**CLDN1 regulates trophoblast apoptosis and proliferation in preeclampsia**

Yu-chen Zhang¹,², Xiao-li Qin², Xiao-ling Ma², Hui-qin Mo³, Shi Qin³, Cheng-xi Zhang¹,², Xiao-wei Wei¹,², Xue-qing Liu¹,², Yan Zhang⁴, Fu-ju Tian¹,² and Yi Lin¹,²

¹Shanghai Key Laboratory of Embryo Original Diseases, The International Peace Maternity & Child Health Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, ²The International Peace Maternity & Child Health Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, ³Department of Obstetrics and Gynecology, Shanghai First Maternity and Infant Hospital, Tongji University of Medicine, Shanghai, China and ⁴Department of Obstetrics and Gynecology, Renmin Hospital of Wuhan University, Wuhan, Hu Bei, China

Correspondence should be addressed to Y Zhang or F Tian or Y Lin; Email: zyan2200@gmail.com or fjtian@sibs.ac.cn or ylinonline@126.com

**Abstract**

Preeclampsia is a gestational hypertensive disease; however, preeclampsia remains poorly understood. Bioinformatics analysis was applied to find novel genes involved in the pathogenesis of preeclampsia and identified CLDN1 as one of the most differentially expressed genes when comparing patients with preeclampsia and healthy controls. The results of the qRT-PCR, Western blotting and immunohistochemistry experiments demonstrated that CLDN1 was significantly downregulated in the chorionic villi in samples from patients with preeclampsia. Furthermore, knockdown of CLDN1 in HTR-B/SVneo cells resulted in the inhibition of proliferation and induction of apoptosis, and overexpression of CLDN1 reversed these effects. In addition, RNA-seq assays demonstrated that the gene BIRC3 is potentially downstream of CLDN1 and is involved in the regulation of apoptosis. Knockdown of CLDN1 confirmed that the expression level of BIRC3 was obviously decreased and was associated with a significant increase in cleaved PARP. Interestingly, the apoptotic effect in CLDN1 knockdown cells was rescued after BIRC3 overexpression. Overall, these results indicate that a decrease in CLDN1 inhibits BIRC3 expression and increases cleaved PARP levels thus participating in the pathogenesis of preeclampsia.

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**Introduction**

Preeclampsia (PE) is a complication of pregnancy characterised by high blood pressure, and it leads to a series of unpleasant outcomes, such as placental abruption, intrauterine growth retardation and preterm birth (*Mol et al. 2016*). According to the 2019 ACOG Practice Bulletin, PE is diagnosed based on abnormal blood pressure after 20 weeks of gestation with nonobligatory confirmation by the presence of proteinuria (*ACOG 2019*). PE can be accompanied by multiorgan dysfunction, including thrombocytopenia, renal insufficiency, impaired liver function and nervous system dysfunction. To date, many studies have investigated the mechanism of this gestational hypertensive disorder; however, detailed molecular mechanism of the disease is unknown. A widely accepted theory describes the pathogenesis of the disease as a ‘two stage’ process: abnormal placentation occurring in the first-trimester of pregnancy and consequent maternal syndrome in the second- or third-trimester of pregnancy. Under physiological circumstances, endovascular extravillous trophoblast entered inside spiral arteries vessel wall after interstitial extravillous trophoblast invasion; shallow invasion of endovascular extravillous trophoblast results in insufficient conversion of spiral arteries and the consequent poor placentation (*Turco & Moffett 2019, Staff et al. 2020*). Insufficient trophoblast invasion results in incomplete spiral artery remodelling and consequent reduced placental perfusion, and decidual vasculopathy and poor uterine decidualisation are potential reasons leading to placental insufficiency (*Fisher 2015, Phipps et al. 2019, Rana et al. 2019*). Increasing evidence has demonstrated that both foetal and maternal factors contribute to abnormal placentation. The development of technologies and numerous tactics assisting with the exploration of the mechanism of the disease can be used to identify additional contributing factors.

Claudin-1 is encoded by the CLDN1 gene and is the first member of the claudin family. CLDN1 is a component of the tight junction complex, which controls epithelial homeostasis by regulating paracellular movement of the molecules (*Garcia-Hernandez*...
et al. 2017). Dysfunction of CLDN1 is involved in the pathogenesis of multiple diseases. For example, CLDN1-deficient mice die soon after birth due to cutaneous dehydration (Furuse et al. 2002). Patients with atopic dermatitis show reduced expression of CLDN1 in the nonlesional epidermis (De Benedetto et al. 2011). A recent study demonstrated that CLDN1 plays a key role in cutaneous wound healing (Volksdorf et al. 2017). In addition, CLDN1 is widely expressed in the intestinal epithelium; increased expression of CLDN1 is observed in intestinal inflammatory disorders, such as Crohn’s disease and ulcerative colitis (Garcia-Hernandez et al. 2017). Abnormal expression levels of CLDN1 were also detected in malignant tumours (Stache et al. 2014, Huang et al. 2015, Zhang et al. 2016). Diminished expression of CLDN1 has been detected in PE placentas in other studies (Lievano et al. 2006, Pirinen & Soini 2014); however, the mechanism behind this phenotype has not been demonstrated.

In the current study, we confirmed that the expression of CLDN1 was downregulated in the placental villus tissue in PE compared to that in uncomplicated pregnancy and identified a novel function of CLDN1 in trophoblasts. Moreover, inhibition of CLDN1 expression results in an increase in cleaved PARP via downregulation of BIRC3 expression.

Materials and methods

GEO data analysis

GEO datasets (GSE66273, GSE75010, GSE96984 and GSE114691) were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). PCA was initially used to evaluate the expression profiles between the two groups (Supplementary Fig. 1, see section on supplementary materials given at the end of this article), and the expression matrix was validated prior to subsequent analysis. Differential genes based on the array data were analysed using the limma package for R. For RNA-seq data, transcripts were filtered and preprocessed using the voom function before differential gene expression analysis using the limma package for R. For RNA-seq data, transcripts were filtered and preprocessed using the voom function before differential gene expression analysis using the limma package for R. Genes with adjusted P values less than 0.05 were considered statistically significant.

Statistical analysis

All data were acquired from at least three independent biological replicates. Data were presented as the mean ± s.d., and statistical analysis was performed using GraphPad Prism 8. For parametric data, a Student’s t-test was used to compare two groups. For nonparametric data, a Mann–Whitney test was used to compare two groups or a Kruskal–Wallis test was used to compare three or more groups. P values <0.05 were considered statistically significant.

Additional materials and methods are provided in Supplementary Information.

Table 1 Clinical information of patients included in this study. Data were presented as the mean ± s.d. and statistical analysis was done performed using GraphPad Prism 8. A Student’s t-test or Mann–Whitney test was used to compare two groups.

| Characteristics                | Healthy controls (n = 27) | Preeclampsia (n = 25) |
|-------------------------------|--------------------------|-----------------------|
| Maternal age, years           | 30.19 ± 3.076            | 33.52 ± 4.656**       |
| Gestational day               | 273.3 ± 12.14            | 256.1 ± 16.70****     |
| Systolic blood pressure, mmHg | 117.1 ± 9.698            | 150.5 ± 15.12****     |
| Diastolic blood pressure, mmHg| 72.41 ± 6.628            | 97.48 ± 8.809****     |
| Infant weight, g              | 3144 ± 470.3            | 2620 ± 703.3**        |
| Placenta weight, g            | 600.2 ± 105.1           | 553.4 ± 162.1**       |

**P < 0.01, **** P < 0.0001.
Results

**CLDN1 was downregulated in the placental villi of patients with preeclampsia**

To determine whether certain genes are involved in the pathogenesis of PE, four GEO datasets of the placental transcriptome in preeclampsia (PE) and healthy controls (HCs) were selected (Leavney et al. 2016, Than et al. 2018, Awamleh et al. 2019). Interestingly, two genes were identified based on the intersection of differentially expressed genes of the datasets, including CLDN1 and BHLHE40 (Fig. 1A). QRT-PCR was performed to confirm the results of bioinformatics analysis, and the level of CLDN1 was found to be significantly decreased in the placental villi of PE patients compared to that in HCs (Fig. 1B); however, the level of BHLHE40 was not significantly different in the present qRT-PCR result; therefore, we chose CLDN1 for further investigation. Western blotting and immunohistochemical staining were used to confirm a decrease in the expression of CLDN1 in the placental villus tissue in PE (Fig. 1C and D). Thus, CLDN1 expression is decreased in trophoblasts of PE patients, suggesting that this decrease may be involved in trophoblast dysfunction.

**Knockdown of CLDN1 inhibits proliferation and induces apoptosis in trophoblasts**

To investigate the role of CLDN1 in PE, we determined the expression level of CLDN1 in three commonly used trophoblast cell lines, HTR-8/SVneo, JAR and Bewo, using qRT-PCR and Western blotting. The results showed that HTR-8/SVneo had the highest level of CLDN1 expression (Fig. 2A), and immunofluorescence result showed the subcellular localisation of CLDN1 in HTR-8/SVneo (Fig. 2B). Therefore, we selected HTR-8/SVneo cells as an in vitro model to study the function of CLDN1 in trophoblasts. We used siRNA to knock down CLDN1 in HTR-8/SVneo cells, and knockdown efficiency was measured by Western blotting (Fig. 2C). Furthermore, the results of the CCK-8 assay indicated that CLDN1 knockdown significantly inhibited the proliferation of trophoblasts (Fig. 2D). Accordingly, the EdU assay demonstrated that knockdown of CLDN1 resulted in a decrease in the proportion of EdU-positive cells, which confirmed that downregulation of CLDN1 impairs trophoblast proliferation (Fig. 2E). The effect of CLDN1 on trophoblast apoptosis was evaluated by flow cytometry, and the results showed that knockdown of CLDN1 promoted trophoblast apoptosis at both early and late stages (Fig. 2F). TUNEL staining confirmed that CLDN1 knockdown induced apoptosis of the cells (Fig. 2G).

**Overexpression of CLDN1 promotes proliferation and alleviates apoptosis in trophoblasts**

We used a CLDN1-overexpressing plasmid to investigate the role of CLDN1 in trophoblasts. The overexpression efficiency was determined by Western blotting (Fig. 3A). The results of the CCK-8 assay demonstrated an increase in cell viability after CLDN1 overexpression (Fig. 3B), and the data of the EdU assay also indicated that CLDN1 overexpression promoted cell proliferation (Fig. 3C). Furthermore, the results of flow cytometry showed that CLDN1 overexpression decreased the proportion of apoptosis mainly in the late stage (Fig. 3D). Correspondingly, the data of the TUNEL assay showed that CLDN1 overexpression reduced apoptosis in trophoblasts though not significant (Fig. 3E).

**CLDN1 downregulation affects apoptosis-associated pathways**

Since CLDN1 has a significant influence on HTR-8/SVneo apoptosis, we tested apoptosis associated
protein BAX and BCL2 in CLDN1 downregulated cells. However, the results showed that CLDN1 may not affect apoptosis through these two proteins (Supplementary Fig. 2). RNA-seq assay was used to determine the molecular mechanism of regulation of trophoblast proliferation and apoptosis by CLDN1; we identified the differences in the transcriptomes of HTR-8/SVneo cells treated with negative control siRNA (siNC) or siRNA against CLDN1. A total of 268 differentially expressed genes were identified, including 144 upregulated and 124 downregulated genes (Fig. 4A and B). Genes with adjusted P values less than 0.05 were used to perform the enrichment analysis (Fig. 4C and D). For KEGG analysis, significantly enriched terms included protein processing in endoplasmic reticulum, ubiquitin-mediated proteolysis, apoptosis and cell cycle. Furthermore, qRT-PCR was used to validate the RNA sequencing results of the top 13 differentially expressed genes (Fig. 4E). BIRC3 was selected for the downstream analysis since it was one of the most prominently differentially expressed genes enriched in the apoptosis pathway. BIRC3 encodes cellular inhibitor of apoptosis protein 2 (cIAP2), a member of the inhibitors of apoptosis protein family (IAPs), which has been reported to indirectly inhibit caspase activity (Bai et al. 2014). Therefore, we detected BIRC3 protein levels in CLDN1-manipulated cells, and the results showed that BIRC3 was downregulated in CLDN1 knockdown cells and upregulated in CLDN1-
overexpressing cells (Fig. 4F). These data indicate that *CLDN1* regulates the expression of *BIRC3* in trophoblasts.

**CLDN1 regulates apoptosis via BIRC3 expression**

Decreased levels of *BIRC3* are commonly accompanied by increased levels of cleaved PARP in apoptotic cells (Zhang et al. 2019). Several small molecule mimetics of second mitochondria-derived activator of caspases (SMAC) have been developed to restore apoptosis in the tumour cells (Bai et al. 2014, Wang et al. 2016). Western blotting was performed to determine whether PARP is involved in trophoblast apoptosis, and the results showed that cleaved PARP was elevated by *CLDN1* downregulation, and the overexpression of *CLDN1* decreased the level of cleaved PARP (Fig. 5A). To verify that the *CLDN1/BIRC3/PARP* pathway regulates apoptosis of HTR-8/SVneo cells, we used flow cytometry to analyse the effect of *BIRC3* overexpression on apoptosis after *CLDN1* knockdown (Fig. 5B). The results showed that apoptosis was successfully reduced after *BIRC3* overexpression. In addition, cleaved PARP levels were decreased after overexpression of *BIRC3* in *CLDN1* knockdown HTR-8/SVneo cells (Fig. 5C). Furthermore, *BIRC3* expression was measured in PE and HC villus tissue using Western blotting, and we found that *BIRC3* is decreased in the PE placentas compared with HC placentas though the difference is not significant (Fig. 5D). Thus, our results suggested that *CLDN1* inhibited *BIRC3* mRNA levels and that downstream PARP participated in trophoblast apoptosis.

**Discussion**

Accumulating studies have demonstrated the transcriptome differences between placenta in preeclampsia (PE) and uncomplicated pregnancy (Nishizawa et al. 2007, Awamleh et al. 2019). In contrast, this study combined four GEO datasets to compare the transcriptome of placenta in preeclampsia and normal
Figure 4 CLDN1 downregulation affects apoptosis-associated pathways. (A and B) Volcano and heatmap plots showing differentially expressed genes after CLDN1 knockdown. (C and D) KEGG and GO enrichment analysis. (E) qRT-PCR results of differentially expressed genes after CLDN1 downregulation. (F) Western blotting assay of BIRC3 after CLDN1 downregulation or overexpression. Each in vitro test was performed three times, Mann Whitney test was used, and the data are presented as the mean ± s.d. *P < 0.05, NS, not significant.
Figure 5 CLDN1 regulates apoptosis through the BIRC3/PARP pathway. (A) Western blotting showing alteration in the levels of cleaved PARP after CLDN1 downregulation or overexpression. (B) BIRC3 overexpression in CLDN1-downregulated cells reduced trophoblasts apoptosis according to the data of flow cytometry. (C) Level of cleaved PARP after BIRC3 overexpression in CLDN1-downregulated cells. (D) Protein level of BIRC3 in HC and PE. Each in vitro test was performed three times, Mann Whitney test were used in (A) and (D) and Kruskal-Wallis test was used in (B), and the data are presented as the mean ± s.d. *P < 0.05, NS, not significant.
pregnancy using bioinformatics analysis to demonstrate potential differential expression of the \textit{CLDN1} gene. Both the mRNA and protein levels of \textit{CLDN1} were decreased in the placental villi tissue of preeclampsia patients. The tight junction complex plays an important role in cellular activities including proliferation, migration and so on (Garcia-Hernandez et al. 2017). As an important protein regulating cell-cell attachment, we assume that the downregulation of \textit{CLDN1} to some extent disrupted the completeness of plasma membrane which is vital to maintain the integrity of the cell function. In this article, we confirmed that a decrease in \textit{CLDN1} leads to the induction of apoptosis in trophoblasts.

Increased apoptosis of villus trophoblasts in placenta pathologies has been observed in many pregnancy-associated diseases, including pregnancy loss, preeclampsia and intrauterine growth restriction (Ishihara et al. 2002, Sharp et al. 2010). Hypoxia-reoxygenation and oxidative stress are associated with trophoblast apoptosis (Soleymanlou et al. 2007). Moreover, Kalkat et al. reported that the balance of BCL2 family proteins was altered towards cell death in preeclampsia (Kalkat et al. 2013). Recent studies have focused on trophoblast apoptosis caused by molecular dysfunction that leads to the disease (Guo et al. 2017, Wang & Yan 2018, Mo et al. 2020). \textit{CLDN1} has been shown to be involved in apoptosis via alterations in the expression levels of Bcl-2 and Bax (Singh et al. 2012, Zheng et al. 2019), which was not detected in our study (Supplementary Fig. 2). We suggest that this difference may be due to the heterogeneity of tissue origin.

The impact of \textit{CLDN1} gene on apoptosis has been investigated in a number of studies. For example, \textit{CLDN1} downregulation was shown to promote apoptosis and hinder cell proliferation in tumour cells (Huang et al. 2015, Zheng et al. 2019). Some studies demonstrated that \textit{CLDN1} was associated with the activation of autophagy (Zhao et al. 2017), and other studies concluded that \textit{CLDN1} regulates anoikis (Singh et al. 2012, Huang et al. 2015). Our data indicated that downregulation of \textit{CLDN1} inhibited the proliferation and induced apoptosis in trophoblasts. Since the detailed mechanism by which \textit{CLDN1} regulates trophoblast apoptosis is unclear, we used RNA-seq in \textit{CLDN1}-knockdown HTR-8/SVneo cells. The results identified BIRC3 as a molecule downstream of \textit{CLDN1}. BIRC3 was shown to be involved in the regulation of both intrinsic and extrinsic apoptosis pathways via indirect regulation of caspase activity (Varfolomeev et al. 2007, Bai et al. 2014, Gressot et al. 2017, Lee et al. 2017, Zhang et al. 2019). Majority of claudins contain a PDZ binding motif that interacts with intercellular molecules that have signalling functions (Garcia-Hernandez et al. 2017). As a tight junction protein, claudin-1 was confirmed to be associated with Src/p-Src in colon cancer cells in modulating cell anoikis (Singh et al. 2012). It was also found that claudin-1 regulates cell anoikis through ZEB-1 (Singh et al. 2011). Evidence has also been found that claudin-1 regulates apoptosis in human breast cancer cells through deactivation of pro-caspase-8 (Liu et al. 2012). Other claudin family member such as claudin-8 interacts with IL22 in colon cell lines to rescue apoptosis induced by LINC00662 knockdown (Cheng et al. 2020). However, the mechanism of how \textit{CLDN1} regulates downstream \textit{BIRC3} remains to be determined.

Accumulated cleaved PARP has been detected in other trophoblast cell lines in a study that demonstrated that increased apoptosis in trophoblasts is accompanied by an increase in the levels of proapoptotic proteins (Chen et al. 2015). We hypothesised that the accumulation of cleaved PARP caused by \textit{BIRC3} downregulation may be involved in the mechanism of PE pathogenesis. Thus, we investigated the effect of \textit{CLDN1} knockdown on the level of cleaved PARP. The results showed that knockdown of \textit{CLDN1} increased the level of cleaved PARP, and subsequent experiments showed that overexpression of \textit{BIRC3} in \textit{CLDN1}-downregulated HTR-8/SVneo cells reduced cell apoptosis. Some studies showed associations between \textit{BIRC3} and necrosis (Geserick et al. 2009, Bai et al. 2014); hence, we examined the impact of \textit{CLDN1} downregulation on trophoblast necrosis. However, the levels of phosphorylated RIP and phosphorylated MLKL showed trends that were not consistent with this assumption (Supplementary Fig. 2).

In conclusion, we demonstrated that downregulation of \textit{CLDN1} regulates trophoblast apoptosis via a decrease in the levels of \textit{BIRC3} and downstream PARP, which is a new phenomenon in the pathogenesis of preeclampsia (Fig. 6). Our study demonstrated the association of \textit{CLDN1} and preeclampsia, which can offer a new potential therapeutic target for future clinical applications. However, current results are based on experiments of trophoblast cell lines which is still quite different to the normal trophoblasts; and further investigation using primary trophoblast cells may help to further verify our conclusion.

![Figure 6 Schematic depiction of CLDN1 in trophoblast apoptosis.](https://rep.bioscientifica.com)
CLDN1 regulates trophoblast apoptosis

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Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/REP-20-0677.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
Y-C Z and F-J T conceived and designed the experiments. Y-C Z, X-L Q, H-Q M, C-X Z helped to collect samples. Y-C Z, H-Q M, S Q, X-W W and X-Q L helped to perform the experiments. Y-C Z and F-J T analysed the data and wrote the manuscript. F-J T, Y Z and Y L contributed reagents and materials and to revise the manuscript.

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