Original Article

Upregulation of ENDOU in cytotrophoblasts from placenta complicated with preeclampsia and fetal growth restriction

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Placental hypoplasia is associated with the pathophysiology of fetal growth restriction and preeclampsia. The placenta consists of differentiated trophoblasts, including cytotrophoblasts, syncytiotrophoblasts, and extravillous trophoblasts. Cytotrophoblasts are thought to have stem-like characteristics and the ability to differentiate into syncytiotrophoblasts and extravillous trophoblasts. However, it is poorly understood whether isolated cytotrophoblasts derived from hypoplastic placentas have specific features compared with those in normal placentas. This study aimed to determine the features of cytotrophoblasts in hypoplastic placentas. Differentially expressed proteins between isolated cytotrophoblasts from hypoplastic placenta with fetal growth restriction and those from the normal placenta were determined by liquid chromatography-tandem mass spectrometry. Among 6,802 proteins, 1,253 and 2,129 proteins were more than 2-fold upregulated and downregulated, respectively. Among them, ENDOU (endonuclease, poly(U) specific), which has high homology with the coronavirus endoribonuclease nonstructural protein 15 (Nsp15), showed a significantly increased expression in cytotrophoblasts from the placenta with fetal growth restriction related to preeclampsia compared with those in normal control placenta. These results provide insight into the pathological mechanisms of placental hypoplasia and additional information on preeclamptic symptoms in cases of SARS-CoV-2 infected placenta, although further investigation is needed.

***Key Words*: LC-MS/MS, preeclampsia, cytotrophoblast, placenta**

Fetal growth restriction (FGR), to date, has no effective therapy *in utero*, meaning treatment must begin after birth in the neonatal intensive care unit, and there have been few improvements in prognosis.[1] Establishing a treatment to improve fetal growth *in utero* is desirable.[2] However, the causes of FGR are variable, including genetic factors, congenital infection, umbilical cord anomaly, placental hypoplasia, and other possibly unknown factors. Recently, pro-inflammatory dietary during pregnancy has also been reported to cause FGR.[3] Among these causes, FGR caused by placental hypoplasia might be improved by antenatal intervention, including improvement of poor placentation because the fetus itself is thought to be normal in those cases. Placental hypoplasia is also a major cause of preeclampsia (PE), which is accompanied by maternal hypertension complicated by organ damage, which is often further complicated by FGR.

Cytotrophoblasts are thought to proliferate and differentiate into distinct types of trophoblasts, syncytiotrophoblasts, and extravillous trophoblasts (EVTs) in the human placenta.[4] Syncytiotrophoblasts mainly produce various humoral factors for maintaining pregnancy, and exchange gases, nutrients, and waste products between the fetus and mother in the intervillous space. EVT invades the maternal uterine decidua and myometrium to remodel the spiral arteries for their dilation, which is involved in maintaining abundant blood flow in the intervillous space. Failure of EVT invasion into the uterus leads to placental hypoplasia, which causes FGR or PE.[5] These findings suggest that cytotrophoblasts could have different characteristics in hypoplastic placentas compared to those in normal placentas.[2] Identifying these characteristics could clarify the pathophysiology of the hypoplastic placenta and develop new *in utero* therapies.

In the present study, we investigated the proteomic profiles of cytotrophoblasts isolated from hypoplastic placentas with FGR and compared them with those from the placentas of healthy pregnant women. Among the differentially expressed proteins (DEPs), we also examined the expression of three proteins relevant to FGR related to PE and compared them with those in healthy control placentas.

Materials and Methods

The study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine (approval number: 2008-0648, 2016-0001, 2017-0302). Study participants comprised pregnant women who had singleton deliveries via cesarean section at Nagoya University Hospital. The control group included women with neonates who had appropriate birth weights for their gestational age. FGR was diagnosed when the estimated fetal weight (EFW) was below −1.5, standard deviation (SD) for their gestational age. FGR cases were excluded as follows: the presence of fetal factors that may restrict growth, including genetic diseases such as trisomy 18 and congenital infections such as cytomegalovirus infection. PE was defined as the presence of hypertension (systolic blood pressure ≥140

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Table 1. Clinical characteristics of the study population

Isolated cytotrophoblast	Control (n = 4)	FGR (n = 5)	p value
Maternal age (years)	36.3 ± 3.6	32.8 ± 6.6	0.357
Nulliparity	1 (25.0%)	2 (40.0%)	1.000
Maternal BMI at delivery	27.0 ± 4.0	26.1 ± 5.7	0.784
Gestational age at birth (weeks)	38.6 ± 0.5	33.7 ± 5.4	0.111
Birth weight (g)	3,323.5 ± 336.6	1,349.4 ± 624.0	<0.001
Placental weight (g)	481.8 ± 110.9	236.6 ± 138.8	0.022
Male newborns	0 (0.0%)	3 (33.3%)	0.167
Immunohistochemistry	Control (n = 13)	PE-FGR (n = 13)	p value
Maternal age (years)	33.3 ± 6.4	33.2 ± 3.6	0.941
Nulliparity	7 (53.9%)	11 (84.6%)	0.202
Maternal BMI at delivery†	25.4 ± 4.8	23.7 ± 2.4§	0.287
Systolic blood pressure (mmHg)†	118.2 ± 15.8	168.5 ± 14.1	<0.001
Diastolic blood pressure (mmHg)†	75.5 ± 12.2	104.9 ± 8.1	<0.001
Gestational age at birth (weeks)‡	37.7 [32.7–38.7]	34.1 [27.1–36.4]	<0.001
Birth weight (g)†	2,803.4 ± 555.2	1,387.5 ± 473.8	<0.001
Placental weight (g)†	470.8 ± 98.8	276.2 ± 101.1	<0.001
Male newborns	8 (61.5%)	9 (69.2%)	1.000

Systolic blood pressures are indicated as the highest value in hospitals for childbirth, and diastolic blood pressures are indicated as the values measured at the same time point. Continuous variables are presented as mean ± SD and p values were calculated by Weltch t test, †Student t test or ‡ as mean [range] by Wilcoxon rank-sum test. Categorical variables are presented as number (percentage) and p values were calculated by Fisher's exact test. §n = 12 as data was absent.

mmHg or diastolic blood pressure ≥90 mmHg) and proteinuria, other maternal organ dysfunction, or uteroplacental dysfunction after 20 weeks of gestation, which are all criteria consistent with the international definition.[6] Inclusion criteria were strictly defined to reduce clinical variance in immunohistochemistry (IHC) study. Only "FGR related to PE" from FGR cases were included in the PE-FGR group. Table 1 shows the clinical information of the study participants, who provided samples for mass spectrometry analysis and IHC.

Human cytotrophoblast isolation. This was established as previously reported.[7,8] Human villous cytotrophoblasts were isolated from the placenta. Briefly, chorionic villous tissues were dissected from the chorionic plate, basal plate, and main vessels. Minced villous tissues (20–30 g) were digested in 100 ml Hank's balanced salt solution (HBSS) supplemented with 0.125% trypsin (Thermo Fisher Scientific, Waltham, MA), 0.5 mg/ml DNase type1, 250 mg Dispase 2 (Sigma-Aldrich, St. Louis, MO), 0.1 mM $CaCl_2$ and 0.8 mM $MgSO_4$ for 20 min at 37°C. The cell suspension was collected, and 5 ml of fetal bovine serum (FBS) (immobilized) was added to inactivate trypsin and was layered over Percoll (GE Healthcare, Chicago, IL) density gradient with four layers (50%, 45%, 25%, and 20% Percoll layers). After centrifugation, the floating cells between the 25% and 45% Percoll layers were collected. The collected cells (1×10^7 cells) were incubated with 0.2 μg/μl of the anti-human leukocyte antigen (HLA)-ABC antibody (Mouse W6/32, eBioscience 14-9983-82; Thermo Fisher Scientific) for 15 min at 4°C, followed by further incubation with 0.2 μg/μl anti-mouse-IgG antibody microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 4°C. After being passed through a 100 μm nylon filter, HLA-ABC negative cells were collected using a Mini MACS™ separator (Miltenyi Biotec) and stored at −20°C until use. Some purified cytotrophoblasts were cultured and confirmed to undergo spontaneous cell fusion to differentiate into syncytiotrophoblasts, as previously reported.[8]

Sample preparation and mass spectrometry analysis. The sample preparation was performed per a previous report.[9] Briefly, cytotrophoblasts were lysed in lysis buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl], cOmplete™ EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich) and sonicated for 5 s on ice. The supernatant fraction was collected by centrifugation for 30 min at 13,000 rpm and sonicated for 5 s. Protein concentrations were evaluated using the bicinchoninic acid assay (Pierce™ BCA Protein Assay kit; Thermo Fisher Scientific) in accordance with the manufacturer's instructions.

The protein samples were digested with trypsin and subjected to mass spectrometry analysis using an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Fisher Scientific) in combination with an UltiMate™ 3000 RSLCnano LC system (Dionex Co., Amsterdam, The Netherlands). The samples were then injected into an UltiMate3000 RSLCnano LC system equipped with a nano HPLC capillary column, 75 μm in diameter and 150 mm in length (Nikkyo Technos Co., Tokyo, Japan). Multiple MS/MS spectra were submitted to the Mascot program, ver. 2.6.0 (Matrix Science Inc., Boston, MA) for the MS/MS ion search. Statistically significant differences in abundance between the PE-FGR and control groups were calculated using a two-tailed t test using Perseus software ver. 1.6.15.0 (http://www.perseus-framework.org/). For the volcano plots, a t test was performed, adjusting S0 to 0.1, the number of randomizations to 250, and the false discovery rate (FDR) to 0.05.[10] DEPs were defined as those proteins showing a 2-fold or greater change in the ratio of average values in the PE-FGR group than the control group average. Pathway analysis was performed on the upregulated and downregulated proteins using Metascape (http://metascape.org). Gene Ontology (GO) terms were considered significantly enriched as follows: p<0.01, minimum count of 3, and enrichment factor >1.5.

Immunohistochemistry. The procedure was performed as previously described.[9] Briefly, paraffin-embedded tissue sections (4 μm thick) were deparaffinized in Tris-EDTA buffer (pH 9.0) and heated at 95°C for 20 min in a microwave oven. Immunohistochemical staining was performed using the Histofine SAB-PO(R) kit (Nichirei Bioscience Inc., Tokyo, Japan) using the avidin-biotin immunoperoxidase technique, according to the manufacturer's instructions. The sections were incubated at

4°C overnight with 5.2 μg/ml of rabbit anti-human apolipoprotein A1 (APOA1) (14427-1-AP; Proteintech, Rosemont, IL), 2.3 μg/ml of anti-human poly(U)-specific endoribonuclease (ENDOU) (18002-1-AP; Proteintech), and 4.5 μg/ml of anti-human myotrophin (MTPN) (13508-1-AP; Proteintech). As a negative control, the primary antibody was replaced with normal goat serum (Nichirei Bioscience Inc.). The sections were then rinsed and incubated for 10 min with biotinylated secondary antibodies (Nichirei Bioscience Inc.). After washing, the sections were incubated for 5 min with horseradish peroxidase-conjugated streptavidin and treated with diaminobenzidine (DAB; Dako Agilent Technologies, Inc., Santa Clara, CA) in 0.01% H_2O_2 for 20 min and counterstained with Meyer's hematoxylin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at approximately 25°C. The intensity of immunoreactivity mainly in cytotrophoblasts in the placenta was evaluated on a four-level scale (0, none; 1, weak; 2, moderate; 3, strong; Fig. 2C). Ten representative fields per section were evaluated by two examiners, and their values were averaged.

Statistical analysis. Statistical analyses were performed using JMP Pro 15 software (SAS Institute Japan, Tokyo, Japan). Fisher's exact test was used to compare the categorical variables. The Wilcoxon rank-sum test and Student's/Welch's *t* test were used to compare continuous variables with non-normal and normal distributions for homoscedasticity/heteroscedasticity, respectively. Statistical significance was set at $p<0.05$.

Results

Protein expression patterns of cytotrophoblasts isolated from placentas with FGR were different from those from the control placenta. Table 1 shows that the clinical characteristics of these two groups were similar, unlike birth and placental weights. Both birth weight and placental weight in the FGR group were significantly lower than those in the control group ($p = 0.022$ and $p<0.001$, respectively). A total of 6,802 proteins were detected. Among them, 1,253 and 2,129 proteins were at least 2-fold upregulated and downregulated, respectively. These proteins were used for the pathway analysis. The top 10 GO pathways were significantly upregulated and downregulated (Fig. 1A, black and white bars, respectively). The upregulated pathways included oxidoreductase activity, but the downregulated pathways included GTPase binding and GTPase regulator activity pathways. The volcano plot was also produced, and the names of proteins were labeled as they were matched to the following criteria: more than 2-fold change, consistent with previous reports (Fig. 1B).[11,12] These proteins were alkaline phosphatase, placental type (ALPP), APOA1, cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1), follistatin-related protein 3 (FSTL3), ENDOU, and MTPN. ALPP, CYP19A1, and FSTL3 were excluded from further analysis as they have been previously reported to be associated with the pathology of PE or FGR.[13–17]

Differential expression of APOA1, ENDOU, and MTPN between PE-FGR and control placenta. The clinical characteristics of the PE-FGR group versus the control group are summarized in Table 1. Maternal age, nulliparity, BMI at delivery, and sex of newborns were similar between the PE-FGR and control group, although both birth weight and placental weight in the PE-FGR group were significantly lower than those in the control group ($p<0.001$). Systolic and diastolic blood pressures were significantly higher and gestational age at birth was significantly earlier in the PE-FGR group than in the control group ($p<0.001$).

The protein expression of APOA1, ENDOU, and MTPN was evaluated by immunohistochemistry. APOA1 was included in the enzyme activator activity pathway and is listed as the 6th most increased pathway (Fig. 1A, shown in red). ENDOU was included in the catalytic activity, acting on RNA pathway (the 9th most increased pathway) and hydrolase activity, acting on ester bonds pathway (the 10th most increased pathway) (Fig. 1A, shown in red). APOA1 (Fig. 2A and B, top panel) and ENDOU (Fig. 2A and B, second panel) expression in the placental cytotrophoblasts in the PE-FGR group were significantly higher than those in the control group ($p = 0.013$ and $p = 0.008$, respectively). MTPN expression was not significantly different between the FGR-PE and control groups ($p = 0.120$, Fig. 2A and B, third panel). No pathway was shown to include MTPN (Fig. 1A).

Discussion

The present study is the first to demonstrate that isolated cytotrophoblasts derived from the placenta with FGR have a unique protein signature compared to those from healthy controls. The oxidoreductase activity pathway was significantly upregulated; however, other pathways, including the GTPase-binding pathway, were significantly downregulated. CYP19A1 is involved in the oxidoreductase activity pathway. GTPases

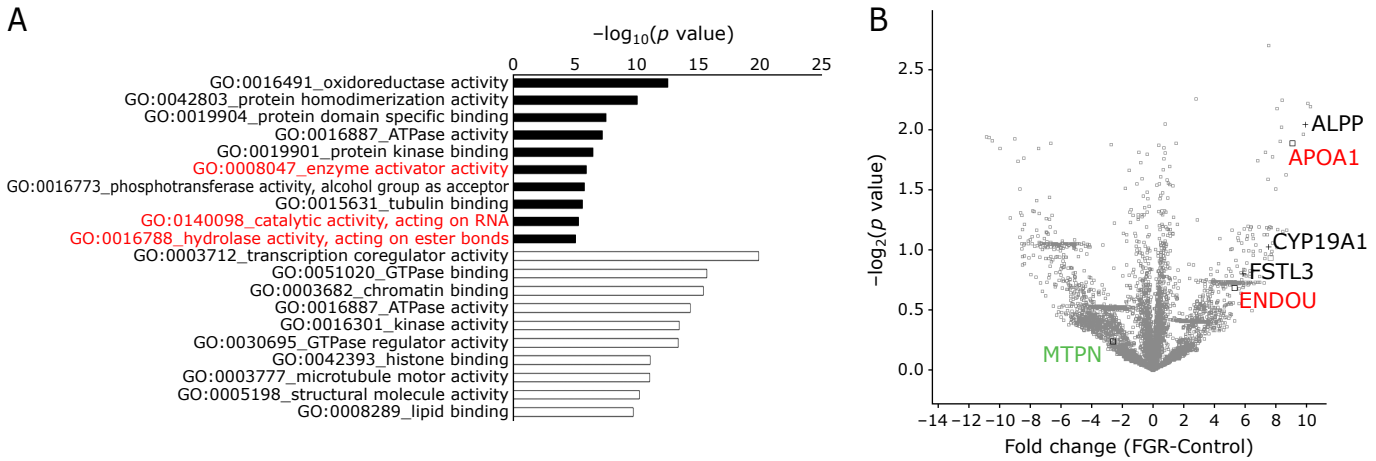

Fig. 1. Proteomic analysis of isolated cytotrophoblasts between FGR and Control. (A) Gene ontology (GO) enrichment analysis for the molecular function. The pathways listed were the top 10 upregulated and downregulated. The pathways including APOA1 and ENDOU are shown in red. Black and white bars indicate upregulated and downregulated pathways respectively. (B) A volcano plot of differentially expressed proteins in isolated cytotrophoblasts. *P* values calculated by *t* test in decadic logarithm for area values are shown in comparison to their average ratio in binary logarithm. FGR, fetal growth restriction. See color figure in the on-line version.

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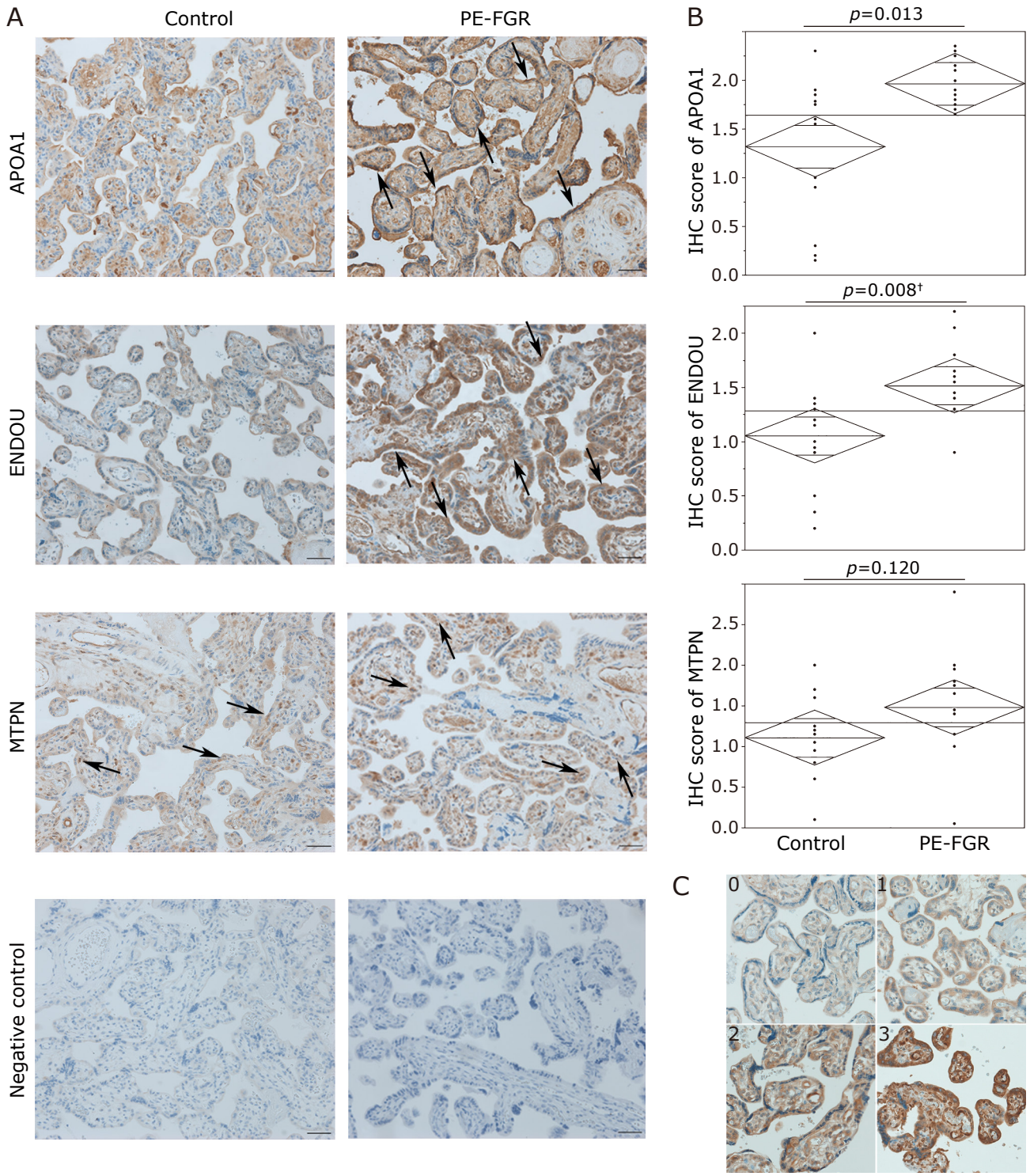

Fig. 2. Immunohistochemistry of the placenta in PE-FGR compared with control groups. (A) Representative images of APOA1 (top panels), ENDOU (second panels), and MTPN expression (third panels) in the placenta. The bottom panels show the negative control. Magnification = 200×. Scale bar = 50 μm. Arrows indicated positive staining of cytotrophoblasts. (B) IHC Scores of APOA1, ENDOU, and MTPN in the cytotrophoblasts from the placenta of PE-FGR ($n = 13$) compared with those from the control ($n = 13$). Data were analyzed by Student's t test or †Welch's t test for homoscedasticity or heteroscedasticity, respectively. (C) Expression levels of immunohistochemistry are shown as IHC scores of 0–3. PE-FGR, preeclampsia related to fetal growth restriction; IHC, immunohistochemistry.

have been previously reported to be associated with placental FGR.[18,19]

The expression levels of ALPP, APOA1, CYP19A1, FSTL3, ENDOU, and MTPN proteins showed greater than 2-fold changes; expression levels of ALPP, APOA1, CYP19A1, FSTL3, and ENDOU proteins were increased and MTPN protein was decreased. The changes in expression observed in this study are consistent with previous reports.[11,12] It has been reported that ALPP, APOA1, CYP19A1, and FSTL3 are significantly increased and MTPN is decreased in the PE placenta, as deter-

mined by proteomics.[11] Microarray analysis has shown that *ENDOU* mRNA is significantly increased in cytotrophoblasts isolated from PE placentas.[12] Of these proteins, ALPP,[15] CYP19A1,[14,16] and FSTL3[13,17] have already been reported to be associated with the pathology of PE; thus, they were excluded from further investigation. In the present study, the expression levels of APOA1, ENDOU, and MTPN were evaluated in PE-FGR placentas by immunohistochemistry.

ENDOU was initially characterized as human placenta protein 11 (PP11)[20] but it was found to have RNA binding capability, allowing it to cleave single-stranded RNA at uridylates in an Mn^{2+}-dependent manner.[21] However, the role of ENDOU in the placenta remains unknown, although ENDOU has been previously reported to regulate B cell survival.[22] Recently, ENDOU inhibitors have been investigated as potential therapeutic agents against COVID-19.[23] ENDOU cleaves the 5'-polyuridines from negative-sense viral RNA, which leads to evasion from the host pattern recognition receptor melanoma differentiation-associated gene 5 (MDA5).[24] Moreover, placental ENDOU has previously been reported to have high homology with the coronavirus endoribonuclease nonstructural protein 15 (Nsp15).[25,26] Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been detected in the placenta of mothers with COVID-19.[27] Moreover, it has been reported that pregnant women with severe COVID-19 can develop a PE-like syndrome, but the abnormal balance of angiogenic factors observed in actual PE has not been observed in these patients.[28] It has been suggested that coronavirus endoribonuclease expression of SARS-CoV-2 might be associated with PE-like symptoms in pregnant women with severe COVID-19. However, several single-institution cohort studies found no association between SARS-CoV-2 infection during pregnancy and adverse pregnancy outcomes, including PE and FGR.[29–32] It remains unknown whether a shared pathology may exist between COVID-19 and PE, and further investigation is needed.[33] A previous study also reported that ENDOU expression is increased in patients with preeclampsia ($n = 4$), but the study population was minimal.[26] The present study supports this finding by including a larger number of cases. In addition, we showed that ENDOU expression was also increased in cytotrophoblasts from FGR placentas. Recently, ENDOU has been reported to play a role in increased CCAAT-enhancer-binding protein homologous protein (CHOP) protein levels upon stress,[34] and CHOP expression was reported to be upregulated in PE and PE-FGR placentas.[35,36] These findings suggest that ENDOU might be related to the pathogenesis of PE-FGR placentas, although further investigation is needed.

APOA1, a major component of high-density cholesterol (HDL), has also been reported to be secreted from the placenta[37] and has been reported to be involved in cholesterol transport to the mother at term.[38] Several studies have reported APOA1 serum levels in women with PE compared with those in healthy controls, but these results contradict with ours; they showed decreased levels or no change in PE.[39–42] The present study showed increased expression in cytotrophoblasts in the PE-FGR placenta. However, cytotrophoblasts exist inside the syncytiotrophoblasts in villi, and syncytiotrophoblasts play a major role in maternal serum secretion. Thus, increased expression in cytotrophoblasts might not be related to maternal serum levels. Previously, only two studies reported APOA1 expression in PE or PE-FGR placentas. The study of severe PE placenta by proteomics showed a significantly increased expression ($n = 20$ in each group) [11] but another study using IHC reported no difference in APOA1 expression in PE placentas compared with normal controls ($n = 17$ in each group).[43] Both study samples were small, which may be a limitation of these results.

MTPN expression was not significantly different between the PE-FGR and control groups. MTPN's function remains unknown, although it is associated with miR-375, a regulator of insulin secretion.[44]

The present study showed different protein profiles in cytotrophoblasts derived from FGR placentas and normal controls. There are various causes associated with the pathology of FGR. In this study, FGR cases related to congenital infections or congenital anomalies were excluded. In the IHC analysis, the samples were further restricted to PE-FGR cases because the pathological factors were unified. PE has been well investigated, and it is well known that the failure of EVT invasion into the maternal decidua causes hypoplastic placenta.[45]

The methodology of the present study could be applied to other obstetric studies. Cytotrophoblasts can also be isolated from other pathological placentas, including cases of placenta accreta and placentas from preterm birth. Findings from other pathologies might lead to the discovery of new therapeutic targets for these diseases.

The present study has several limitations. First, the sample size was small. Second, the protein levels gained from isolated cytotrophoblasts were limited and difficult to analyze for validation. Thus, the protein levels were evaluated by IHC. Third, cytotrophoblasts were derived from the placenta after birth and at the end of placentation. Thus, the protein profile may show placental hypoplasia's result rather than its cause. However, ENDOU, which showed increased expression in PE-FGR, might play a pathological role in poor placentation, although further investigation is required to confirm this.

In conclusion, we found an altered protein profile between cytotrophoblasts derived from the hypoplastic FGR placentas and those from control placentas. ENDOU, which is highly homologous to coronavirus endoribonuclease Nsp15, showed increased expression in PE-FGR, although its pathological role should be determined in the future.

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Abbreviations

ALPP	alkaline phosphatase, placental type
APOA1	apolipoprotein A1
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1
CHOP	CCAAT-enhancer-binding protein homologous protein
ENDOU	poly(U)-Specific Endoribonuclease
EVT	extravillous trophoblast
FBS	fetal bovine serum
FGR	fetal growth restriction
FSTL3	follistatin-related protein 3
HBSS	Hank's balanced salt solution
HLA	human leukocyte antigen
IHC	immunohistochemistry
MDA5	melanoma differentiation-associated gene 5
MTPN	myotrophin
Nsp15	nonstructural protein 15
PE	preeclampsia
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2

Conflict of Interest

No potential conflicts of interest were disclosed.

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