Center for Advancing Translational Sciences of the National Institutes of Health (UL1TR002378).

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TABLES

Table 1. Clinical characteristics of study population

Variable	Normal Pregnancy (n=7) (mean or number)	Preeclampsia (n=6) (mean or number)
Maternal age (years)	35.7 ± 5.3	29.8 ± 4.3
Race	Caucasian = 4	Caucasian = 1
	Black = 3	Black = 4
	Asian = 0	Asian = 1
Maternal body mass index (kg/m2)	30.6 ± 5.1	35.0 ± 3.3
Systolic blood pressure (mmHg)	118 ± 8	155 ± 18**
Diastolic blood pressure (mmHg)	70 ± 5	92 ± 4**
Gestational age at delivery (weeks)	39.8 ± 0.6	34.0 ± 4.0***
Placental weight (g)	589 ± 77	376 ± 167*
Neonatal weight (g)	3401 ± 210	1989 ± 1001*
Proteinuria	N/A	5/6
Cesarean section	4/7	4/6

Table 1: Clinical characteristics of enrolled subjects in normal pregnancy and preeclamptic groups. Quantitative characteristics compared between groups with Mann-Whitney *U* test and are described as mean ± standard deviation. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Table 2. Primer sequences used for mRNA expression quantification.		
Primer	**Direction**	**Sequence**
hRPL17	Forward	5’-TGAACAAAGCACCTAAGATGCGCC-3’
	Reverse	5’-TGGGCAACCTCCTCTTCTGGTTTA-3’
Flt1	Forward	5’-GTTTAAAAGGCACCCAGCAC-3’
	Reverse	5’-TGTTTGCCATTTCTTCCACA-3’
PRL	Forward	5’-CACCCCCGAAGACAAGGA-3’
	Reverse	5’-CCAGGATCGCAATATGCTGAC-3’
IGFBP1	Forward	5’-TACCTGCCAAACTGCAACAAGA-3’
	Reverse	5’-CCATGGATGTCTCACACTGTCTG-3’
VEGF	Forward	5’-TGCCTGGAAGATTCAGGAGCCT-3’
	Reverse	5’-GAGCAGGAAGAGGATGAGG-3’

Table 1: Primer sequences used for mRNA expression quantification via reverse transcription polymerase chain reaction (RT-PCR) and quantitative polymerase chain reaction (qPCR). Genes studied include housekeeping gene human 60S ribosomal protein L17 (hRPL17), fms-like tyrosine kinase-1 (Flt1), prolactin (PRL), insulin-like growth factor-binding protein 1 (IGFBP1), and vascular endothelial growth factor (VEGF).

FIGURES

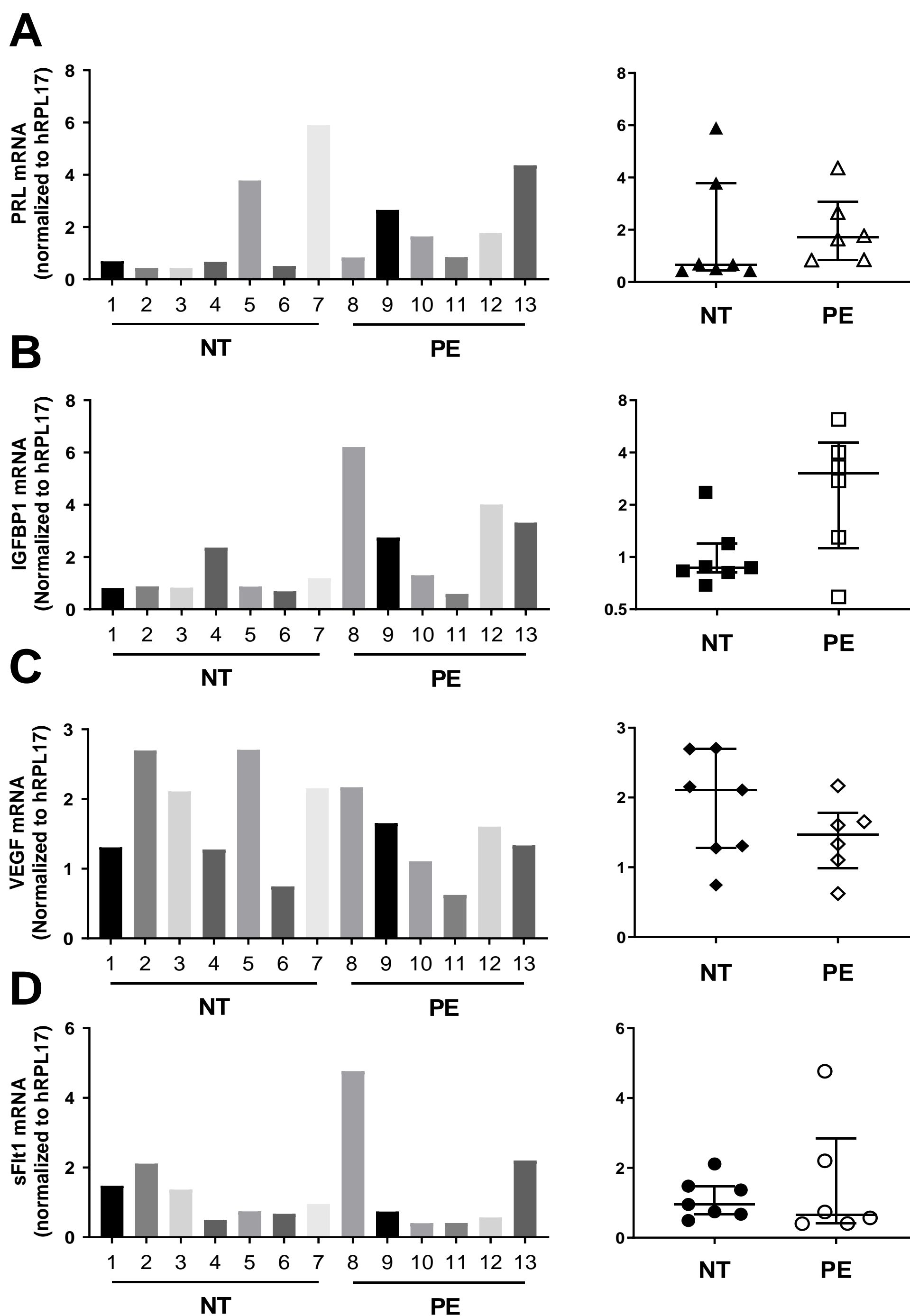

Figure 1: Baseline decidualization marker (A-C) and sFlt1 (D) expression levels of normotensive decidual stromal cells (NT-DSCs) and preeclamptic decidual stromal cells (PE-DSCs) prior to treatment, shown across individual cell lines and as grouped data with overlying mean. Mean expression levels compared using Kruskal-Wallis *H* test.

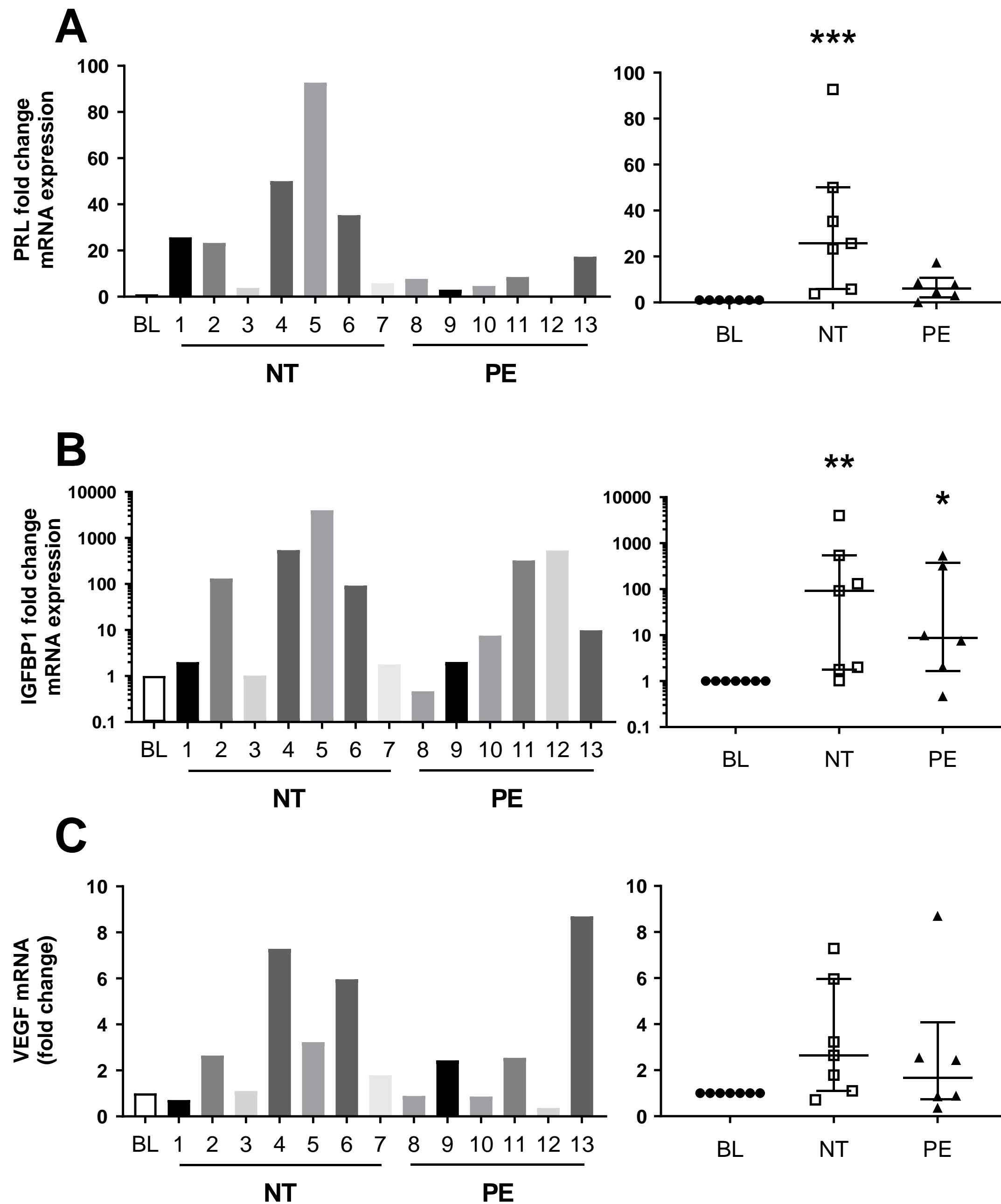

Figure 2: Effect of cyclic adenosine monophosphate (cAMP) treatment on markers of decidualization in normotensive decidual stromal cells (NT-DSCs) and preeclamptic decidual stromal cells (PE-DSCs). Cells were treated for 8 days with 0.5 mM cAMP and expression level was normalized to human 60S ribosomal protein L17 (hRPL17) via the $2^{-\Delta\Delta C(T)}$ method. Fold change was obtained by dividing expression level when treated (treatment day 8, D8) by expression level of untreated duplicates from the same cell line (baseline, labeled 'BL', normalized to 1.0). Fold change in RNA expression of (A) *PRL*, (B) *IGFBP1*, (C) *VEGF* at D8 are shown both individually and as grouped median with overlying interquartile range. n=13 (7 NT-DSCs and 6 PE-DSCs). *p<0.05, **p<0.01, ***p<0.001 using Kruskal Wallis H test.

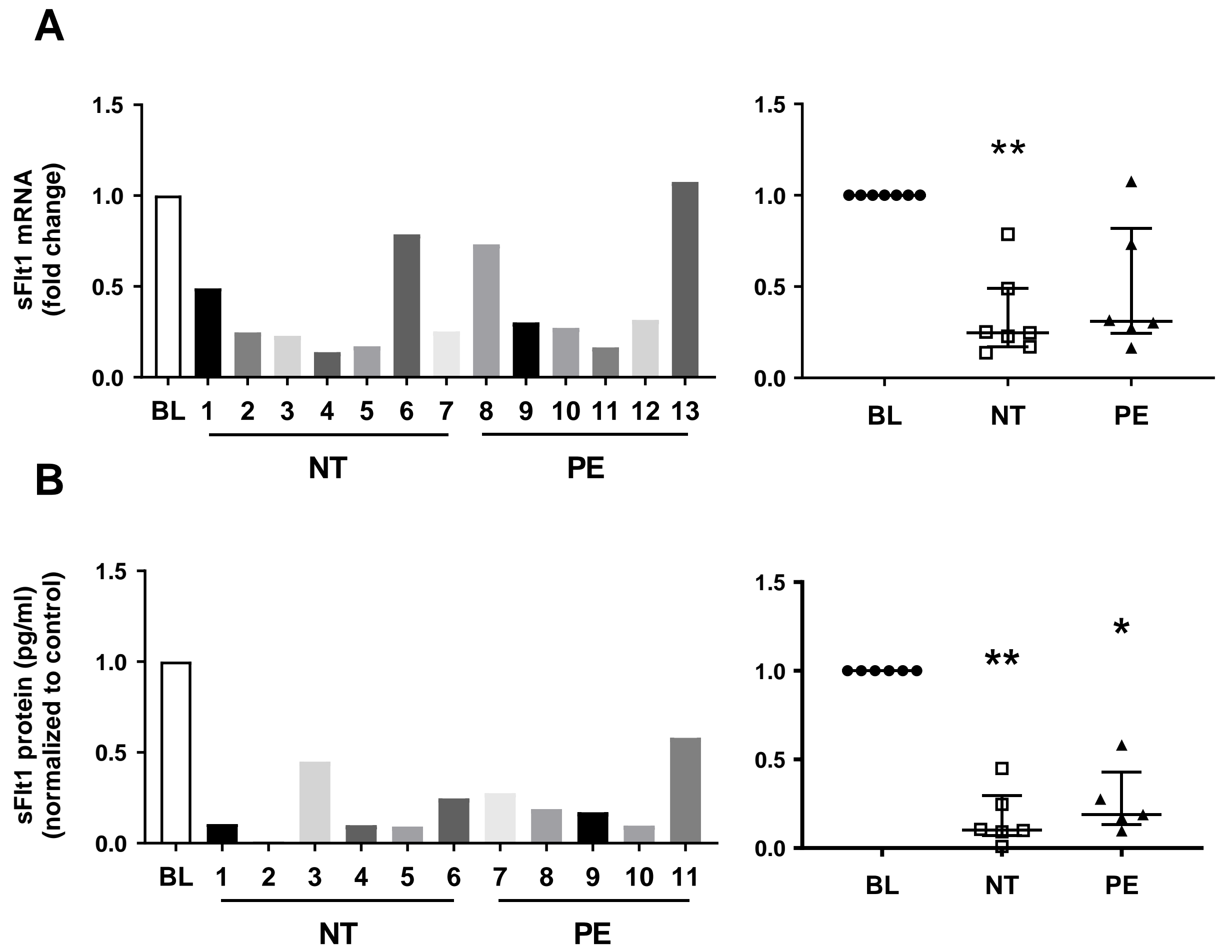

Figure 3: Effect of decidualization treatment on sFlt1 protein and mRNA expression levels in normotensive (NT) and preeclamptic (PE) decidual stromal cells. (A) represents fold changes in mRNA expression and (B) secreted protein quantification of sFlt1 protein levels using ELISA. Individual levels within each cell line and mean levels are shown. Significant changes at D8 are relative to untreated duplicates from the same cell line (baseline, labeled 'BL'). Analysis was conducted using Kruskal Wallis *H* test to compare each group. *$p<0.05$, **$p<0.01$.

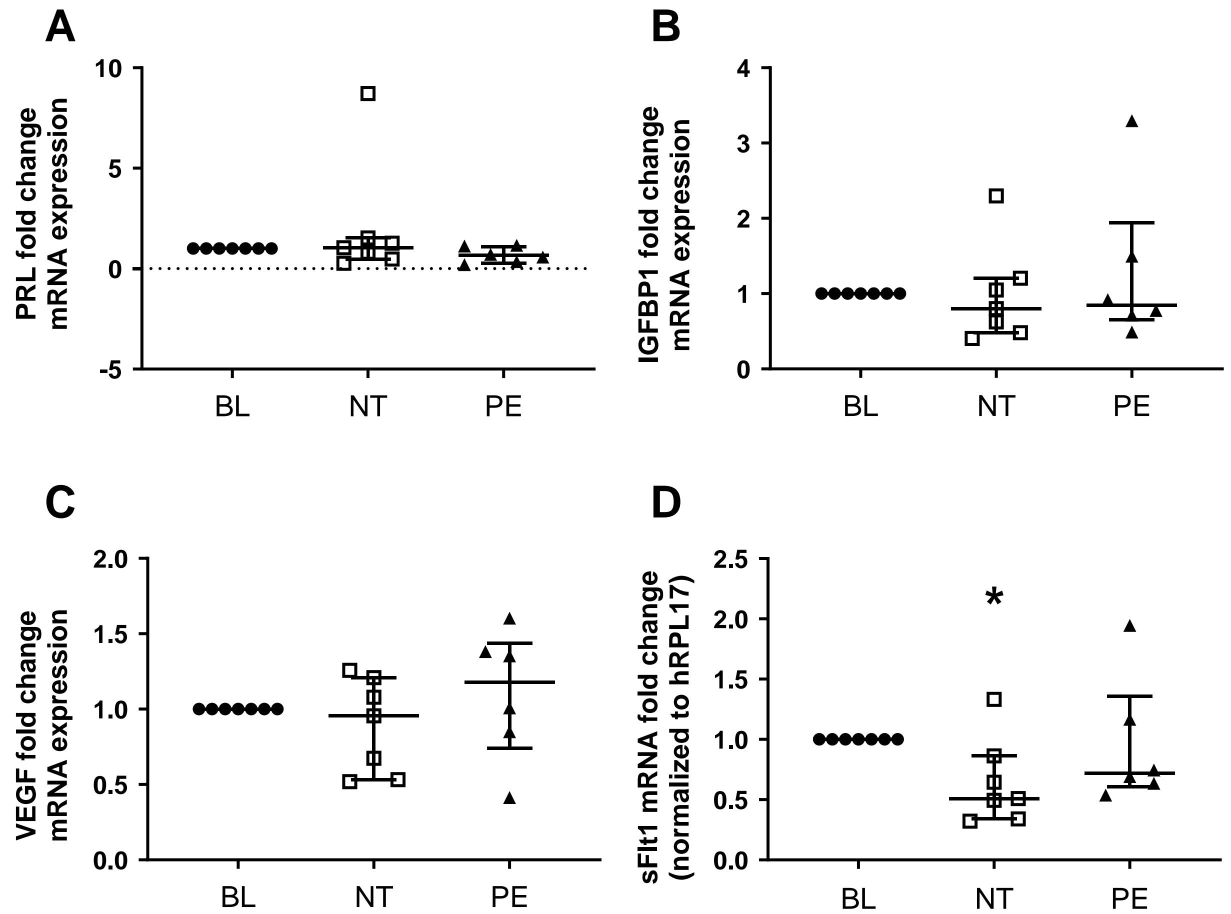

Figure 4: Effect of decidualization treatment reversal on decidualization marker (A-C) and sFlt1 (D) expression levels in normotensive (NT) and preeclamptic (PE) decidual stromal cells. Significant changes at RD8 are relative to untreated duplicates from the same cell line (baseline, labeled 'BL'). Analysis was conducted using Kruskal Wallis H test to compare each group. *p<0.05.