

FULL LENGTH ARTICLE

The metabolic role of LncZBTB39-1:2 in the trophoblast mobility of preeclampsia

Yamin Liu ^{a,b,1}, Ting-Li Han ^{a,b,c,1}, Xiaofang Luo ^{a,b},
Yuxiang Bai ^{a,b}, Xuehai Chen ^{a,b}, Wei Peng ^{a,b}, Xi Xiong ^{a,b},
Philip N. Baker ^{a,b,d}, Chao Tong ^{a,b,**}, Hongbo Qi ^{a,b,*}

^a Canada - China - New Zealand Joint Laboratory of Maternal and Fetal Medicine, Chongqing Medical University, Chongqing, 400016, China

^b Department of Obstetrics and Gynaecology, The First Affiliated Hospital of Chongqing Medical University, China

^c Liggins Institute, The University of Auckland, Auckland, New Zealand

^d College of Medicine, Biological Sciences and Psychology, University of Leicester, UK

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Abstract Preeclampsia is characterized by new onset of hypertension and proteinuria after 20 weeks' gestation and is a leading cause of maternal and neonatal morbidity and mortality. The pathogenesis of preeclampsia is often associated with aberrant trophoblast function that leads to shallow placental implantation. However, the exact underlying mechanisms remain unclear. Placental LncZBTB39-1:2 expression level was investigated in 20 healthy placentae and 20 placentae with preeclampsia using qRT-PCR, and the metabolic profile of trophoblasts overexpressing LncZBTB39-1:2 *in vitro* was analysed using gas chromatography-mass spectrometry (GC–MS). In this study, we found that the expression of LncZBTB39-1:2 was significantly higher in preeclamptic placentae than in healthy placentae. Our metabolomics results have shown that tricarboxylic acid cycle intermediates and metabolites related to carbohydrate metabolism were decreased with the overexpression of LncZBTB39-1:2 in HTR8/SVneo cells. These findings were validated by detecting a lower level of intracellular ATP in HTR8/Vneo cells. Furthermore, the migration of HTR8/SVneo cells was compromised when cells were transfected with a plasmid encompassing LncZBTB39-1:2 overexpression. From these results, we conclude that abnormal levels of LncZBTB39-1:2 expression might lead to aberrant

* Corresponding author. International Collaborative Laboratory of Reproduction and Development, Ministry of Education, Chongqing Medical University, Chongqing, 400016, China; Department of Obstetrics, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China

** Corresponding author. International Collaborative Laboratory of Reproduction and Development, Ministry of Education, Chongqing Medical University, Chongqing, 400016, China.

E-mail addresses: 522010433@qq.com (C. Tong), CQmuqihongbo@yeah.net (H. Qi).

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¹ Equally contributing first authors.

conditions in HTR-8/SVneo trophoblast cells. Aberrant conditions might be associated with dys-regulated trophoblast migration and subsequent failure of uterine spiral artery remodelling, a pathogenesis recognised as a contributing factor in the aetiology of preeclampsia.

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Introduction

Preeclampsia (PE) is defined as new onset hypertension and proteinuria after 20 weeks' gestation and is a leading cause of perinatal morbidity and pregnancy-associated mortality, especially in underdeveloped countries. Termination of pregnancy is the only treatment for PE.¹ Many theories have been established to explain the pathogenesis of PE such as inflammatory cytokines,² endothelial dysfunction,³ and imbalance between proangiogenic and antiangiogenic factors.⁴ At present, the "Two-stage Disorder"⁵ theory of PE is accepted. Trophoblasts extensively invade the spiral arteries of the uterus during the first stage of gestation, thus remodeling the uterine spiral arteries. The vessel diameter enlarges and reduces the flow resistance, increasing uteroplacental perfusion.⁶ Trophoblasts play an important role in the spiral artery remodeling. Abnormal trophoblast invasion of uterine vessels, as hypothesised to occur in PE, causes recasting obstacles for the spiral arteries and shallow placental implantation.⁷ Furthermore, there is an increasing number of studies demonstrating the role of disordered cellular energy metabolism in the pathophysiology of PE. Many researchers reported that ATP level was significantly reduced in PE placentae compared to normal placentae. This phenomenon may have resulted by the accumulation of mitochondrial oxidative stress, dysregulated mitochondrial fusion, autophagy, biogenesis and abnormal lipid metabolism.^{8–10} However, the specific pathological mechanisms leading to PE development remain undefined.

Yu et al. (2009) reported that Long noncoding RNA (LncRNA)H19 was related to the pathogenesis of PE¹¹. Long noncoding RNA (LncRNA) is a nucleotide (longer than 200 base pairs) which is unable to code protein and does not have an open reading frame (ORF). Recently, LncRNA has been found to participate in many disease processes including cancer invasion, cancer migration, and apoptosis.^{12–14} Some biological functions of trophoblasts resemble cancer cells, and some LncRNAs have been shown to play important roles in tumorigenesis¹⁵ as well as the regulation of PE, such as LncRNAH19, LncRNASPRY4-IT1^{16,17}, LncNAMEG3¹⁸, and LncRNAMALAT.¹⁹ These LncRNAs are involved in the cellular modulation of trophoblasts, including important processes such as proliferation, migration, apoptosis, invasion, and angiogenesis.

In this study we found that the expression of a particular type of LncRNA called LncZBTB39-1:2 (<http://www.lncipedia.org>) was increased in severe PE placental tissue compared with normal placental tissue, analysed using qRT-PCR. Furthermore, The LncZBTB39-1:2 transcript appears to be a splice variant of the tachykinin 3 (TAC3) gene. The encoded peptide is neurokinin B (NKB). This gene product has been studied relatively well including in the placenta of pre-

eclampsia patients. These neuropeptides have been found to contribute to the elevation of blood pressure and in raising pressor sensitivity as observed in PE. They have also been shown to inhibit several proteins that participate in the metabolic response to oxidative and hypoxia stress.²⁰ Based on this reason, we applied a metabolomics approach to investigate the metabolic effects of LncZBTB39-1:2 over-expression in an HTR8/SVneo cell line with plasmids *in vitro*.

Methods

Selection criteria for participants

All experiments were approved by the Ethics Board of the First Affiliated Hospital of Chongqing Medical University and informed consent was obtained from all participants. All clinical investigations were performed according to the principles expressed in the Declaration of Helsinki.

Villi at 6–8 gestational weeks were collected from healthy pregnant women who underwent induced abortion for nonmedical causes. All chorionic villus samples with acute and chronic diseases were excluded. PE was diagnosed following the America College of Obstetricians and Gynaecologists (ACOG) criteria. Both blood pressure and proteinuria were required to meet the following criteria to define PE diagnosis: systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg and/or 24-h urinary protein excretion exceeding 300 mg. Patients were not eligible for the study if they were experiencing any other pregnancy complications such as severe intrauterine growth retardation (IUGR), diabetes, chemical dependency, or fetal congenital abnormalities.

All placental tissues were collected between 2015 and 2016 from the Department of Obstetrics and Gynaecology at the First Affiliated Hospital of Chongqing Medical University, China. PE placentae were delivered from the womb by cesarean without extrusion of uterine contraction. Specimens were washed with cold normal saline and stored in liquid nitrogen prior to RNA purification.

RNA extraction

Total RNA was extracted from the placental, cells and villi using RNAiso plus reagent (Takara, Japan). The cDNA was synthesised from 1 μ g of total RNA by using a reverse transcription Kit (Takara, Japan).

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

qRT-PCR was performed using an SYBR Green PCR Kit (Roche, Germany), according to the manufacturer's

instructions. The thermocycling conditions were 95 °C for 10 min, 95 °C for 10 s, 57 °C for 30 s, and 72 °C for 10 s, which were repeated for 40 cycles. The primers (Invitrogen) of LncZBTB39-1:2 and β -actin were as follows: LncZBTB39-1:2: Forward: 5'-TCTGTAAGGAGCCACAGGA-3', Reverse: 5'-CAAGAGATAGGGAGGGAGAAGA-3' and β -actin: Forward: 5'-TGGCACCCAGCACAATGAA-3', Reverse: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'.

Cell culture

The HTR8/SVneo cell line, which is derived from an invasive human extra-villi trophoblast of the first trimester, is widely used as a model for trophoblast invasion and migration. The HTR8/SVneo cells used in this study were gifted by Dr. Charles Graham (Queen's University, Kingston, Ontario, Canada). The cells were cultured in RPMI1640 medium that comprised of 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 μ g/mL streptomycin, and humidified atmosphere consisting of 5% CO₂ at 37 °C. 2.0×10^5 HTR8/SVneo cells were seeded onto 6-well culture plates with three replicates. After 24 h, 1 μ g of both plasmid (pHB-EF1-MCS-GFP, Hanbio, China) with LncZBTB39-1:2 and an empty vector control were transferred into HTR8/SVneo cells via a lipofectamine2000 transfection kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. After 48 h of transfection, the overexpression of LncZBTB39-1:2 was confirmed by qRT-PCR.

Metabolite extraction from cell culture

At 48 h after the cell line was transfected with plasmid and empty vector, cells in culture dishes were washed with 10 ml of deionized water at 37 °C and quenched by adding 15 mL of liquid nitrogen. After the evaporation of liquid nitrogen, 1.2 ml of cold extraction solvent 9:1 methanol:chloroform with 20 μ l of 2,3,3,-d4-alanine (0.3 mol/ μ L) was added to each culture dish. Cells were scraped with a cell lifter and transferred into a 1.5 ml Eppendorf tube²¹. After vortexing for 1 min, the supernatant was separated from the cell debris via centrifugation and the metabolites were concentrated using a SpeedVac (LABCONCO) for 2 h at room temperature.

Metabolomic analysis

The cell extracts were resuspended in 200 μ l of sodium hydroxide (1 M) and underwent methyl chloroformate (MCF) derivatization based on the published protocol.²² The chemically modified metabolites were analysed by an Agilent GC7890B system coupled to a MSD5977A mass selective detector (EI) with an electron beam energy at 70 eV. The GC column incorporated into the GC oven was the ZB-1701 GC capillary column (30 m $\times$ 250 μ m id $\times$ 0.15 μ m with 5 m guard column, Phenomenex). The GC oven settings and mass spectrometry parameters were implemented according to Smart et al (2010). Chromatogram deconvolution and compound identification was achieved by AMDIS in conjunction with an in-house MCF-derivatised mass spectrum library and

commercially available NIST mass spectrum library (<http://www.nist.gov/srd/nist1a.cfm>). The relative abundances of identified metabolites were extracted using an automated XCMS-R-script.²³

ATP measurement

The ATP levels of the HTR8/SVneo trophoblast cells were measured, according to the manufacturer's instructions (Beyotime, Shanghai, China). The HTR8/SVneo cells were seeded into a 6-well plate, cells were lysed after 48 h of plasmid transfection, followed by centrifugation at 4 °C and 12000 g for 5 min. The supernatant was collected and 50 μ l was added into 100 μ l of working solution. The fluorescence value was measured using a plate reader (GloMax20/20 promega). Calibration curves were created using pure ATP.

Transwell migration assay

For the trophoblast migration assay, a transwell insert was not pre-coated with matrigel. 5×10^4 HTR8/SVneo cells transfected with plasmid were seeded into the upper chamber in 100 μ l medium without FBS. Meanwhile, 600 μ l medium with 10%FBS was added into the lower compartment. After 24 h, the transwell insert was fixed with cold methanol and stained with 0.1% crystal violet, while the cells inside the inserts were wiped out using a cotton swab. Cells that passed through the chamber were counted randomly from five selected fields, using light microscopy (Olympus, Japan).

Data normalisation and statistical analysis

An internal standard (2,3,3,3-d4-alanine) was added into the cell culture prior to metabolite extraction to normalise the relative concentration of metabolites, correcting for human error and batch variation. The different cell populations were also corrected using their corresponding biomass. The pair-wise comparisons of metabolite profiles between the groups: overexpression by plasmid, cells containing vector only, and cell culture without plasmid, was performed using multivariate ANOVA and Tukey's Honest significant difference test. False discovery rates were calculated to account for multiple comparison testing by using the qvalue R package. Important metabolites were selected by constructing a Random Forest model via the randomForest R package. Student's *t*-tests were performed to calculate the statistical differences between groups for qRT-PCR results and ATP measurements.

Results

Participant characteristics

A total of 40 participants were recruited for this study; 20 PE cases and 20 healthy controls. Systolic blood pressure, diastolic blood pressure, and proteinuria were significantly higher in PE cases when compared to controls (Table 1). Placental weight and neonatal birth weight were lower in

Table 1 Participant clinical characteristics.

Category	Preeclampsia (n = 20)	Normal pregnancy (n = 20)
Maternal age	28.16 ± 2.35	27.28 ± 3.43
BMI(kg/m ²)	29.16 ± 5.53	27.33 ± 2.46
Length of gestation (weeks)	35.52 ± 2.69	38.27 ± 0.95
Smoking history	None	None
Drinking history	None	None
Placental weight (g)	411.69 ± 52.31**	541.00 ± 42.06
Neonatal birth weight (g)	2617.15 ± 177.81*	3156.00 ± 267.73
Systolic blood pressure (mmHg)	162 ± 11**	108 ± 7
Diastolic blood pressure (mmHg)	101 ± 9*	69 ± 5
Proteinuria level (g/ 24 h)	>100–300 mg	none

* $P < 0.05$, ** $P < 0.01$.

Values are shown as the mean ± SD. All statistical analyses were performed using nonparametric Mann–Whitney test on SPSS v19.0.

the PE cases when compared to controls. No significant differences were observed between PE subjects and normal pregnant women in regards to gestational age, maternal age, and maternal body mass index (BMI), as illustrated in Table 1.

The endogenous expression of LncZBTB39-1:2 in chorionic villi and placental tissues

To research the potential function of LncZBTB39-1:2 in the pathogenesis of PE, we first examined the expression of LncZBTB39-1:2 in the placenta of PE and normal subjects. The expression level of LncZBTB39-1:2 in the PE placenta was significantly higher than that in the healthy placenta (Fig. 1A). The expression of LncZBTB39-1:2 was also significantly higher in normal placenta than in the early chorionic villi (Fig. 1B).

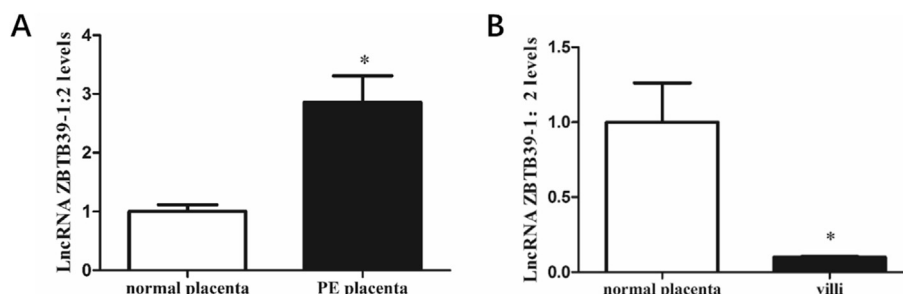


Figure 1 qRT-PCR of LncZBTB39-1:2 in normal placenta, PE placenta, and Villi. **A.** Relative expression of LncZBTB39-1:2 in PE placenta was significantly higher than in normal placenta. **B.** Relative expression of LncZBTB39-1:2 in normal placenta was significantly higher than in early chorionic villi. The expression level is determined by real-time reverse transcription polymerase chain reaction (qRT-PCR). The height of the bar graph represents the mean value of LncZBTB39-1:2 expression, with standard deviation represented by error bars. * represents $P < 0.05$ following a student's t -test.

The expression level of LncZBTB39-1:2

The level of LncZBTB39-1:2 was overexpressed in cells transfected with plasmid compared to controls and negative controls, measured using qRT-PCR (Fig. 2).

The metabolite profiles of cell cultures containing overexpression of LncZBTB39-1:2, empty plasmid, and non-transfected cells

A total of 137 metabolite peaks were detected in the cell cultures, 80 of which were identified by the in-house MCF-derivatised library of standards. Partial least squares discriminant analysis (PLSDA) showed that cell lines containing LncZBTB39-1:2 overexpression plasmid were separated from normal and negative control samples (Fig. 3). Normal and negative control samples were overlapped, indicating similar metabolomic profiles. There were 28 metabolites significantly different between the cells with LncZBTB39-1:2 overexpression and control samples, with p -values and q -values below 0.05 (Fig. 4); only seven metabolites were significantly different between controls and negative controls. We found that most of the significant metabolites were reduced in the cell culture with overexpression of LncZBTB39-1:2, including amino acids, tricarboxylic acid (TCA) cycle intermediates, vitamins, antioxidants, and unsaturated fatty acids. On the other hand, a few saturated fatty acids and organic acids were elevated in cells with the overexpression of LncZBTB39-1:2. Interestingly, five TCA cycle intermediates (citric acid, fumaric acid, malic acid, succinic acid, and 2-oxoglutaric acid) were significantly reduced when cells demonstrated overexpression of LncZBTB39-1:2. In addition, glutathione, an antioxidant, was also reduced in the cell line that overexpressed LncZBTB39-1:2.

Furthermore, we used a Random Forest model to rank the metabolites with the most significant contributions to the separation of LncZBTB39-1:2 from empty plasmid and non-transfected cells (Fig. 5). The results showed that TCA cycle intermediates including malic acid, 2-oxoglutaric acid, malic acid, fumaric acid, succinate were among the top 12 highest ranked metabolites separating the cell lines containing LncZBTB39-1:2 overexpression plasmid from the normal

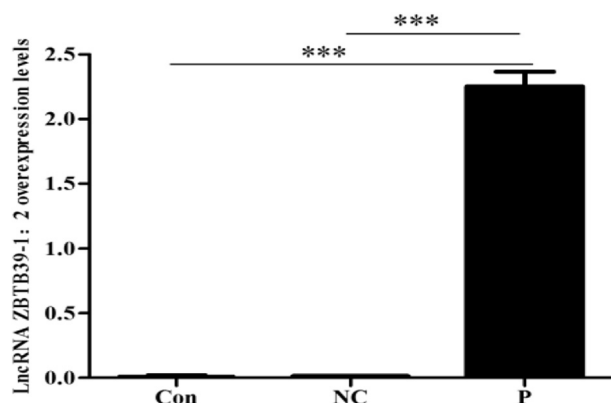


Figure 2 qRT-PCR of overexpression with plasmid. Relative expression of LncZBTB39-1:2 transfected with plasmid was significantly higher than control and negative controls ($P < 0.0001$ (***) following a student's *t*-test), as determined by qRT-PCR. The height of the bar graph represents the mean value of LncZBTB39-1:2 expression, with standard deviation represented by error bars.

cells. Whereas, TCA cycle intermediates were much less influential between the LncZBTB39-1:2 overexpression plasmid and negative control. These findings might indicate that energy metabolism is downregulated by LncZBTB39-1:2.

The metabolic pathways affected by the overexpression of LncZBTB39-1:2, when compared to empty plasmid and non-transfected cells

The metabolic pathways affected by the overexpression of LncZBTB39-1:2 were analysed using the intracellular metabolites identified in the cell lines cultured in the presence or absence of LncZBTB39-1:2 overexpression. When compared to cells transfected with empty plasmids, there were 14 metabolic pathways that appeared to be downregulated in response to LncZBTB39-1:2 overexpression, while only one

metabolic pathway was upregulated (Fig. 6). Only three metabolic pathways were downregulated when comparing cells with the overexpression of LncZBTB39-1:2 to non-transfected cells. Interestingly, most of the downregulated metabolic pathways were involved in carbohydrate metabolism; this may indicate that reduced substrate catabolism was associated with compromised energy production.

Adenosine Triphosphate(ATP) level

To validate the reduced energy production hypothesised from the metabolic pathway analysis results, we analysed the ATP levels of HTR8/SVneo cells in the cell line with the overexpression of LncZBTB39-1:2, the empty plasmid, and non-transfected vector (Fig. 7). The intracellular ATP levels were significantly reduced when HTR8/SVneo cells were transfected with empty plasmids and LncZBTB39-1:2 overexpression.

The effect of LncZBTB39 overexpression on HTR8/SVneo migration

Since the impaired mobility of trophoblasts is one of the major suggested routes leading to the pathogenesis of preeclampsia, we implemented a transwell experiment to test if LncZBTB39-1:2 modulates HTR8/SVneo cell migration. Fig. 8 demonstrated that migration of HTR8/SVneo cells was significantly suppressed when cells were transfected with a plasmid containing LncZBTB39-1:2 overexpression.

Discussion

Historically, long noncoding RNA (LncRNA) had been suggested to be "transcriptional noise" without any biological significance.²⁴ An increasing number of studies are revealing that the cellular structure and regulatory roles of

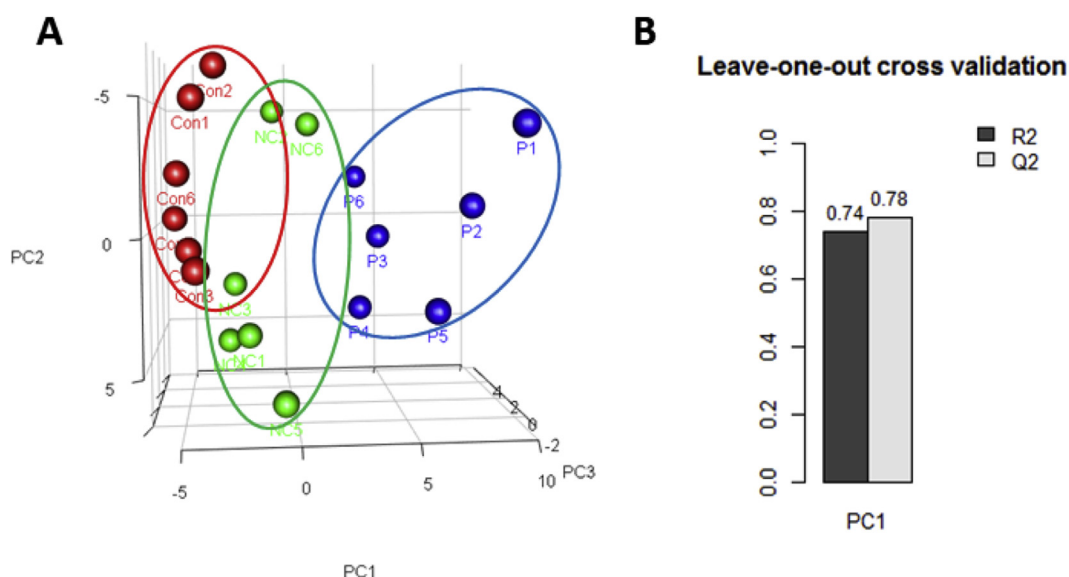


Figure 3 A. PLS-DA analysis. Red circles represent normal cells; green circles represent cells with plasmids (negative control); and blue circles represent cells containing the LncRNAZBTB39-1:2 overexpression plasmid. B. leave-one-out cross validation of PLS-DA.

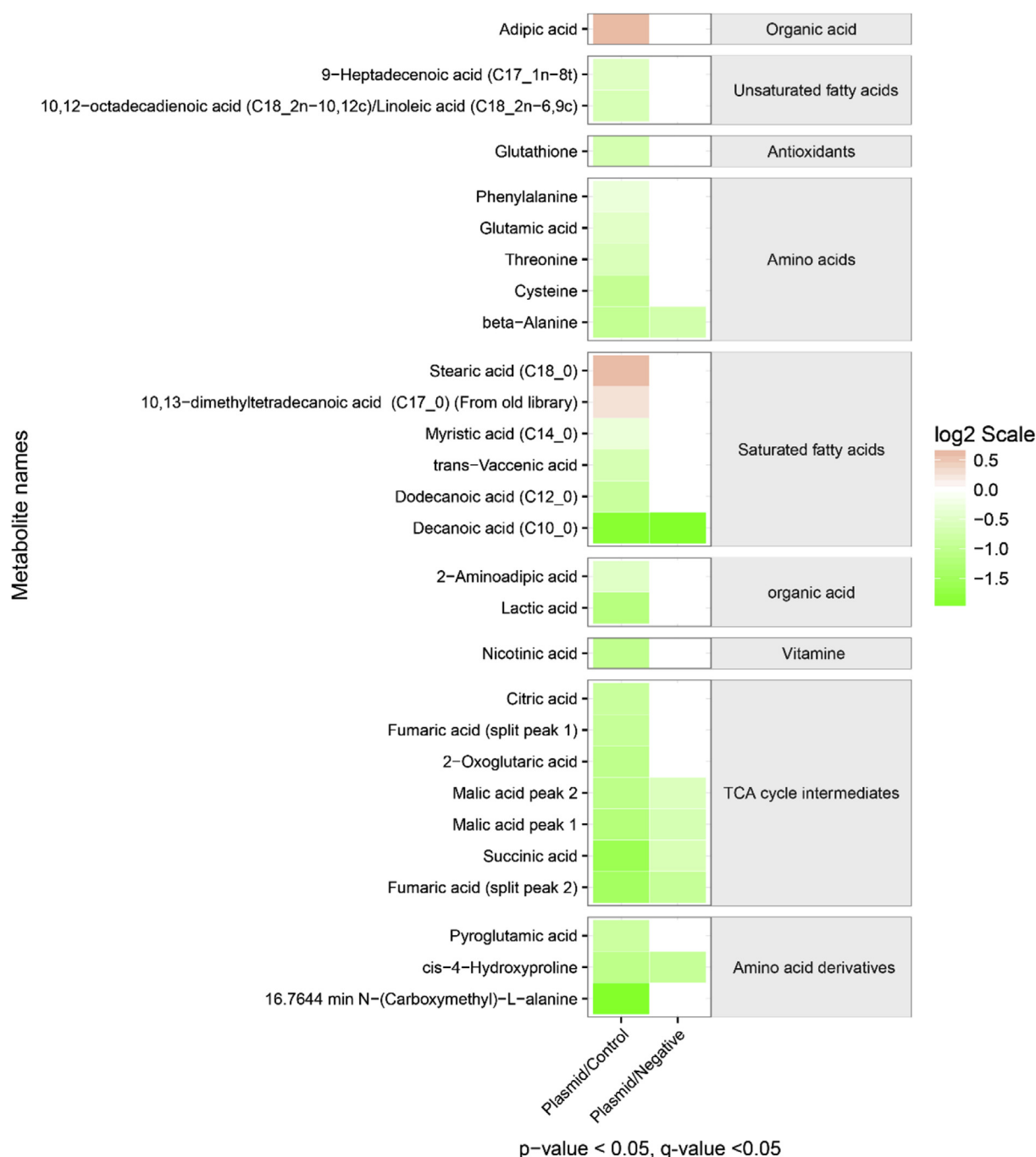


Figure 4 Metabolite profile of HTR8 cell lines comparing overexpressed LncRNAZBTB39-1:2 and controls. The heatmap colours indicate the relative concentration abundance ratio of intracellular metabolites for cells overexpressing the LncRNAZBTB39-1:2 gene (plasmid) to those with normal expression (control, negative) using a log2 scale. Red colour indicates metabolite levels that were elevated in response to overexpression of LncRNAZBTB39-1:2. Green colour indicates metabolite levels that were reduced in response to the overexpression of LncRNAZBTB39-1:2. Only the compounds with p- and q-values below 0.05 were included.

LncRNA are involved in important physiological processes such as embryonic development,²⁵ X-chromosome inactivation,²⁶ and genomic imprinting.²⁷ In our research, we found that the expression level of LncZBTB39-1:2 in PE placenta was significantly higher than in normal control placenta and the migration of trophoblasts was compromised by LncZBTB39-1:2 overexpression. For this reason,

we investigated the metabolic changes in trophoblasts when LncZBTB39-1:2 was overexpressed by transfection with plasmids. We utilized GC-MS profiling to detect metabolites in HTR8/SVneo cells after the overexpression of LncZBTB39-1:2. The results indicated that metabolites involved in the TCA cycle such as malic acid, succinic acid, formic acid and citric acid, as well as metabolites involved

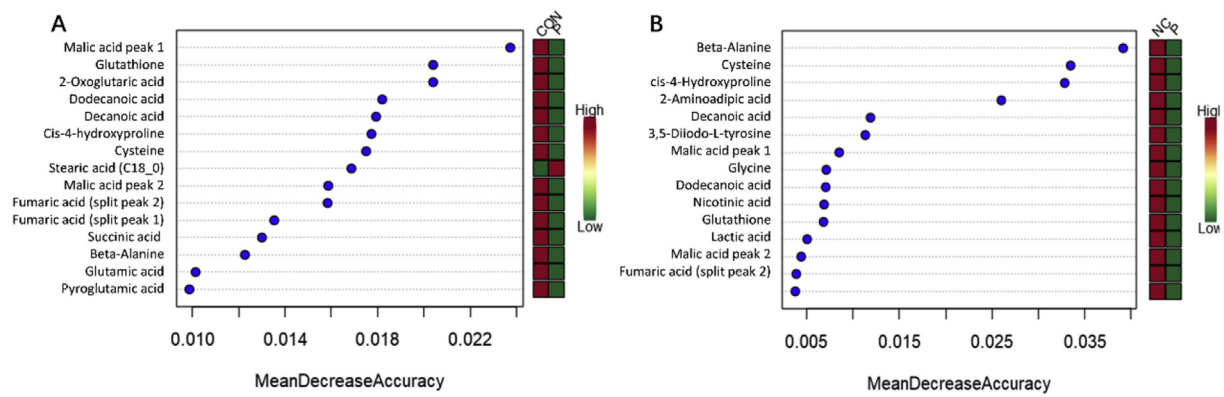


Figure 5 The metabolites of HTR8 cell lines ranked by their ability to discriminate between overexpressed LncZBTB39-1:2 (P), controls (CON), and negative controls (NC). A. The Random forest model was implemented to rank the important metabolites via mean decrease accuracy (MDA) between CON and P. B. The Random forest model was implemented to rank the important metabolites via MDA between NC and P. The greater MDA means the greater ability to separate the two groups. The red colour indicates higher concentration, while the green colour indicates lower concentration.

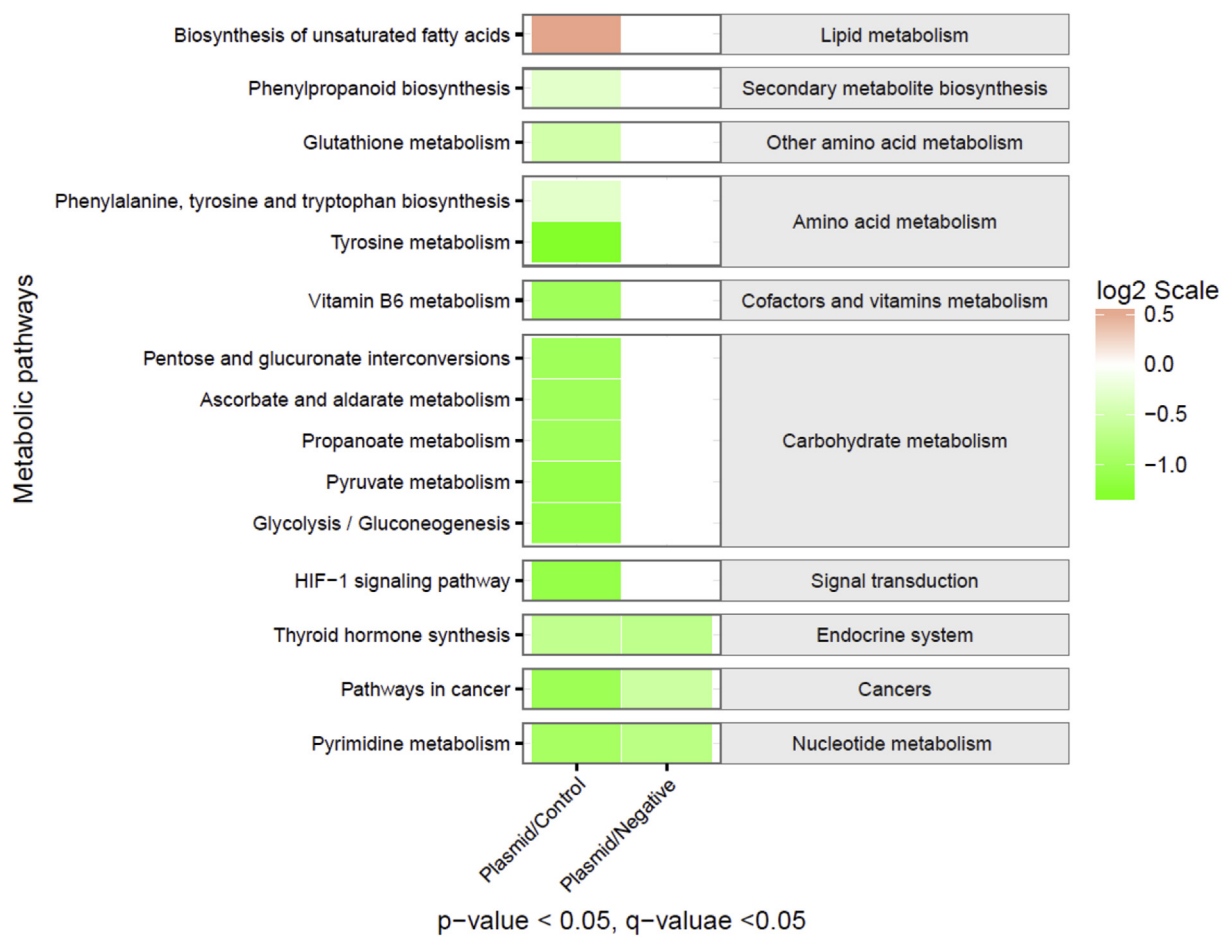


Figure 6 Metabolic pathways of HTR8/SVneo cell lines affected by the overexpression of LncZBTB39-1:2. The heatmap colours indicate the relative metabolic activity ratio of metabolic pathways for cells overexpressing the LncRNAZBTB39-1:2 gene (plasmid) to those with normal expression (control, negative) using a log2 scale. Red colour indicates that metabolic activities were upregulated in response to the overexpression of LncRNAZBTB39-1:2. Green colour indicates metabolic activities were down-regulated in response to the overexpression of LncRNAZBTB39-1:2. Only the metabolic pathways with p- and q-values below 0.05 were included.

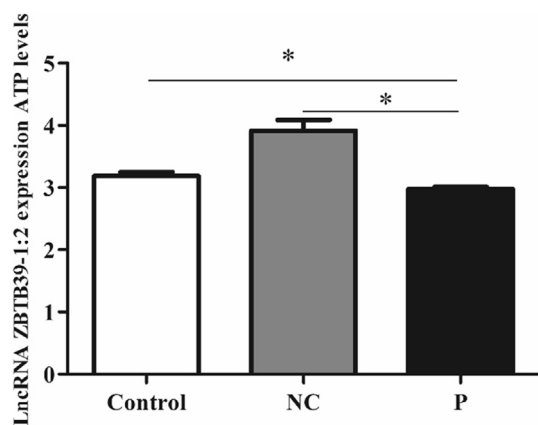


Figure 7 A comparison of ATP levels between HTR8/SVneo cells with overexpression of LncZBTB39-1:2 (P), empty plasmid (Con), and non-transfected vector (NC). The total ATP levels in cells with LncZBTB39-1:2 overexpression and empty plasmid were significantly lower than those with non-transfected cells ($P < 0.05$ (*)).

in carbohydrate metabolism, were significantly reduced when HTR8/SVneo cells overexpressed LncZBTB39-1:2. We also found intracellular ATP levels to be significantly lower when HTR8/SVneo cells overexpressed LncZBTB39-1:2.

These findings suggest that LncZBTB39-1:2 overexpression is associated with the downregulation of cellular energy metabolism. The lower concentration of TCA cycle intermediates compromises the production of NADH and FADH, which are precursors for the production of ATP via the electron transport chain. It has been reported that disturbance of energy metabolism is associated with the pathogenesis of late PE²⁸. Fumarate deficiency has also been shown to impact the invasiveness of cancer cells by promoting mRNA expression of HIF-1 α through a non-canonical NF- κ B-dependent pathway.²⁹ In our study of the metabolic pathways affected by LncZBTB39-1:2 overexpression, the HIF-1 signaling pathway was suppressed significantly, which may result in the reduction of trophoblast invasion. Furthermore, it has been demonstrated in prior work that cellular motility and apoptosis is associated with ATP production.^{30,31} We also found that LncZBTB39-1:2 was expressed differentially at the early and late stages of pregnancy. The expression of LncZBTB39-1:2 was lower in early chorionic villi than in normal fully grown placenta, while the expression of LncZBTB39-1:2 was markedly lower in the healthy placenta compared to PE placenta. This observation is consistent with the varied invasiveness of trophoblasts at different stages of pregnancy. The results of the transwell migration assay demonstrated that LncZBTB39-1:2 overexpression restrains trophoblast migration *in vitro*. Based on our results, we propose that

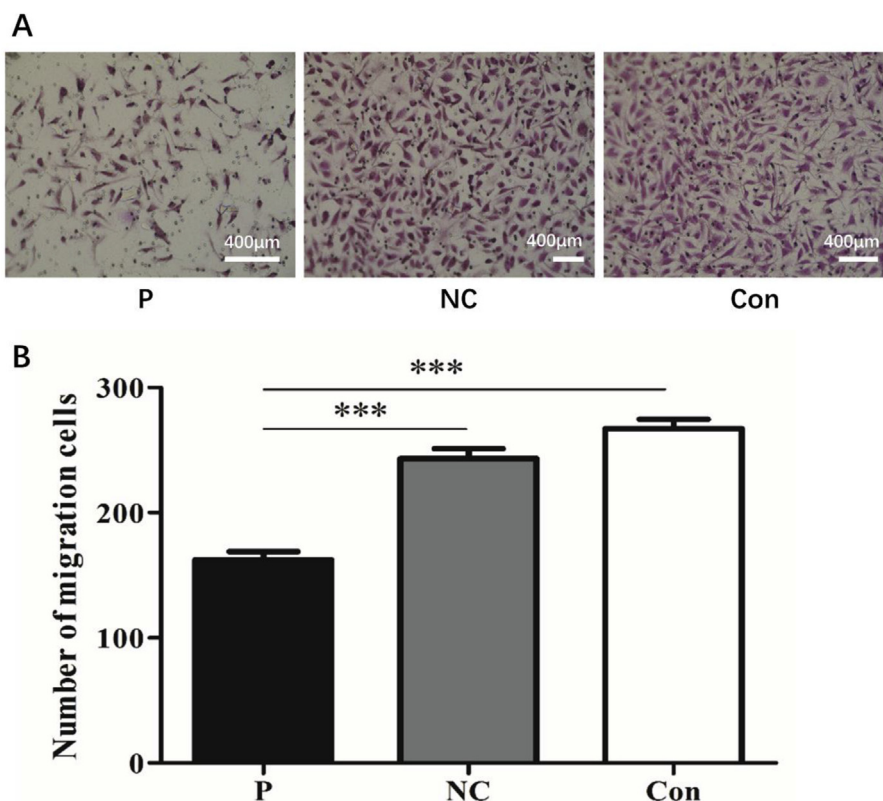


Figure 8 The effect of LncZBTB39-1:2 overexpression on trophoblast migration analysed via a Transwell experiment. **A.** HTR-8/SVneo cells transfected with plasmid (P) demonstrated significantly reduced migration capability compared with cells transfected with an empty vector (NC) and without plasmid (Con). Cells were stained by crystal violet to visualise transwell migration assay. Scale bars represent 400 μ m. **B.** The bar graphs indicate that the number of migrated cells in the P, NC, and Con groups (Values are mean $\pm$ SEM; *** indicates $P < 0.0001$).

LncZBTB39-1:2 in human trophoblast cells would reduce cellular mobility and other biological functions that are known to be associated with the pathogenesis of PE. However, future investigations are needed to validate our hypothesis on a larger sample size and further explore the mechanisms of the role LncZBTB39-1:2 plays in trophoblast invasion, apoptosis, and proliferation.

Conclusion

In conclusion, our results demonstrated that placental LncZBTB39-1:2 expression was increased in PE placentae when compared to healthy placentae. To model this phenomenon *in vivo*, we studied the metabolic changes of human trophoblast cells in response to overexpressed LncZBTB39-1:2. Our results suggest that LncZBTB39-1:2 may attenuate trophoblast mobility by compromising its energy metabolism, potentially contributing to the pathophysiology of PE development.

Conflicts of interest

All authors declare no conflicts of interest related to this study.

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