Methylation pattern of *H19* exon 1 is closely related to preeclampsia and trophoblast abnormalities

LINSHAN LU[1*], ZHENG HOU[2*], LI LI[3*], YANHONG YANG[1], XIAOHONG WANG[1], BEILEI ZHANG[1], MO REN[1], DAN ZHAO[3], ZHUO MIAO[1], LILI YU[3] and YUANQING YAO[1,4]

[1]Department of Obstetrics and Gynecology, Tangdu Hospital, [2]Department of Pharmacology, School of Pharmacy, Fourth Military Medical University, Xi'an, Shaanxi; [3]Department of Obstetrics and Gynecology, Research Institute of Surgery and Daping Hospital, Third Military Medical University, Chongqing, Sichuan; [4]Department of Obstetrics and Gynecology, General Hospital of the Chinese People's Liberation Army, Beijing, P.R. China

Received December 24, 2013; Accepted June 6, 2014

DOI: 10.3892/ijmm.2014.1816

Abstract. Preeclampsia (PE) is a pregnancy-induced disorder characterized by the overproliferation of trophoblasts. Hydatidiform moles, which are associated with a high risk of developing PE, are characterized by the excessive proliferation of trophoblastic tissue. *H19* is highly expressed in placental tissue; however, its biological function remains unclear. A fundamental modification of the *H19* gene is DNA methylation, which typically occurs in CG-rich regions at the promoter or the first exon region. In this study, in order to investigate the DNA methylation pattern of the *H19* exon 1 region in placental tissues and trophoblast cells, placental specimens were collected from women in the first trimester of pregrancy (FTP) and the third trimester of pregnancy (TTP), as well as from from women with severe preeclampsia (sPE). We found that the DNA methylation levels of *H19* exon 1 were significantly higher in the tissues obtained from women in TTP than from those obtained from women in FFP. The methylation status of CpG 1 sites within exon 1 of *H19* was markedly higher in the placental tissues obtained from women with sPE than in the tissues obtained from women in TTP. In addition, we used the human choriocarcinoma cell line, JEG-3, and treated the cells with the methylation inhibitor, 5-aza-2'-deoxycytidine (5-Aza-Dc). Following treatment with 5-Aza-Dc, the methylation levels at this CpG site showed marked hypomethylation. In addtion, the cell proliferative, migratory and invasive capacities of the cells were remarkably inhibited. Our data suggest that hypermethylation at individual CpG sites within exon 1 of *H19* may be involved in the dysfunction of trophoblasts and the pathogenesis of PE.

Correspondence to: Professor Yuanqing Yao, Department of Obstetrics and Gynecology, Tangdu Hospital, Fourth Military Medical University, 1 Xinsi Road, Xi'an, Shaanxi 710038, P.R. China
Email: yyqyao@yeah.net

Professor Lili Yu, Department of Obstetrics and Gynecology, Daping Hospital, 10 Changjiang Zhilu, Chongqing, Sichuan 400042, P.R. China
Email: lililiyuyu@sina.com

*Contributed equally

Key words: preeclampsia, *H19* exon 1, DNA methylation, 5-aza-2'-deoxycytidine

Introduction

The placenta, a unique endocrine organ during pregnancy, plays a critical role in embryonic and fetal development. The main functions of the placenta are executed by trophoblasts, which originate from the trophectoderm layer of the blastocyst. During morula-to-blastocyst transition, the surface cells become trophoblasts and give rise to extraembryonic structures, including the placenta (1). The trophoblast, which behaves like a 'pseudotumor', proliferates, differentiates, migrates and invades the endometrium in a controlled manner (2). However, implantation and placentation are strictly regulated in time and space; they are intimately linked to and enable the embryo to be anchored to the uterine wall, and thus ensure a normal pregnancy and fetal growth through the placenta. Any abnormal factors promoting or inhibiting this process may cause pregnancy-related diseases. One of these diseases is preeclampsia (PE).

As one of the first imprinted genes, the *H19* gene was initially isolated in 1984 (3) and further cloning and sequence determination was carried out in 1988 (4). This gene is composed of 5 exons and 4 small introns (4). *H19* RNA is transcribed by RNA polymerase II, then spliced and exported into the cytoplasm. Due to the lack of a long conserved open reading frame between the murine and human genome, the product of *H19* appears to be a non-coding RNA (5). The allele-specific expression profile of the *H19* gene in human placental tissue may result from dynamic alternations during fetal development (6). The *H19* gene is highly expressed during mammalian embryonic development (7).

In the murine placenta, the *H19* gene is the second most abundant transcript (7), which is activated in the cells of the trophectoderm at the time of implantation (8) and accumulates to extremely high levels in the cells. These cells exclusively ontribute to the formation of extraembryonic tissue. Moreover, the *H19* transcript from human placental tissue during the first and third trimester may play a regulatory role in trophoblast

differentiation (9). In a previous study of ours, we indicated that the knockdown of *H19* inhibits the proliferation and apoptosis of human trophoblast-derived choriocarcinoma cells (10). Besides, the lack of *H19* gives rise to placental overgrowth (7). Thus, the pattern of *H19* expression suggests that it may regulate the function of trophoblasts during the stage of embryogenesis and placental development. We thus hypothesized that exon 1 is the longest exon and functional region of the *H19* gene, which may contribute to the modulation and function of the placenta via trophoblasts.

The methylation patterns of specific genes have been found to undergo dynamic changes in the germ line and early embryo (11). Variations in DNA methylation are involved in the developmental process occurring in normal and abnormal human placentas (12). The demethylating agent, 5-azacytidine, has been shown to change methylation profiles in pregnant rats and to induce a marked reduction in placental growth (13). Using methylation-sensitive high resolution melting, Gao *et al* found that the promoter region of the *H19* gene was hypermethylated in early-onset PE placentas compared with third-trimester normal controls (14). Moreover, the *H19* promoter and exon 1 were differentially methylated in embryonic and somatic tissues in mice (15).

Although our understanding of the intricate role of the *H19* gene in the regulation of the placental function in mice has improved, the methylation status of *H19* exon 1 in human placental development and trophoblast function remains unclear. Furthermore, little is known about the temporal methylation variation of *H19* exon 1 across gestation, and whether the methylation status in the region is stable throughout the first trimester and during the later stages of gestation. Thus, in the present study, we aimed to assess the DNA methylation pattern of *H19* exon 1 in trophoblasts and human placental tissues obtained from women undergoing normal pregnancy and in pregnant women withPE.

Materials and methods

Subjects and sample collection. The experimental protocols in this study were reviewed and approved by the Institutional Review Boards of the corresponding hospitals and written informed consent was obtained from all participants. A total of 37 subjects (21-39 years old) at different stages of gestation were recruited from outpatient and inpatient services at the Department of Obstetrics and Gynecology, Daping Hospital, Xinan Hospital and Xinqiao Hospital of the Third Military Medical University, the First Affiliated Hospital of Chongqing Medical University and the Reproductive Centre of the District of Banan, Chongqing, China, from August 2007 to March 2008. Six subjects in the first trimester of pregnancy (FTP), 16 subjects in the third trimester of pregnancy (TTP) and 15 women with severe PE (sPE) were included in the present study. The placental villous tissues from women undergoing normal pregnancy between 6 and 9 weeks of gestation were collected during the procedure of induced abortion, while the placental tissues from women undergoing normal full-term pregnancy or from women with sPE in the third trimester were obtained by cesarean section. Patients with sPE were diagnosed according to the criteria stated in the American College of Obstetricians and Gynecologists (ACOG) Practice Bulletin (2002), which included blood pressure of ≥160/110 mmHg on 2 occasions at least 6 h apart after 20 weeks of gestation and proteinuria (≥5 g/24 h or ≥3+ on 2 random urine samples collected at least 4 h apart). Patients with a history of chronic hypertension, diabetes mellitus, nephropathy or a recent urinary tract infection were excluded from this study. All the specimens were quickly dissected, snap-frozen in liquid nitrogen and stored at -80˚C until further analyses.

Cell culture. The human choriocarcinoma cell line, JEG-3, was obtained from the American Type Culture Collection (ATCC, Mannasas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 medium (DMEM/F-12; Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) at 37˚C in a humidified atmosphere containing 5% CO_2. To assess the effects of methylation inhibition, the cells were treated with 5-aza-2'-deoxycytidine (5-Aza-Dc; Sigma-Aldrich, St. Louis, MO, USA).

DNA methylation assay. The cells were pelleted following treatment with 5-Aza-Dc at concentrations of 1, 10, or 100 μmol/l for 24, 48 and 72 h. Cells treated with 0.05% DMSO were used as controls. Genomic DNA was extracted from the cells or frozen tissues using the phenol-chloroform method. The bisulphite conversion of DNA was performed using a EZ DNA Methylation-Gold™ kit (Zymo Research, Orange, CA, USA) following the manufacturer's instructions. DNA methylation analysis was conducted following bisulfite sequencing PCR (BSP). The bisulfite sequence of the primers was as follows: 5'-TTGGAGTTTGGTAGGAGTGATG-3' (forward) and 5'-CCCAAACCCTAAAATCAAACCCT-3' (reverse). PCR amplification was conducted under the following conditions: 94˚C for 5 min, then 34 cycles of 94˚C for 30 sec, 61˚C for 30 sec, 72˚C for 1 min, followed by 72˚C for 10 min for the final extension. All products were confirmed to be single bands by 2% agarose gel electrophoresis. PCR products were sequenced by Shanghai Invitrogen Biotechnology Co.,Ltd. (Shanghai, China).

RNA extraction and real-time reverse transcription PCR (RT-qPCR). The cells were treated with 5-Aza-Dc at concentrations of 0.1, 1, 10 or 100 μmol/l for 24, 48 or 72 h and pelleted. Cells treated with 0.05% DMSO were used as controls. Total RNA from the tissues and cells was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and reverse transcribed into cDNA using the RT kit (BioBRK, Chengdu, China) according to the manufacturer's instructions. Quantitative (real-time) PCR was performed using the SYBR-Green PCR kit (Takara Bio Inc., Shiga, Japan). The primer sequences were as follows: H19 forward, 5'-GGCAAG AAGCGGGTCTGT-3' and reverse, 5'-GCTGCTGTTCCG ATGGTGT-3'; and GAPDH forward, 5'-ACCCATCACC ATCTTCCAGGAG-3' and reverse, 5'-GAAGGGGCGGAG ATGATGAC-3'.

The reactions in triplicate were first denatured at 94˚C for 5 min and then subjected to 35 cycles of 94˚C for 30 sec, 59˚C for 30 sec and 72˚C for 30 sec. Data were normalized to those of *GAPDH*. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method.

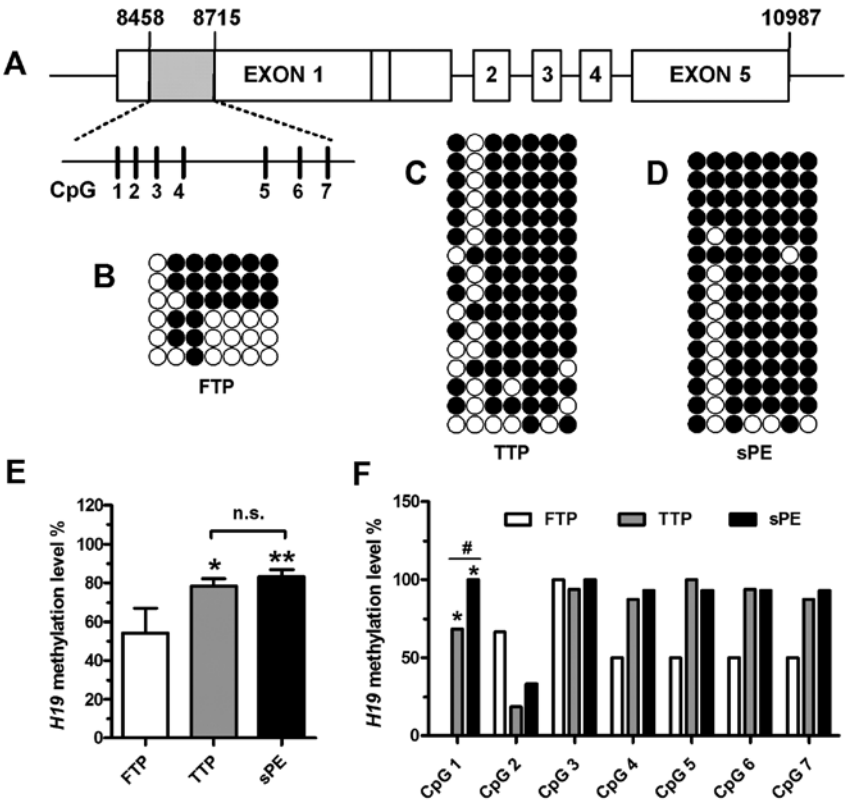

Figure 1. DNA methylation status of *H19* exon 1 in placental tissue. (A) Schematic diagram of human *H19* exonic region. The region (grey box), containing 7 CpG sites (vertical black bars) of the *H19* exon 1 region was analyzed. Bisulfite sequencing regions are shown with individual CpG sites underneath. Data represent the placental tissue from women in (B) the first trimester of pregnancy (FTP), (C) the third trimester pregnancy (TTP) and (D) women with severe preeclampsia (sPE). Open and closed circles indicate unmethylated and methylated CpG sites, respectively. (E) DNA methylation levels across 7 CpG sites in placental tissue specimens from women in FTP and TTP, as well as from women with sPE. The data are presented as the means ± standard error of the mean (SEM): *P<0.05, **P<0.01 vs. results for FTP. (F) Mean methylation for each CpG site in placental tissue specimens from women FTP and TTP and women with sPE (*P<0.05 vs. results for FTP, $^{\#}$P<0.05 vs. results for TTP).

MTT assay. The effects of 5-Aza-Dc on cell proliferation were determined by MTT assay. The cells ($3.5x10^3$ cells/well) were seeded in 96-well plates with 100 μl DMEM/F-12 with 10% FBS in each well in sextuplicate for 24 h, and then treated without or with 5-Aza-Dc at concentrations of 0.01, 0.1, 1, 10 or 100 μmol/l for 24, 48 or 72 h. Cells treated with 0.05% DMSO were used as controls. On the day of the assay, the growth medium was replaced with serum-free medium containing 5 mg/ml MTT (Sigma-Aldrich) and incubated at 37˚C for 4 h. At the end of the incubation period, the cells were solubilized in 150 μl of DMSO, and colorimetric determination was performed at 570 nm absorbance with a plate reader. The data are presented as the mean values from 3 independent experiments.

Cell migration and invasion assays. A cell migration assay were was performed using Transwell inserts (6.5 mm diameter; 8 μm pore size polycarbonate membrane; BD Biosciences, Franklin Lakes, NJ, USA). For cell invasion assay, Transwell plates were coated with 50 μl Matrigel (1:10 dilution; BD Biosciences) solution per well and dried for 30 min at 37˚C with 5% CO_2. Cells ($1x10^5$ cells/well) in 0.2 ml serum-free medium with or without 5-Aza-Dc (100 μmol/l) were placed in the upper chamber, whereas the lower chamber was loaded with 0.8 ml medium containing 10% FBS. The cells were allowed to migrate or invade for 24 h at 37˚C with 5% CO_2. After removing the upper Transwell, the non-migrated or non-invaded cells on the inner surface of the Transwell were carefully removed using a cotton swab. The cells that had penetrated to the bottom side of the membrane were then fixed in buffered formalin and stained using haematoxylin. The number of stained cells/well was counted using an inverted microscope (Olympus, Tokyo, Japan).

Statistical analysis. Analysis of variance (ANOVA) or Fisher's exact test was used for statistical analysis. A probability (P)-value <0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS software version 13.0 or GraphPad Prism version 5.01.

Results

The methylation status at the H19 exon 1 region in human placental tissue. As shown in Fig. 1A, we used BSP assay to quantitatively measure the levels of methylation at 7 CpG sites (CpG 1, 8,547 bp; CpG 2, 8,558 bp; CpG 3, 8,571 bp; CpG 4, 8,587 bp; CpG 5, 8,637 bp; CpG 6, 8,658 bp; CpG 7, 8,675 bp) in *H19* exon 1 (GenBank accession no. AF087017) in human placental tissues obtained from women at different stages of gestation. Methylation levels in the first exon of the *H19* gene in placental tissue from women in FTP (Fig. 1B), TTP (Fig. 1C) and from women with sPE (Fig. 1D) were 54.17±12.81%, 78.44±15.63% and 83.21±14.33%, respectively. The region showed significant hypomethylation in the placental tissue

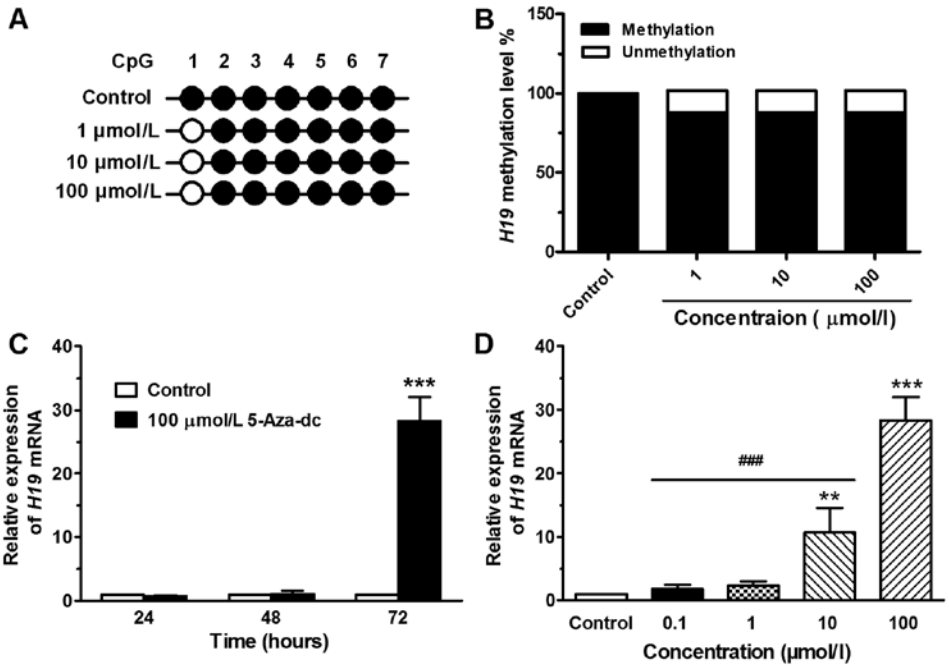

Figure 2. DNA methylation level and *H19* mRNA expression in JEG-3 cells. (A) DNA methylation status of individual CpG sites in JEG-3 cells treated with 5-aza-2'-deoxycytidine (5-Aza-Dc) for 72 h. (B) DNA methylation levels of *H19* exon 1 across 7 CpG sites in JEG-3 cells from different treatment groups. (C) *H19* expression level of JEG-3 cells treated with 100 μmol/l 5-Aza-Dc for 24, 48 or 72 h. The data are presented as the means ± SD: ***P<0.001 vs. results for control (DMSO-treated cells). (D) *H19* expression level in JEG-3 cells treated with 5-Aza-Dc for 72 h at concentrations of 0.1, 1, 10 or 100 μmol/l. The data are presented as the means ± SD: **P<0.01, ***P<0.001 vs. results for control; $^{###}$P<0.001 vs. results for 100 μmol/l 5-Aza-Dc treatment.

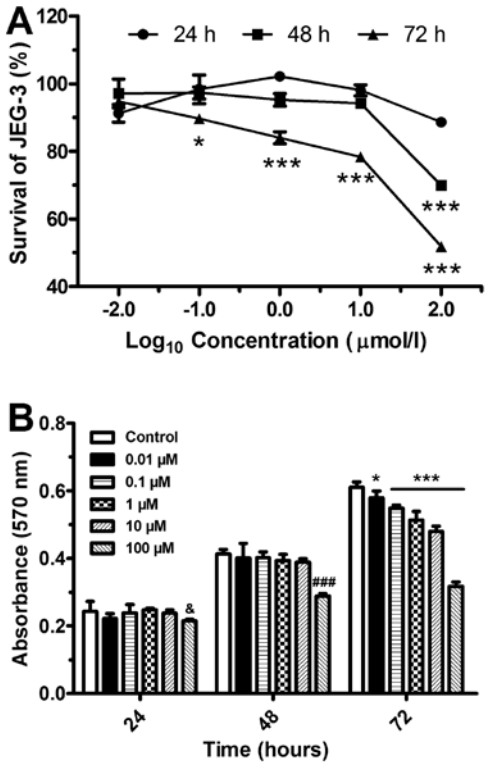

Figure 3. Inhibition of JEG-3 cell proliferation following demethylation treatment. (A) Concentration-response curves of JEG-3 cells treated with 5-aza-2'-deoxycytidine (5-Aza-Dc) at a concentration of 100 μmol/l for 24, 48 or 72 h. Cell proliferation was determined by MTT assay. The data are presented as the means ± SEM: *P<0.05, ***P<0.001 vs. results for control. (B) The absorbance at 570 nm of JEG-3 cells following incubation without or with various concentrations of 5-Aza-Dc for 24, 48 and 72 h. The data are presented as the means ± SD: $^{\&}$P<0.05 vs. results for control of 24 h, $^{###}$P<0.001 vs. results for control of 48 h; *P<0.05, ***P<0.001 vs. results for control of 72 h.

from women in FTP compared with that from women in TTP or women with sPE (P<0.05) (Fig. 1E). When each CpG site was analyzed independently, the methylation level at CpG 1 of *H19* exon 1 displayed significant demethylation in the placental tissue from women in FTP in comparison to that from women in TTP (P<0.01) (Fig. 1F). The methylation levels at this site of *H19* were significantly increased in the placental tissue from women with sPE compared with the levels in the placental tissue from women in TTP (P<0.01).

Methylation level of the H19 exon 1 region in JEG-3 cells treated with 5-Aza-Dc. The chemical agent, 5-Aza-Dc, a cytidine analog, has been reported to effectively result in the demethylation of DNA (16). In this study, the region (H19 exon 1) containing 7 CpG sites, spanning 258 bp, was analyzed (Fig. 2A). DNA from the JEG-3 cells treated without or with 5-Aza-Dc at concentrations of 1, 10 or 100 μmol/l was extracted at 72 h and then analyzed by BSP assay. The methylation level of the exon 1 region was signaficantly demethylated in the cells treated with 5-Aza-Dc compared with the control (DMSO-treated) cells, and the effect was dose-independent (Fig. 2B). However, the demethylation effect of 5-Aza-Dc was only observed at the CpG 1 site and not at the other 6 CpG sites (Fig. 2A).

Expression level of H19 in JEG-3 cells treated with 5-Aza-Dc. Subsequently, we examined whether *H19* exon 1 demethylation leads to an increase in the *H19* mRNA level in JEG-3 cells. Following treatment with 5-Aza-Dc at 100 μmol/l for 24, 48 or 72 h, the *H19* mRNA expression was 0.71±0.07, 1.05±0.49 and 28.35±3.72, respectively (Fig. 2C). Following treatment for 72 h with 5-Aza-Dc at concentrations of 0.1, 1, 10 or 100 μmol/l, the

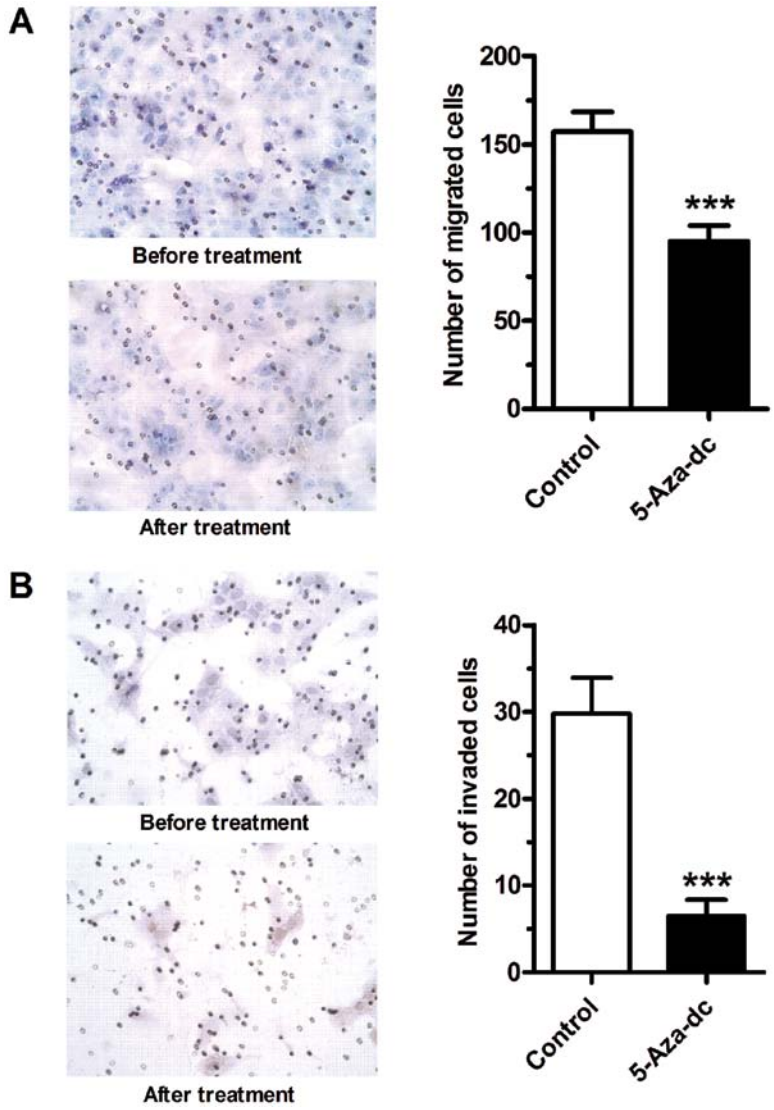

Figure 4. Inhibition of JEG-3 cell migration and invasion following demethylation treatment. (A) Migration of JEG-3 cells in the absence or presence of 5-aza-2'-deoxycytidine (5-Aza-Dc) at 100 μmol/l for 24 h. Migrated cells were examined with hematoxylin staining (original magnification, x200) and then quantified by manual counting. The data are presented as the means ± SD: ***P<0.001 vs. results for control. (B) Invasion of JEG-3 cells in the absence or presence of 5-Aza-Dc at 100 μmol/l for 24 h. Invaded cells were examined with hematoxylin staining (original magnification, x200) and then quantified by manual counting. The data are presented as the means ± SD: ***P<0.001 vs. results for control.

H19 mRNA expression was 1.82±0.64, 2.32±0.69, 10.70±3.89 and 28.35±3.72, respectively (Fig. 2D). As the treatment time or concentration increased, a marked increase in *H19* expression was observed.

Inhibition of cell proliferation by 5-Aza-Dc. We then investigated JEG-3 cell viability following treatment with 5-Aza-Dc at various concentrations and different periods of time points. Cell proliferation was suppressed in a concentration-dependent manner (Fig. 3A). In comparison to the control (DMSO-treated) group, the cells treated for 24 and 48 h displayed a significant inhibition of proliferation at the concentration of 100 μmol/l (P<0.001) (Fig. 3B). By contrast, the proliferation of the cells treated for 72 h with various concentrations of 5-Aza-Dc was significantly inhibited.

Demethylation by 5-Aza-Dc inhibits trophoblast-derived cell migration and invasion. The effects of 5-Aza-Dc on the migratory and invasive properties of JEG-3 cells were then examined. Following treatment with 5-Aza-Dc at 100 μmol/l for 24 h, both the migration (Fig. 4A) and invasion (Fig. 4B) abilities of the JEG-3 cells were markedly inhibited (P<0.001).

Discussion

The placenta is a unique endocrine organ during pregnancy that, although it is only transiently required, plays a critical role in protecting and nourishing the growing fetus (17). Trophoblasts developing into placental tissue are exclusive to this type of tissue. During normal gestation, these trophoblasts act as tumor-like cells, with enhanced proliferative and invasive activities, but behave in a moderate and balanced way (2). Once this balance is broken, corresponding diseases may occur. Excessive invasion may lead to invasive moles or choriocarcinoma (18). On the other hand, poor invasion may cause obstetric complications, including miscarriage, intra-

uterine growth restriction and PE (19). PE is a life-threatening, pregnancy-specific disorder characterized by hypertension and proteinuria (20). It is generally agreed that the presence of the placenta, and more specifically the presence of trophoblasts, is a major cause of this disorder (17). Elucidating the modulation of trophoblast development is key to understanding the pathogenesis of PE.

Previous studies on DNA methylation of the *H19* gene have mainly focused on the imprinting control region (ICR), the *H19* promoter region, or the *H19* transcription start site (21-23). In the present study, we investigated the methylation status of *H19* exon 1 in placental tissue from women undergoing normal pregnancy during the first and third trimesters, as well as pregnant women with PE. As one of the first imprinted genes (3), the *H19* transcript is abundantly expressed in mouse placental tissue and human intermediate trophoblasts and cytotrophoblasts. Mice that carry a deletion of *H19* display placental overgrowth (7). In a previous study of ours, we demonstrated that the *H19* gene imprinting status in human placental tissue is markedly altered during normal pregnancy, but imprinting is lost in the placental tissue of PE patients (24). These differences suggest that epigenetic changes in the *H19* gene may be relevant to the pathogenesis of PE. DNA methylation is fundamental for epigenetic modulation in mammalian development (25). It typically occurs at the C5 position of cytosine residues in a CpG dinucleotide context. CG-rich regions, known as CpG islands, most often localize at the promoter and exon 1 regions (26). *H19* exon 1 is highly conserved in both marsupials and eutherians (27), suggesting that this region may play some unknown important role in mammalian development.

In this study, we assessed the methylation status in *H19* exon 1 in placental tissue from women in FTP, TTP, as well as in pregnant women with sPE by BSP. We found that the DNA methylation level of *H19* exon 1 was significantly increased in placental tissue from women in TTP in comparison to that in placental tissue from women in FTP. It was observed that, compared with the placental tissue from women in TTP, the methylation levels in placental tisssue from women with sPE were only slightly enhanced in this region (P>0.05) (Fig. 1E). We further analyzed the methylation status of each CpG site independently. The methylation levels at the CpG 1 site were significantly increased in the placental tissue from women in TTP in comparison to those in placental tissue from women in FTP. More importantly, hypermethylation at the CpG 1 site was markedly increased in the placental tissue from women with sPE when compared with the placental tissue from women in TTP (Fig. 1F). These results indicate that the methylation levels of *H19* exon 1 in the human placenta are dynamically altered during gestation. Furthermore, there was marked hypermethylation at individual CpG sites of *H19* exon 1 in the placental tissue from women with PE. Hence, the alteration of the methylation status in individual CpG sites may be associated with abnormal placentation and the pathogenesis of PE.

In this study, to investigate the effects of demethylation on trophoblasts, 5-Aza-Dc, a cytidine analog, we used as a DNA methylation inhibitor. The CpG 1 site rather than the other sites in this region showed marked demethylation in the treated cells (Fig. 2A and B). These results indicated that the demethylation agent, 5-Aza-Dc, does not act on each CpG site but on some individual positions in DNA sequences. Besides, the relative expression of *H19* mRNA increased following treatment of the trophoblasts with 5-Aza-Dc (Fig. 2C and D), indicating that demethylation at some individual CpG sites may correlate with the enhanced mRNA expression of *H19*.

Originating from trophoblasts, the hydatidiform mole has a propensity to malignancy. The expression of *H19* has been shown to be decreased during the transition from a complete hydatidiform mole to choriocarcinoma (28), which indicates an inhibitory role for *H19* during the malignant transformation of trophoblastic diseases. This is consistent with our present results that the proliferation (Fig. 3), migratory and invasive (Fig. 4) abilities of the choriocarcinoma cells were suppressed following demethylation treatment, which induced the upregulation of *H19* expression.

In conclusion, in this study, we demonstrate that the CpG 1 site in *H19* exon 1 is hypermethylated in placental tissue from pregnant women with PE. Following treatment of the cells with the methylation inhibitor, 5-Aza-Dc, the methylation levels at this site in the trophoblasts were markedly decreased and the mRNA levels of the *H19* gene were increased. Furthermore, the proliferative, migratory and invasive abilities of the trophoblasts were significantly inhibited. Therefore, our data suggest that hypermethylation at the CpG 1 site of *H19* exon 1 may be associated with the overproliferation of trophoblasts and may contribute to the pathogenesis of PE.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (nos. 81070505 and 81070496).

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