Preeclampsia (PE) is characterized by abnormal placentation in the early stages of pregnancy. Adequate migration and invasion of trophoblasts into the uterine wall and spiral arteries to form a functional maternal-fetal interface are pivotal for normal placentation, but the exact mechanism remains unclear. Growing evidence has revealed that special AT-rich sequence-binding protein 1 (SATB1) is a tumor promoter that participates in cancer cell migration and invasion. However, the expression and function of SATB1 in trophoblasts is unknown. Here, we characterize the stimulatory effect of SATB1 on the migration and invasion of trophoblasts and identify the regulatory events and downstream signaling components. Down-regulated SATB1 was detected in PE placentae and villous explants cultured under hypoxia/re-oxygenation (H/R) conditions. H/R-treated trophoblasts with lower SATB1 levels exhibited weaker invasive and growth capacities, whereas up-regulation of the SATB1 level with recombinant SATB1 restored these impairments. This restoration was especially
apparent with the sumoylation-deficient SATB1 variant, which contained a mutated site that blocked sumoylation. Moreover, the elevated concentration of SATB1 also increased the expression of β-catenin, which is involved in human placental trophoblast invasion and differentiation and is down-regulated in PE. However, a specific activator, namely, lithium chloride (LiCl), increased β-catenin expression but had no evident influence on SATB1 expression. Furthermore, up-regulated SATB1 failed to restore trophoblast function when Wnt/β-catenin was suppressed by Dickkopf1 (DKK1). Together, these data show that SATB1 expression in the human placenta is affected by oxidative stress and might regulate the migration and invasion of trophoblasts via β-catenin signaling.

**Suggested Reviewers:**

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**Opposed Reviewers:**

Hongbo Qi  
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Responses to the Reviewers’ Comments

Dear Prof. Wei Yan and Prof. Hugh Clarke,

Thank you very much for your letter of Sep 19, 2017 regarding our manuscript entitled “Sumoylation-dependent SATB1 disintegration induced by oxidative stress participates in trophoblast invasion by regulating β-catenin” (Decision on BIOLRE-2017-0360), which was submitted to *Biology of Reproduction* for publication.

We feel that the comments and suggestions of the reviewers are very helpful. As suggested, we have revised our manuscript carefully, and the amendments have been highlighted in red in the revised manuscript. We believe that with the revision according to the comments of the reviewers, the manuscript has been significantly improved. We have submitted the revised manuscript and appended in below the point-by-point responses, which address the issues raised by the reviewers. We hope that this revised version is acceptable for publication in your journal.

Thanks again for your reconsideration of publishing our manuscript in your journal. We are looking forward to your response.

With best wishes,
Sincerely,
Hong-bo Qi

**Reviewer 1:**

1. The placentas for PE and controls were at different gestational weeks (34W vs 39W). Although the authors gave brief explanation in Discussion, I still suggest the authors to use gestational-week matched control placentas, for examples, derived from unexplained preterm birth.

   **Response:** Thank you for noting this issue. We completely understand your concern about the impacts of samples at different gestational weeks on the reliability of the results. However, it is difficult to obtain samples of matched gestational weeks in the clinic. Considering that cases of premature delivery are mostly related to fetal placental defects or maternal complications and that these known or unknown factors could affect the results of placental study, we chose uncomplicated term placenta as the control samples. To eliminate the confounding variables in the comparisons between the preeclampsia (PE) and control groups, a fixed-effects model (Mantel–Haenszel method) was adopted for data analysis. The differences between groups at different gestational weeks in late stages of gestation were not statistically significant. In addition, un-matched control placentas have been used in previous studies[1, 2], and the effects of this variability on results were insignificant. However, if you insist that gestational-week matched controls are essential for our study, we are happy to follow your suggestion accordingly.
2. In Figure 1, the SATB1 exhibited evident nuclear staining in trophoblast cells. Any evidence showing this protein can translocated from cytoplasma to nuclear? What’s the significance of its nuclear localization?

Response: Thank you for your insightful comment. SATB1 is a nuclear matrix attachment-binding protein. It has been shown that the N-terminal residues 20-40 of SATB1 represent a novel signal of nuclear targeting[3]. The SATB1 gene is located on human chromosome 3p23 and encodes five important functional domains, namely, the nuclear localization signal (NLS), PSD-95, Disc-large, and ZO-1 (PDZ), nuclear matrix-targeting sequences (NMTS), MARs-binding domain (MD) and homeodomain (HD). Thus, SATB1 specifically binds MARs with high affinity and provides docking sites for transcription factors, proteins and DNA sequences to control the expression of genes and regulate biological functions, such as the development and differentiation of T cells, as well as the growth and metastasis of various cancers. Based on our study, the nuclear localization of SATB1 might be associated with the stabilization and nuclear localization of β-catenin, which can result in the consequent regulation of trophoblast functions. However, the exact potential mechanism needs to be validated by additional experiments in future studies.

3. For β-catenin in the Wnt pathway, the de-phosphorylation and nuclear localization indicate its activation. In the present study, only the expression levels of β-catenin was measured by WB or IF. However, this is not adequate to prove the activation of Wnt/β-catenin pathway.

Response: Thank you for your insightful comment. According to your comments, we have conducted additional experiments to measure the level of nuclear β-catenin by WB to evaluate the activation of the Wnt pathway in our study, and the data are shown in revised Figures 2 and 4.

4. In Figure 1, the nuclear β-catenin should be counted and compared between PE and Control, N and H/R group.

Response: Thank you for your constructive comment. The quantification of nuclear β-catenin protein levels in tissues has been provided in the revised manuscript(Figure 1 C and D).

5. One major concern is regarding the experimental group, especially the K+LiCl+H/R group. It seems in this group, SATB1 and β-catenin overexpression were simultaneous induced in the cells, and the effects on cell migration and invasion appeared to be stronger than any of the treatment alone. With these data, it's possibly to speculate that SATB1 and Wnt/β-catenin are two independent pathway, and can work synergistically. However, the purpose of the authors seemed to prove that β-catenin is the downstream of SATB1. If so, they should use the "rescue" strategy, to knockdown β-catenin expression when overexpression STAB1 under H/R condition.

Response: Thank you for insightful and constructive suggestion that has definitely improved the quality of our manuscript. We completely agree with you that a "rescue" strategy is essential to validate our speculation that β-catenin is a downstream target of SATB1. Accordingly, we employed an antagonist of the Wnt signaling pathway, Dickkopf-related protein 1 (DKK1), in additional experiments. The details of cell treatments and classification of groups are shown in
The results are included in the revised manuscript. In brief, when SATB1 or β-catenin was over-expressed in cells under conditions of hypoxia/reperfusion (H/R), cell migration and invasion were increased relative to that in the control group. Furthermore, when SATB1 and β-catenin were simultaneously over-expressed in cells, the improvements in cell migration and invasion ability were even more obvious. However, the up-regulation of SATB1 failed to improve cell functions when β-catenin was inhibited by DKK1. These data demonstrated that SATB1 affects trophoblast migration and invasion by regulating β-catenin expression. Please note that the original figures have been replaced with revised versions on account of changes in experimental groups.

6. To follow the above concern, LiCl could not reverse the cell apoptosis caused by H/R, while STAB1 could. Is that because STAB1 and Wnt/β-catenin are not in the same pathway?  
**Response:** Thank you for the helpful and insightful question. We have conducted additional experiments in which cells were treated with DKK1 in the presence of SATB1 over-expression under conditions of H/R and confirmed that β-catenin is a downstream target of SATB1. In our previous study, we treated HTR8/SVneo cells with 40 µM LiCl as references described[4, 5]. However, despite its positive effects on cell functions, LiCl could not reverse cell apoptosis caused by H/R. To determine whether this phenomenon was caused by excessive LiCl, we conducted MTT assays to identify the optimal concentration (30 µM) of LiCl for treating HTR8/SVneo cells under conditions of H/R and then measured the apoptosis rate of each group (see Supplementary materials). The results have been updated in the revised version of the manuscript (Figure 6A). In brief, apoptosis rates of HTR8/SVneo cells were reduced by LiCl treatments under conditions of H/R.

7. Although the H/R treatment could lower down the level of SATB1, but at present the authors had little evidence to show this was due to the induce of sumoylation-dependent degardation of SATB1 by oxidative stress. Therefore, the conclusion is not evidence-supported.  
**Response:** Thank you for your critical comment. Investigators have demonstrated a novel mechanism for caspase-mediated cleavage of the critical regulatory protein SATB1, in which the post-translational conjugation of SATB1 to the ubiquitin-like SUMO-1 protein was shown to target SATB1 for caspase cleavage. Of the six potential consensus SUMO-1 modification sites within SATB1, mutation at only Lys744 can prevent the in vitro conjugation of SUMO-1 to SATB1 and subsequent cleavage[6]. Considering the reduction in SATB1 levels in PE and under conditions of H/R, we constructed lentiviral vector-based plasmids containing two variants of SATB1 gene sequences and found that compared with wild-type SATB1 sequences, SUMOylation-deficient SATB1sequences could result in the up-regulation of the level of SATB1 under oxidative stress. However, we agree with you that the evidence may not be strong enough to irrevocably prove the hypothesis that the reduction of SATB1 was due to the induction of SUMOylation-dependent degradation in response to oxidative stress. Nevertheless, based on your comments, we have amended the conclusion as shown in red in the revised version of the manuscript. We will keep your insightful comments in mind and carry out relevant experiments to investigate the possibility of SUMOylation-dependent degradation of SATB1 in our future study.
8. There are lots of grammatical problems. Language editing is needed.
Response: Thank you for carefully and patiently reviewing our manuscript. The language of the manuscript has been edited during the revision process, and we have carefully examined the manuscript to reduce typos and grammar errors as much as possible.

Reviewer 2:
1. The English writing in the Abstract should be more precisely, such as the "placental implantation" and "expression of SATB1 in human placenta affected by sumoylation";
Response: Thank you for bringing this issue to our attention. The writing in the Abstract has been professionally edited. We are also grateful to you for noting the errors regarding "placental implantation" and "expression of SATB1 in human placenta affected by sumoylation" and have changed them in the revised version of the manuscript. Furthermore, we have carefully examined the English writing in the revised manuscript.

2. The title is not suitable, since the data just show the oxidative stress could reduce the SATB1 expression, and it's not clear whether this downregulation is Sumoylation-dependent or not;
Response: Thank you for the constructive and insightful comments. We completely agree with you that the evidence in our study was not strong enough to irrevocably conclude that the down-regulation of SATB1 caused by oxidative stress was SUMOylation-dependent. The conclusion is indeed farfetched, and further studies are needed to prove this hypothesis. We have thus reconsidered the evidence and revised the title of the revised manuscript.

Response: Thank you for carefully and patiently reviewing our manuscript. We have corrected those errors in the revised version of the manuscript. And we have carefully examined the manuscript to reduce errors in spelling and grammar. All the modifications have been highlighted in red in the revised version of the manuscript.

4. Line115, reference should be included for the PE diagnose criteria;
Response: Thank you very much for this suggestion. We have carefully re-checked the references, and the reference for the PE diagnosis criteria has been appended to the revised version of the manuscript.

5. The catalog NO, should be provided for the antibodies used;
Response: Thank you for bringing this to our attention. Accordingly, in the revised version of the manuscript, we have added the catalog numbers of the antibodies used.

6. Line342, it's the expression level but not the activities;
Response: Thank you very much for your comment. We have revised the wording in the revised
version of the manuscript and have highlighted the modified parts in red.

7. **There should be more description for SATB1 expression in placenta as which cell type express the target proteins.**

**Response:** Thank you for your insightful comment. We have provided detailed descriptions of the expression and localization of SATB1 in placenta and have highlighted the changes in red in the revised version of the manuscript (page 15, lines 320-326).

SATB1 down-regulation induced by oxidative stress participates in trophoblast invasion by regulating β-catenin

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ABSTRACT

Preeclampsia (PE) is characterized by abnormal placentation in the early stages of pregnancy. Adequate migration and invasion of trophoblasts into the uterine wall and spiral arteries to form a functional maternal-fetal interface are pivotal for normal placentation, but the exact mechanism still remains distinct unclear. Growing evidence has revealed that special AT-rich sequence-binding protein 1 (SATB1) is a tumor promoter that participates in cancer cells migration and invasion—as a tumor
promoter. However, the expression and function of SATB1 in trophoblasts is unknown. Here, we characterize the stimulatory effect of SATB1 on the migration and invasion of trophoblasts and identify the regulatory events and the downstream signaling components. Down-regulated SATB1 was detected in preeclamptic PE placentae and villous explants cultured under hypoxia-re-oxygenation (H/R) conditions. H/R-treated trophoblasts with lower SATB1 levels exhibited weaker invasion and growth capacities, while—whereas up-regulation of the SATB1 level by recombinant SATB1 can restored those impaired capacities. This restoration was, especially apparent with the sumoylation-deficient SATB1 variant, which contained a mutated site for blocking sumoylation of SATB1. Moreover, the elevated concentration of SATB1 also increased the expression of β-catenin, which is involved in human placental trophoblast invasion and differentiation and be observed down-regulated in PE. However, a specific activator, namely, lithium chloride (LiCl), could improve increased β-catenin expression but had no evident influence on the expression of SATB1. Furthermore, up-regulated SATB1 failed to improve-troblast function when Wnt/β-catenin was suppressed by
Dickkopf1 (DKK1). Together, these data showed that the SATB1 expression of SATB1 in the human placenta is affected by oxidative stress, and might regulate the migration and invasion of trophoblasts via β-catenin signaling.

Keywords: preeclampsia; trophoblast; SATB1; sumoylation; β-catenin

INTRODUCTION

Preeclampsia (PE) is a pregnancy-specific disorder, which is characterized by new-onset hypertension, proteinuria and maternal endothelial dysfunction and affecting 3–8% of all pregnancies [1]. Although numerous research studies have attempted to illuminate understand this disease, the exact etiopathogenesis still remains a mystery, and except for placental delivery, there is no specific effective treatment for PE exists except placental delivery [2]. It is clear that the process of placenta tion is affected by oxidative stress caused by ischaemia ischemia reperfusion in the placenta, but excessive oxidative stress might induce dysfunction of trophoblast dysfunction and insufficient maternal-fetal interface formation, ultimately leading to the development of PE [3, 4]. Therefore, it is crucial to disclose understanding the molecular
mechanism underlying oxidative stress-induced trophoblast dysfunction is crucial.
The Special AT-rich sequence-binding protein 1 (SATB1), which was first identified in T-cells, is a global chromatin organizer and transcription factor. SATB1 specifically binds to MARs with high affinity and provides docking sites for proteins and DNA sequences to control the expression of hundreds of genes; moreover, SATB1 is essential for T cell differentiation and erythroid development, and also can induce gene silencing in embryonic cells. Previous studies have demonstrated that SATB1 can facilitate the invasion and metastasis of cancer cells in multiple tissues, such as breast, gastric and colorectal tissues, and that SATB1 knockdown of SATB1 can decrease the invasion of cancer cells. It has also been reported that SATB1 is abundantly expressed in rat trophoblast stem cells of rats and decreases following differentiation. However, the expression and function of SATB1 in human trophoblasts remains unclear.
As a posttranslational protein modification, SUMO can conjugate to specific substrates to mediate numerous physical and pathological processes, including regulation of transcriptional regulation, cell growth, differentiation and apoptosis. It has been shown that SUMO can be conjugated to SATB1 at lysine-744 and
regulates the caspase-6-mediated cleavage of SATB1, while a
sumoylation-deficient SATB1 variant which contains a mutant
arginine-744 failed to exhibit the characteristic cleavage
pattern[16, 17]. These observations together suggested a
novel SUMO-dependent modification and
disintegration degradation manner mechanism for SATB1.

The Wnt/β-catenin signaling pathway belongs to comprises an
extensive functional network that has developed around a group of
proteins, and deregulation of the components involved in this
signaling network has been implicated in many
pathological process[18-20]. The armadillo protein β-catenin is the
central denominator player in Wnt/β-catenin signaling, and the
regulation of the presence, stability and nuclear accumulation of
β-catenin provides a measurement of Wnt signaling. It has been
proved β-catenin participates in the placentation of murine
mice and regulates the migration and invasion of trophoblasts[21-23].
More interestingly, β-catenin signaling was indicated taking
part participates in the epithelial–mesenchymal transition and
invasion invasive behavior of colorectal cancer cells and is
correlated with SATB1 levels[24], and Additionally SATB1 can also
orchestrate TH2 lineage commitment by mediating
Wnt/β-catenin signaling[25]. However, the interrelation between
SATB1 and Wnt signaling in human trophoblasts still needs to be settled.

In the present study, we aimed to obtain evidence of the participation of SATB1 and β-catenin signaling in the pathogenesis of PE by comprehensively investigating the impact of oxidative stress on the expression of SATB1 and β-catenin expression, the effects of SATB1 and β-catenin on trophoblast migration and invasion, and the correlation between SATB1 and β-catenin during the early stage of human placentation. We attempted to obtain some evidences for the participation of SATB1 and β-catenin signaling in the pathogenesis of PE.

MATERIALS AND METHODS

Clinical materials

Preeclampsia (PE) was diagnosed based on both clinical and laboratory findings according to the American College of Obstetrics and Gynecology criteria [26]. All participants were women with singleton pregnancies without a history of chronic hypertension, pregnancy--gestational diabetes mellitus, thrombophilias and chronic renal disease, et al. Informed consent was obtained from all of the participants. In addition, all procedures were approved by the Ethics Committee of the First Affiliated
Hospital of Chongqing Medical University. First-trimester villous samples (6-10 weeks gestation) were collected from healthy pregnant women (n=10) who accepted elective pregnancy termination for non-medical reasons. Term (n=25) and preeclamptic PE (n=20) placental tissues were obtained from pregnant women who underwent elective cesarean section. Placental tissue samples were dissected from five cotyledons of the maternal side without visible infarction, calcification, hematoma, or tears and had a general dimension of approximately 1-2 cm³ and a wet weight of 100 mg. Each sample was located midway between the umbilical cord insertion site and the peripheral edge of the placenta[27].

Cell and villous explants cultures

The extravillous trophoblast cell line HTR8/Svneo was kindly provided by Dr. Charles Graham (Queen's University, Kingston, Ontario, Canada) and routinely maintained in RPMI 1640 (Gibco) containing 10% fetal bovine serum (FBS, Gibco) and cultured under normal conditions (37°C, 20% oxygen) as the control group. The H/R treatment (8 hours at 2% oxygen, followed by 16 hours at 20% oxygen) was performed to mimic the oxidative stress in the preeclamptic PE placenta in vitro[28]. First-trimester placental tissues were washed in 0.9% saline and dissected from
the tips of the villi, and then explanted as previously mentioned under normoxic or H/R conditions [29]. Briefly, small pieces of tissue (2–3 mm mm) were explanted in Millicell-CM culture dish inserts—pre-coated with a phenol-red-free matrigel substrate and placed into 24-well culture dishes. The explants were maintained in serum-free F12/DMEM media containing various intervention factors. The proportion level of trophoblast sprouting and migration from the distal ends of the tips were measured. The eCell treatment and the group classification of groups are shown in Table 1.

Up-regulation of SATB1 and inhibition of Wnt/β-catenin signaling

The lentiviral vector-based plasmids containing the gene sequences of full-length (K) SATB1 or of SATB1 containing a specific mutation of at the sumoylation site (R, the lysine at position 744 site was replaced by with arginine) of SATB1 (NM_002971.4) were constructed, sequenced and packaged (GenePharma, Shanghai, China). For HTR8/Svneo, lentivirus was transfected into cells, and the cells were cultured in complete medium containing puromycin following the manufacturer’s instructions of manufacturer to obtain stably transfected cells. For the villous explants, lentivirus
were added into the inserts directly. For the inhibition of Wnt/β-catenin signaling, cells were transfected with pcDNA3-DKK-1 (DKK-1) using the Fugene HD transfection agent (Promega, Madison, WI, USA) according to the manufacturer's instructions.

**Lithium chloride treatment and the**

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cytotoxicity assay

Cells were incubated with lithium chloride (LiCl; Sigma-Aldrich, St. Louis, MO, USA) was incubated with cells for 6 h before the H/R intervention as described[30]. The cell viability of the LiCl-treated HTR8/Svneo under normoxic or H/R conditions at different concentrations (10, 20, 30, 40, 50 μM) cells with LiCl treatment were measured by MTT assay, and the cell viability of each group was assessed after 24, 48, and 72 hours as described[31]. Thereafter, the optimal cultivating concentration (30 μM) of LiCl was obtained (see Supplementary materials).

**Immunohistochemistry**

Immunohistochemistry was performed as previously described[32].
Formalin-fixed sections of placental tissues were deparaffinized in xylene and rehydrated in a serial gradient of ethanol. Endogenous peroxidase was quenched by incubation with 3% hydrogen peroxide for 10 minutes. After blocking with normal goat serum for 15 min, sections were incubated with a primary SATB1 antibody (1:500 dilution; product no. ab109100; Abcam, Inc. Cambridge, UK) or β-catenin antibody (1:500 dilution; product no. ab32572; Abcam, Inc., Cambridge, UK) overnight at 4°C. Non-immunized rabbit IgG was used as a negative control. After washing with PBS, the sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:500 dilution; product no. AP307P; Millipore, Inc. Dallas, Texas, USA) for 1 h at 37°C. Hematoxylin was used as a nuclear counter-stain. The sections were observed with a microscope system (Olympus LX70; Olympus, Middlesex, UK) after dehydration with a serial gradient of ethanol. Immunohistochemical staining was quantitated using IPP 6.0 image analysis software (Media Cybernetics, USA) as described[33].

Immunofluorescence

Cells with different indicated treatments were permeabilized with 0.2% Triton X-100 fixed in ice-cold methanol and blocked with
1% bovine serum albumin (BSA) for 1 hour. The primary antibodies used were anti-SATB1 antibody (1:1000 dilution; product no. ab109100; Abcam, Inc.; Abcam, Cambridge, UK) and anti-β-catenin antibody (1:1000 dilution; product no. ab32572; Abcam, Inc.; Abcam, Cambridge, UK). The secondary antibody was a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (1:50; Santa Cruz Biotechnology, Dallas, TX, USA). Nuclei were stained with propidium iodide (PI) (3 mg/mL). Images were acquired with an Olympus BMX-60 microscope equipped with a cooled charge-coupled device Sensicamera (Cooke, Auburn Hills, MI) and Slidebook software (Intelligent Imaging Innovations, Denver, CO, USA).

**Western blotting**

Tissues and cells were lysed in RIPA lysis buffer (Sigma, USA) as per the manufacturer's instructions. The extracted proteins were quantified with a Bradford reagent (Bio-Rad, Richmond, California, USA) and then subjected to western blot analyses. The membranes were blotted using primary antibodies against human SATB1 (1:1000 dilution; product no. ab109100, Abcam, Inc.), β-catenin (1:1000 dilution; product no. ab32572, Abcam, Inc.), matrix metalloproteinase (MMP)-9 (1:500 dilution;
product no. sc-10737, Santa Cruz Biotechnology, Inc.), MMP-2 (1:500 dilution; product no. sc-10736; Santa Cruz Biotechnology, Inc.), TIMP1 (1:2500 dilution; product no. 04-1133; Millipore, Inc.), TIMP2 (1:1000 dilution; product no. MAB13446; Millipore, Inc.), β-actin (1:1000 dilution; product no. sc-130656; Santa Cruz Biotechnology, Inc.) at 4°C overnight and then incubated with the horseradish peroxidase-conjugated secondary antibody (1:2000 dilution; product no. sc-2357; Santa Cruz Biotechnology, Inc.). Densitometric analysis was performed by enhanced chemiluminescence reagents and analyzed by a Chemi-doc image analyzer (Bio-Rad).

Migration assay and matrigel invasion assay

For the cell migration assays, HTR8/SVneo cells with different indicated treatments (1 × 10^5) were planted into the upper chambers of transwell inserts (Costar, Cambridge, MA, USA), then and the inserts were placed into 24-well culture dishes. For the migration assays, the inserts were pre-coated with matrigel (BD Biosciences). To induce the cell migration and invasion of cells, 200 µl of serum-free medium was added into the inserts, while 600 µl of medium with 10% FBS was
was added to 24-well culture dishes, and Cells were cultured under normal or H/R conditions, respectively. To estimate the number of invaded or migrated cells, the cells on the upper chambers of the inserts were gently wiped away with a cotton swab, then and the inserts were fixed in methanol and stained with crystal violet. Then, cell numbers were counted determined by light microscopy (Olympus IX51, Japan) in five random fields.

Flow cytometric analysis of apoptosis and intracellular reactive oxygen species (ROS) formation

The apoptosis of cells was detected visualized by with the Annexin V-FITC and PI Apoptosis Detection Kit (Key-Gen Bio tech, Nanjing, China) as previously described[34]. HTR8/SVneo cells with different under indicated treatments were seeded on to 6-wellplates and exposed to H/R or normal conditions for 48 h. After twice two washes with PBS, the cells were incubated in binding buffer for 30 min in the dark. The apoptosis rate was quantified with the FACS Vantage SE flow cytometer (BD Biosciences, San Jose, CA, USA).

Intracellular ROS formation was assessed by using a fluorogenic reagent, 2',7'-dichlorodihydrofluorescein diacetate
(Invitrogen), according to the manufacturer’s protocols. Cells from each group were seeded into 96-well plates at a density of 5000 cells per well and incubated with 10 μM DCFH-DA at 37°C for 30 min. Then, the cells were harvested and underwent analysis by flow cytometry. To evaluate the changes in oxidative stress, the fluorescence intensity at a wavelength of 530 nm was measured by a microplate reader to reflect the changes of oxidative stress.

**Statistical analysis**

All the data are presented as mean ± standard deviations (SD). Differences between two groups were analyzed by independent t-test assuming a Gaussian distribution, and statistical differences among multiple groups were evaluated by one-way analysis of variance. P ≤ 0.05 was considered significantly different.

**RESULTS**

Aberrant expression of SATB1 and β-catenin in placental tissues from pregnancies complicated by preeclampsia and in villous explants cultured under H/R conditions

Patient characteristics were collected and are shown in Table 2.
No significant differences were evident for age, BMI, and nulliparity (%) in the women of normal term pregnancy (NP) group or preeclampsia PE pregnancy group. Immunohistochemical analysis of placental tissues from the early and late stages of pregnancy showed that SATB1 and β-catenin were both expressed in the trophoblast cells in most of the samples. Strongly stained SATB1 was observed in the cytotrophoblasts and syncytiotrophoblasts in the early-stage samples; staining was, mostly observed in the cytotrophoblasts. In addition, positive staining appeared in the syncytiotrophoblasts of the samples from late-stage of pregnancy, when cytotrophoblasts transformed to syncytiotrophoblasts physiologically. Compared with the NP samples, the aberrant expression of SATB1 and β-catenin in the trophoblasts were observed in the PE placental tissues from PE was aberrant compared with that in the NP samples compared with the samples from NP (Figure 1). To simulate the microenvironment of PE in vitro, villous tissues from human first-trimester placenta were cultured with H/R intervention treatment. Immunohistochemical analysis showed weakened expression of SATB1 and β-catenin in the villous tissues treated with H/R (Figure 1) compared with that in the villous tissues cultured under normoxic conditions. Statistical analysis indicated aberrant decreases of
in SATB1 (data not shown) and nuclear β-catenin (Figure 1 C and D) expression indicated aberrant decrease in the PE samples. Western blot analysis of the placental protein revealed that the levels of SATB1 and nuclear β-catenin decreased significantly in the PE placental samples from PE and in the villous tissues cultured under H/R conditions (Figure 2). These data suggest that SATB1 and β-catenin were down-regulated in PE and this that these changes might be associated with the oxidative stress caused by H/R.

SATB1 up-regulation in HTR8/SVneo enhances the expression of β-catenin and improves the invasion of cells.

To ameliorate the attenuation of SATB1 caused by oxidative stress, HTR8/SVneo cells were transfected with lentivirus containing recombinant SATB1. We employed to transfec HTR8/SVneo. Immunofluorescence staining (Figure 3) and Western blot (Figure 4) analysis revealed that the H/R intervention could lessen the expression of SATB1 and β-catenin expression in the HTR-8/SVneo cells. However, after the treatment with lentivirus (K and R), the intracellular SATB1 and nuclear β-catenin levels were increased under H/R conditions, and higher protein levels were detected in the...
cells transfected with R than in the control cells. This data indicated that improved SATB1 promotes the nuclear accumulation of β-catenin, and interrupting disrupting the sumoylation of SATB1 appears to relieved the H/R-induced caused consumption of SATB1. LiCl-mediated a Activation of Wnt/β-catenin signaling by pre-treating with LiCl could elevateds the level of β-catenin level in the cells exposed to H/R condition but had little impact on SATB1 (the data off for the negative control groups are not shown). To evaluate the effects of SATB1 and β-catenin on trophoblast cell migration and invasion, HTR8/SVneo cells of each group were subjected to transwell assays (Figure 5). The results revealed a significant attenuation of the cell migration migratory and invasion invasive abilities under H/R conditions, while the up-regulation of SATB1 or treatment with LiCl can partially alleviated the impaired migratory and invasive impairments migration and invasion of the trophoblasts. The cells transfected with K and pre- precultured with LiCl before subject to the H/R intervention or and the cells transfected with R showed greater alleviation compared with those only transfected with K. These data strongly suggested that SATB1 up-regulation on f SATB1 and Wnt signaling activation of Wnt signalling could improve trophoblast migration and invasion in H/R conditions.
Moreover, SATB1 could up-regulate the expression of $\beta$-catenin, but $\beta$-catenin could not the opposite up-regulate SATB1 expression.

SATB1 up-regulation of SATB1 and the LiCl treatment with LiCl decreased the intracellular ROS formation and apoptosis caused by the H/R intervention.

It has been demonstrated that hypoxia/re-oxygenation could generate oxidative stress in cells and results in apoptosis. ROS accumulation of ROS in trophoblasts plays a pivotal role in oxidative stress-induced cell injury. To determine the impacts effects of increased SATB1 and activation of Wnt signaling on the H/R-induced ROS formation of ROS and trophoblast apoptosis of trophoblast induced by H/R, flow cytometric analysis was performed. The results showed that compared with that in the control group, the ROS fluorescence intensity of ROS were in the cells cultured under H/R conditions was significantly enhanced compared with that in the control group in cells cultured under H/R conditions. Compared with control group, HTR8/SVneo cells transfected with K presented showed a remarkably reduced fluorescence intensity when subjected to after the H/R intervention. An attenuated intensity was also detected in the cells pretreated with LiCl. Moreover, the cells that underwent
concomitant performed—ectopic transfectioned with K and pretreatmensted with LiCl or—and the cells that were transfected with R before subjected to the H/R intervention showed a dramatically reductions in ROS fluorescence intensity of ROS compared with those—the intensity in the cells only transfected with K or only treated with LiCl(Figure 6 A). Similarly, compared with the control group, the apoptotic rate increased when the cells were objected—subjected to the H/R treatment compared with that in the control group compared with the control group. Overexpression expression of SATB1 or treatment with LiCl could decreases the apoptotic rates of the trophoblasts exposed to H/R (Figure 6 B).

The effects of SATB1 and β-catenin on the expression of MMPs and TIMPs

Undoubtedly, matrix metalloproteinases MMPs (MMP2/9) and their special tissue inhibitors TIMPs (TIMP1/2) directly participate in the regulation of trophoblast migration and invasion. Thus we—Thus, we also detected enumerated the expression of these proteins in the placental tissues and HTR8/SVneo cells (Figure 2, 4). As we suspected, reduced expression of MMP2/9 and increased expression of TIMP1/2 were found—observed in
preeclamptic the PE placenta and in H/R-treated villous tissues or and cells. Additionally, SATB1 up-regulation of SATB1 or and the LiCl treatment with LiCl could both each alleviate these changes in the HTR8/SVneo cells. Therefore, based on these data, we have sufficient reasons to believe suggest that SATB1 and Wnt signaling are involved in the regulation of trophoblast migration and invasion.

SATB1 up-regulation and activation of Wnt signaling promote the outgrowth of trophoblasts in villous explants under H/R conditions. To verify the results obtained described in our previous study above and the hypothetical regulatory route, trophoblast outgrowth and migration of trophoblast were measured in the villous explants cultured in different experimental conditions in vitro. As shown in Figure 7, the outgrowth proportion of the H/R-treated villous explants was significantly less than that of the control group. However, this impaired migratory potency was effectively restored by SATB1 overexpression of SATB1 or LiCl treatment. Explants in which concomitantly performed transfection treatments with K and pretreated with LiCl before subjected to the H/R intervention were concomitantly performed showed more significant improvement compared with than any of the treatments alone. And
In addition, similar phenomena were observed in the villous explants transfected with R.

SATB1 overexpression expression of SATB1 failed to improve trophoblast the function of trophoblast when Wnt signaling is inhibited by DKK1.

To figure out determine the cascade connection between SATB1 and Wnt/β-catenin signaling, DKK1 was used to treat cells containing improved increased SATB1 under H/R conditions. As the data shown show, little β-catenin were was detected in the cells even though SATB1 was highly expression of SATB1 existed expressed (Figure 3, 4). And, In addition, the impaired cell functions cannot were be not relieved by SATB1 up-regulation when Wnt signaling inhibition was inhibited under H/R conditions(Figure 5, 6, 7). Combining Based on with the results described above, we have established a regulatory signaling pathway in through which H/R-induced oxidative stress causes SATB1 disintegration degradation of SATB1, leading to the down-regulation of β-catenin signaling and ultimately to trophoblast the dysfunction of trophoblast. In addition, the abnormal down-regulation of SATB1 might be mediated by sumoylation.
DISCUSSION

The process of placentation is regulated by an intricate network that includes massive gene expression and multiple signaling pathways, cytokines and growth factors. Invasive trophoblasts can degrade cells capable of degrading the extracellular matrix, can invade into the uterine wall and remodel the interstitial spiral arteries to form an effective maternal-fetal interface. Overwhelming oxidative stress has been recognized as a vital factor in the pathogenesis of pregnancy complications such as PE and fetal growth restriction[35]. Although the clinical signs and symptoms of PE present during late pregnancy, the molecular mechanism of trophoblast dysfunction occurs during the early stages of gestation[36]. Therefore, we examined not only the placenta from PE but also the immortalized human trophoblast HTR8/SVneo cell line and first-trimester villous explants were also employed in our research. The gestational age of the patient could not be matched because of the consideration of different optimal termination times of pregnancy between normal and preeclamptic PE pregnancy in the clinic. In this study, we investigated the potential roles and mechanisms of SATB1 and β-catenin signaling in the regulation of trophoblast function in vitro.
Oxidative stress in that was analogous to that of preeclamptic PE placenta was generated in cells and villous explants by H/R intervention.

SATB1 has been extensively studied as a tumor promoter in many organs and tissues [13, 37-39]. Although it has been reported to facilitate the invasion and metastasis of tumor cells, its role in trophoblast function and placental development still remains unexplored. Our study has demonstrated that SATB1 proteins are histologically detectable in the trophoblast cells of the placenta and that SATB1 expression of SATB1 is lessened in the preeclamptic PE placenta and in villous explants cultured under H/R conditions. The results indicated that SATB1 is involved in the pathogenesis of PE; however, the underlying biomolecular mechanism requires further study. Sumoylation of SATB1 at lysine-744 by SUMO can mediate caspase hydrolysis and reduce SATB1 presence level and protein stabilization of SATB1. To figure out whether the SUMO-dependent consumption degradation of SATB1 existed in the pathogenesis of PE, lentivirus containing a wild-type or sumoylation-deficient sequence of SATB1 were employed to improve the
levels of SATB1 in HTR8/SVneo cells. We found that cells transfected with R showed higher intracellular levels of SATB1 and resulting in increased activation of Wnt signaling and decreased cell functional injury impairment caused by due to the H/R interventions. These data suggest that oxidative stress might induce disintegration degradation of SATB1 by via a sumoylation-dependent mechanism. However, more research studies that focus on the association between sumoylation and SATB1 during PE development should be done. Gelatinases and their specific inhibitors has been demonstrated to participate in invasion of trophoblast [40]. In accordance with As shown in a previous study [41], it is clear that the activation of MMPs is suppressed in PE. To explore the regulatory mechanism, diverse signaling pathways involved in cell invasion have been investigated. The Wnt/β-catenin signaling pathway has been demonstrated to be an upstream pathway of MMPs [42, 43]. In our previous studies, we have demonstrated that the reduced β-catenin is associated with impaired function of trophoblast [22]. In this study, we also found decreases in the levels of β-catenin in the preeclamptic PE placental tissues and H/R-treated cells. Combined with results mentioned above, based on these data, we have every reason to believe that there are
some relevances among SATB1 and Wnt signaling are relevant to the pathogenesis of PE. Here, we conducted a series of study experiments to explore the potential roles of these players. The results showed that SATB1 up-regulation promoted increased the level of nuclear β-catenin and enhanced the trophoblast migration and invasion both of trophoblast cells in culture and of trophoblasts in human first-trimester villous tissues under H/R conditions. The intracellular ROS and cell apoptosis of cells were detected and measured, and the results showed that higher SATB1 levels activate increased β-catenin levels and reduced the ROS accumulation and cells apoptosis. Then, The detection of matrix metalloproteinases further verified changed the changes in trophoblast migration and invasion of trophoblast. In the end, we measured the outgrowth of trophoblasts in villous explants in vitro, obtained favorable results and reconfirmed our previous speculation. We additionally employed a specific activator (LiCl) and inhibitor (DKK1) of Wnt signaling to investigate the cascade connection between SATB1 and β-catenin. The activation of Wnt signaling by LiCl could improve the trophoblast function but failed to affect SATB1 expression of SATB1. Moreover, the inhibition of Wnt signaling by DKK1 could disturb the healing effects of up-regulated SATB1 on the
impaired cell functions. These findings proved confirm that β-catenin is the downstream of SATB1.

In this paper, we have shown a potential regulatory pathway in regulation of trophoblast migration and invasion. Taken together, these results above remind us indicate that SATB1 might act via β-catenin in response to oxidative stress-induced responder of the functional damage of trophoblasts via mediating β-catenin, but however, more elaborate studies in vivo are needed for more extensively research. Additionally, the expression developable changes expression of β-catenin molecule in our study implied the suppression and activation of the Wnt/β-catenin signaling pathway in trophoblasts cells. But, whether it make sense that SATB1 regulates cell function by via the Wnt signaling pathway in the pathogenesis of PE should be explored further.

**ACKNOWLEDGMENTS**

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SATB1 down-regulation induced by oxidative stress participates in trophoblast invasion by regulating β-catenin

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ABSTRACT

Preeclampsia (PE) is characterized by abnormal placentation in the early stages of pregnancy. Adequate migration and invasion of trophoblasts into the uterine wall and spiral arteries to form a functional maternal-fetal interface are pivotal for normal placentation, but the exact mechanism remains unclear. Growing evidence has revealed that special AT-rich sequence-binding protein 1 (SATB1) is a tumor promoter that participates in cancer cell migration and invasion. However, the expression and function of
SATB1 in trophoblasts is unknown. Here, we characterize the stimulatory effect of SATB1 on the migration and invasion of trophoblasts and identify the regulatory events and downstream signaling components. Down-regulated SATB1 was detected in PE placentae and villous explants cultured under hypoxia/re-oxygenation (H/R) conditions. H/R-treated trophoblasts with lower SATB1 levels exhibited weaker invasive and growth capacities, whereas up-regulation of the SATB1 level with recombinant SATB1 restored these impairments. This restoration was especially apparent with the sumoylation-deficient SATB1 variant, which contained a mutated site that blocked sumoylation. Moreover, the elevated concentration of SATB1 also increased the expression of β-catenin, which is involved in human placental trophoblast invasion and differentiation and is down-regulated in PE. However, a specific activator, namely, lithium chloride (LiCl), increased β-catenin expression but had no evident influence on SATB1 expression. Furthermore, up-regulated SATB1 failed to restore trophoblast function when Wnt/β-catenin was suppressed by Dickkopf1 (DKK1). Together, these data show that SATB1 expression in the human placenta is affected by oxidative stress and might regulate the migration and invasion of trophoblasts via β-catenin signaling.
Keywords: preeclampsia; trophoblast; SATB1; sumoylation; β-catenin

INTRODUCTION

Preeclampsia (PE) is a pregnancy-specific disorder. PE is characterized by new-onset hypertension, proteinuria and maternal endothelial dysfunction and affects 3–8% of all pregnancies[1]. Although numerous studies have attempted to understand this disease, the exact etiopathogenesis remains a mystery, and except for placental delivery, no effective treatment for PE exists [2]. The process of placentation is affected by oxidative stress caused by ischemia reperfusion in the placenta, but excessive oxidative stress might induce trophoblast dysfunction and insufficient maternal-fetal interface formation, ultimately leading to the development of PE[3, 4]. Therefore, understanding the molecular mechanism underlying oxidative stress-induced trophoblast dysfunction is crucial.

Special AT-rich sequence-binding protein 1 (SATB1), first identified in T-cells, is a global chromatin organizer and transcription factor. SATB1 specifically bind to MARs with high affinity and provides docking sites for proteins and DNA sequences to control the expression of hundreds of genes; moreover, SATB1 is essential for T
cell differentiation[5-7] and erythroid development[8] and can induce gene silencing in embryonic cells[9]. Previous studies have demonstrated that SATB1 facilitates the invasion and metastasis of cancer cells in multiple tissues, such as breast, gastric and colorectal tissues, and that SATB1 knockdown can decrease the invasion of cancer cells[10-13]. SATB1 is abundantly expressed in rat trophoblast stem cells and decreases following differentiation[14]. However, SATB1 expression and function in human trophoblasts remains unclear.

As a posttranslational protein modification, SUMO is conjugated to specific substrates to mediate numerous physical and pathological processes, including transcriptional regulation, cell growth, differentiation and apoptosis[15]. SUMO can be conjugated to SATB1 at lysine-744 and regulate the caspase-6-mediated cleavage of SATB1, while a sumoylation-deficient SATB1 variant that contains a mutant arginine-744 fails to exhibit the characteristic cleavage pattern[16, 17]. These observations suggest a novel SUMO-dependent modification and degradation mechanism for SATB1.

The Wnt/β-catenin signaling pathway comprises an extensive functional network that has developed around a group of proteins, and deregulation of the components involved in this signaling
network has been implicated in many pathological process[18-20]. The armadillo protein β-catenin is the central player in Wnt/β-catenin signaling, and the regulation of the presence, stability and nuclear accumulation of β-catenin provides a measure of Wnt signaling. β-catenin participates in the placentation of mice and regulates the migration and invasion of trophoblasts[21-23]. More interestingly, β-catenin signaling participates in the epithelial–mesenchymal transition and invasive behavior of colorectal cancer cells and is correlated with SATB1 levels[24]. Additionally, SATB1 can orchestrate TH2 lineage commitment by mediating Wnt/β-catenin signaling[25]. However, the interrelation between SATB1 and Wnt signaling in human trophoblasts still needs to be clarified. In the present study, we aimed to obtain evidence of the participation of SATB1 and β-catenin signaling in the pathogenesis of PE by comprehensively investigating the impact of oxidative stress on SATB1 and β-catenin expression, the effects of SATB1 and β-catenin on trophoblast migration and invasion, and the correlation between SATB1 and β-catenin during the early stage of human placentation.

MATERIALS AND METHODS
Clinical materials

PE was diagnosed based on clinical and laboratory findings according to the American College of Obstetrics and Gynecology criteria[26]. All participants were women with singleton pregnancies without a history of chronic hypertension, gestational diabetes mellitus, thrombophilia or chronic renal disease. Informed consent was obtained from each participant. In addition, all procedures were approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. First-trimester villous samples (6-10 weeks gestation) were collected from healthy pregnant women (n=10) who underwent pregnancy termination for non-medical reasons. Term (n=25) and PE(n=20) placental tissues were obtained from pregnant women who underwent elective cesarean section. Placental tissue samples were dissected from five cotyledons of the maternal side without visible infarction, calcification, hematoma, or tears and had a general dimension of approximately 1-2 cm$^3$ and a wet weight of 100 mg. Each sample was located midway between the umbilical cord insertion site and the peripheral edge of the placenta[27].

Cell and villous explant cultures

The extravillous trophoblast cell line HTR8/Svneo was kindly
provided by Dr. Charles Graham (Queen's University, Kingston, Ontario, Canada), routinely maintained in RPMI 1640 (Gibco) containing 10% fetal bovine serum (FBS, Gibco), and cultured under normal conditions (37°C, 20% oxygen) as the control group. The H/R treatment (8 hours at 2% oxygen, followed by 16 hours at 20% oxygen) was performed to mimic the oxidative stress of the PE placenta in vitro [28]. First-trimester placental tissues were washed in 0.9% saline, dissected from the tips of the villi, and explanted as previously described under normoxic or H/R conditions [29]. Briefly, small pieces of tissue (2-3 mm) were explanted in Millicell-CM culture dish inserts precoated with a phenol-red-free Matrigel substrate and placed into 24-well culture dishes. The explants were maintained in serum-free F12/DMEM containing various intervention factors. The level of trophoblast sprouting and migration from the distal ends of the tips was measured. The cell treatment and group classification are shown in Table 1.

*Up-regulation of SATB1 and inhibition of Wnt/β-catenin signaling*

Lentiviral plasmids containing the gene sequences of full-length (K) SATB1 or of SATB1 containing a specific mutation at the sumoylation site (R, the lysine at position 744 was replaced with arginine) (NM_002971.4) were constructed, sequenced and
packaged (GenePharma, China). For HTR8/Svneo, lentivirus was transfected into cells, and the cells were cultured in complete medium containing puromycin following the manufacturer’s instructions to obtain stably transfected cells. For the villous explants, lentivirus was added into the inserts directly. For the inhibition of Wnt/β-catenin signaling, cells were transfected with pcDNA3-DKK-1 (DKK-1) using the Fugene HD transfection agent (Promega) according to the manufacturer’s instructions.

*Lithium chloride treatment and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay*

Cells were incubated with lithium chloride (LiCl; Sigma) for 6 h before the H/R intervention as described[30]. The cell viabilities of the LiCl-treated HTR8/Svneo under normoxic or H/R conditions at different concentrations (10, 20, 30, 40, 50μM) were measured by MTT assay, and the cell viability of each group was assessed after 24, 48, and 72 hours as described[31]. Thereafter, the optimal cultivating concentration (30 μM) of LiCl was obtained (see Supplementary materials).

*Immunohistochemistry*
Immunohistochemistry was performed as previously described[32].
Formalin-fixed sections of placental tissues were deparaffinized in xylene and rehydrated in a serial gradient of ethanol. Then, endogenous peroxidase was quenched by incubation with 3% hydrogen peroxide for 10 minutes. After blocking with normal goat serum for 15 min, sections were incubated with a primary SATB1 antibody (1:500 dilution; product no.ab109100; Abcam, Inc.) or β-catenin antibody (1:500 dilution; product no.ab32572; Abcam, Inc.) overnight at 4°C. Non-immunized rabbit IgG was used as a negative control. After washing with PBS, the sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:500 dilution; product no.AP307P;Millipore,Inc.) for 1 h at 37°C. Hematoxylin was used as a nuclear counter stain. The sections were observed with a microscope system (Olympus LX70; UK) after dehydration with a serial gradient of ethanol. Immunohistochemical staining was quantitated using IPP 6.0 image analysis software (Media Cybernetics, USA) as described[33].

**Immunofluorescence**

Cells under indicated treatments were permeabilized with 0.2% Triton X-100 fixed in ice-cold methanol and blocked with 1% bovine serum albumin (BSA) for 1 hour. The primary antibodies used were
anti-SATB1 antibody (1:1000 dilution; product no. ab109100; Abcam, Inc.) and anti-β-catenin antibody (1:1000 dilution; product no. ab32572; Abcam, Inc.). The secondary antibody was a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (1:50; Santa Cruz, Inc.). Nuclei were stained with propidium iodide (PI) (3 mg/ml). Images were acquired with an Olympus BMX-60 microscope equipped with a cooled charge-coupled device Sensicam (Cooke, Auburn Hills, MI) and Slidebook software (Intelligent Imaging Innovations, USA).

**Western blotting**

Tissues and cells were lysed in RIPA lysis buffer (Sigma) per the manufacturer's instructions. The extracted proteins were quantified with a Bradford reagent (Bio-Rad) and subjected to Western blot analysis. The membranes were blotted using primary antibodies against human SATB1 (1:1000 dilution; product no. ab109100, Abcam, nc.), β-catenin (1:1000 dilution; product no. ab32572, Abcam, Inc.), matrix metalloproteinase (MMP)-9 (1:500 dilution; product no. sc-10737, Santa Cruz, Inc.), MMP-2 (1:500 dilution; product no. sc-10736; Santa Cruz, Inc.), TIMP1 (1:2500 dilution; product no. 04-1133; Millipore, Inc.), TIMP2 (1:1000 dilution; product no. MAB13446; Millipore, Inc.), β-actin (1:1000 dilution; product
no.sc-130656; Santa Cruz Biotechnology, Inc.) at 4°C overnight and then incubated with the horseradish peroxidase-conjugated secondary antibody (1:2000 dilution; product no.sc-2357; Santa Cruz, Inc.). Densitometric analysis was performed with enhanced chemiluminescence reagents and a Chemi-doc image analyzer (Bio-Rad).

Migration assay and Matrigel invasion assay

For the cell migration assays, HTR8/SVneo cells under indicated treatments (1×10⁵) were seeded into the upper chambers of transwell inserts (Costar), and the inserts were placed into 24-well culture dishes. For the migration assays, the inserts were precoated with Matrigel (BD Biosciences). To induce cell migration and invasion, 200 µl of serum-free medium was added to the inserts, while 600 µl of medium with 10% FBS was added to the 24-well culture dishes. Cells were cultured under normal or H/R conditions. To estimate the number of invaded or migrated cells, the cells in the upper chambers of the inserts were gently wiped away with a cotton swab, and the inserts were fixed in methanol and stained with crystal violet. Then, cell number was determined by light microscopy (Olympus IX51) in five random fields.
Flow cytometric analysis of apoptosis and intracellular reactive oxygen species (ROS) formation

Cell apoptosis was visualized with the Annexin V-FITC and PI Apoptosis Detection Kit (Key-Gen Bio tech) as previously described[34]. HTR8/SVneo cells under indicated treatments were seeded to 6-wellplates and exposed to H/R or normal conditions for 48 h. After two washes with PBS, the cells were incubated in binding buffer for 30 min in the dark. The apoptosis rate was quantified with the FACS Vantage SE flow cytometer (BD Biosciences).

Intracellular ROS formation was assessed by using a fluorogenic reagent, 2’,7’-dichlorodihydrofluorescein diacetate (Invitrogen), according to the manufacturer’s protocols. Cells from each group were seeded into 96-well plates at a density of 5000 cells per well and incubated with 10 μM DCFH-DA at 37°C for 30 min. Then, the cells were harvested and analyzed by flow cytometry. To evaluate the changes in oxidative stress, the fluorescence intensity at a wavelength of 530 nm was measured with a microplate reader.

Statistical analysis

All data are presented as the mean ± standard deviation(SD). Differences between two groups were analyzed by
independent t-test assuming a Gaussian distribution, and significant
differences among multiple groups were evaluated by one-way
analysis of variance. P<0.05 was considered significantly different.

RESULTS

Aberrant expression of SATB1 and β-catenin in placental tissues
from pregnancies complicated by preeclampsia and in villous
explants cultured under H/R conditions

Patient characteristics were collected and are shown in Table 2. No
significant differences were evident for age, BMI, and nulliparity (%) in
the normal term pregnancy (NP) group or PE pregnancy group. Immunohistochemical analysis of placental tissues from the early
and late stages of pregnancy showed that SATB1 and β-catenin were
expressed in the trophoblasts of most of the samples. Strongly
stained SATB1 was observed in the cytotrophoblasts and
syncytiotrophoblasts of the early-stage samples; staining was mostly
observed in the cytotrophoblasts. In addition, positive staining
appeared in the syncytiotrophoblasts of the samples from late-stage
pregnancy, when cytotrophoblasts transformed to
syncytiotrophoblasts physiologically. The expression of SATB1 and
β-catenin in the trophoblasts of the PE placental tissues was aberrant
compared with that in the NP samples(Figure 1). To simulate the
microenvironment of PE in vitro, villous tissues from human first-trimester placentae were cultured with H/R treatment. Immunohistochemical analysis showed weakened expression of SATB1 and β-catenin in the villous tissues treated with H/R(Figure 1) compared with that in the villous tissues cultured under normoxic conditions. Statistical analysis indicated aberrant decreases in SATB1 (data not shown) and nuclear β-catenin (Figure 1 C and D) expression in the PE samples. Western blot analysis of the placental protein revealed that the levels of SATB1 and nuclear β-catenin decreased significantly in the PE placental samples and in the villous tissues cultured under H/R conditions(Figure 2).These data suggest that SATB1 and β-catenin are down-regulated in PE and that these changes might be associated with the oxidative stress caused by H/R.

SATB1 up-regulation in HTR8/SVneo enhances β-catenin expression and improves cell invasion

To ameliorate the attenuation of SATB1 caused by oxidative stress, HTR8/SVneocells were transfected with lentivirus containing recombinant SATB1. Immunofluorescence staining (Figure 3) and Western blot (Figure4) analysis revealed the H/R intervention lessened SATB1 and β-catenin expression in the HTR-8/SVneo cells.
However, after the treatment with lentivirus (K and R), the intracellular SATB1 and nuclear β-catenin levels were increased under H/R conditions, and higher protein levels were detected in the cells transfected with R than in the control cells. These data indicated that improved SATB1 promotes the nuclear accumulation of β-catenin and the disrupting the sumoylation of SATB1 relieved the H/R-induced consumption of SATB1. LiCl-mediated activation of Wnt/β-catenin signaling elevated the β-catenin level in the cells exposed to H/R but had little impact on SATB1 (the data for the negative control groups are not shown). To evaluate the effects of SATB1 and β-catenin on trophoblast cell migration and invasion, HTR8/SVneo cells from each group were subjected to transwell assays (Figure 5). The results revealed a significant attenuation of the cell migratory and invasive abilities under H/R conditions, while the up-regulation of SATB1 or treatment with LiCl partially alleviated the migratory and invasive impairments of the trophoblasts. The cells transfected with K and precultured with LiCl before the H/R intervention and the cells transfected with R showed greater alleviation than those only transfected with K. These data strongly suggested that SATB1 up-regulation and Wnt signaling activation could improve trophoblast migration and invasion in H/R conditions. Moreover, SATB1 could up-regulate the expression of
β-catenin, but β-catenin could not up-regulate SATB1 expression.

SATB1 up-regulation and the LiCl treatment decreased the intracellular ROS formation and apoptosis caused by the H/R intervention

Hypoxia/re-oxygenation generates oxidative stress in cells and results in apoptosis. ROS accumulation in trophoblasts plays a pivotal role in oxidative stress-induced cell injury. To determine the effects of increased SATB1 and activation of Wnt signaling on H/R-induced ROS formation and trophoblast apoptosis, flow cytometric analysis was performed. The results showed that the ROS fluorescence intensity in the cells cultured under H/R conditions was significantly enhanced compared with that in the control group. HTR8/SVneo cells transfected with K showed a remarkably reduced fluorescence intensity after the H/R intervention. An attenuated intensity was also evident in the cells pretreated with LiCl. Moreover, the cells that underwent concomitant ectopic transfections with K and pretreatments with LiCl and the cells that were transfected with R before the H/R intervention showed dramatic reductions in ROS fluorescence intensity compared with the intensity in the cells only transfected with K or only treated with LiCl(Figure 6 A). Similarly, the apoptotic rate increased when the cells were subjected to the H/R
treatment compared with that in the control group. Overexpression of SATB1 or treatment with LiCl decreases the apoptotic rates of the trophoblasts exposed to H/R (Figure 6 B).

The effects of SATB1 and β-catenin on the expression of MMPs and TIMPs

Undoubtedly, MMPs (MMP2/9) and their special tissue inhibitors (TIMP1/2) directly participate in the regulation of trophoblast migration and invasion. Thus, we evaluated the expression of these proteins in the placental tissues and HTR8/SVneo cells (Figure 2, 4). As we suspected, reduced expression of MMP2/9 and increased expression of TIMP1/2 were observed in the PE placenta and in H/R-treated villous tissues and cells. Additionally, SATB1 up-regulation and the LiCl treatment each alleviated these changes in the HTR8/SVneo cells. Based on these data, we suggest that SATB1 and Wnt signaling are involved in the regulation of trophoblast migration and invasion.

SATB1 up-regulation and activation of Wnt signaling promote the outgrowth of trophoblasts in villous explants under H/R conditions

To verify the results described above and the hypothetical regulatory route, trophoblast outgrowth and migration were measured in the
villous explants cultured in different experimental conditions in vitro. As shown in Figure 7, the outgrowth proportion of the H/R-treated villous explants was significantly less than that of the control group. However, this impaired migratory potency was effectively restored by SATB1 overexpression or LiCl treatment. Explants in which transfections with K and pretreatments with LiCl before the H/R intervention were concomitantly performed showed more significant improvements than any of the treatments alone. In addition, similar phenomena were observed in the villous explants transfected with R.

_SATB1 overexpression fails to improve trophoblast function when Wnt signaling is inhibited by DKK1_

To determine the cascade connection between SATB1 and Wnt/β-catenin signaling, DKK1 was used to treat cells containing increased SATB1 under H/R conditions. As the data show, little β-catenin was detected in the cells even though SATB1 was highly expressed (Figure 3, 4). In addition, the impaired cell functions were not relieved by SATB1 up-regulation when Wnt signaling was inhibited under H/R conditions(Figure 5, 6, 7). Based on the results described above, we have established a regulatory signaling pathway through which H/R-induced oxidative stress causes SATB1 degradation, leading to the down-regulation of β-catenin
signaling and, ultimately, to trophoblast dysfunction. In addition, the abnormal down-regulation of SATB1 might be mediated by sumoylation.

**DISCUSSION**

The process of placentation is regulated by an intricate network that includes massive gene expression and multiple signaling pathways, cytokines and growth factors. Invasive trophoblasts can degrade the extracellular matrix, invade into the uterine wall and remodel the interstitial spiral arteries to form an effective maternal-fetal interface. Overwhelming oxidative stress is a vital factor in the pathogenesis of pregnancy complications, such as PE and fetal growth restriction[35]. Although the clinical signs and symptoms of PE present during late pregnancy, the molecular mechanism of trophoblast dysfunction is active during the early stages of gestation[36]. Therefore, we examined not only the placenta from PE but also the immortalized human trophoblast HTR8/SVneo cell line and first-trimester villous explants in our research. The gestational age of the patient could not be matched due to the different optimal termination times of pregnancy between normal and PE pregnancies in the clinic. In this study, we investigated the potential roles and mechanisms of SATB1 and β-catenin signaling in the regulation of trophoblast function in
vitro. Oxidative stress that was analogous to that of the PE placenta was generated in cells and villous explants by H/R intervention.

SATB1 has been extensively studied as a tumor promoter in many organs and tissues [13, 37-39]. Although SATB1 facilitates the invasion and metastasis of tumor cells, its role in trophoblast function and placental development remains unexplored. Our study has demonstrated that SATB1 proteins are histologically detectable in the trophoblasts of the placenta and that SATB1 expression is lessened in the PE placenta and in villous explants cultured under H/R conditions. The results indicate that SATB1 is involved in the pathogenesis of PE; however, the underlying biomolecular mechanism requires further study. Sumoylation of SATB1 at lysine-744 with SUMO mediates caspase hydrolysis and reduces the SATB1 level and protein stability. To determine whether SUMO-dependent degradation of SATB1 existed in the pathogenesis of PE, lentivirus containing a wild-type or sumoylation-deficient sequence of SATB1 was employed to increase the levels of SATB1 in HTR8/SVneo cells. We found that the cells transfected with R showed higher intracellular levels of SATB1, resulting in increased activation of Wnt signaling and decreased cell functional impairment due to the H/R intervention. These data suggest that oxidative stress might induce SATB1 degradation via a sumoylation-dependent
mechanism. However, more studies that focus on the association between sumoylation and SATB1 during PE development should be performed.

Gelatinases and their specific inhibitors participate trophoblast invasion[40]. As shown in a previous study[41], the activation of MMPs is suppressed in PE. To explore the regulatory mechanism, diverse signaling pathways involved in cell invasion have been investigated. The Wnt/β-catenin signaling pathway is an upstream pathway of MMPs[42, 43]. In our previous studies, we have demonstrated that reduced β-catenin is associated with impaired trophoblast function[22]. In this study, we found decreases in the levels of β-catenin in the PE placental tissues and H/R-treated cells. Based on these data, we suggest that SATB1 and Wnt signaling are relevant to the pathogenesis of PE. Here, we conducted a series of experiments to explore the potential roles of these players. The results showed that SATB1 up-regulation increased the level of nuclear β-catenin and enhanced the migration and invasion of trophoblast cells in culture and of trophoblasts in human first-trimester villous tissues under H/R conditions. Intracellular ROS and cell apoptosis were measured, and the results showed that higher SATB1 levels increased β-catenin levels and reduced ROS accumulation and cell apoptosis. The detection of matrix
metalloproteinases further verified the changes in trophoblast migration and invasion. In the end, we measured the outgrowth of trophoblasts in villous explants in vitro, obtained favorable results and reconfirmed our previous speculation. We additionally employed a specific activator (LiCl) and inhibitor (DKK1) of Wnt signaling to evaluate the cascade connection between SATB1 and β-catenin. The activation of Wnt signaling by LiCl improved trophoblast function but failed to affect SATB1 expression. Moreover, the inhibition of Wnt signaling by DKK1 attenuated the healing effects of up-regulated SATB1 on the impaired cell functions. These findings confirm that β-catenin is downstream of SATB1.

In this paper, we have shown a potential regulatory pathway of trophoblast migration and invasion. Taken together, these results indicate that SATB1 might act via β-catenin in response to oxidative stress-induced functional damage in trophoblasts; however, more elaborate studies in vivo are needed to more extensively examine the roles of SATB1 and β-catenin in the development of PE. Additionally, the expression changes in β-catenin in our study implied the suppression and activation of the Wnt/β-catenin signaling pathway in trophoblasts. However, whether SATB1 regulates cell function via the Wnt signaling pathway in the pathogenesis of PE should be explored further.
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FIG.1. Aberrant expression of SATB1 and β-catenin in preeclamptic placentae and H/R-treated villous. (A) SATB1 expression in the placenta of normal pregnancy (NP) or PE pregnancy (PE) and in first-trimester villous cultured under normoxic (N) or H/R


conditions. (B) β-catenin expression in placentae and villous. (400x magnification) (C) Quantification of immunostained nuclear β-catenin in a placenta and villous (D). (Five fields on each slide were observed. MQS=percentage of positive nucleus × meanintensity. P*<0.05 compared with the NP group).

FIG.2. Aberrant protein levels in pre-eclamptic placentae and H/R-treated villous explants. (A) Western blot of SATB1, nuclear β-catenin, MMP2/9 and TIMP1/2 in the placental tissues from the NP and PE groups and in the villous explants cultured under N and H/R conditions. B, C, D, Quantifications of the relative protein levels in the placenta and villous explants. (P*<0.01 compared with the NP group, P#<0.05 compared with the N group).

FIG.3. Protein expression in HTR8/SVneo cells under H/R conditions after SATB1 up-regulation, LiCl treatment or DKK1 treatment. (A) Immunofluorescence microscopy of SATB1 in HTR8/SVneo cells. SATB1 is indicated in green, and nuclei are indicated in red. (B) Immunofluorescence of β-catenin in HTR8/SVneo cells. β-catenin is indicated in green. (Scale bar, 75 µm).

FIG.4. The protein levels in HTR8/SVneo cells under H/R conditions after SATB1 up-regulation, LiCl treatment or DKK1 treatment. (A) Western blot of proteins in HTR8/SVneo cells. (B, C, D) Quantifications of the relative protein expression levels in the cells from each group. (P*<0.05 compared with the control group, P<0.05 compared with the H/R group, P**<0.05 compared with the K+H/R group).

FIG.5. The migratory and invasive ability of HTR8/SVneo cells under H/R conditions after SATB1 up-regulation, LiCl treatment or DKK1 treatment. (A) Images of the Matrigel invasion by HTR8/SVneo cells. (B) Representative statistical bar graphs of migrated cells. (P*<0.05 compared with the control group, P<0.05 compared with the H/R group, P**<0.05 compared with the K+H/R group). (Scale bar, 200 µm).

FIG.6. Intracellular reactive oxygen species (ROS) formation and HTR8/SVneo cell apoptosis for each group. (A) The bar graph shows the quantification of intracellular ROS formation in trophoblast cells. (B) The apoptotic rate of the cells in each group. (P<0.05 compared with the control group, P<0.05 compared with the H/R group, P**<0.05 compared with the K+H/R group).

FIG.7. The outgrowth and migration of trophoblasts in villous explants under H/R conditions after SATB1 up-regulation, LiCl treatment or DKK1 treatment. (A) Pictures of the outgrowth distance of trophoblasts in villous explants (200x magnification). (B) The statistical bar graph shows the relative distance of trophoblast outgrowth. (P<0.05 compared with the control group, P<0.05 compared with the H/R group, P**<0.05 compared with the K+H/R group).
<table>
<thead>
<tr>
<th>Treatments</th>
<th>Experimental groups</th>
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<tr>
<td></td>
<td>N</td>
<td>H/R</td>
<td>LiCl+</td>
<td>K+H/R</td>
<td>R+H/R</td>
<td>K+D+</td>
</tr>
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<td>-</td>
<td>-</td>
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<tr>
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<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
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<td>+</td>
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<td>+</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>+</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>Transfected with DKK1</td>
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<td>-</td>
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Table 2. Clinical information of the normal term pregnancy (NP) and preeclampsia (PE) groups.

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<tr>
<td>Number</td>
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</tr>
<tr>
<td>Age (years)</td>
<td>27.68±5.7</td>
<td>28.90±4.32</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.95±1.48</td>
<td>26.59±1.98</td>
</tr>
<tr>
<td>Nulliparity (%)</td>
<td>61.23%</td>
<td>49.91%</td>
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<tr>
<td>Smoking history</td>
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<td>None</td>
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<tr>
<td>Gestational age (weeks)</td>
<td>39.27±1.19</td>
<td>34.13±1.75*</td>
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<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>108.6±6.67</td>
<td>154.3±9.08*</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>88.5±5.89</td>
<td>108.45±6.15*</td>
</tr>
<tr>
<td>Neonatal birth weight (g)</td>
<td>2893.47±574.21</td>
<td>2426.97±441.65*</td>
</tr>
</tbody>
</table>

*P<0.05
Figure 5

A

B

Percentage of migrated cells (% control)

N  H/R  LiCl+H/R  K+H/R  R+H/R  K+D+H/R  K+LiCl+H/R