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Original Article

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# Cooperation between NSPc1 and DNA methylation represses HOXA11 expression and promotes apoptosis of trophoblast cells during preeclampsia

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## Abstract

Accumulating evidence has shown that the apoptosis of trophoblast cells plays an important role in the pathogenesis of preeclampsia, and an intricate interplay between DNA methylation and polycomb group (PcG) protein-mediated gene silencing has been highlighted recently. Here, we provide evidence that the expression of nervous system polycomb 1 (NSPc1), a BMI1 homologous polycomb protein, is significantly elevated in trophoblast cells during preeclampsia, which accelerates trophoblast cell apoptosis. Since NSPc1 acts predominantly as a transcriptional inactivator that specifically represses HOXA11 expression in trophoblast cells during preeclampsia, we further show that NSPc1 is required for DNMT3a recruitment and maintenance of the DNA methylation in the HOXA11 promoter in trophoblast cells during preeclampsia. In addition, we find that the interplay of DNMT3a and NSPc1 represses the expression of HOXA11 and promotes trophoblast cell apoptosis. Taken together, these results indicate that the cooperation between NSPc1 and DNMT3a reduces HOXA11 expression in preeclampsia pathophysiology, which provides novel therapeutic approaches for targeted inhibition of trophoblast cell apoptosis during preeclampsia pathogenesis.

**Key words** preeclampsia, nervous system polycomb 1, DNA methyltransferase 3a, HOXA11, apoptosis

## Introduction

Preeclampsia is one of the major causes of maternal and fetal mortality and morbidity and is characterized by new-onset hypertension and proteinuria after 20 weeks of gestation [1]. Unfortunately, preeclamptic women and children born from preeclamptic pregnancies are at greater risk of developing severe cardiovascular complications and metabolic syndromes [2]. The main causes of preeclampsia are still unknown and remain a problem for obstetricians. Since delivery of the placenta is the only cure for preeclampsia, disorders of placental function are both necessary and sufficient to cause the condition [3]. In recent years, some studies have suggested that trophoblast cells are the main functional cells in the placenta, and it is found that the placental ischemia-hypoxia state during preeclampsia triggers the phase of oxidative stress and produces ROS, leading to increased apoptosis of placental trophoblast cells, which may be one of the important

mechanisms of preeclampsia [4–6]. Therefore, exploring the pathogenesis of preeclampsia will help to find a more effective treatment approach for preeclampsia patients.

Nervous system polycomb 1 (NSPc1, also named polycomb group ring finger 1), a member of the PRC1 family, shares high homology with the PcG proteins Bmi-1 and Mel-18 [7]. It has been reported that NSPc1 is elevated in stem cells, similar to side population cells, in oral squamous cell carcinomas [8]. In addition, it has also been reported that NSPc1 affects glioma cell proliferation and apoptosis and is associated with the malignant potential of various glioma cell lines [9], suggesting that the function of NSPc1 varies depending on host cell lineage. However, to date, NSPc1 has not been studied in the apoptosis of trophoblast cells, and thus, understanding the function of NSPc1 in the regulation of trophoblast cell apoptosis may provide a potential novel therapeutic target for preeclampsia. A previous report showed that NSPc1 negatively regulates transcription and promotes cell cycle progression by altering the homeobox (HOX) gene family [10]. HOX genes, a highly conserved subgroup of the homologous box superfamily, play crucial roles in cell development and organ formation [11]. It has been discovered that HOX can act as a tumor regulator, and its members are involved in tumorigenesis by regulating receptor signaling, cell differentiation, apoptosis, migration, EMT, and angiogenesis [12]. It has also been reported that HOX genes are expressed in specific adult tissues, such as the placenta, to control extraembryonic development of the placenta and cancers [13]. Therefore, it is important to understand the molecular mechanisms by which NSPc1 participates in trophoblast cell apoptosis by regulating HOX genes.

DNA methylation is one type of repressive modification of gene expression, and its close relationship with PcG-mediated gene silencing has been widely studied in recent years [14]. DNA methylation is a crucial epigenetic modification of the genome that is involved in regulating many cellular processes, including transcription, chromatin structure, X chromosome inactivation, genomic imprinting and chromosome stability [15]. Genomic DNA is enzymatically methylated by a family of DNA methyltransferases (DNMTs), including DNMT1, DNMT3a and DNMT3b [16]. Given the strong evidence that gene expression is altered in preeclamptic pregnancies [17], it is reasonable to hypothesize that preeclampsia may also be associated with dysregulated DNA methylation in key regulatory regions.

In the present study, we aimed to investigate the potential mechanism of NSPc1 in regulating HOX gene expression in the apoptosis of placental trophoblast cells. Our results showed that NSPc1 represses HOXA11 expression through coordination with DNA methylation in trophoblast cells, which promotes apoptosis of trophoblast cells under hypoxic conditions. Our findings revealed the involvement of a new regulatory mechanism of NSPc1-mediated HOXA11 gene silencing in trophoblast cell apoptosis, which might provide a potential therapeutic strategy for preeclampsia pathophysiology.

## Materials and Methods

### Placental samples and clinical characteristics

All placental samples were collected from the General Hospital of Ningxia Medical University between 2018 and 2020. Collection and experimentation of human tissues were approved by the Clinical Research Ethics Committee of Ningxia Medical University, and written informed consent was obtained from all enrolled subjects. All cases were delivered by cesarean section before delivery. Placenta in the normal control group (gestational age at delivery is approximately 39 weeks) were obtained from pregnancies delivered by cesarean sections due to breech presentation, horizontal position, or pregnancy with scar uterus. Placenta in the preeclampsia group (gestational age at delivery is approximately 37 weeks) were obtained from pregnancies who delivered with early-onset preeclampsia ( < 34 weeks gestation) and late-onset PE (≥34 weeks of gestation). Preeclampsia was diagnosed based on the standard criteria [18]: a systolic blood pressure ≥140 mmHg and/or a diastolic blood pressure ≥90 mmHg in two successive measurements four to six hours apart and a urinary protein level > 0.3 g in a 24 h urine collection. Additionally, subjects enrolled in the two groups did not have any other complications, including maternal history of hypertension and/or renal disease, severe intrauterine growth retardation (IUGR), diabetes, smoking, alcoholism, chemical dependency, or fetal congenital abnormalities. The specimens were then washed with sterile phosphate-buffered saline before being stored in liquid nitrogen for further analyses of RNA and protein.

### Cell culture and cell treatment

Human placental trophoblast cells (HTR-8/SVneo) and HEK293T cells were both obtained from Fudan IBS Cell Center (FDCC; Shanghai, China) and grown in RPMI 1640 medium (Gibco, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 units/mL penicillin (Gibco) and 100 μg/mL streptomycin (Gibco). Before treatment, trophoblast cells were cultured in RPMI 1640 medium supplemented with 5% FBS for 24 h and then cultured under hypoxia (1% $O_2$ and 5% $CO_2$) for 48 h. Cells were transiently transfected with lentiviruses overnight. To establish stable cell lines, trophoblast cells were selected with 2 μg/mL puromycin after lentivirus infection. Adenoviruses encoding NSPc1 (Ad-NSPc1), HOXA11 (Ad-HOXA11) or a negative control (Ad-GFP) were purchased from Genechem (Shanghai, China), and the short heparin RNAs of NSPc1 (sh-NSPc1-1, 2 and 3), HOXA11 (sh-HOXA11-1, 2 and 3), DNMT3a (sh-DNMT3a-1, 2 and 3) and short heparin RNA of scrambled (sh-NC) were purchased from GenePharma (Shanghai, China). The sequences of these shRNA were shown in the Table 1.

### Transmission electron microscopy

Biopsied placental tissues were cut into pieces (1 mm × 1 mm × 5 mm) and fixed with glutaraldehyde in 0.1 M phosphate buffer (PBS; pH 7.2) for 2 h at 4°C. After being washed with PBS, they were postfixed in 1% osmium tetroxide for 1 h at room temperature,

**Table 1. Sequences of shRNAs used in this study**

| Name | Sequence (5′→3′) |
|---|---|
| sh-NSPc1-1 | ACTTTCTGCAAGAGTTGTATT |
| sh-NSPc1-2 | AGCAGCTTTGACCACTCTAAA |
| sh-NSPc1-3 | AGGTTCGAGTGAAGATCAAAG |
| sh-HOXA11-1 | GCGUCUACAUUAACAAAGATT |
| sh-HOXA11-2 | GAGACCGUUUACAGUACUATT |
| sh-HOXA11-3 | GCAGUCUCGUCCAAUUUCUTT |
| sh-DNMT3a-1 | GCCAAGGUCAUUGCAGGAATT |
| sh-DNMT3a-2 | CCAUGUACCGCAAAGCCAUTT |
| sh-DNMT3a-3 | GCAGAACAAGCCCAUGAUUTT |

followed by dehydration with alcohol. Tissues were then embedded and sectioned with a diamond knife, stained with 1% uranyl acetate and 1% lead citrate, and examined with a transmission electron microscope (JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV.

### TUNEL assay

Apoptosis of placental tissues was detected by terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick and labelling (TUNEL) using a fluorescein-based kit from Roche Applied Science (Basel, Switzerland) according to the manufacturer's instructions. Nuclei were counterstained with 5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; Bioss, Beijing, China). Fluorescent images were acquired with an Olympus Fluoview 1000 confocal laser-scanning microscope (Olympus, Tokyo, Japan).

### Immunofluorescence staining

The placental tissues were permeabilized for 5 min with PBS containing 0.2% Triton X-100 and 10% goat serum, and then incubated with anti-NSPc1 antibody (Abcam, Cambridge, USA) or anti-CK-7 antibody (Abcam) overnight at 4°C. After being washed with PBS, the sections were incubated with a fluorescein isothiocyanate (FICT)-conjugated or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (Abcam) for 1 h at 37°C, followed by washing with PBS and nuclear counterstaining with DAPI. The expressions of target proteins were viewed under an Olympus Fluoview 1000 confocal laser-scanning microscope (Olympus).

### Quantitative real-time RT-PCR (qRT-PCR)

The relative expressions of NSPc1 and HOX genes were examined by qRT-PCR analysis using cDNA synthesized from RNA isolated from trophoblast cells as described previously [19] on an FTC3000 qRT-PCR detection system (Funglyn Biotech Inc., Toronto, Canada) under the following conditions: 95°C for 5 min, 45 cycles at 95°C for 10 s, annealing for 30 s, extension at 72°C for 30 s, and 4°C indefinitely. The relative expression of the target gene was calculated by the $2^{-\Delta\Delta CT}$ method and normalized to *GAPDH*. The primer sequences are listed in Table 2.

### Western blot analysis

The placental tissues and trophoblast cells were lysed in NP40 lysis buffer (Beyotime, Shanghai, China). The concentration of the protein in the lysates was determined using the BCA Assay kit (Beyotime). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, USA). After blocking and incubation with antibodies against NSPc1, DNMT3a, HOXA9, HOXA11, HOXA13, HOXB7, HOXD3, HOXD8, Bax, Bcl-2, caspase-3 and caspase-9 (Abcam) overnight at 4°C, the membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h. Protein bands were visualized using enhanced chemiluminescence (ECL) reagent and signal intensities of the target bands were analyzed by Bio-Rad image analysis (Bio-Rad, Hercules, USA). β-Actin was used as the loading control.

### Dual-luciferase reporter assay

Various lengths of the *HOXA11* promoter (–2000/ + 72, –1500/ + 72, –1100/ + 72, –700/ + 72, –300/ + 72 and –50/ + 72) were amplified and inserted into the luciferase reporter plasmid pGL3-basic (Promega, Madison, USA). The vectors were transfected into HEK293T cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions, and the transfected cells were used for the luciferase assay with 0.4 μg of the firefly luciferase reporter vector and 0.08 μg of the control vector containing *Renilla* luciferase pRL-CMV vector (Promega). *Firefly* and *Renilla* luciferase activities were measured consecutively using the dual-luciferase assay kits (Promega) 48 h after transfection. Relative luciferase activity was normalized to *Renilla* luciferase activity.

**Table 2. Sequences of primers used for qRT-PCR analysis**

| Name | Sequence (5′→3′) | Tm (°C) | Product size (bp) |
|---|---|---|---|
| *NSPc1* | F: GGCTGGGATCAGCCTTTAAGAT<br>R: ACCTCCTCCTCGTTCCGTA | 59.3 | 121 |
| *HOXA1* | F: CTCAAGTTGTGGTCCAAGCTAT<br>R: TGGGTCTGCTTCCTGATTTAAC | 58.7 | 82 |
| *HOXA3* | F: AAAAAGCGACCTACTACGACAG<br>R: GCTGATTGGCATTATAAGCGAA | 59.5 | 87 |
| *HOXA4* | F: TCCACTTCAATCGATACCTGAC<br>R: TCTTTCTTCCACTTCATCCTCC | 59.4 | 118 |
| *HOXA5* | F: CTACAATGGCATGGATCTCAGC<br>R: GAGTTGCTTAGGGAGTTTTTCC | 58.4 | 231 |
| *HOXA7* | F: TTTTTAGCAAATATACGGCGGG<br>R: CTGTTGACATTGTATAAGCCCG | 59 | 148 |
| *HOXA9* | F: ACTTTGTCCCTGACTGACTATG<br>R: AGGGTCTGGTGTTTTGTATAGG | 58.8 | 206 |
| *HOXA10* | F: TGGAGAAGGAGTTTCTGTTCAA<br>R: CAGATTTTCACTTGTCTGTCCG | 60 | 97 |
| *HOXA11* | F: CGTGGTCCCTGCTCCTCTAAC<br>R: GGCAGGTTGGAGGAGTAGGAG | 59.2 | 140 |
| *HOXA13* | F: CTAAGGAGTTCGCCTTCTACC<br>R: CTCTTTGGGGCAGTACATTTG | 58.5 | 209 |
| *HOXB2* | F: GATGAAAGAGAAGAAATCCGCC<br>R: AAGTGGAATTCCTTCTCCAGTT | 59.8 | 201 |
| *HOXB7* | F: CGAGAGTAACTTCCGGATCTAC<br>R: GGTCTTGTTCTCCTTTTTCCAC | 59.4 | 235 |
| *HOXB9* | F: CGAGTACAGTTTGGAAACTTCG<br>R: CGCAAATTTTATTGTCCCCGTA | 58.9 | 83 |
| *HOXB13* | F: CTGATGCCTGCTGTCAACTAT<br>R: CTCCAAAGTAACCATAAGGCAC | 59.8 | 130 |
| *HOXC6* | F: ACCCTTCCTTATCCTGCCACCTC<br>R: GCCGCTCCATAGGTCGAGAAATG | 59.8 | 109 |
| *HOXC8* | F: GGCCACTTAAATCAAAACTCGT<br>R: GAGACTTCAATCCGACGTTTTC | 59.4 | 170 |
| *HOXD3* | F: GCTATGGCTACAGCAAAACTAC<br>R: CATTGAGTTCAGCTCCTTTGTG | 57.5 | 171 |
| *HOXD8* | F: TGACCAGGAAAAGAAGAATCGA<br>R: GAAACGGGAAATTTGTCCTTGT | 59 | 127 |
| *HOXD9* | F: CAGCAACTTGACCCAAACAAC<br>R: GTCTGGTATTTGGTGTAGGGAC | 58.5 | 89 |
| *HOXD10* | F: GCATGTATTCTGATAAGCGCAA<br>R: CCTTCGGGGCTATTATTGTACT | 59 | 175 |
| *HOXD11* | F: CCAAGTACCAGATCCGCGAACTG<br>R: AGTGAGGTTGAGCATCCGAGAGAG | 59 | 98 |

### Nested methylation-specific polymerase chain reaction (nMS-PCR)

Genomic DNA was isolated from placental tissues and trophoblast cells using the TIANamp Genomic DNA kit (Promega). DNA denaturation and bisulfite conversion processes were integrated into one step using the EZ DNA Methylation-Gold kit (Zymo Research, Irvine, USA). nMS-PCR consisted of two-step PCR amplifications after a standard sodium bisulfite DNA modification. The first step of nMS-PCR used an outer primer pair. The second-step PCR was carried out with a methylation primer and an unmethylation primer. The primers used in the nMS-PCR assays are listed in Table3. The PCR products were separated in a 2% agarose gel containing ethidium bromide. DNA bands were visualized with ultraviolet light, and methylation was calculated by the formula: methylation(%) = methylation/(methylation + unmethylation) × 100%.

### MassARRAY methylation analysis

Genomic DNA from trophoblast cells was bisulfite-converted using the EpiTect Bisulfite kit (QIAGEN, Beijing, China) according to the manufacturer's instructions, followed by PCR to amplify the *HOXA11* promoter. The primers for *HOXA11* covered the region with the most CpG sites. For each cell, at least 3 PCR product clones were randomly selected for DNA sequencing and methylation analysis by MethTools (SpectroCHIP Bioarray; Agena, San Diego, USA). The mass spectra were collected using a MassARRAY Compact MALDI-TOF (SEQUENOM; BioMiao Biological Technology, Beijing, China), and the spectra' methylation ratios were generated by EpiTYPER software.

### Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed using the EZ-ChIP kit (Millipore) according to the manufacturer's instructions. Anti-DNMT3a antibodies (Abcam) were used to immunoprecipitate chromatin in trophoblast cells. Subsequently, the immunoprecipitated DNA was subject to PCR analysis using primers that span the promoter regions of *HOXA11*. The forward primer was 5′-CCCGAGTCCTCAGCTCCAGAAG-3′, and the reverse primer was 5′-ATCTTCCCTGCCTACCTCTTCATCC-3′. Nonimmune IgG was used as a control to measure nonspecific background in immunoprecipitation.

### Co-immunoprecipitation (Co-IP) assay

The trophoblast cells were washed 3 times with ice-cold PBS and then lysed in ice-cold NP40-based lysis buffer containing protease inhibitor cocktail and phenylmethanesulfonyl fluoride (PMSF) for 30 min, followed by centrifugation at 15,800 *g* for 15 min at 4°C. The cell lysates were then incubated with antibody against NSPc1 at 4°C overnight and then incubated with protein G-agarose beads (Beyotime) for 2 h at 4°C. The immunoprecipitated complexes were washed 5 times with ice-cold lysis buffer and then boiled in 1 × SDS loading buffer for 5 min at 100°C, followed by western blot analysis.

### Statistical analysis

GraphPad Prism 6.0 was used for statistical analysis. Data are expressed as the mean ± SD. One-way ANOVA followed by Student-Newman-Keul's test (for comparisons among multiple groups) or unpaired Student's *t* test (between two groups) was used as appropriate. $P < 0.05$ was considered statistically significant.

## Results

### Apoptosis is enhanced in trophoblast cells in preeclampsia

Considering that abnormal apoptosis of trophoblast cells may contribute to the pathophysiology of preeclampsia, we first collected placentas from normal (PC) and preeclampsia (PE) pregnancies. TUNEL staining indicated that the number of TUNEL-positive nuclei was significantly increased in the placentas of PE pregnancies (Figure 1A). In addition, the ratios of Bax/Bcl-2, cleaved caspase-3 (c-caspase-3) and cleaved caspase-9 (c-caspase-9) expression were significantly elevated in the placentas of PE pregnancies (Figure 1B). Next, we simulated preeclampsia by establishing an *in vitro* hypoxia model in HTR-8/SVneo cells. Transmission electron microscopy (TEM) further showed signs of apoptosis with a shrunken nucleus and condensed, fragmented and marginated nuclear chromatin in trophoblast cells under hypoxia (Figure 1C). Similarly, flow cytometry analysis showed that apoptotic cells were significantly increased in trophoblast cells under hypoxia (Figure 1D). Moreover, the ratios of Bax/Bcl-2, c-caspase-3 and c-caspase-9 were increased in trophoblast cells under hypoxia (Figure 1E). These results demonstrated that apoptosis is markedly increased in trophoblast cells during preeclampsia.

### NSPc1 is involved in the regulation of trophoblast cell apoptosis in preeclampsia

A previous study demonstrated that NSPc1 promotes cell cycle progression by repressing the transcription of p21 (cyclin-dependent kinase inhibitor) [20]. To determine whether NSPc1 is involved in trophoblast cell apoptosis in preeclampsia, we performed co-immunofluorescent staining of cytokeratin-7 (CK-7, a marker for trophoblast cells) and NSPc1 in the placentas of PC and PE pregnancies. Interestingly, co-localization of NSPc1 and CK-7 was significantly increased in the placentas of the PE group compared to that in the PC group (Figure 2A). Consistently, both qRT-PCR and western blot analysis demonstrated that NSPc1 expression was upregulated in the placentas of PE pregnancies and

**Table 3. Sequences of primers used for nMS-PCR analysis of HOXA11**

| Name | Sequence (5′→3′) | Tm (°C) | Product size (bp) |
|---|---|---|---|
| HOXA11-O | F: TTAAGGTAGTTTAATAATGGATTTTGATGA<br>R: TTAAACCTAAAACAAATTAAAAAAATAAAA | 58 | 181 |
| HOXA11-M | F: TTTCGGTTATATTGAGGATAAGGTC<br>R: TAAACATACGAAACAATTACAAACG | 58.5 | 161 |
| HOXA11-U | F: TTTGGTTATATTGAGGATAAGGTTG<br>R: AACATACAAAACAATTACAAACACT | 56 | 158 |

O, out primer; M, methylation primer; and U, unmethylation primer.

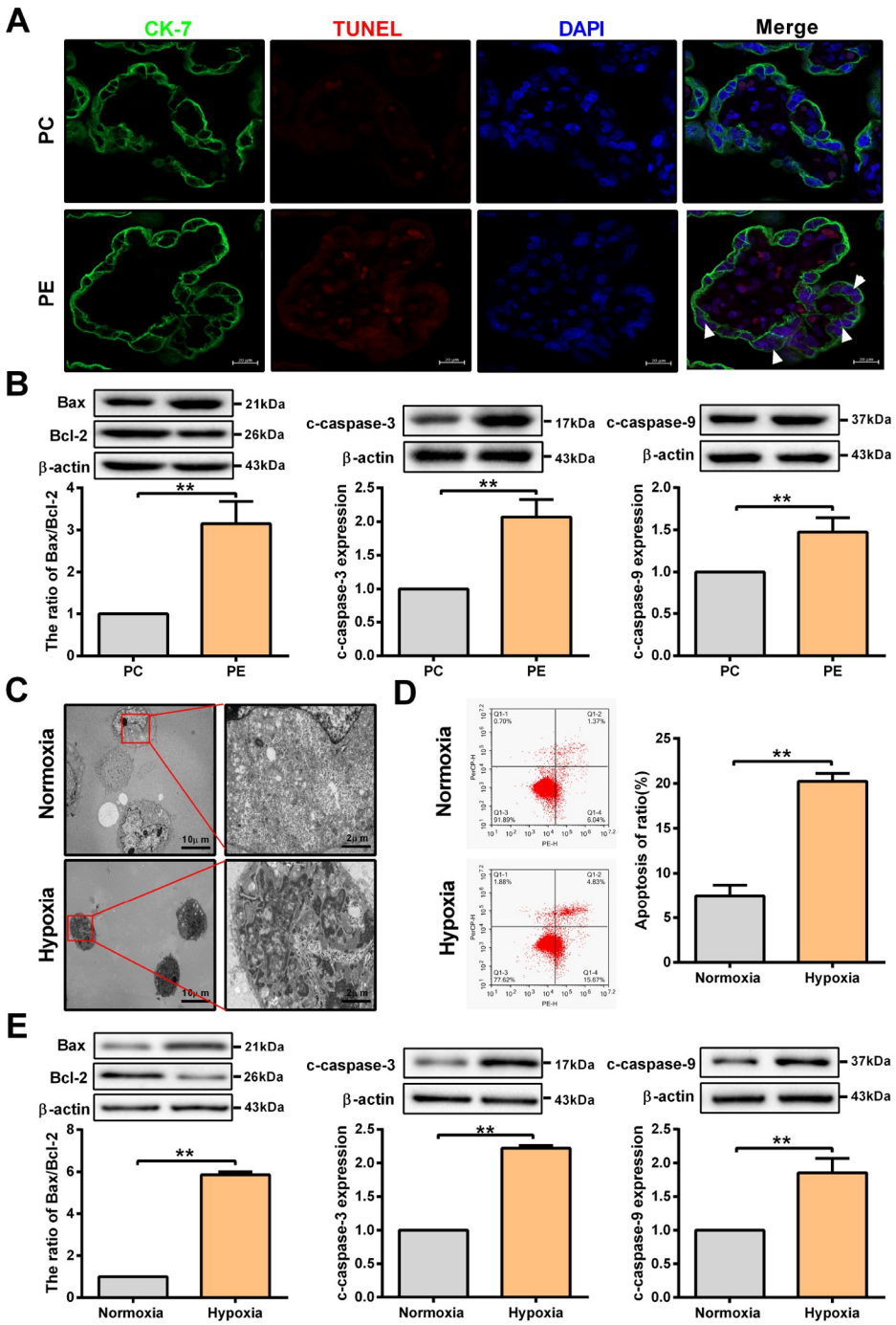


**Figure 1. Enhanced apoptosis in the placenta of preeclampsia pregnancies and hypoxia-induced trophoblast cells** (A) Representative images showing cytokeratin-7 (CK-7, a marker for trophoblast cells, green) and TUNEL (red) double staining in the placenta of normal (PC) and preeclampsia (PE) pregnancies. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue). The white arrows refer to TUNEL-positive trophoblast cells. Scale bar, 20 μm. (B) The protein expressions of Bax, Bcl-2, cleaved caspase-3 (c-caspase-3) and cleaved caspase-9 (c-caspase-9) in the placentas of PC and PE pregnancies were detected by western blot analysis ($n=6$). (C) Transmission electron microscopy (TEM) was performed to observe the ultrastructure of apoptosis in trophoblast cells after treatment with hypoxia for 48 h. Scale bar, 10 μm (left); scale bar, 2 μm (right). (D) The apoptosis rate of trophoblast cells was measured by flow cytometry analysis after treatment with hypoxia for 48 h ($n=3$). (E) The protein expressions of Bax, Bcl-2, c-caspase-3 and c-caspase-9 were detected by western blot analysis in trophoblast cells after treatment with hypoxia for 48 h ($n=3$). Data are shown as the mean ± SD from 3 independent experiments. $^{**}P<0.01$.

trophoblast cells under hypoxia (Figure 2B,C). To further explore the role of NSPc1 in trophoblast cell apoptosis, NSPc1 was overexpressed or knocked down in trophoblast cells (Supplementary Figure S1). The results showed that NSPc1 overexpression markedly increased the ratios of Bax/Bcl-2, c-caspase-3 and c-caspase-9 protein expression in trophoblast cells under hypoxia, which was contrary to the results from cells with *NSPc1* knockdown (Figure 2D). Additionally, flow cytometry analysis showed

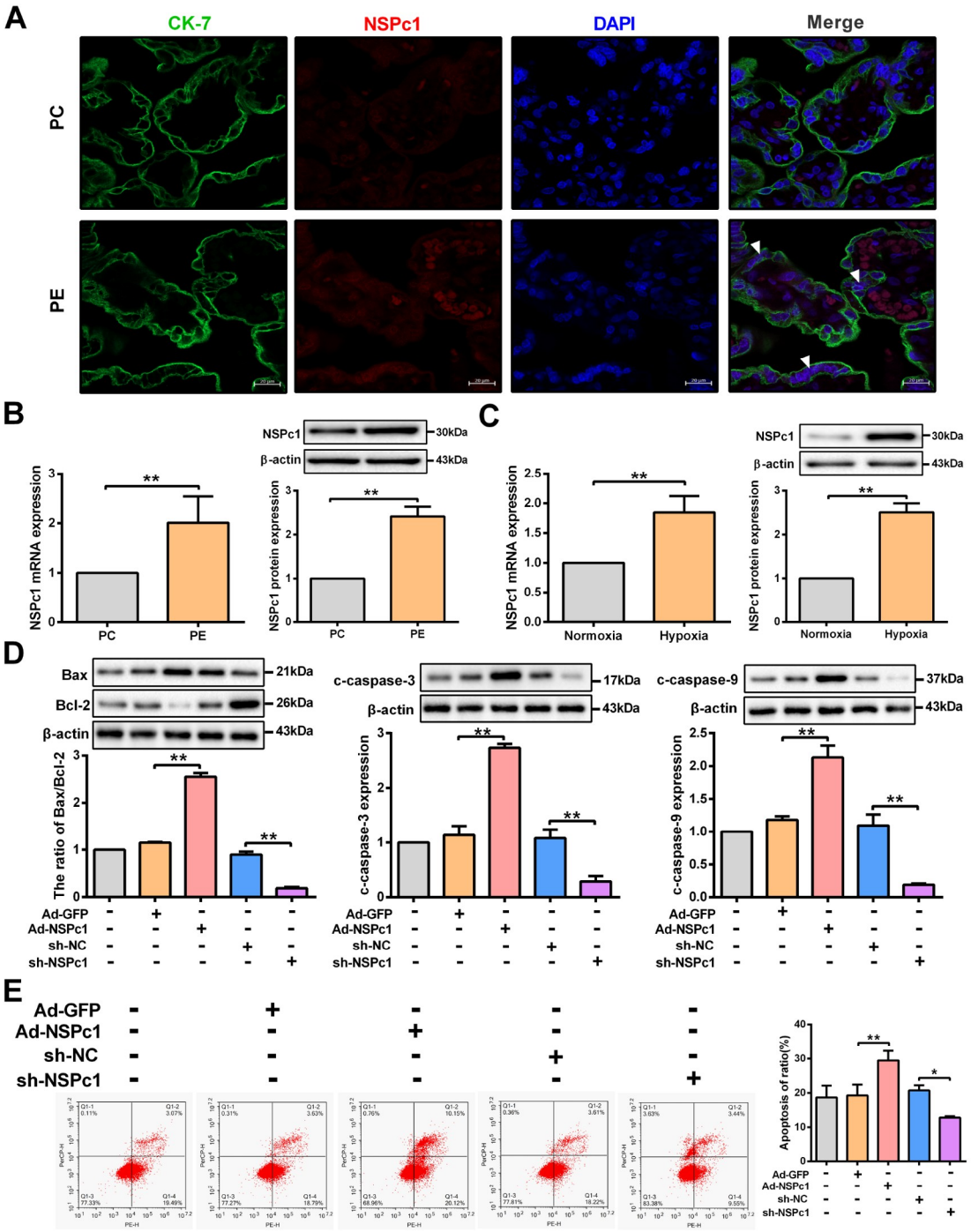


**Figure 2. NSPc1 promotes apoptosis of trophoblast cells during preeclampsia** (A) Immunofluorescent staining of NSPc1 (red) and CK-7 (green) in the placentas of PC and PE pregnancies. Nuclei were stained with DAPI (blue). The white arrows refer to NSPc1-positive trophoblast cells. Scale bar, 20 μm. (B,C) qRT-PCR and western blot analysis were performed to detect the mRNA and protein expression of NSPc1 in the placentas of PE pregnancies ($n=6$) and trophoblast cells under hypoxia ($n=3$). (D) Western blot analysis was used to detect the protein expressions of Bax, Bcl-2, c-caspase-3 and c-caspase-9 in trophoblast cells infected with adenoviruses encoding NSPc1 (Ad-NSPc1) and short heparin RNA of NSPc1 (sh-NSPc1) under hypoxia ($n=3$). (E) Flow cytometry analysis was used to detect the apoptosis rate of trophoblast cells after infection with Ad-NSPc1 and sh-NSPc1 under hypoxia ($n=3$). Data are shown as the mean ± SD from 3 independent experiments. $^*P<0.05$, $^{**}P<0.01$.

that NSPc1 overexpression markedly increased the apoptosis of trophoblast cells under hypoxia, while knockdown of *NSPc1* had the opposite effects (Figure 2E). These results suggested that NSPc1 promotes apoptosis of trophoblast cells during preeclampsia.

## NSPc1 downregulates HOXA11 expression in apoptotic trophoblast cells in preeclampsia

To understand the underlying mechanism by which NSPc1 regulates trophoblast cell apoptosis, it is critical to characterize NSPc1-regulated *HOX* genes. We first examined the mRNA

expression of *HOX* genes in cell lines under hypoxia when *NSPc1* was knocked down and observed that the expressions of HOX genes such as *HOXA9*, *HOXA13*, *HOXB7* and *HOXD3* were repressed, while the expressions of *HOXA11* and *HOXD8* were significantly upregulated (Figure 3A). The effects of *NSPc1* knockdown on the protein expressions of HOXA9, HOXA11, HOXA13, HOXB7, HOXD3 and HOXD8 were further detected. The results showed that HOXA11 protein expression was significantly increased, while HOXA9 protein expression was downregulated, and the expressions of other HOX proteins (HOXA13, HOXB7, HOXD3 and HOXD8) were not significantly changed (Figure 3B). We further found that HOXA11 expression was suppressed in trophoblast cells over-expressing NSPc1 under hypoxia (Figure 3C). Meanwhile, HOXA11 expression was significantly decreased in the placentas of PE pregnancies and hypoxia-treated trophoblast cells (Figure 3D,E). Considering that HOXA11 is related to apoptosis, we overexpressed or knocked down *HOXA11* in trophoblast cells (Supplementary Figure S2), and further studied the role of HOXA11 in the pathogenesis of preeclampsia with respect to trophoblast cell apoptosis. As expected, downregulated expressions of Bax/Bcl-2, c-caspase-3 and c-caspase-9 were observed in trophoblast cells with HOXA11 overexpression, which is opposite to the results obtained from cells with *HOXA11* knockdown (Figure 3F). Flow cytometry results also supported our point that HOXA11 overexpression attenuates apoptosis of trophoblast cells, while knockdown of *HOXA11* has the opposite effect (Figure 3G). Collectively, these results suggested that NSPc1 acts predominantly as a transcriptional inactivator of *HOXA11* to regulate the apoptosis of trophoblast cells in preeclampsia.

## Hypermethylation of the *HOXA11* promoter is responsible for the inhibition of HOXA11 expression

DNA methylation is an important epigenetic regulation of gene expression, and the *HOXA11* gene cluster was frequently found to be hypermethylated in tumors [21]. In this study, we treated trophoblast cells with the DNMT inhibitor 5-aza-2-deoxycytidine (5-Aza) to investigate whether HOXA11 downregulation is regulated by DNA methylation in preeclampsia. The results indicated that the expression of HOXA11 was significantly increased in 5-Aza-treated trophoblast cells (Figure 4A), suggesting that DNA methylation is associated with *HOXA11* transcriptional repression. Subsequently, the DNA sequence of the *HOXA11* promoter was analyzed by MethPrimer 2.0. The CpG island searcher program displayed 2 CpG islands at the promoter of *HOXA11* (Supplementary Figure S3A). Based on these predictions, several fragments of the *HOXA11* promoter (–2000/ + 72, –1500/ + 72, –1100/ + 72, –700/ + 72, –300/ + 72, and –50/ + 72) were synthesized and inserted into the *Firefly* luciferase vector pGL3-Basic (Basic) to measure promoter activity. The results confirmed that the promoter region (–1500/ + 72) had basic core promoter activity, which provided the structural basis for methylation modification (Figure 4B). To determine whether DNA methylation of the CpG sites silences the expression of HOXA11, DNA methylation of *HOXA11* was detected by nMS-PCR, and the results showed that the DNA methylation of *HOXA11* was enhanced in the placenta of PE pregnancies compared with that in the PC pregnancies (Figure 4C). Meanwhile, the DNA methylation status examined by MassArray indicated an increased DNA methylation level in trophoblast cells under hypoxia (Figure 4D). These results implied that DNA methylation contributes to the suppression of *HOXA11* promoter activity in trophoblast cells during preeclampsia.

DNMTs (DNMT1, DNMT3a and DNMT3b) are the key enzymes for DNA methylation, which catalyzes the transfer of the methyl group from S-adenosyl-L-methionine (SAM or AdoMet) to the C5 position of cytosine [22]. To determine which DNMTs contribute to *HOXA11* promoter methylation, we examined the DNA methylation level and expression of HOXA11 in trophoblast cells after treatment with DC-05 (DNMT1-specific inhibitor), theaflavin-3 (TFD, DNMT3a-specific inhibitor) or nanaomycin A (NA, DNMT3b-specific inhibitor). The results showed that TFD strongly decreased the DNA methylation level of *HOXA11* and increased HOXA11 expression, while no significant difference was observed in DC-05- and NA-treated trophoblast cells (Figure 4E,F). Additionally, we also found that DNMT3a was significantly enriched in the promoter of *HOXA11* by ChIP analysis (Figure 4G).

To further explore whether DNMT3a participates in the DNA methylation of HOXA11, we downregulated the expression of DNMT3a in trophoblast cells (Supplementary Figure S3B). Consequently, *DNMT3a* knockdown significantly repressed the methylation level of *HOXA11* but increased the expression of HOXA11 in trophoblast cells under hypoxia (Figure 4H,I). Therefore, DNMT3a-mediated DNA methylation leads to a decrease in HOXA11 expression, which may be one of the mechanisms of preeclampsia.

## NSPc1 facilitates DNMT3a binding to the *HOXA11* promoter to inhibit HOXA11 expression

Given that downregulation of HOXA11 expression is DNMT3a-dependent and the suppressive effect of NSPc1 on the transcription of HOXA11, we further explored whether there is a crosstalk between DNMT3a and NSPc1 in regulating HOXA11 expression. Co-IP analysis showed that endogenous HOXA11 cannot directly bind to NSPc1 in trophoblast cells under hypoxia (Figure 5A). The interaction between endogenous NSPc1 and endogenous DNMT3a was further detected in trophoblast cells under hypoxia by Co-IP. The results showed that NSPc1 could be co-immunoprecipitated with DNMT3a (Figure 5B), implying the potential of the interaction between NSPc1 and DNMT3a. Interestingly, ChIP assays in *NSPc1*-knockdown and NSPc1-overexpressing trophoblast cells showed that NSPc1 overexpression promoted DNMT3a binding to the *HOXA11* promoter, while the enrichment of DNMT3a on the *HOXA11* promoter was decreased after knockdown of *NSPc1*(Figure 5C). In addition, inhibition of DNMT3a reversed the repression of HOXA11 induced by NSPc1 (Figure 5D). These data suggested that NSPc1 facilitates DNMT3a binding to the *HOXA11* promoter to inhibit HOXA11 expression.

## Inhibition of DNMT3a reverses NSPc1-induced trophoblast cell apoptosis

Since the binding of DNMT3a to the *HOXA11* promoter can inhibit HOXA11 expression, we next examined whether DNMT3a is involved in the apoptosis of trophoblast cells induced by NSPc1. As expected, western blot analysis results showed that inhibition of DNMT3a reduced the elevated expression of Bax/Bcl-2, c-caspase-3 and c-caspase-9 induced by NSPc1 (Figure 6A). In addition, flow cytometry analysis further showed that inhibition of DNMT3a decreased the apoptosis of trophoblast cells induced by NSPc1 (Figure 6B), suggesting that the combination of DNMT3a and NSPc1 further promotes the apoptosis of trophoblast cells. These data suggested that NSPc1 facilitates DNMT3a binding to the *HOXA11*

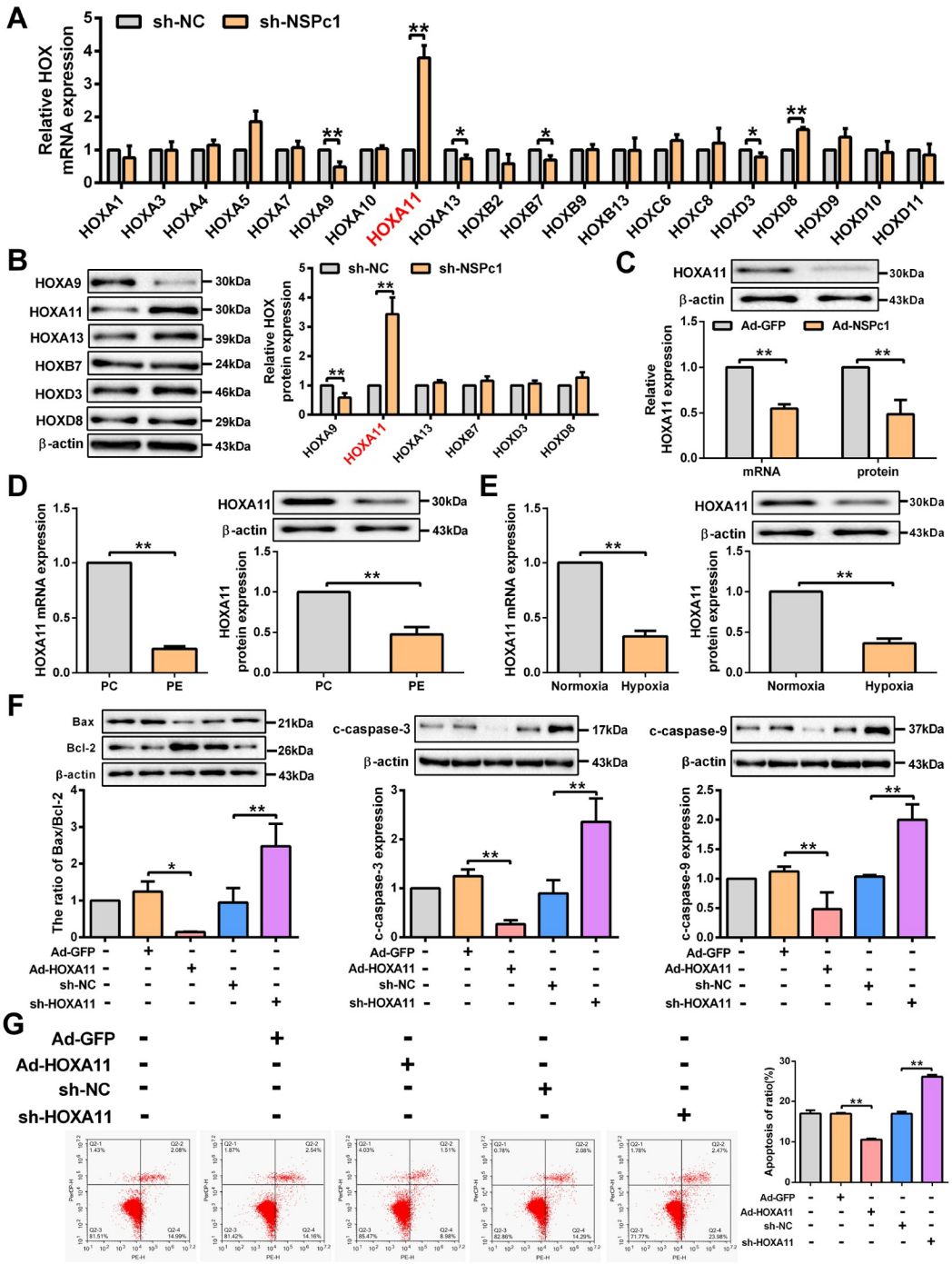


**Figure 3. NSPc1 specifically represses HOXA11 expression in trophoblast cells during preeclampsia** (A) qRT-PCR was used to detect *HOX* gene cluster mRNA expression in trophoblast cells after infection with sh-NSPc1 under hypoxia, and the expression level of each *HOX* mRNA was normalized to that of *GAPDH* ($n=3$). (B) The protein expressions of HOXA9, HOXA11, HOXA13, HOXB7, HOXD3 and HOXD8 were detected by western blot analysis in trophoblast cells after infection with sh-NSPc1 under hypoxia ($n=3$). (C) The mRNA and protein expression of HOXA11 in trophoblast cells after infection with Ad-NSPc1 under hypoxia was measured by qRT-PCR and western blot analysis, respectively ($n=3$). (D,E) The mRNA and protein expression levels of HOXA11 were detected by qRT-PCR and western blot analysis in the placentas of PE pregnancies ($n=6$) and trophoblast cells under hypoxia ($n=3$). (F) Western blot analysis was used to measure the protein expressions of Bax, Bcl-2, c-caspase-3 and c-caspase-9 in trophoblast cells after infection with adenoviruses encoding HOXA11 (Ad-HOXA11) and short heparin RNA of HOXA11 (sh-HOXA11) under hypoxia ($n=3$). (G) Flow cytometry analysis was used to detect the apoptosis rate of trophoblast cells infected with Ad-HOXA11 and sh-HOXA11 under hypoxia ($n=3$). Data are shown as the mean ± SD from 3 independent experiments. $^*P<0.05$, $^{**}P<0.01$.

promoter to inhibit HOXA11 expression, which further promotes the apoptosis of trophoblast cells during preeclampsia.

## Discussion

Preeclampsia is a pregnancy syndrome characterized by hyperten-

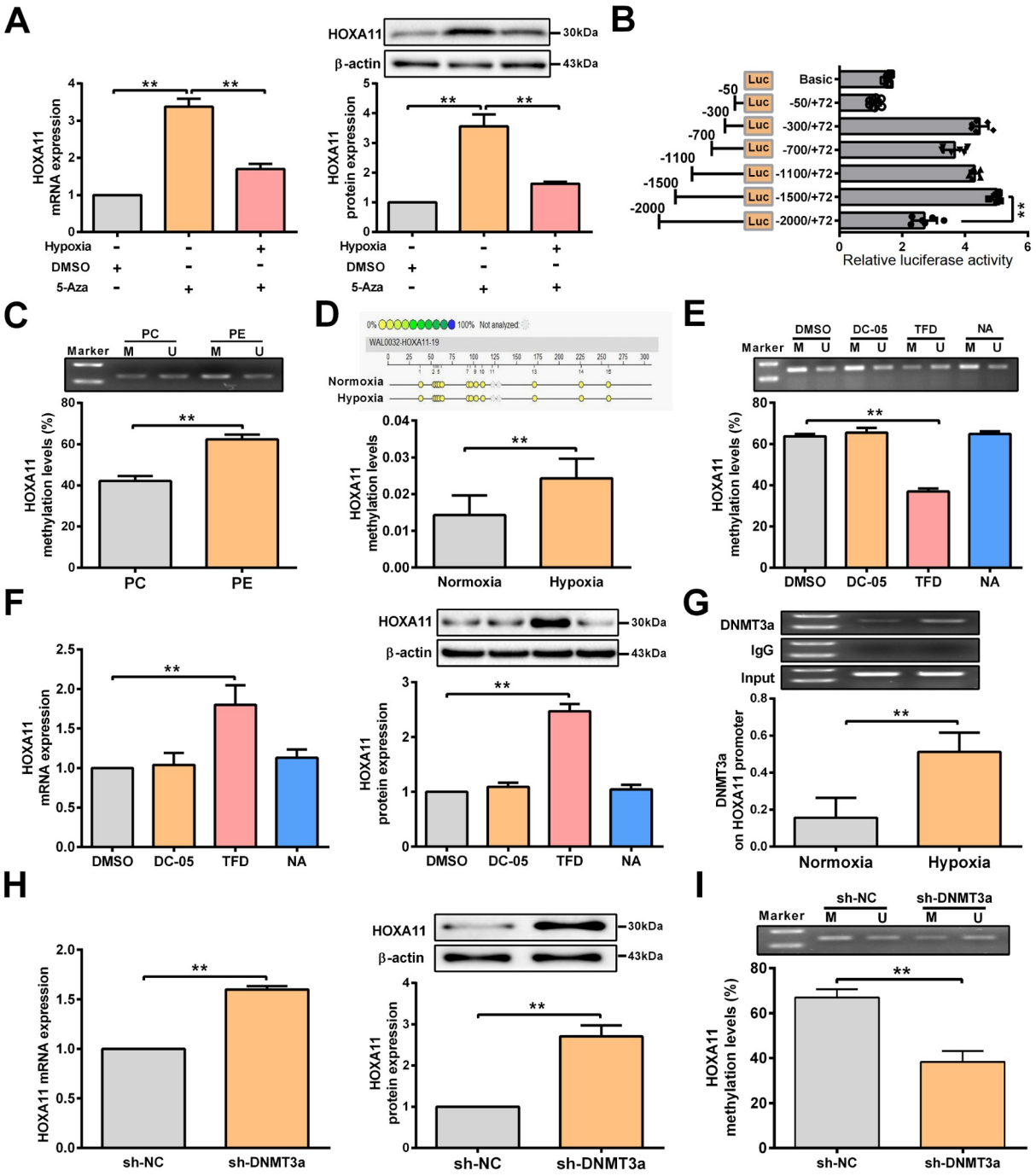


**Figure 4. HOXA11 is downregulated in a DNA methylation-dependent manner mediated by DNMT3a** (A) Relative expression of HOXA11 was determined by qRT-PCR and western blot analysis after the trophoblast cells were treated with the DNMT inhibitor 5-Aza-2′-deoxycytidine (5-Aza) under hypoxia. (B) A luciferase assay was used to measure HOXA11 promoter activity after several fragments (–2000/+72, –1500/+72, –1100/+72, –700/+72, –300/+72, and –50/+72) were cloned into a pGL3-basic reporter vector (Basic) and transfected into HEK293T cells. (C) The DNA methylation level of the *HOXA11* promoter was detected by nMS-PCR in the placentas of PC and PE pregnancies ($n=6$). M, methylated status; U, unmethylated status. (D) MassARRAY methylation analysis was used to measure the methylation level of the *HOXA11* promoter in trophoblast cells under hypoxia ($n=3$). (E) The methylation level of the *HOXA11* promoter was validated by nMS-PCR after trophoblast cells were treated with DC-05 (DNMT1-specific inhibitor), theaflavin-3 (TFD, DNMT3a-specific inhibitor) or nanaomycin A (NA, DNMT3b-specific inhibitor) ($n=3$). (F) Relative expression of HOXA11 was validated by qRT-PCR and western blot analysis in trophoblast cells treated with DC-05, TFD or NA ($n=3$). (G) The enrichment of DNMT3a on the *HOXA11* promoter was assessed by chromatin immunoprecipitation (ChIP) assay in trophoblast cells under hypoxia ($n=3$). (H) Relative expression of HOXA11 was validated by qRT-PCR and western blot analysis in trophoblast cells transfected with short heparin RNA of DNMT3a (sh-DNMT3a) under hypoxia ($n=3$). (I) The DNA methylation level of the *HOXA11* promoter was analyzed by nMS-PCR in trophoblast cells transfected with sh-DNMT3a under hypoxia ($n=3$). Data are shown as the mean ± SD from 3 independent experiments. $^{**}P<0.01$.

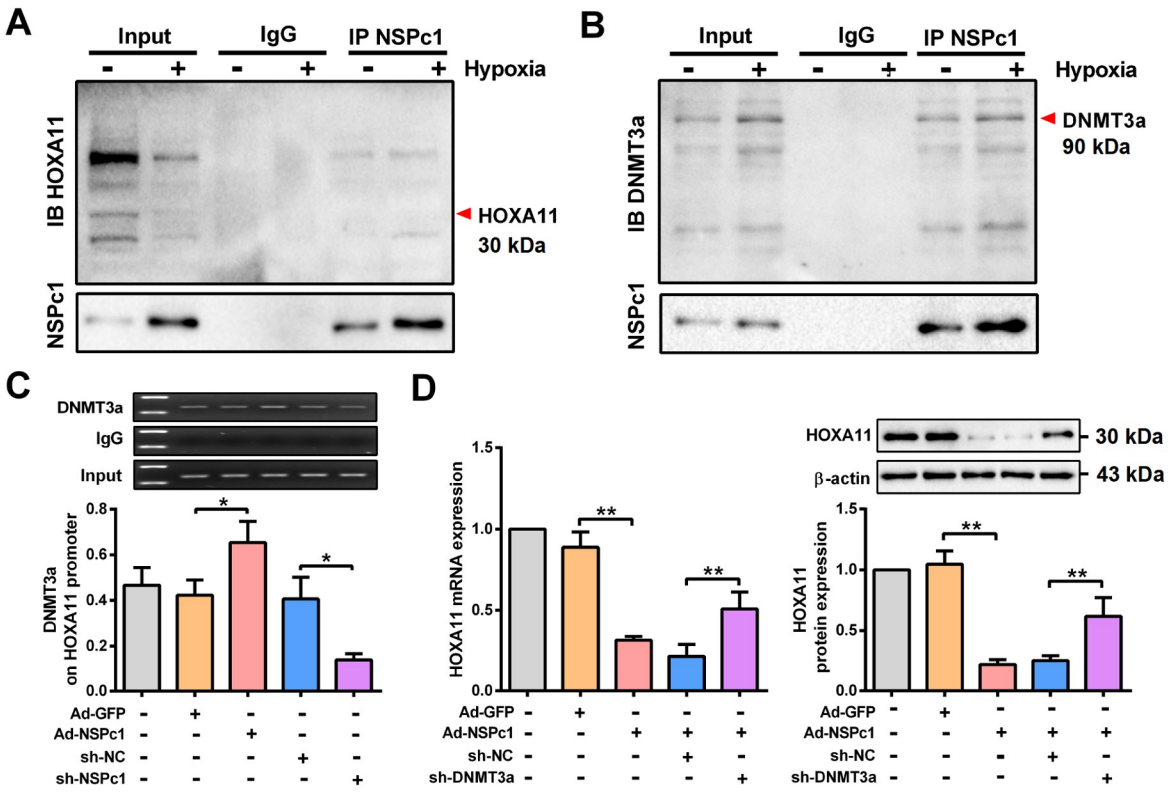


**Figure 5. NSPc1 inhibits HOXA11 expression by promoting the binding of DNMT3a to the HOXA11 promoter** (A) Total cell lysates of trophoblast cells under hypoxia were immunoprecipitated with NSPc1 antibodies or control IgG and subjected to western blot analysis using anti-HOXA11 antibodies. (B) A Co-IP assay was performed to determine the endogenous interaction between NSPc1 and DNMT3a in trophoblast cells using antibodies against NSPc1 and DNMT3a. IgG was used as a negative control. (C) ChIP assay was used to detect DNMT3a enrichment at the *HOXA11* promoter after trophoblast cells were infected with Ad-NSPc1 or sh-NSPc1 under hypoxia. The ChIP-enriched DNA fragments of the *HOXA11* promoter using IgG and anti-DNMT3a antibody were amplified by PCR. Total input (5%) was used as a positive control ($n=3$). (D) The mRNA and protein expression levels of HOXA11 in trophoblast cells after transfection with Ad-NSPc1 and sh-DNMT3a under hypoxia were measured by qRT-PCR and western blot analysis ($n=3$), respectively. Data are shown as the mean ± SD from 3 independent experiments. $^{*}P<0.05$, $^{**}P<0.01$.

sion and proteinuria after 20 weeks of pregnancy [23]. Now, the termination of pregnancy is the only definitive cure [24]. As an important organ to maintain communication between the mother and infant, the placenta has complex physiological functions, such as nutritional metabolism, biosynthesis, immune regulation, and substance exchange [25]. Trophoblast cells, the main types of cells that make up the placenta, are the basis for the establishment and maintenance of pregnancy [26]. Disturbance of the perfusion or blood supply of the placenta is usually considered the underlying cause of placental dysfunction [27]. At present, an increasing number of studies have found that placental dysfunction caused by excessive apoptosis of trophoblast cells is an important factor in preeclampsia [28]. Meanwhile, it has been reported that hypoxia promotes trophoblast cell apoptosis and triggers placental dysfunction by regulating the expressions of hypoxia inducible factor 1 (HIF1) and its target genes [29]. Our study demonstrated elevated apoptosis of trophoblast cells during preeclampsia. However, it is necessary to elucidate the mechanism of trophoblast cell apoptosis during preeclampsia.

According to a previous study, polycomb group (PcG) proteins are epigenetic modifiers involved in gene repression [30]. Organized within multiprotein complexes, they regulate developmental genes in multiple cell types and tissues, especially in embryonic tissues, and are essential for cell fate transitions and proper development [31]. NSPc1 is a newly discovered transcriptional suppressor in the PcG family. A previous study pointed out that NSPc1 is highly expressed in the early developing nervous system and is involved in the differentiation of neural crest cells [32]. The expression of NSPc1 is significantly altered in gliomas and is related to the maintenance of the stemness of cancer stem cells [33]. In addition, decreased expression of NSPc1 promotes apoptosis of glioma cells [34], which emphasizes the important role of NSPc1 in cancer. In this study, we showed that NSPc1 overexpression increases trophoblast cell apoptosis under hypoxia, implying that NSPc1 may play a crucial role in the apoptosis of trophoblast cells during preeclampsia.

In addition, HOX genes exert their function by regulating cell proliferation and differentiation during embryogenesis and organogenesis [35]. Many HOX genes display altered expression patterns in a variety of tumors. For example, HOXB13 overexpression suppresses the growth of prostate cancer cells [36]. HOXA11 potently promotes peritoneal metastasis of gastric cancer cells through the formation of a positive feedback loop between HOXA11 and STAT3 [37]. However, HOX genes can thus far be defined only as tumor modulators rather than as oncogenes or tumor suppressors. To further understand the role of NSPc1 in *HOX* gene silencing, we established *NSPc1*-knockdown cell lines and found that NSPc1 acts predominantly as a transcriptional inactivator of HOXA11 to

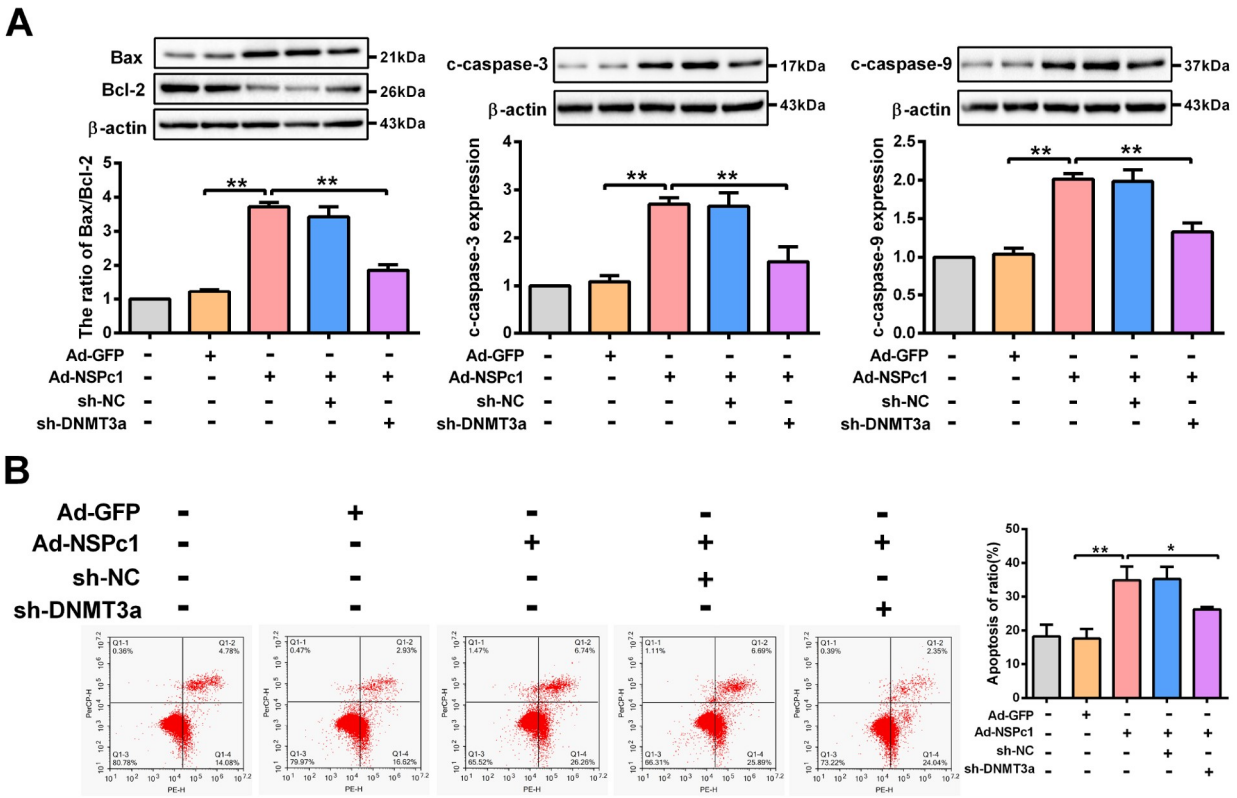


**Figure 6. NSPc1 and DNMT3a synergistically regulate apoptosis of trophoblast cells** (A) The protein expressions of Bax, Bcl-2, c-caspase-3 and c-caspase-9 in trophoblast cells transfected with Ad-NSPc1 and sh-DNMT3a under hypoxia were measured by western blot analysis ($n=3$). (B) Flow cytometry analysis was used to detect the apoptosis rate of trophoblast cells transfected with Ad-NSPc1 and sh-DNMT3a under hypoxia ($n=3$). Data are shown as the mean ± SD from 3 independent experiments. $^{*}P<0.05$, $^{**}P<0.01$.

regulate trophoblast cell apoptosis during preeclampsia. Unlike others, our study found that HOXA11 expression is downregulated in the placenta of patients with preeclampsia and in trophoblast cells under hypoxia, which concluded that its ability to enhance apoptosis of trophoblast cells is precisely due to silencing of HOX genes. DNA methylation is a dynamic process involving both *de novo* DNA methylation and demethylation during placental development, with genome changes in methylation patterns between the first- and third-trimester placentas. DNA methylation is catalyzed by a family of DNMTs (including DNMT1, DNMT3a and DNMT3b) that transfer a methyl group from S-adenyl methionine (SAM) to the fifth carbon of a cytosine residue to form 5-methylcytosine (5mC) [38]. The ten eleven translocation (TET) enzymes oxidize 5mC and promote locus-specific reversal of DNA methylation to activate DNA demethylation [39]. We sought to understand the HOXA11 regulation mechanism in trophoblast cells during preeclampsia, and an intriguing finding is that the DNA hypermethylation of HOXA11 promoter is caused by the increased binding with DNMT3a at its promoter, implying that DNA methylation-dependent repression of HOXA11 is mediated by DNMT3a in trophoblast cells under hypoxia. Although DNMT3a has been identified as a *de novo* DNA methyltransferase, several studies have shown that in addition to affecting DNA methylation, DNMTs can also act as corepressors to silence gene expression [40]. Our data showed that an increase in NSPc1 promotes the binding of DNMT3a to the *HOXA11* promoter and *de novo* methylation of specific CpG islands, causing robust silencing of *HOXA11* and thereby predisposing trophoblast cells to apoptosis during preeclampsia.

In summary, our study suggested that NSPc1 promotes apoptosis of trophoblast cells by repressing HOXA11 expression under hypoxic conditions. More importantly, DNMT3a binds with the *HOXA11* promoter and induces hypermethylation of *HOXA11*, and NSPc1 interacts with DNMT3a to suppress HOXA11 expression. In this study, we demonstrated a synergistic effect between NSPc1 and DNMT3a in silencing *HOXA11* in trophoblast cells under hypoxic conditions, which provides a promising molecular target for the development of novel therapeutic strategies for preeclampsia. Considering that epigenetic regulation is generally reversible, further identification and functional study of DNA methylases will bring us new strategies for preeclampsia treatment and prevention.

## Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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