Preeclampsia

Dysregulated DNA Methyltransferase 3A Upregulates IGFBP5 to Suppress Trophoblast Cell Migration and Invasion in Preeclampsia

Yuanhui Jia,* Ting Li,* Xiaojie Huang, Xiangdong Xu, Xinyao Zhou, Linyan Jia, Jingping Zhu, Dandan Xie, Kai Wang, Qian Zhou, Liping Jin, Jiqin Zhang, Tao Duan

Abstract—Preeclampsia is a unique multiple system disorder during human pregnancy, which affects ≈5% to 8% of pregnancies. Its risks and complications have become the major causes of maternal and fetal morbidity and mortality. Although abnormal placentation to which DNA methylation dysregulation is always linked is speculated to be one of the reasons causing preeclampsia, the underlying mechanisms still remain elusive to date. Here we revealed that aberrant DNA methyltransferase 3A (DNMT3A) plays a critical role in preeclampsia. Our results show that the expression and localization of DNMT3A are dysregulated in preeclamptic placenta. Moreover, knockdown of DNMT3A obviously inhibits trophoblast cell migration and invasion. Mechanistically, IGFBP5 (insulin-like growth factor–binding protein 5) known as a suppressor, is upregulated by decreased DNMT3A because of promoter hypomethylation. Importantly, IGFBP5 downregulation can rescue the defects caused by DNMT3A knockdown, thereby, consolidating the significance of IGFBP5 in the downstream of DNMT3A in trophoblast. Furthermore, we detected low promoter methylation and high protein expression of IGFBP5 in the clinical samples of preeclamptic placenta. Collectively, our study suggests that dysregulation of DNMT3A and IGFBP5 is relevant to preeclampsia. Thus, we propose that DNMT3A and IGFBP5 can serve as potential markers and targets for the clinical diagnosis and therapy of preeclampsia.

Key Words: DNMT3A ■ DNA methylation ■ IGFBP5 ■ migration and invasion ■ preeclampsia ■ trophoblast

Received September 18, 2016; first decision October 10, 2016; revision accepted December 3, 2016.

From the Clinical and Translational Research Center (Y.J., X.X., X.Z., L.J.), Department of Obstetrics (T.L., T.D.), Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, China; and Shanghai Key Laboratory of Regulatory Biology, the Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai, China (J. Zhang).

The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.116.08483/D1.

© 2017 American Heart Association, Inc.

Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.116.08483
study demonstrated the importance of Dnm3a for a cluster of genes involved in placental development.23 Furthermore, it has been proved that maternal deletion of Dnm3ab causes the defects of trophoblast development at embryonic day 9.5, which is represented by a deficient labyrinthine layer and a low-density trophoblast giant cell layer, possibly as a result of decreased cell adhesion and migration.24 However, little is known about the role of DNMT3A in PE.

IGFBP5 (insulin-like growth factor–binding protein 5) is a member of one protein family, which is characterized by specifically binding to insulin. The role of IGFBP5 has been uncovered in cancer, although the conclusions are paradoxical in different types. For example, IGFBP5 serves as an antiproliferative factor in breast cancer, whereas it augments IGF-dependent and IGF-independent cell survival and proliferation in retinoblastoma and pancreatic cancer.25–27 Moreover, casstration-induced IGFBP5 upregulation accelerates progression to androgen independence in prostate cancer.28 Interestingly, several data implicate that IGFBP5 may function in placenta as well. It is localized in the syncytiotrophoblast layer of first trimester placental villi and attenuates the effects of IGF-1 and IGF-2 on promoting trophoblast cell migration.29 Additionally, increased transcriptional level of IGFBP5 has been detected in preeclamptic placenta.30 Nevertheless, the current understanding of IGFBP5 in placenta is still limited.

Here, we found that DNMT3A is aberrantly expressed and localized in preeclamptic placenta. Further study demonstrated that knockdown of DNMT3A inhibits the migration and invasion of trophoblast cells. In mechanism, downregulated DNMT3A elevates IGFBP5 expression by reducing its promoter methylation. The significant role of IGFBP5 as a factor downstream of DNMT3A is further supported by the rescue experiments. Moreover, our data of the clinical samples reveal promoter hypomethylation and high protein expression of IGFBP5 in preeclamptic placenta. Taken together, our study suggests that dysregulated DNMT3A plays a critical role in PE by upregulating IGFBP5.

Materials and Methods

Patients and Samples Collection

Placenta tissues were obtained immediately (<30 mins) from a portion of normal pregnancies (38±0.24 weeks, n=14) and PE patients (37±0.39 weeks, n=12) after delivery by caesarean section. The clinical characteristics of the full-term pregnancies and PE patients are shown in Table S1 in the online-only Data Supplement. Pe group was defined as severe PE according to the National High Blood Pressure Education Program Working Group’s report.31 PE group was defined as severe PE according to the National High Blood Pressure Education Program Working Group’s report.31 PE patients are shown in Table S1 in the online-only Data Supplement. PE group was defined as severe PE according to the National High Blood Pressure Education Program Working Group’s report.31 PE patients are shown in Table S1 in the online-only Data Supplement.

The placental samples were approved by the Scientific and Ethical Committee of the Shanghai First Maternity and Infant Hospital affiliated with Tongji University. All the samples were collected with a written informed consent provided by the participants.

Cell Culture

The HTR-8/SVneo cell line is derived from first trimester human trophoblasts and introduced with gene encoding simian virus 40 large T antigen, which was described by Graham et al.32 It was a kind gift from Dr C.H. Graham at Queen’s University, Canada. The JAR and JEG3 cell lines were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and characterized by short tandem repeat detection. The HTR-8/SVneo and JAR cells were cultured in DMEM/F12 medium (GIBCO), and JEG3 was cultured in αMEM medium (GIBCO), which were supplemented with 10% fetal bovine serum at 37°C under 5% CO₂ humidified air according to the standard procedures.

Bisulfite Sequencing and Pyrosequencing

Genomic DNA of cells and tissues was extracted using the standard phenol–chloroform technique followed by proteinase K treatment to prevent protein contamination. Bisulfite conversion was performed using an EpiTect Bisulfite Kit (Qiagen) according to the manufacturer’s instruction.

For bisulfite sequencing, bisulfite-treated DNA was amplified, cloned into vector pEASYT1 (TransGen Biotech), and sequenced. The primers for IGFBP5 amplification were as follows: forward 5′-GAAAAGGTATAGAGGTAGTA-3′; reverse 5′-CAAAATCTCCCAAATTCATCC-3′.

For pyrosequencing, the amplified DNA products were detected by PyroMark Q96 ID (Qiagen). The primers for IGFBP5 amplification were as follows: forward 5′-AGAGATGTATAGAGGTAGTA-3′; reverse 5′-CAAAATCTCCCAAATTCATCC-3′. The primer for sequencing was as follow: 5′-GGTG AGAGATGTTAGTGTAG-3′.

Statistical Analysis

Values are presented as mean±SEM. Statistical data were analyzed by Student’s t test, 1-way analysis of variance, and 2-way analysis of variance. Significant differences were considered when P<0.05. The statistical analyses were performed by using software SPSS 16.0.

Results

DNMT3A Is Aberrantly Expressed and Localized in Preeclamptic Placenta

Several groups have reported that DNA methylation is aberrant in preeclamptic placenta.32–34 To dissect the underlying mechanisms, we analyzed DNMT3A protein expression in the placentas of first trimester, full-term pregnancies, and PE patients by using Western blot. Although the protein level of DNMT3A was comparable between first trimester villi and full-term pregnant placentas, its protein expression was significantly decreased in preeclamptic placentas (Figure 1A and 1B). In addition, we performed immunohistochemistry to detect DNMT3A localization in placenta. It was observed that DNMT3A was localized in both the syncytiotrophoblast and cytotrophoblast layers of first trimester villi and in the syncytiotrophoblast layer of placentas from full-term pregnancies. However, DNMT3A was localized rarely in the syncytiotrophoblast layer and found mainly in villous stroma cells of placentas from PE patients (Figure 1C and
Altogether, these data reveal that the protein expression and localization of DNMT3A are abnormal in preeclamptic placenta.

DNMT3A Downregulation Suppresses the Migration and Invasion of Trophoblast Cells

On the basis of the above observations, we speculated that loss of DNMT3A function in trophoblast is critical for PE. To test this hypothesis, we attempted to investigate the role of DNMT3A in trophoblast. First, we detected DNMT3A expression in 3 different trophoblast cell lines and found that it was highly expressed in HTR-8/SVneo cells and lowly expressed in JAR and JEG3 cells (Figure 2A). Thus, we used 2 specific shRNAs to reduce DNMT3A expression in HTR-8/SVneo cells. The knockdown effects of those shRNAs on DNTM3A protein expression were verified by Western blot (Figure 2B). Then, we performed MTS assay to determine trophoblast cell proliferation. The representative results showed that a slight decrease in cell number was observed in only one DNMT3A knockdown group.
after 48 or 72 hours, which implies that DNMT3A down-regulation has a little effect on trophoblast cell proliferation (Figure 2C). In addition, the migration and invasion of trophoblast cells were assessed by transwell assays. It was seen that decreased DNMT3A led to an obvious inhibition of cell migration and invasion of ≈70% to 80% (Figure 2D and 2E). Taken together, these results suggest that DNMT3A is required for the migration and invasion of trophoblast cells.

**Overexpression of DNMT3A Promotes the Migration and Invasion of Trophoblast Cells**

To further validate the important functions of DNMT3A in trophoblast cells by another way, we stably overexpressed DNMT3A in the JAR cell line (Figure 3A). As shown in Figure 3B, upregulated DNMT3A did not affect trophoblast cell proliferation evidently. In contrast, it significantly promoted the migration and invasion of trophoblast cells (Figure 3C and 3D). These observations were compatible with the findings in DNMT3A knockdown cell lines, thereby, further supporting the significance of DNMT3A in trophoblast cells.

**IGFBP5 Is Regulated by DNMT3A Through Promoter Methylation Alteration in Trophoblast Cells**

IGFBP5 has been reported to inhibit trophoblast cell migration and be highly transcribed in PE, which suggests that it may play a significant role in PE. Intriguingly, IGFBP5 has been found to be upregulated by a DNMTs inhibitor (5-AzadC) as well. These findings prompted us to explore whether DNMT3A modulates IGFBP5 expression in trophoblast cells. To this end, we first examined mRNA transcription and protein expression of IGFBP5.
after DNMT3A was knocked down in HTR-8/SVneo cells. The results of quantitative polymerase chain reaction and Western blot showed that both the mRNA and protein levels of IGFBP5 were elevated by decreased DNMT3A (Figure 4A and 4B). Consistently, we also observed that overexpressed DNMT3A resulted in the reduction of IGFBP5 mRNA transcription in JAR cells (Figure 4C). These data implicate that IGFBP5 expression is controlled by DNMT3A in trophoblast cells.

Next, to clarify whether this influence is owing to promoter methylation alteration, we detected the changes of IGFBP5 transcription after adding 1, 5, and 10 μmol/L 5-AzadC to HTR-8/SVneo cells, respectively. The results illustrated that mRNA level of IGFBP5 was gradually raised by increased 5-AzadC after 24 hours (Figure 4D). Furthermore, we performed bisulfite sequencing of some CpG sites in IGFBP5 promoter and found that the percentage of DNA methylation was diminished from 20% to 8.2% after DNMT3A downregulation (Figure 4E). Consistent with the observation, pyrosequencing results indicated that DNA methylation levels of several specific CpG sites (5, 9, and 10) were obviously decreased in DNMT3A knockdown HTR-8/SVneo cells (Figure 4F). Collectively, these findings implicate that DNMT3A suppresses mRNA transcription and protein expression of IGFBP5 by increasing its promoter methylation.

IGFBP5 Serves as a Key Factor Downstream of DNMT3A to Regulate Trophoblast Cell Migration and Invasion

To understand the importance of IGFBP5 as a factor downstream of DNMT3A, we sought to perform rescue experiments in trophoblast cells. For this purpose, we transfected specific siRNA against IGFBP5 into DNMT3A-downregulated HTR-8/SVneo cells and then performed cell proliferation, migration, and invasion assays. The knockdown effects on IGFBP5 transcription and expression were confirmed by quantitative polymerase chain reaction and Western blot (Figure 5A and 5B). The results of cell proliferation assay showed that decreased IGFBP5 did not have a significant influence on the proliferation of HTR-8/SVneo cells (Figure 5C). In contrast, IGFBP5 reduction partially reversed the inhibitory effects of
DNMT3A knockdown on trophoblast cell migration and invasion (Figure 5D and 5E).

One study previously reported that IGFBP5 inhibits the migration of human breast cancer cells by altering the p53-FAK (focal adhesion kinase) pathway. To test whether the DNMT3A-IGFBP5 axis also affects cell migration and invasion through this pathway in trophoblast, we determined the phosphorylation of p53 and FAK by Western blot. As shown in Figure 5F, S46 phosphorylation rather than S392 phosphorylation of p53 was elevated, and T397 phosphorylation of FAK was reduced by DNMT3A downregulation in HTR-8/SVneo cells, which were similar to the alterations when recombinant IGFBP5 was added exogenously. Notably, these changes could be reversed by IGFBP5 knockdown, thus, implying that DNMT3A influences the p53-FAK pathway by modulating IGFBP5 in trophoblast cells. Altogether, these data indicate that IGFBP5 is a key factor downstream of DNMT3A to regulate the migration and invasion of trophoblast cells.

Low Promoter Methylation and High Protein Expression of IGFBP5 Are Found in PE

To further evaluate the role of IGFBP5 in PE, we measured the mRNA and protein levels of IGFBP5 in clinical placental samples from full-term pregnancies and PE patients by quantitative polymerase chain reaction, immunohistochemistry, and Western blot. We observed that IGFBP5 was highly transcribed and expressed in the placentas from PE patients compared with those from full-term pregnancies (Figure 6A through 6C). It is also noteworthy that the phosphorylation
levels of p53 at S46 and FAK at T397 were higher and lower, respectively, in the placentas of PE patients relative to full-term pregnancies, which were in accordance with the change of IGFBP5. Furthermore, we examined the status of IGFBP5 promoter methylation by bisulfite sequencing and pyrosequencing in 3 pairs of independent samples. The results indicated that the IGFBP5 promoter was hypomethylated in PE (Figure 6D and 6E). Taken together, these findings reveal that IGFBP5 is dysregulated in PE.

During embryonic development, epigenetic changes are elaborately regulated, which is coupled with the alterations of intricate signaling networks and transcriptional events. It is thought that dysregulation of epigenetic changes is the possible reason causing various diseases during this period. As one of these diseases, PE is a unique multiple system disorder leading to maternal and fetal morbidity and mortality. Although aberrant DNA methylation has been found in PE,
its influence on PE is yet unclear. In this study, we uncovered that DNMT3A is indispensable for trophoblast cell migration and invasion. The molecular mechanism is that DNMT3A modulates the expression of IGFBP5 through altering its promoter methylation. Importantly, we further found aberrant DNMT3A and IGFBP5 in PE. In conclusion, our study demonstrates that dysregulation of DNMT3A and IGFBP5 is relevant to PE.

Recent studies in mouse model report that maternal deletion of Dnmt3a/b causes the defects of trophoblast development at embryonic day 9.5, and loss function of Dnmt3a in mouse embryonic stem cells disrupts the expression of a cluster of genes involved in placenta development. In agreement with these results, we found that DNMT3A is necessary for the migration and invasion of trophoblast cells and is dysregulated in PE. In addition, the functions

Figure 6. IGFBP5 (insulin-like growth factor–binding protein 5) is dysregulated in preeclampsia (PE). A, Quantitative PCR analysis of IGFBP5 mRNA level in the placentas of full-term pregnancies (n=14) and PE patients (n=12). B, Representative images of IGFBP5 expression and localization in the clinical samples by immunohistochemistry (IHC) analysis. C, The clinical samples were lysed and then subjected to Western blot by using antibodies as indicated. Densitometry quantification of IGFBP5/β-actin, p-p53 (S46)/p53 and p-FAK (T397)/FAK levels are shown. D and E, The percentages of DNA methylation in the specific CpG sites of the IGFBP5 promoter, as described in Figure 4, were analyzed by bisulfite sequencing (D) and pyrosequencing (E) in 3 pairs of independent samples. All the statistical data were analyzed by Student’s t test (2 groups) or 1-way analysis of variance (ANOVA; above 2 groups). All data are means±SEM. *P<0.05; **P<0.01; ***P<0.001.
of other DNMTs in embryonic development have also been studied. For example, Dnmt1-deficient mice showed genome-wide demethylation and increased triacylglycerol levels in placenta.\textsuperscript{40} Increased DNMT1 and DNMT3B correlate with decreased expression of syncytin-1, GATA1, and HLA-G in preeclamptic placentas, which are key factors in trophoblast.\textsuperscript{41–43} These findings stimulate interest in investigating the roles of DNMT1 and DNMT3B in PE in future studies.

Although DNMT3A has been identified as a de novo DNA methyltransferase, several studies have demonstrated that it is also required for maintaining the DNA methylation patterns of some specific sites.\textsuperscript{44–46} Here, we found that DNMT3A downregulation results in IGFBP5 promoter hypomethylation in trophoblast cells. Consistently, this correlation is further detected in clinical samples of PE. These results suggest that DNMT3A plays a significant role in the DNA methylation maintenance of specific genomic regions in trophoblast. Moreover, our data also present the first direct evidences showing that IGFBP5 expression is controlled by promoter methylation. It should be noted that IGFBP5 reduction fails to fully restore the deficiencies caused by DNMT3A knockdown, which implies that DNMT3A probably regulates other downstream modulators to influence the migration and invasion of trophoblast. Thus, future research about DNMT3A-mediated promoter methylation alteration of other key factors may provide further insight into the function of DNMT3A in PE.

IGFBP5, as one of the members of the IGFBP family, has been suggested to play an important role in embryonic development.\textsuperscript{47} In line with this finding, we found that IGFBP5 works as a critical factor downstream of DNMT3A to regulate cell migration and invasion in trophoblast. Additionally, we further observed that both mRNA transcription and protein expression of IGFBP5 are upregulated in placentas of PE patients. This finding substantiates the significance of IGFBP5 in PE. Notably, our data are partially inconsistent with the preceding result that mRNA but not protein level of IGFBP5 is elevated in PE.\textsuperscript{30} We suppose that this discrepancy is likely because of different methods for detection. Ligand blot assay was applied formerly, whereas we determined IGFBP5 protein expression by using the specific antibody in Western blot. Regarding downstream factors, we found that the p53-FAK pathway is influenced by the DNMT3A-IGFBP5 axis in trophoblast cells. This observation is consistent with the previous report showing that IGFBP5 represses cell migration by inducing sustained S46 phosphorylation of p53 and decreased T397 phosphorylation of FAK in breast cancer.\textsuperscript{37,48} In addition, we detected the similar changes in clinical placental samples from PE patients, which indicates that the p53-FAK pathway plays a crucial role in PE.

To date, the causes of PE still remain enigmatic. One significant finding by us was that DNMT3A is aberrantly expressed and localized in preeclamptic placenta. We provide the first evidence that dysregulation of DNMT3A is associated with PE and, thus, support the results of previous studies showing the important influence of dysregulated DNA methylation on PE. Furthermore, we observed low promoter methylation and high protein expression of IGFBP5 in the clinical samples of preeclamptic placenta, which suggests the critical role of IGFBP5 in PE. Based on these results, we propose that DNMT3A and IGFBP5 can serve as potential markers for the clinical diagnosis of PE. Notably, IGFBP5 is a secreted protein, which makes it a good candidate for convenient diagnosis. On the other hand, because upregulated IGFBP5 suppresses trophoblast cell migration and invasion, it presents a new strategy for PE therapy by applying specific inhibitors or antibodies to block IGFBP5 pathway. The feasibility and availability of these methods need to be tested by laboratory and clinical experiments in the future.

In summary, this study indicates that DNMT3A plays important roles in trophoblast cell migration and invasion by changing promoter methylation to modulate IGFBP5 expression. Furthermore, our evaluation of clinical samples reveals that dysregulation of DNMT3A and IGFBP5 is associated with PE. Thus, these findings suggest that DNMT3A and IGFBP5 are potential markers and targets for clinical diagnosis and therapy of PE.

**Perspectives**

In the current study, we demonstrated that DNMT3A is required for the migration and invasion of trophoblast cells by modulating IGFBP5 expression. In theory, our data present new evidences supporting the significant roles of DNMT3A and IGFBP5 in embryonic development. In addition, we are the first to disclose that IGFBP5 is directly regulated by DNMT3A through promoter methylation alteration. Importantly, we also observed that DNMT3A and IGFBP5 are dysregulated in PE. These findings are valuable for understanding the pathogenesis of PE. Thus, we propose that DNMT3A, and especially IGFBP5, as a secreted protein, can serve as potential ideal markers for the clinical diagnosis of PE. Furthermore, given that IGFBP5 is aberrantly highly expressed in PE, it is reasonable to develop strategies to treat PE by using specific inhibitors or antibodies to block the IGFBP5 pathway in future studies.

**Sources of Funding**

This work was supported by National Natural Science Foundation of China (81401217 to Y. Jia; 81301819 to J. Zhang; 81501273 to T. Li; 81501270 to X. Xu; 81601312 to X. Zhou) and the project of Excellent Academic Leaders of Shanghai (14XD1403200 to T. Duan).

**Disclosures**

None.

**References**


What Is New?

- Our study demonstrates that DNA methyltransferase 3A (DNMT3A) is required for trophoblast cell migration and invasion by regulating IGFBP5 (insulin-like growth factor–binding protein 5) expression.
- We provide the first evidence that IGFBP5 is directly regulated by DNMT3A through promoter methylation alteration.
- Our data indicate that the DNMT3A–IGFBP5 axis influences the downstream p53-FAK (focal adhesion kinase) pathway in trophoblast.
- This is the first report revealing dysregulation of DNMT3A and IGFBP5 in preeclampsia.

What Is Relevant?

- Aberrant DNMT3A and IGFBP5 are correlated with preeclampsia.

Summary

Dysregulation of DNMT3A and IGFBP5 is associated with preeclampsia.
Dysregulated DNA Methyltransferase 3A Upregulates IGFBP5 to Suppress Trophoblast Cell Migration and Invasion in Preeclampsia
Yuanhui Jia, Ting Li, Xiaojie Huang, Xianghong Xu, Xinyao Zhou, Linyan Jia, Jingping Zhu, Dandan Xie, Kai Wang, Qian Zhou, Liping Jin, Jiqin Zhang and Tao Duan

Hypertension. 2017;69:356-366; originally published online January 3, 2017;
doi: 10.1161/HYPERTENSIONAHA.116.08483

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/69/2/356

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2017/01/03/HYPERTENSIONAHA.116.08483.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Dysregulated DNA Methyltransferase 3A Up-regulates IGFBP5 to Suppress Trophoblast Cell Migration and Invasion in Preeclampsia

Yuanhui Jia¹*, Ting Li²*, Xiaojie Huang¹, Xianghong Xu¹, Xinyao Zhou¹, Linyan Jia¹, Jingping Zhu¹, Dandan Xie¹, Kai Wang¹, Qian Zhou¹, Liping Jin¹, Jiqin Zhang³†, Tao Duan²†

¹Clinical and Translational Research Center, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai, 200040, China.
²Department of Obstetrics, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai, 200040, China.
³Shanghai Key Laboratory of Regulatory Biology, the Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai, 200241, China.

†Corresponding authors:
Jiqin Zhang, Ph.D., Shanghai Key Laboratory of Regulatory Biology, the Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, No. 500, Dongchuan Road, Shanghai, China. Phone: +86 24207054. Email: jqzhang@bio.ecnu.edu.cn.
Tao Duan, M.D., Ph.D., Department of Obstetrics, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, No. 536, Changle Road, Shanghai, China. Phone: +86 54037438. Email:tduan@yahoo.com.

*These authors contributed equally to this work.

Short title: Aberrant DNMT3A and IGFBP5 Link to Preeclampsia
Supplementary Methods

Establishment of stable cell lines and transient transfection
DNMT3A knockdown was performed by using a short hairpin RNA (shRNA) lentiviral vector (pGreenPure™, System Biosciences) according to the manufacturer’s instructions. Briefly, HTR-8/SVneo cells were seeded at 2 × 10⁵ cells/well in a 6-well plate. After adherent cells reached ~30% confluence, they were infected with specific DNMT3A shRNA viruses (shDNMT3A#1 or shDNMT3A#2) or a scramble shRNA (shCtrl) virus, respectively. Then, DNMT3A knockdown stable cell lines were selected with 0.2 μg/ml puromycin (sigma). IGFBP5 gene knockdown was performed by transient transfection of siRNA in the use of Lipofectamin™ 3000 (Invitrogen) according to the manufacturer’s instructions. For DNMT3A over-expression, JAR cells were infected by using the lentiviral vector pCDH (System Biosciences) and next selected with 0.5 μg/ml puromycin (sigma).

For rescue experiment, HTR-8/SVneo cells with or without DNMT3A knockdown were transiently transfected with IGFBP5 siRNA for 48 hours, then the cells were subject to the following experiments.

The RNAi sequences targeting human DNMT3A were as follows: DNMT3A shRNA#1 5’-CTACTACATCAAGGCAGCAA-3’; shRNA#2 5’-CCAGATGTTCTTCGCTAAT-3’; scramble sequence 5’-AATCGCATAGCGTATGCCGTT-3’. The RNAi sequences targeting human IGFBP5 were as follows: IGFBP5 siRNA 5’-GAUUCUAAGAGAAGCAGUGCTT-3’; scramble sequence 5’-UUCUCCGAGCCUGACGUTT-3’.

Immunohistochemistry

Four-millimeter thick sections were cut from the paraffin-embedded tissue and mounted onto 3-amino-propyl-tri-ethoxy-silane (APES)-coated glass slides. For immunohistochemical detection, tissue slides were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide/methyl alcohol for 10 mins. Nonspecific binding sites were blocked with 5% BSA for 30 mins. Slides were incubated with primary polyclonal antibody against DNMT3A (Santa Cruz) or IGFBP5 (Santa Cruz) at 4°C overnight, then incubated for 30 mins with secondary rabbit antibody conjugated with horseradish peroxidase and exposed to DAB (Gene Technology Shanghai Company).

The approach of distinguishing different cell types in placenta is as following: syncytiotrophoblast overlies a layer of cytotrophoblast and interstitial tissue in early pregnancy. After pregnancy for about 14 weeks, cytotrophoblastic column structures beneath the syncytiial layer gradually disappear. By parturition the predominant structure in villous surface is a layer of syncytiurn with sparse cytотrophoblast.

Quantitative PCR

Total RNA was extracted using Trizol reagent (Invitrogen) and RNAsimple Total RNA kit (Tiangen Biotech) according to the manufacturer’s instructions. RNA was quantified by UV absorption, and 0.5 μg total RNA was reverse-transcribed using the PrimeScript RT Reagent kit (TaKaRa) to generate cDNA. Quantitative PCR was carried out by using SYBR Premix Ex Taq (TaKaRa) and the Applied Biosystems StepOnePlus PCR System (Life Technologies) according to the manufacturer’s instructions. The relative levels of mRNA were analyzed using the 2⁻ΔΔCT method and normalized to β-actin.

The primers were as follows: IGFBP5 forward 5’-GACCCCTGAACCTTCTCTCC-3’,
reverse 5’-ATGTCTGCCTGGGGAATGAA-3’; β-actin forward 5’-CGTCTTCCCCCTCCATCG-3’, reverse 5’-CTCGTTAATGTCACGCAC-3’.

**Western blot**

Cells and tissues were lysed by cold lysis buffer (20 mM Tris-HCl pH=7.4, 150 mM NaCl, 10mM EDTA, 1% NP40, 1% Triton X-100, sodium deoxycholate) with complete ULTRA tablets (Roche) and PhosSTOP EASYpack tablets (Roche). The supernatants were harvested and protein concentration was determined by the BCA Protein Assay Reagent (Thermo Scientific). For IGFBP5 detection in cells, the supernatants of HTR-8/SVneo cells were collected for 100-fold protein concentration by Amicon Ultra (Milipore, MWCO=10000Da). The lysates were then separated by 10% SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride membranes. After blocked with 5% non-fat milk in TBST, membranes were incubated with the primary antibodies against human DNMT3A (Santa Cruz), IGFBP5 (R&D), p53 (Cell Signaling Technology), phosphorylated p53 at serine 46 (Cell Signaling Technology), phosphorylated p53 at serine 392 (Cell Signaling Technology), FAK (Bioworld Technology), phosphorylated FAK at Tyr397 (Bioworld Technology) or β-actin (Abcam). After incubation with an HRP-conjugated secondary antibody (Cell Signaling Technology), the signals were measured by using ECL reagents (Millipore). The densitometry of the target protein band was quantified by Image J software. β-actin was used as an endogenous loading control.

**Treatment with 5-Aza-2’-deoxycytidine (5-AzadC)**

HTR-8/SVneo cells were seeded for 12-24 hours and then treated by 5-AzadC (Sigma) with different concentrations (0 μmol/L, 1 μmol/L, 5 μmol/L, 10 μmol/L) for 24 hours, respectively.

**Cell proliferation assay**

Cells were seeded in 96-well plates at 2000 cells/well. The cell proliferation was determined after 24, 48 and 72 hours, respectively, by using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega) according to the manufacturer's instruction.

**Cell migration and invasion assays**

For migration assay, a 24-well transwell insert system (8 μm pore size, BD Biosciences) was used. Cells were seeded in the upper chamber of the insert (4x10^4 cells/well) and cultured in 300 μl DMEM/F12 medium supplemented with 2% FBS. The lower chambers were filled with 800 μl DMEM/F12 medium supplemented with 10% FBS. After 16 hours, 80 μl fluorescent stain (calcein-AM) was added to each lower chamber and incubated for 30 mins. The migrated labeled cells were detected by an inverted microscope mounted with a CCD camera and counted by using Metamorph image analysis software (Molecular Devices, Sunnyvale, CA).

For invasion assay, the upper chamber of the insert was precoated with 100 μl Matrigel (400 μg/ml, Corning) in serum-free medium for 30 mins at 37°C. Cells were seeded in the upper chamber of the insert (5x10^4 cells/well), and the following procedures were performed as the migration assay.
**Supplementary Table**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Term (n=14)</th>
<th>PE (n=12)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient age (years)</td>
<td>30±0.95</td>
<td>31±1.07</td>
<td>0.76</td>
</tr>
<tr>
<td>Gestation age (weeks)</td>
<td>38±0.24</td>
<td>37±0.39</td>
<td>0.004</td>
</tr>
<tr>
<td>Systolic pressure (mmHg)</td>
<td>117±1.24</td>
<td>157±3.48</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diastolic pressure (mmHg)</td>
<td>77±1.14</td>
<td>100±2.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>-</td>
<td>+++-++++</td>
<td>-</td>
</tr>
<tr>
<td>Fetal weights (g)</td>
<td>3330±74.62</td>
<td>2401±133.1</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

**Supplementary Table S1**: Characteristics of full term pregnancies and PE patients. All data are means ± SEM. *Obtained using nonparametric Mann-Whitney test on SPSS 16.0.