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Placenta-specific protein 1 (PLAC1) expression is significantly down-regulated in preeclampsia *via* a hypoxia-mediated mechanism

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Abstract

Objective: Examine a mechanism of PLAC1 regulation and its potential role in preeclampsia (PE).

Materials and methods: Placental tissue samples and detailed clinical information were obtained through the University of Iowa Maternal Fetal Tissue Bank (IRB# 200910784) from gestational and maternal age-matched control (n=17) and PE affected pregnancies (n=12). PLAC1 and PLAC1 promoter-specific expression was measured using quantitative polymerase chain reaction (qPCR) and differences were assessed *via* the standard ΔΔCt method. In addition, the role of hypoxia in PLAC1 transcription was investigated through the exposure of HTR8/SVneo human trophoblast cells to the hypoxia mimic dimethyloxaloylglycine (DMOG).

Results: PLAC1 expression is seen to be 8.9-fold lower in human placentas affected by preeclampsia in comparison with controls ($p < .05$). Further, this decrease is paralleled by a significantly lower expression of the P2 or proximal PLAC1 promoter ($p < .05$). Expression of mediator complex subunit 1 (MED1), a known hypoxia-sensitive transcription coactivator and PLAC1 effector, is significantly correlated with PLAC 1 expression (r^2=0.607, $p < .001$). These data suggest that PLAC1 expression is significantly down-regulated in preeclampsia at least in part *via* a MED1 hypoxia-mediated mechanism.

Conclusions: We confirm that PLAC1 transcription is suppressed in the placentae of women affected by preeclampsia. We further demonstrate that this suppression is driven through the P2

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Author contributions

E.J.D., M.K.S., and D.A.S. conceived and designed the project. E.J.D., A.W., and S.M.S. performed the experiments. E.J.D. and M.K.S. analyzed and interpreted data. All authors contributed to drafting the manuscript and approved its final version.

Disclosure statement

The authors declare no conflicts of interest, financial or otherwise, in relation to this work.

or proximal PLAC1 promoter. This demonstration led to the identification of the MED1-TRAP cofactor complex as the hypoxia-sensitive driver.

Keywords

Preeclampsia placental dysfunction; placenta-specific protein 1 (PLAC1); mediator complex subunit 1 (MED1); hypoxia; dimethyloxaloylglycine (DMOG)

Introduction

Preeclampsia affects up to 8% of pregnancies world-wide leading to 76,000 maternal and 500,000 infant deaths per year [1,2]. Appropriate prediction and therapeutic interventions for preeclampsia have been limited by complicated pathogenesis which may be initiated by the immune system but leads to clear vascular, renal, angiogenic, and oxidative dysfunction. Central to the onset of preeclampsia is placental dysfunction [3]. Assessment of placental function in real-time, using *in vivo* technologies, such as MRI, is being actively pursued [4–7] but the basic pathogenic processes underlying dysfunction remain unclear.

Placenta-specific protein 1 (PLAC1) is a small (212 amino acid) protein encoded by an intronless gene located at chromosome Xq26, a region containing the hypoxanthine phosphoribosyltransferase 1 (HPRT) gene and known to be linked to placentomegaly and intra-uterine growth restriction (IUGR) [8,9]. Normally, PLAC1 is almost exclusively expressed in trophoblast cells of the placenta where it appears to support the endometrial invasion and placental attachment [10,11]. However, numerous investigators have shown that the PLAC1 gene is switched on in a variety of solid tumors including breast [12] as well as uterus, cervix, and ovaries [13–15]. Following the discovery that there are two distinct PLAC1 promoters, termed P1 (or distal) and P2 (or proximal), it was found that the proximal promoter is responsible for the vast majority of messages in placental tissues while the distal promoter is responsible for the vast majority of message in cancers and fetal tissues [16–19].

PLAC1 expression and its regulation raise the question of its role in other placenta-driven gestational disorders, particularly in preeclampsia. Several studies have reported increased levels of PLAC1 mRNA in peripheral blood of women with preeclampsia [20–23]. To date, however, changes in PLAC1 gene expression in placental tissues of women with preeclampsia have only been reported once [24]. In that study, PLAC1 transcription was shown to be suppressed in severe preeclampsia and that hypoxia plays a role in transcriptional suppression. Here, we present evidence confirming that PLAC1 expression is significantly lower in a mixed ethnicity cohort of women who developed preeclampsia. Moreover, we identify *in vitro* that PLAC1 transcriptional suppression is driven from the P2, or proximal, promoter mediated through the MED1/TRAP transcription mediator complex in response to hypoxia.

Subjects and methods

Cohort assembly

Placental tissues from 12 women diagnosed with preeclampsia and from 17 women having had full-term, uncomplicated, spontaneous labor, vaginal deliveries were obtained from the

large Maternal Fetal Tissue Bank (MFTB) maintained by the Department of Obstetrics and Gynecology of the University of Iowa Carver College of Medicine [25]. Placentas are uniformly processed and frozen according to published protocols [25]. All biosamples and clinical data are coded and supplied to the investigators for analysis under IRB# 200910784 and IRB# 201603838. As shown in Table 1, the assembled groups were well-matched for maternal age, race, ethnicity, mode of delivery, presence of chronic hypertension, and diabetes. In addition, birth outcomes were similar with regard to the sex of the fetus and APGAR scores. Expectedly, the gestational age at delivery (37.2 vs. 38.6 years, $p = .04$) was significantly lower in preeclamptic mothers vs. controls and birth weight (3149 vs. 3645 g, $p = .06$) correspondingly trended lower in children of preeclamptics. Yet, the average gestational age of delivery for both groups was still in the term period.

RNA purification, qPCR, and analysis

Total cellular RNA was prepared from frozen tissue samples using the mirVana miRNA isolation kit (Life Technologies) according to manufacturers' instructions. RNA yield and quality were assessed using a NanoDrop 1000 as well as an Agilent Model 21 Bioanalyzer in the Genome Division of the University of Iowa Institute of Human Genetics.

All mRNA expression levels were determined using fixed starting RNA mass (250 ng each sample). RNAs were reverse transcribed with SuperScript III following the manufacturer's recommendations (Life Technologies). Total PLAC1 as well as P1- (Distal Promoter) and P2- (Proximal Promoter) specific mRNA expression was assessed *via* SYBR Green qPCR assays using the primers sequences shown in Table 2. Also shown in Table 2 are the primer sequences used to determine both MED1 mRNA expression levels and that of the 18S rRNA endogenous control. Individual mRNA expression levels (Ct) were normalized (ΔCt) using an 18S rRNA message. Fold change was calculated *via* the conventional expression $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (\text{Mean } \Delta Ct_{PE}) - (\text{Mean } \Delta Ct_{CTRL})$ and significance was assessed using a standard *t*-test with unequal variances [26–28].

All qPCR assays were carried out on an Applied Biosystems Model 7900HT Real-Time PCR system in the Genomics Division of the University of Iowa Institute of Human Genetics.

Cell culture and DMOG treatment and analysis

HTR8/SVneo trophoblast cells (ATCC, CRL-3271) were grown under optimal conditions in RPMI-1640 media supplemented with 10% Fetal Bovine Serum (Gibco) and 1% antibiotic (Pen-Strep). Six well plates were seeded with 10^5 cells per well in 3 ml of fresh RPMI-1640 and grown for 24 h. After incubation, the media was replaced with fresh RPMI-1640 media in which 1 M dimethyloxaloylglycine (DMOG, TOCRIS Bioscience) was added to final concentrations of 0.5, 1.0, and 2.0 mM. Cells were then grown for an additional 24 h before harvesting and RNA purification. All cell treatments were done in triplicate with controls composed of HTR8/SVneo cells in unaltered RPMI-1640 media. SYBR Green qPCR assays for PLAC1 and MED1 were carried out on HTR8/SVneo RNAs as described above for placental tissues. Fold change for MED1 and PLAC1 for control vs. DMOG treated cells was calculated as above. The dose-response relationship between DMOG concentration

and Fold Change was evaluated using ANOVA with a Bonferroni corrected $p = .016$ for statistical significance.

Results

PLAC1 mRNA expression in placental tissues of women with preeclampsia as compared with placental tissues of women experiencing an uncomplicated full-term vaginal delivery is shown in Figure 1. As can be seen, PLAC1 expression in placental tissues of women who developed preeclampsia is 8.9-fold lower than it is among placentas from women who did not develop preeclampsia and this decrease is statistically significant ($p = .041$). Further, the relative expression of PLAC1 message attributable to the proximal promoter (P2) is 16.8-fold lower among women who develop preeclampsia ($p = .043$) while that attributable to the distal promoter (P1) is 6.9-fold lower ($p = .012$). This suggests that the vast majority of the decrease in total PLAC1 message in the placentas of women who develop preeclampsia is due to the decrease in proximal promoter-initiated transcription.

We also investigated potential mechanisms influencing this differential promoter-initiated transcription. Mouillet et al. [29] demonstrated that hypoxia regulates the transcription coactivator MED1. One of the known direct clients of MED1 is PLAC1 [29,30]. We found that MED1 is under-expressed in the placental tissues of women with preeclampsia by nearly 4-fold (−3.7-fold) compared with the control placental tissues. Moreover, using 18S rRNA normalized MED1 and PLAC1 expression (ΔCt) from all 29 placental tissues returned a highly significant correlation (Figure 2, $r^2 = 0.607$; $p < .01$).

To further examine the relationship between hypoxia, MED1, and PLAC1, HTR8/SVneo human trophoblast cells were exposed to increasing concentrations of dimethyloxalylglycine (DMOG), a hypoxia emulator that activates the hypoxia-inducible factor (HIF) system responsible for molecular oxygen sensing [31]. Exposure of HTR8/SVneo cells to increasing DMOG concentrations produced a significant dose-dependent decrease in PLAC1 expression (Figure 3). The HTR8 cells also had a correspondingly significant dose-dependent decrease in the expression of MED1 (Figure 3). The high correlation between normalized MED1 and normalized PLAC1 expression seen in the placental tissues was observed in the HTR8/SVneo cells to an even greater degree ($r^2 = 0.92$, $p < .05$).

Discussion

Data presented here support a clear negative relationship between preeclampsia and transcription of placenta-specific protein 1 (PLAC1) in placental tissues. We also show that this decreased expression of PLAC1 is driven by the P2, or proximal, promoter. Further, the mechanism underlying these changes can be explained, at least in part, by a hypoxia-driven mechanism operating through the ME1/TRAP mediator transcription coactivator complex.

Koslowski et al. [18] demonstrated that C/EBPβ2 is important in regulating PLAC1 transcription and ChiP-Seq data reported in the UCSC Browser (https://genome.ucsc.edu/) confirms the presence of C/EBPβ binding sites in both PLAC1 promoters. We suggest that the link between C/EBPβ2 and PLAC1 that trans-activates the latter is the mediator complex protein MED1 which has been shown to bind C/EBPβ (Figure 4) [32]. Mouillet et

al. [33] propose that hypoxia down-regulates MED1 which, in turn, down-regulates PLAC1 transcription. Thus, our current data, in particular, the highly correlated dose-dependent response of PLAC1 and MED1 to the hypoxia-mimetic DMOG in HTR8/SVneo trophoblast cells, provides substantial initial support for a model in which MED1 binds C/EBPβ2 and trans-activates PLAC1 expression. Moreover, it is the hypoxia sensitivity of MED1 that is driving PLAC1 down-regulation in preeclampsia. Of course, such a mechanism must be more thoroughly explored both *in vitro* and *in vivo.*

Extensive genomics analysis of the PLAC1 gene in twenty-five placental mammals and four marsupial and monotreme species demonstrated that this gene appeared soon after the emergence of placental mammals some 165 million years ago and that it has been under strong purifying selection since its' emergence [34]. Given the tight genomic control on this gene and because PLAC1 is normally almost exclusively expressed in trophoblast cells of the placenta where it appears to promote endometrial invasion and placental attachment [10,11], the consensus view is that PLAC1 is an essential element in the establishment and maintenance of a healthy placenta [8,17,35]. Thus, clinically, PLAC1 represents a novel potential circulating marker for placental health. PLAC1 mRNA levels in the blood are increased in preeclampsia compared with controls. The reason for this is unclear at present but it has been suggested that apoptotic changes in placental villous trophoblasts could be the source [20,21]. Regardless, we suggest that decreased PLAC1 expression in placental tissues coupled with increased mRNA levels in peripheral blood in preeclampsia is yet another marker of compromised placental health in this disorder.

The association of PLAC1 activation by hypoxia also identifies it as a novel pathway for potential targeting in the therapeutics of preeclampsia. This finding is reinforced by the strengths of our study. We used a prospectively enrolled cohort of samples in which women were enrolled early in the pregnancy without regard to disease status. We did not introduce bias by selecting which women to enroll. Although our cohort came from a relatively homogenous population, added to the report by Wan et al. [24] whose cohort was exclusively Asian, there is sufficient ethnic diversity to generalize the results of both studies. This can serve to apply our findings to all cases of preeclampsia, such as those that occur very early in gestation, and to a larger, more diverse population of women.

Conclusions

Placenta-specific protein 1 (PLAC1) has been linked to a variety of gestational disorders including preeclampsia. Our study strengthens the implication of PLAC1 in the pathogenesis of preeclampsia as well as a mechanistic link to hypoxia through the mediator complex MED1. This knowledge may be useful in determining effective interventions that will ultimately lessen or eliminate preeclampsia.

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Data availability statement

Data supporting the findings reported in this study are available from EJD upon reasonable request.

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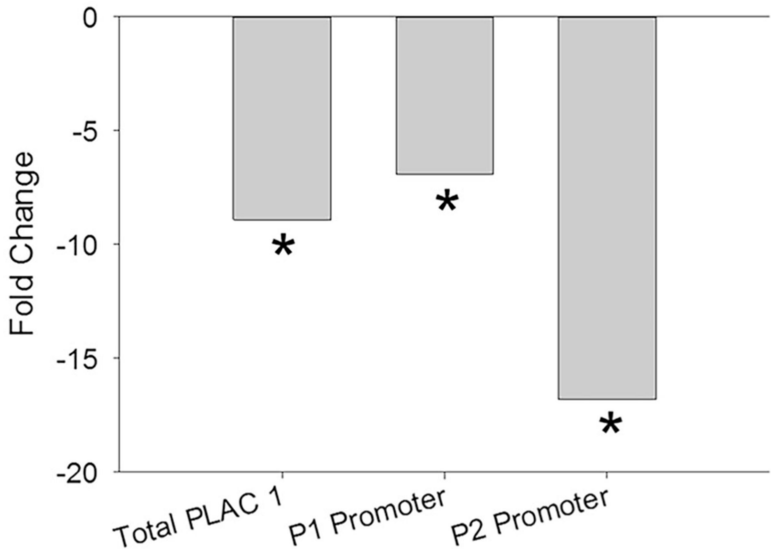

Figure 1.

Expression of total PLAC1 message in placentas from preeclampsia affected pregnancies as compared with controls. Also shown is the expression of both P1- and P2-specific promoter-driven transcription from the same samples.*$p < .05$.

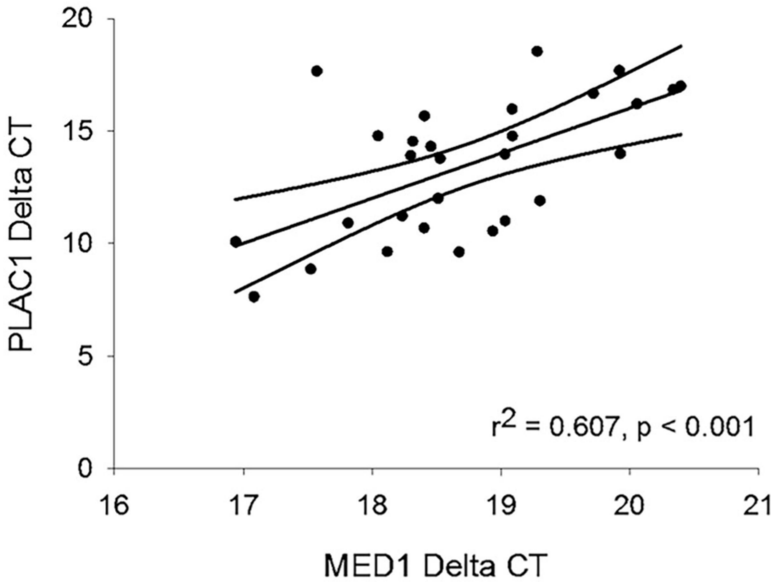

Figure 2.

Normalized PLAC1 and MED1 expression in placental tissues is significantly correlated (r^2 = 0.607, $p < .001$, $df = 28$).

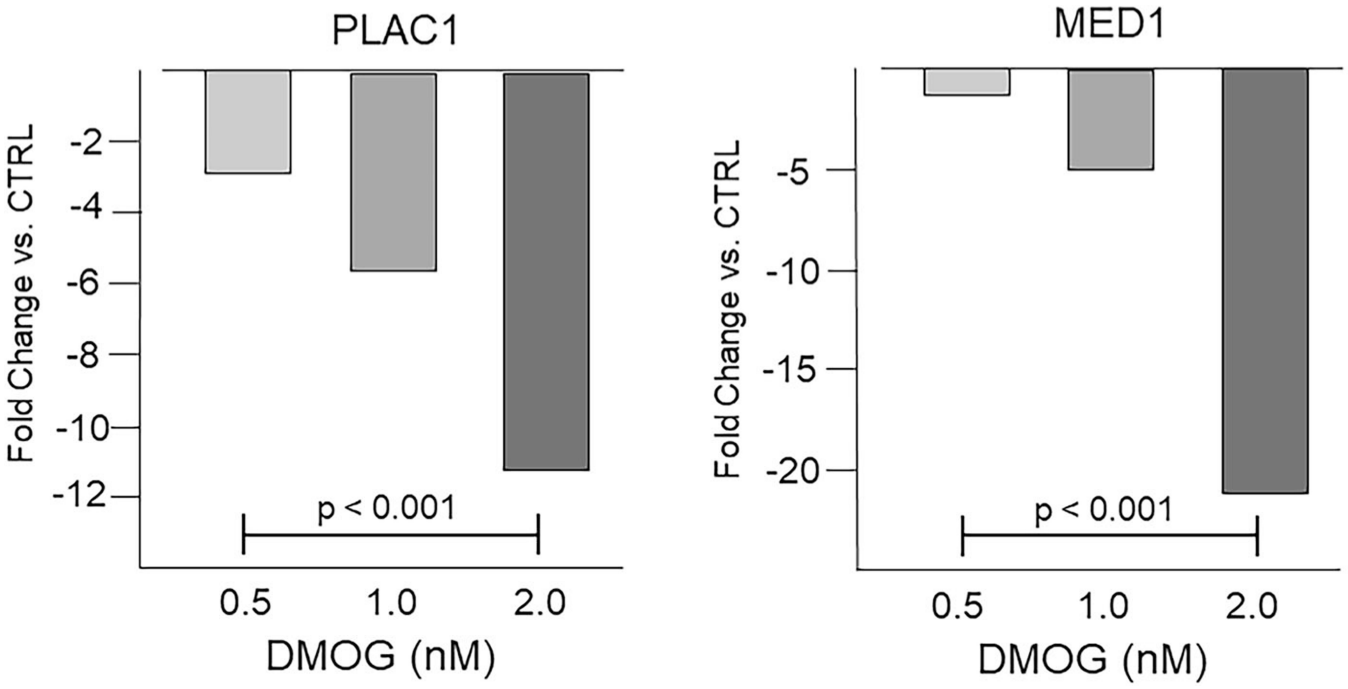

Figure 3.

Changes in PLAC1 (left) and MED1 (right) expression in HTR8/SVneo trophoblast cells in response to dimethyloxaloylglycine (DMOG) exposure. Fold change is presented in relation to untreated cells and statistical significance refers to the dose-response over all three dosing levels.

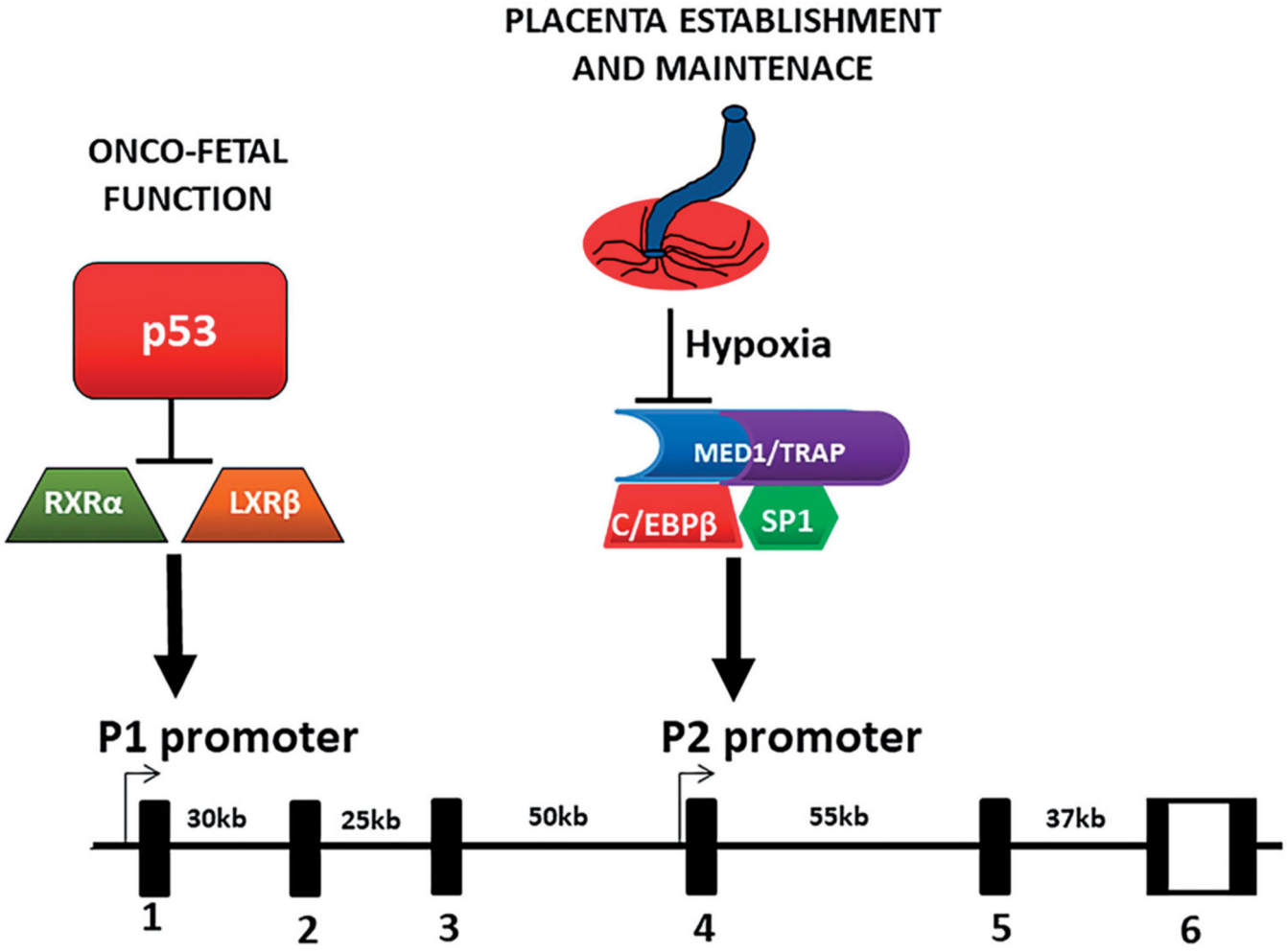

Figure 4.

A model of PLAC1 expression. Message is transcribed from two separate promoters, P1 and P2. PLAC1 message in cancers is primarily driven by the P1 promoter *via* the transcription factors RXRα and LXRβ and is regulated, in part, by the p53 tumor suppressor [36]. PLAC1 message in the placenta is primarily driven by the P2 promoter *via* the transcription factor C/EBPβ which is regulated, in part, by the MED1/TRAP transcription mediator complex..

Table 1.

Clinical features of the patients and controls used in this study.

Character	Controls (*n* = 17)	Preeclamptic (*n* = 12)	*p*-Value
Race/ethnicity			
White	13 (76.5%)	11 (91.6%)	.50 *
Black	1 (5.9%)	1 (8.3%)	
Asian	2 (11.8%)		
Native American	1 (5.9%)		
Maternal age at delivery	28.9 years	32.6 years	.11 #
Gestational age at delivery	38.6 weeks	37.2 weeks	.04 #
Mode of delivery Vaginal Cesarean	 (47.1%) (52.9%)	 5 (41.7%) 7 (58.3%)	 .90 *
Fetal gender Female Male	 11 (64.7%) 6 (35.3%)	 6 (50.0%) 6 (50.0%)	 .71 *
Maternal chronic hypertension	2 (11.8%)	3 (25.0%)	.62 *
Diabetes			
Type I	1 (5.9%)		.67 *
Type II	1 (5.9%)		
Gestational	2 (11.8%)	2 (16.7%)	
Fetal birthweight (g)	3645 ± 587	3149 ± 713	.06 #
APGAR (median) 1 min	 8	 9	 .523 ¶
5 min	8	9	.523 ¶

All clinical information is logged into the Maternal–Fetal Tissue Bank (MFTB) and linked *via* de-identified tags in compliance with University of Iowa IRB regulations.

* Fisher's exact test.

Student's t-test.

¶ Mann–Whitney Rank Sum Test.

Table 2.

Primer sequences were used in the SYBR Green qPCR assays reported in this study.

Target	Amplicon	Sequence
Total PLAC1	232 bp	Forward: 5′-CACCAGTGAGCACAAAGCCACATT-3′ Reverse: 5′-CCATGAACCAGTCTATGGAG-3′
P1-specific	370 bp	Forward: 5′-AAACTTACACGAGGAGTCTGTC-3 Reverse: 5′-CTGTGACCATGAACCAGTCTAT-3
P2-specific	361 bp	Forward: 5′-GTGACTCTCCTATGAAGGTAAAGG-3′ Reverse: 5′-CCATGAACCAGTCTATGGAG-3′
MED1	319 bp	Forward: 5′-CAGACACCAAGTGGCCTATAAC-3′ Reverse: 5′-GGTTTCAGCTTTCCTCCGAATA-3′
18S rRNA	104 bp	Forward: 5′-AACTTTCGATGGTAGTCGCCG-3′ Reverse: 5′-CCTTGGATGTGGTAGCCGTTT-3′