HSPB8 binding to c-Myc alleviates hypoxia/reoxygenation-induced trophoblast cell dysfunction

LING CHEN, MEITING WU and YU ZHOU

Department of Gynecology and Obstetrics, Fujian Medical University Union Hospital, Fuzhou, Fujian 350001, P.R. China

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Abstract. Preeclampsia (PE) is a pregnancy-specific syndrome with complex pathogenesis. The present study aimed to explore the role of heat shock protein B8 (HSPB8) and c-Myc in trophoblast cell dysfunction using a hypoxia/reoxygenation (H/R)-treated HTR8/SVneo cell model. HSPB8 expression in tissues of patients with PE was analyzed using the Gene Expression Omnibus database. Following detection of HSPB8 expression in H/R-stimulated HTR8/SVneo cells, HSPB8 was overexpressed by transfection of the gene with a HSPB8-specific plasmid. Cell Counting Kit-8, wound healing and Transwell assays were used to evaluate the proliferation, migration and invasion of HTR8/SVneo cells exposed to H/R conditions. Reactive oxygen species (ROS) were determined by 2,7-dichlorodihydrofluorescein diacetate staining. 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbo-cyanine iodide (JC-1) staining was applied to assess mitochondrial membrane potential. Malondialdehyde (MDA) and superoxide dismutase (SOD) levels were detected using the corresponding commercial kits. In addition, the induction of apoptosis was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Moreover, the Biogrid database predicted that HSPB8 was bound to c-Myc, and a co-immunoprecipitation (Co-IP) assay was used to verify this interaction. Subsequently, c-Myc expression was silenced to conduct rescue experiments in HTR8/SVneo cells exposed to H/R conditions and upregulated HSPB8 expression. Notably, reduced HSPB8 expression was noted in PE tissues and H/R-stimulated HTR8/SVneo cells. HSPB8 enforced expression promoted the proliferation, migration and invasion of HTR8/SVneo cells. Moreover, H/R caused an increase in ROS and MDA levels as well as in TUNEL staining and a decrease in aggregated JC-1 fluorescence and SOD activity levels, which were restored following HSPB8 overexpression. Co-IP confirmed the interaction between HSPB8 and c-Myc. Moreover, knockdown of c-Myc expression compromised the effects of HSPB8 upregulation on trophoblast cell dysfunction following induction of H/R. Collectively, the data indicated that HSPB8 could improve mitochondrial oxidative stress by binding to c-Myc to alleviate trophoblast cell dysfunction. The findings may provide new insights into the pathogenesis of PE and highlight the role of HSPB8/c-Myc in the prevention and treatment of PE in the future.

Correspondence to: Dr Ling Chen, Department of Gynecology and Obstetrics, Fujian Medical University Union Hospital, 29 Xinquan Road, Fuzhou, Fujian 350001, P.R. China
E-mail: chenlin830526@163.com

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Introduction

Preeclampsia (PE) is a complex pregnancy-associated disorder that occurs after 20 weeks of gestation (1). This condition is characterized by the presence of new-onset hypertension, proteinuria and edema (2). As a complex medical disorder, the incidence of PE ranges from 2-8% in pregnancies worldwide, eventually leading to maternal and perinatal deaths (3). Significant scientific efforts have been made to elucidate this complex multifactorial disease; however, the pathogenesis of PE is still not fully understood. Therefore, an in-depth understanding of the molecular mechanism involved in the occurrence of PE is of crucial importance to the development of novel therapeutic strategies.

The invasion of fetal-derived extravillous trophoblasts (EVTs) into the myometrium of the uterine wall is considered to be a key step in placental development (4). Invading trophoblast perform multiple essential functions including the anchoring of the placenta to the uterus, regulating maternofetal immune tolerance and conversion of the maternal spiral arterioles, ensuring adequate blood supply to the intervillous space (5,6). The inadequate invasion will cause the anchoring villi to break down, forming a globular-shaped placenta, resulting in damage to the villi structure and impaired placental function (7,8). Inadequate invasion has been associated with a number of obstetric syndromes, including PE (9). Previous studies have shown that trophoblast cell proliferation, invasion and migration ensure the normal development of placental metabolites, nutrient supply and excretion, and are key processes that successfully establish the maternal-fetal circulation and allow the continuation of pregnancy (10,11). During PE in pregnancy, alterations in the physiological transformation of the spiral arteries lead to placental hypoperfusion and hypoxia, eventually leading to placental insufficiency (12). An important feature of PE is

oxidative stress in the trophoblast, which may be the result of placental ischemia and hypoxia due to poor spiral artery invasion (13,14). In this process, the increased levels of ROS induces apoptosis through the mitochondrial pathway and affects the functions of cell proliferation, invasion and migration (15-17).

Heat shock protein B8 (HSPB8), a ubiquitous member of small heat shock proteins family, has been demonstrated to be involved in the regulation of multiple cellular functions, such as growth, aging, oxidative stress, migration and apoptosis (18-20). As a stress-related protein, HSPB8 accelerates the migration and invasion of hepatocellular carcinoma, breast cancer and cholangiocarcinoma (21-23). A previous study performed by Li *et al* suggests that HSPB8 overexpression protects against brain ischemia/reperfusion (I/R) injury by improving oxidative stress and apoptosis (24). It is worth noting that HSPB8 plays a significant role in the progression of human BeWo cytotrophoblasts to syncytiotrophoblasts (25). However, to the best of our knowledge, the role of HSPB8 in trophoblast cell dysfunction during PE has not been reported. In addition, the Biogrid database predicts that c-Myc, a well-known oncogene that has been shown to regulate tumor cell invasion and migration, can interact with HSPB8 (26). A previous study that examined PE revealed that upregulation of c-Myc expression enhances trophoblast cell migration and invasion (27). Therefore, the present study aimed to elucidate whether HSPB8 could affect trophoblast cell dysfunction by regulating c-Myc expression.

In the present study, HTR-8/SVneo, a human first-trimester trophoblast cell line that has a similar function to EVTs, was stimulated by hypoxia/reoxygenation (H/R), to simulate a PE model *in vitro*. Additional experiments were conducted to explore the association between HSPB8 and c-Myc expression and their regulatory effects on trophoblast cell dysfunction.

Materials and methods

Bioinformatical analysis. The expression levels of HSPB8 in placental tissues of patients with preterm PE and normal preterm pregnant women were analyzed using Gene Expression Omnibus (GEO) datasets (GSE102897 and GSE147776) downloaded from the national center of biotechnology information website (https://www.ncbi.nlm.nih.gov/) (28-30). GSE102897 dataset included the placenta tissues from normal human (n=3) and patients with severe preeclampsia (n=3). GSE147776 dataset included placenta tissues from normal human (n=8) and patients with severe preeclampsia (n=7). The analysis platform for GSE102897 and GSE147776 datasets were GPL22120 and GPL20844, respectively. The Biogrid database (https://thebiogrid.org) was used to predict the proteins that could interact with HSPB.

Cell culture and H/R induction. The human trophoblast cell line HTR-8/SVneo was acquired from American Type Culture Collection. The cells were routinely maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (MACGENE Biotechnology) at 37˚C in the presence of 5% CO_2. For the H/R treatment, when HTR-8/SVneo cells reached 50-60% confluence, they were exposed to 2% O_2, 5% CO_2 and 93% N_2 to achieve hypoxic conditions for 8 h. Subsequently, the cells were transferred to an atmosphere containing 20% O_2 and incubated for 16 h. A PE cell model was established by two cycles of H/R (31). The cells in the control group were cultured under standard culture conditions with 5% CO_2 for 48 h.

Cell transfection. To overexpress HSPB8, HTR-8/SVneo cells were transfected with a HSPB8 plasmid (Ov-HSPB8) or the empty vector plasmid (Ov-NC). c-Myc was silenced by transfection of the cells with short interference (si) RNA sequences specific to c-Myc (siRNA-c-Myc-1, 5'-GAGCTAAAACGG AGCTTTTTTGC-3'; siRNA-c-Myc-2, 5'-GAGGAAGAA ATCGATGTTGTTTC-3'). The cells transfected with a scrambled sequence [siRNA-negative control (NC)] (5'-AAGACA UUGUGUGUCCGCCTT-3') were considered to be the negative control. The aforementioned plasmids were all provided by Shanghai GenePharma Co., Ltd. The siRNA sequences were provided by OBiO Technology (Shanghai) Corp., Ltd. Subsequently, 4 μg Ov-HSPB8, Ov-NC, 100 nM siRNA-c-Myc-1/2 and siRNA-NC were transfected into HTR-8/SVneo cells with the application of Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. At 48 h post transfection, the cells were collected for subsequent experiments.

Cell Counting Kit-8 (CCK-8) assay. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) obtained from Beyotime Institute of Biotechnology. Following transfection and H/R exposure, HTR-8/SVneo cells were seeded into 96-well plates ($1x10^4$/well). Following incubation at 37˚C for 24, 48 and 72 h, a CCK-8 solution was added to each well for an additional 2 h incubation at 37˚C. The optical density was recorded at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Wound healing assay. The wound healing assay was conducted to evaluate the migratory ability of HTR-8/SVneo cells following the indicated treatment. Briefly, HTR-8/SVneo cells were seeded into six-well plates at a density of $1x10^5$ cells/well. When HTR-8/SVneo cells reached 90% confluence, a straight wound was created on the bottom of the plates using a sterile 100 μl pipette tip. Subsequently, the cells were incubated in serum-free RPMI-1640 medium for 24 h. The gap area was imaged using an inverted light microscope (Olympus Corporation) and the width of the wound was analyzed by ImageJ software (version 1.48v; National Institutes of Health).

Transwell assay. Invasion of HTR-8/SVneo cells was assessed with the application of Transwell assay. A total of $5x10^4$ HTR-8/SVneo cells were suspended in 200 μl serum-free RPMI-1640 medium and were plated into the upper compartments of Transwell chambers (8 μm pore size; Corning, Inc.) coated with Matrigel (BD Biosciences) at 37˚C for 1 h. The lower chambers were subsequently filled with the normal RPMI-1640 medium containing 10% FBS. Following culture of the cells for 48 h at 37˚C, they were fixed with 4% paraformaldehyde for 20 min at 37˚C and stained with 0.1% crystal violet for 10 min at room temperature. The images were obtained with an inverted light microscope (Olympus Corporation) and the invading cells were counted using ImageJ software.

Measurement of cellular ROS. The content of ROS in HTR-8/SVneo cells was determined utilizing a ROS Assay kit (Beyotime Institute of Biotechnology) and the fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) according to the instructions provided by the manufacturer. DCFH-DA (10 μmol/l) was applied for the staining of the cells for 20 min at 37˚C in the dark. The content of cellular ROS was evaluated by detecting the fluorescence intensity of the cells in each group using a confocal microscope (Olympus Corporation) with 488 nm excitation and 525 nm emission filters.

Mitochondrial membrane potential assay. The mitochondrial membrane potential was quantified by 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbo-cyanine iodide (JC-1) staining (Thermo Fisher Scientific, Inc.). HTR-8/SVneo cells were subjected to 10 mg/ml JC-1 staining for 10 min at 37˚C away from light. The normal mitochondrial potential exhibited red fluorescence (JC-1 aggregates) and the damaged mitochondrial potential displayed green fluorescence (JC-1 monomers). The images (five fields of view) were obtained using a confocal microscope (Olympus Corporation).

Detection of oxidative stress indicators. After H/R exposure and/or transfection, HTR-8/SVneo cells were washed three times with cold PBS, and then cell lysis buffer (Beyotime Institute of Biotechnology) was added for cell lysis. Following centrifugation at 1,000 x g for 10 min at 4˚C, the supernatant was collected for detection. The content of malondialdehyde (MDA; cat. no. A003-4-1) and the activity of superoxide dismutase (SOD; cat. no. A001-3-2) in the supernatant were detected using the corresponding commercial kits (Nanjing Jiancheng Bioengineering Institute) according to the instructions provided by the manufacturer. The optical density was measured using a microplate reader (Bio-Rad Laboratories, Inc.).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. The induction of apoptosis of the transfected cells was assessed by the TUNEL assay (Beyotime Institute of Biotechnology). HTR-8/SVneo cells were cultured on glass coverslips overnight. Following fixation with 4% paraformaldehyde at room temperature for 1 h, the cells were blocked with 3% H_2O_2 (dissolved) for 20 min at room temperature. Following permeation with 0.1% Triton X-100 at 4˚C for 2 min, the slides were incubated with 50 μl TUNEL reaction mixture for 1 h at 37˚C, mounted with Vectashield® mounting medium. Following addition of DAPI solution at 37˚C for 10 min away from light, the cell samples were analyzed using an inverted fluorescence microscope (Olympus Corporation) in five random fields.

Co-immunoprecipitation (Co-IP) assay. HTR-8/SVneo cells were lysed on ice for 30 min in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology) containing protease inhibitors. The lysed protein samples (500 μg cell lysate) were incubated with antibodies against 2 μg anti-IgG (cat. no. ab172730; Abcam), anti-HSPB8 (cat no. 15287-1-AP; ProteinTech Group, Inc.) or anti-c-Myc (cat no. 10828-1-AP; ProteinTech Group, Inc.) antibodies and incubated overnight at 4˚C. Then, 50 μg protein A magnetic beads (cat. no. #sc-2003; Santa Cruz Biotechnology, Inc.) were added for capturing the complexes of HSPB8 and c-Myc. After the IP reaction, 50 μg protein G/A agarose beads were centrifuged at 1,000 x g for 3 min at 4˚C to the bottom of the tube. The supernatant was then carefully absorbed, and the agarose beads were washed three times with PBS. A total of 15 μl 2X SDS sample buffer was finally added for boiling at 100˚C for 5 min. Western blotting was used to analyze the expression levels of the target proteins.

Reverse transcription-quantitative PCR (RT-qPCR). TRIzol® reagent (Thermo Fisher Scientific, Inc.) was used to extract the total RNA from HTR-8/SVneo cells. The RNA was reverse transcribed to complementary DNA (cDNA) using the PrimeScript RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. Quantitative PCR for the associated genes was conducted by RT-qPCR using SYBR Green master mix (Vazyme Biotech Co., Ltd.) on an ABI Prism 7500 Sequence Detector (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: Initial denaturation for 2 min at 94˚C; followed by 35 cycles for 30 sec at 94˚C and 45 sec at 55˚C. β-actin was selected as the endogenous control. The calculation of the expression values was performed using the $2^{-\Delta\Delta Cq}$ method (32). Specific primers were shown below: HSPB8 forward, 5'-TTC CCAGACGACTTGACAGC-3', and reverse, 5'-GCCAATTGC GCTATCCTGTG-3'; c-Myc forward, 5'-GCAATGCGTTGC TGGGTTAT-3', and reverse, 5'-TCCCTCCGTTCTTTTTCC CG-3'; β-actin forward, 5'-GCCTCGCCTTTGCCGAT-3', and reverse, 5'-AGGTAGTCAGTCAGGTCCCG-3'.

Western blotting. Total proteins were extracted from HTR-8/SVneo cells using a RIPA lysis buffer (Beyotime Institute of Biotechnology). The concentration levels of the proteins were determined with the application of a bicinchoninic acid assay (BCA) kit (Beyotime Institute of Biotechnology). Equal amounts of proteins (40 μg proteins per lane) were separated using 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature, followed by probing with primary antibodies at 4˚C overnight. Following addition of the HRP-conjugated goat anti-rabbit secondary antibody (1:2,000; cat. no. 7074S; Cell Signaling Technology, Inc.) for 1 h at room temperature, the bands were visualized using an ECL Plus Chemiluminescence Reagent kit (Pierce; Thermo Fisher Scientific, Inc.). The gray values of the protein bands were determined by ImageJ software (version 1.48v). The intensity of β-actin was used as a loading control. Anti-HSPB8 (1:2,000; cat. no. ab151552) and anti-c-Myc (1:2,000; cat. no. ab185656) antibodies were acquired from Abcam. Anti-Bcl-2 (1:1,000; cat. no. 4223T), anti-Bax (1:1,000; cat. no. 41162S), anti-cleaved caspase3 (1:1,000; cat. no. 9664T), anti-Caspase3 (1:1,000; cat. no. 9662S) and anti-β-actin (1:1,000; cat. no. 4970T) antibodies were provided by Cell Signaling Technology, Inc.

Statistical analysis. Data from three independent replicates were presented as the mean ± standard deviation and analyzed using GraphPad 8.0 statistical software (GraphPad Software,

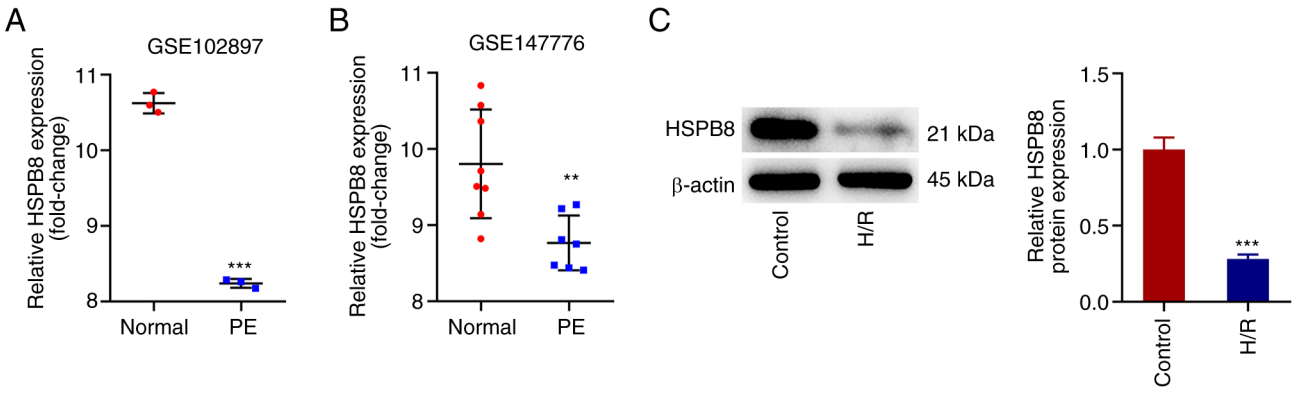

Figure 1. HSPB8 expression is downregulated in placental tissues of patients with preterm PE and HTR-8/SVneo cells exposed to H/R conditions. The expression levels of HSPB8 in placental tissues of patients with preterm PE and in normal preterm pregnant women were analyzed by GEO datasets. (A) GSE102897 and (B) GSE147776. **P<0.0 and ***P<0.001 vs. normal group. (C) HSPB8 expression in HTR-8/SVneo cells exposed to H/R conditions was detected using western blotting. ***P<0.001 vs. control group. HSPB8, heat shock protein B8; PE, preeclampsia; H/R, hypoxia/reoxygenation; GEO, Gene Expression Omnibus.

Inc.; Dotmatics). The differences between the two groups were calculated by the unpaired Student's t-test. One-way analysis of variance (ANOVA) followed by Tukey's test was appointed for data comparison among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

HSPB8 expression is downregulated in placental tissues of patients with preterm PE and H/R-induced HTR-8/SVneo cells. In order to identify the role of HSPB8 in PE, the GEO datasets (GSE102897 and GSE147776) were used for analysis and HSPB8 expression was found to be significantly reduced in placental tissues of preterm patients with PE compared with that noted in normal preterm pregnant women (Fig. 1A and B). Following exposure of HTR-8/SVneo cells to H/R conditions to mimic the PE pathology, HSPB8 expression was also significantly decreased compared with that of the control group (Fig. 1C). These data revealed the abnormal low expression of HSPB8 in placental tissues of patients with preterm PE and H/R-induced HTR-8/SVneo cells.

HSPB8 overexpression promotes the proliferation, migration and invasion of H/R-induced HTR-8/SVneo cells. Subsequently, HSPB8 was overexpressed by transfection with HSPB8 plasmid and the transfected efficiency was assessed by RT-qPCR and western blotting. Significantly elevated HSPB8 expression was observed in the Ov-HSPB8 group compared with that noted in the empty vector control group (Fig. 2A and B). The results of the CCK-8 assay indicated that H/R stimulation decreased the viability of HTR-8/SVneo cells compared with control cells, which was significantly increased following transfection of the cells with the HSPB8 plasmid compared with the H/R + Ov-NC group (Fig. 2C). Moreover, overexpression of HSPB8 significantly restored H/R-induced limitations on the migration and invasion of HTR-8/SVneo cells compared with those noted in the H/R + Ov-NC group (Fig. 2D and E). The aforementioned results demonstrated that under H/R conditions, upregulation of HSPB8 expression accelerated the proliferation, migration and invasion of HTR-8/SVneo cells.

HSPB8 overexpression alleviates the induction of oxidative stress and apoptosis of HTR-8/SVneo cells exposed to H/R conditions. DCFH-DA staining indicated notably enhanced fluorescence intensity in HTR-8/SVneo cells in response to H/R stimulation, suggesting the elevated ROS levels in the H/R group (Fig. 3A). By contrast, HSPB8 overexpression markedly reduced the fluorescence intensity induced by H/R conditions compared with the H/R + Ov-NC group. In addition, in comparison with the control group, H/R stimulation increased JC-1 aggregate formation and decreased JC-1 monomer levels in HTR-8/SVneo cells, indicating the damaged mitochondrial potential in H/R-induced HTR-8/SVneo cells (Fig. 3B). Subsequent overexpression of HSPB8 caused elevated levels of JC-1 aggregates and reduced the levels of JC-1 monomers (Fig. 3B). Concomitantly, H/R caused a significant increase in the MDA content and a decrease in SOD activity, which were significantly reversed following transfection of the cells with the HSPB8 plasmid (Fig. 3C and D). Moreover, in contrast with the control group, significantly enhanced TUNEL staining was noted in the H/R group in HTR-8/SVneo cells compared with the control group, as determined by downregulation of Bcl-2 expression and upregulation of Bax and cleaved caspase 3 expression (Fig. 4A and B). However, overexpression of HSPB8 attenuated the effect of H/R on TUNEL staining and the expression levels of the aforementioned apoptotic proteins compared with the H/R + Ov-NC group (Fig. 4A and B). These results implied that HSPB8 overexpression alleviated oxidative stress and induction of apoptosis of HTR-8/SVneo cells exposed to H/R conditions.

HSPB8 binds to c-Myc to promote proliferation, migration and invasion of H/R-induced HTR-8/SVneo cells. To explore the potential mechanism of HSPB8 in the regulation of H/R-induced HTR-8/SVneo cells, the Biogrid database was used to predict the proteins that could interact with HSPB8; c-Myc was found to interact with HSPB8 (Fig. 5A). It was also shown that c-Myc expression was significantly downregulated in HTR-8/SVneo cells under H/R conditions compared with the control cell, and that HSPB8 overexpression significantly upregulated c-Myc expression compared with the Ov-NC group (Fig. 5B). The subsequent Co-IP data demonstrated the

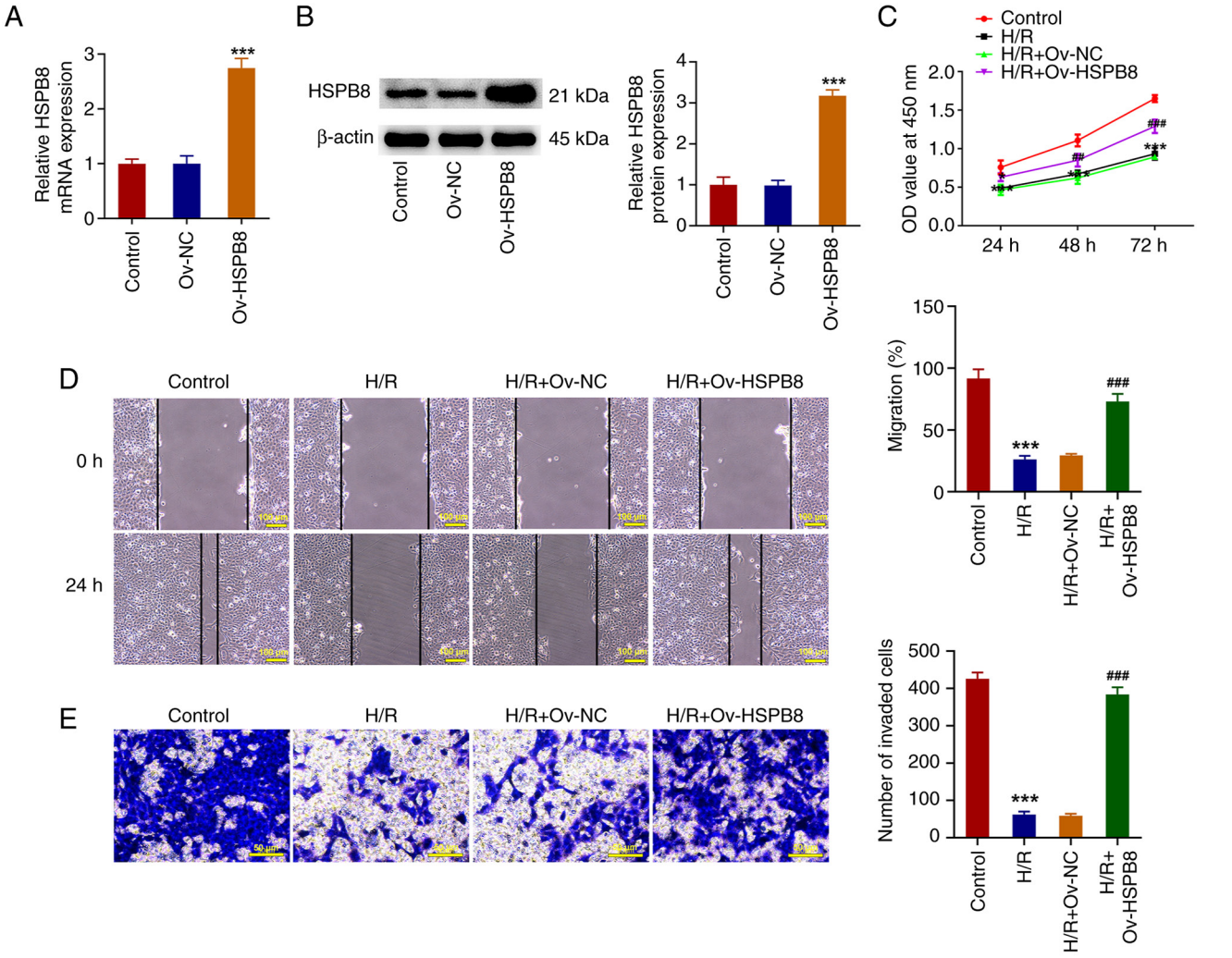

Figure 2. HSPB8 overexpression promotes the proliferation, migration and invasion of H/R-induced HTR-8/SVneo cells. Following transfection of the cells with a HSPB8 plasmid, HSPB8 expression was assessed by (A) RT-qPCR and (B) western blotting. ***P<0.001 vs. Ov-NC group. (C) Cell viability was detected using the Cell Counting Kit-8 assay. The migration and invasion of HTR-8/SVneo cells were evaluated using (D) wound healing (Scale bars, 100 μm) and (E) Transwell invasion assays (scale bars, 50 μm). *P<0.05, ***P<0.001 vs. the control group; ##P<0.01, ###P<0.001 vs. the H/R + Ov-NC group. HSPB8, heat shock protein B8; H/R, hypoxia/reoxygenation; RT-qPCR, reverse transcription-quantitative PCR; Ov, overexpression; NC, negative control.

binding between HSPB8 and c-Myc (Fig. 5C). Subsequently, c-Myc expression was silenced. HTR-8/SVneo cells transfected with siRNA-c-Myc-1/2 displayed significantly decreased c-Myc expression levels compared with that of the siRNA-NC group (Fig. 5D and E). Due to the lower c-Myc expression in the siRNA-c-Myc-1 group, HTR-8/SVneo cells transfected with siRNA-c-Myc-1 were selected to perform the following experiments. It was observed in Fig. 5F-H that knockdown of c-Myc expression partially attenuated the elevated effects of HSPB8 overexpression on the proliferation, migration and invasion of H/R-induced HTR-8/SVneo cells. Collectively, these findings confirmed that HSPB8 binds to c-Myc to regulate the proliferation, migration and invasion of HTR-8/SVneo cells exposed to H/R conditions.

HSPB8 can bind to c-Myc to inhibit the induction of oxidative stress and apoptosis of HTR-8/SVneo cells exposed to H/R conditions. In contrast to the H/R + Ov-HSPB8 + siRNA-NC group, HTR-8/SVneo cells in the H/R + Ov-HSPB8 + siRNA-c-Myc group exhibited the highest ROS fluorescence intensity, the lowest level of JC-1 aggregate formation and the highest level of JC-1 monomers (Fig. 6A and B). In addition, c-Myc-silencing elevated MDA levels and reduced SOD activity compared with that noted in the negative control group (Fig. 6C and D). Consistent with these observations it was found that the decreased TUNEL levels, the upregulated Bcl-2 expression levels and the downregulated Bax and cleaved caspase3 expression levels in the H/R + Ov-HSPB8 group compared with the H/R group were all reversed by knockdown of c-Myc expression in HTR-8/SVneo cells exposed to H/R conditions compared with the siRNA-NC group (Fig. 7A and B). These data provide evidence that HSPB8 binding to c-Myc inhibited the induction of oxidative stress and apoptosis of HTR-8/SVneo cells exposed to H/R.

Discussion

During the entire phase of pregnancy, the placenta is considered to be a crucial organ that assists the development of the mammalian fetus. The occurrence of PE may be related to

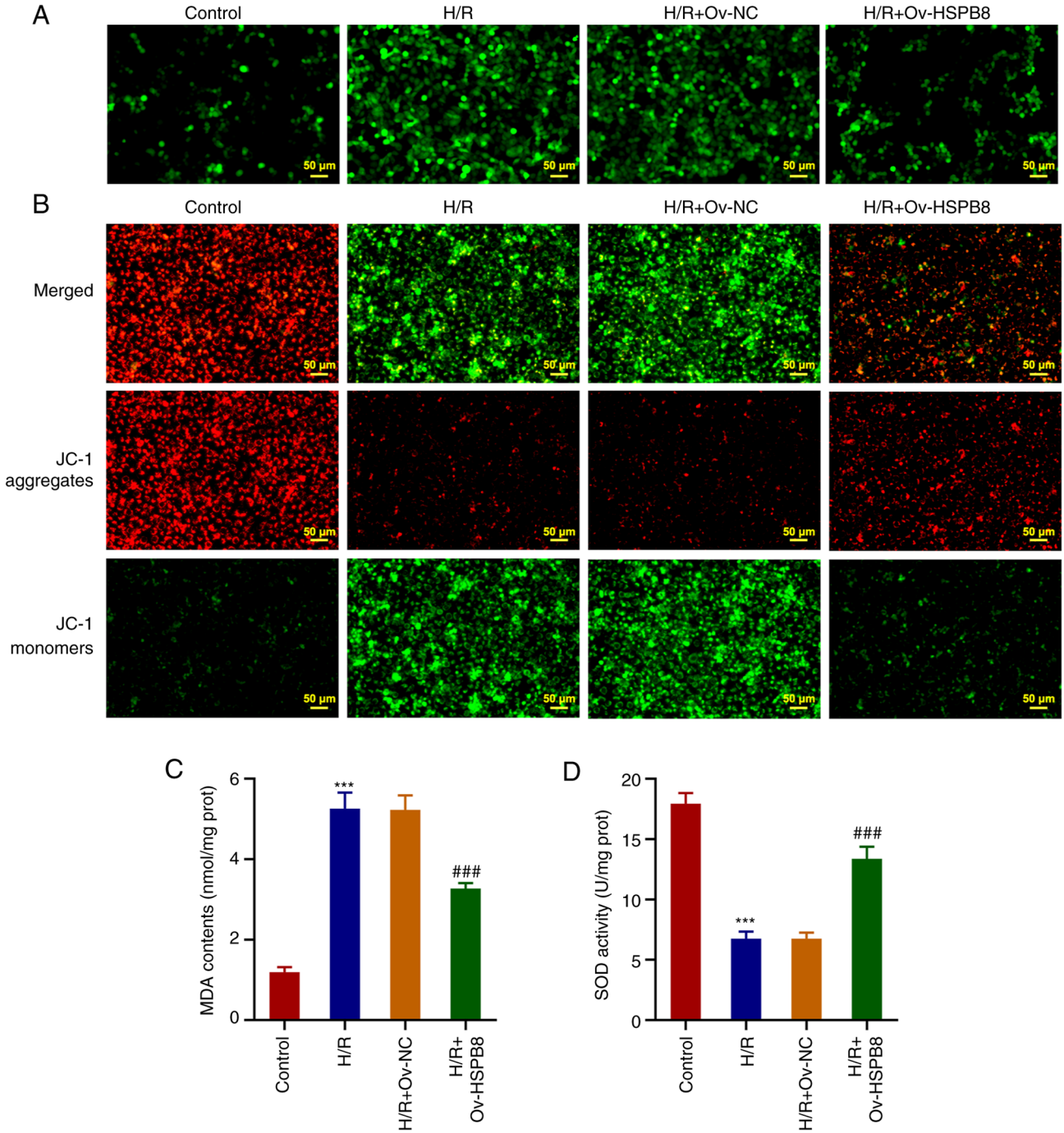

Figure 3. HSPB8 overexpression alleviates the induction of oxidative stress of HTR-8/SVneo cells exposed to H/R conditions. (A) Levels of ROS were evaluated using DCFH-DA staining. (B) Mitochondrial membrane potential was analyzed using JC-1 staining. Specific kits were employed for the measurement of (C) MDA and (D) SOD levels. Scale bars, 50 μm. ***P<0.001 vs. the control group; $^{###}$P<0.001 vs. the H/R + Ov-NC group. HSPB8, heat shock protein B8; H/R, hypoxia/reoxygenation; ROS, reactive oxygen species; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbo-cyanine; MDA, malondialdehyde; SOD, superoxide dismutase; Ov, overexpression; NC, negative control.

inadequate blood perfusion and ischemia caused by defective placental formation (33). PE is a hypertensive disorder of pregnancy and has been demonstrated to have various acute and long-term complications in pregnant women and neonates (34). It has been reported to be responsible for the increase in maternal and perinatal morbidity and mortality in recent years, seriously affecting maternal and child health (35). The molecular mechanism of PE occurrence and progression is important in searching for potential target molecules; it can also be used to design targeted therapy. HTR-8/SVneo is the human first-trimester trophoblast cell line and has a similar function to EVTs. As a result, HTR-8/SVneo cells subjected to H/R have been widely used to simulate PE *in vitro* and to investigate the pathogenesis of PE. The present study discovered that HSPB8 expression was significantly downregulated in placental tissues of patients with preterm PE and H/R-induced HTR-8/SVneo cells. HSPB8 alleviated oxidative stress by binding to c-Myc to improve trophoblast cell dysfunction.

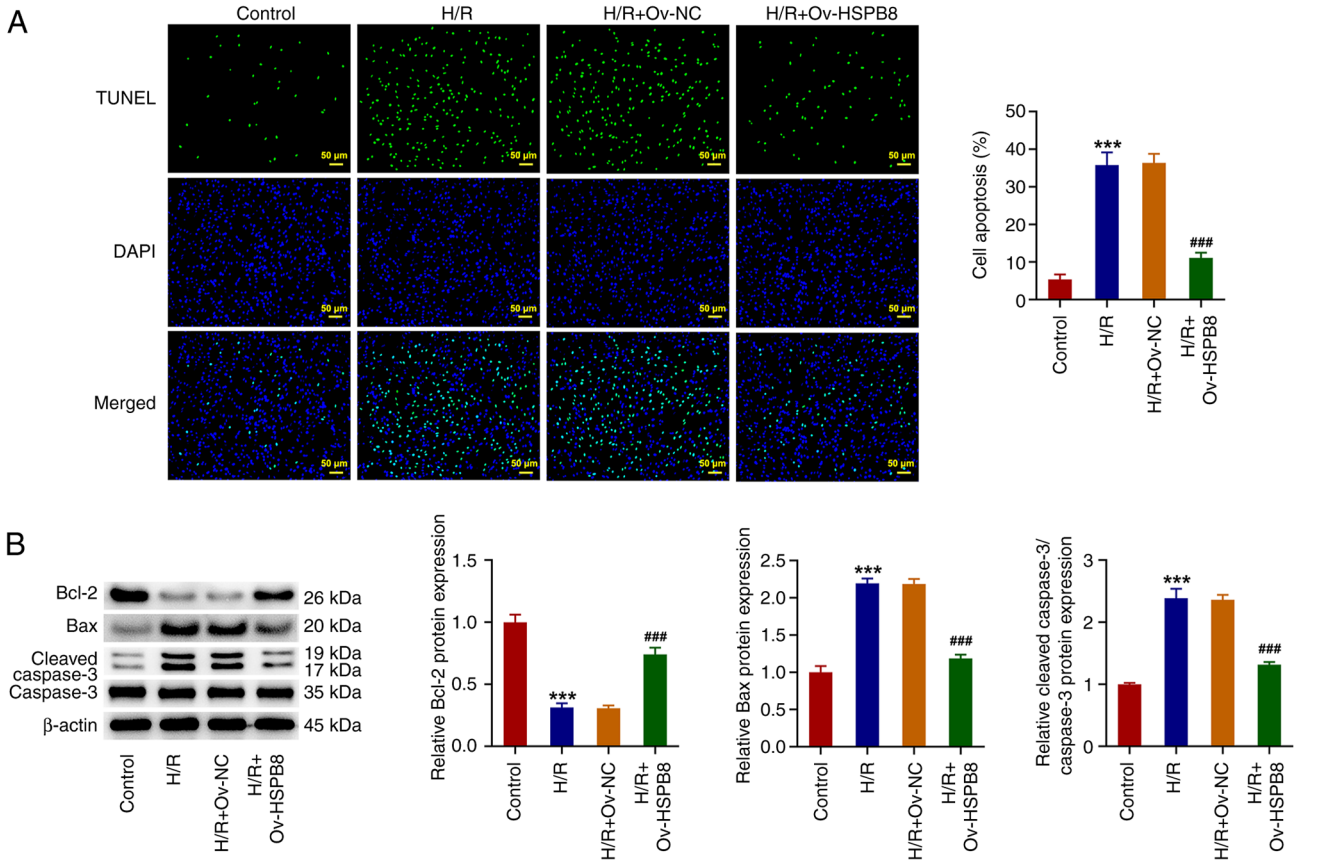

Figure 4. HSPB8 overexpression attenuates the induction of apoptosis of HTR-8/SVneo cells exposed to H/R conditions. (A) TUNEL was used to evaluate the induction of apoptosis of HTR-8/SVneo cells Scale bars, 50 μm. (B) Expression levels of apoptosis-related proteins were detected using western blotting. ***P<0.001 vs. the control group; $^{###}$P<0.001 vs. the H/R + Ov-NC group. HSPB8, heat shock protein B8; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; H/R, hypoxia/reoxygenation; Ov, overexpression; NC, negative control.

It is widely accepted that trophoblast proliferation, invasion and migration are essential for placental development; poor migration and invasion capacities of trophoblast cells are considered to be the main causes of PE (36,37). As the main component cells of the placenta, trophoblast cells exhibit invasive ability similar to tumor invasion, while the proliferation, invasion and migration of trophoblast cells ensure the normal development of the placenta (38). An increasing number of studies have validated that excessive oxidative stress results in an increase in ROS levels during the development of PE, which further leads to induction of apoptosis and loss of trophoblast invasion and migration (39,40). It has been reported that mitochondria are the main sites of ROS production and mitochondrial membrane integrity is sensitive to the cellular ROS levels (41). In the present study, H/R stimulation led to suppressed proliferation, migration, invasion and elevated ROS and JC-1 monomer levels of HTR-8/SVneo cells, suggesting the damage caused by trophoblast cell dysfunction and induction of oxidative stress, which were consistent with the results of previous studies (17,42,43). At present, HSPB8 has been confirmed to be highly expressed in various human tumors and HSPB8 overexpression has been demonstrated to facilitate the proliferation, migration and invasion of these cancer cells, such as prostate cancer, gastric cancer and lung adenocarcinoma (44-46). HSPB8 has also been shown to improve the H/R or I/R-induced damage of the myocardium and brain by repressing oxidative stress, apoptosis and restoring mitochondrial function (47,48). Particularly, a previous study has suggested that HSPB8 plays a significant role in the progression of human BeWo cytotrophoblasts to syncytiotrophoblasts (25). The present study observed significantly downregulated expression levels of HSPB8 in placental tissues of patients with preterm PE and HTR-8/SVneo cells exposed to H/R conditions. It was also demonstrated that HSPB8 gain-of-function facilitated the proliferation, migration and invasion of HTR-8/SVneo cells exposed to H/R and inhibited the induction of their oxidative stress and apoptosis.

To study the molecular mechanisms of HSPB8 in the regulation of trophoblast cell dysfunction under H/R conditions, the Biogrid database was used in the present study to predict the proteins that could interact with HSPB8; c-Myc was found to interact with HSPB8. Numerous studies have revealed that c-Myc is considered to be a target for the treatment of multiple human cancers (49,50). Upregulation of c-Myc expression accelerates the proliferation, migration and invasion of cervical, pancreatic and lung cancers (51-53). In addition, c-Myc has been reported to inhibit oxidative stress during ventricular remodeling of rats with myocardial infarction (54). c-Myc may restore appropriate levels of antioxidant proteins to ensure optimal mitochondrial function while maintaining ROS levels (55). Kfoury *et al* proposed that ROS levels are significantly increased in melanoma cells following knockdown of c-Myc expression, which

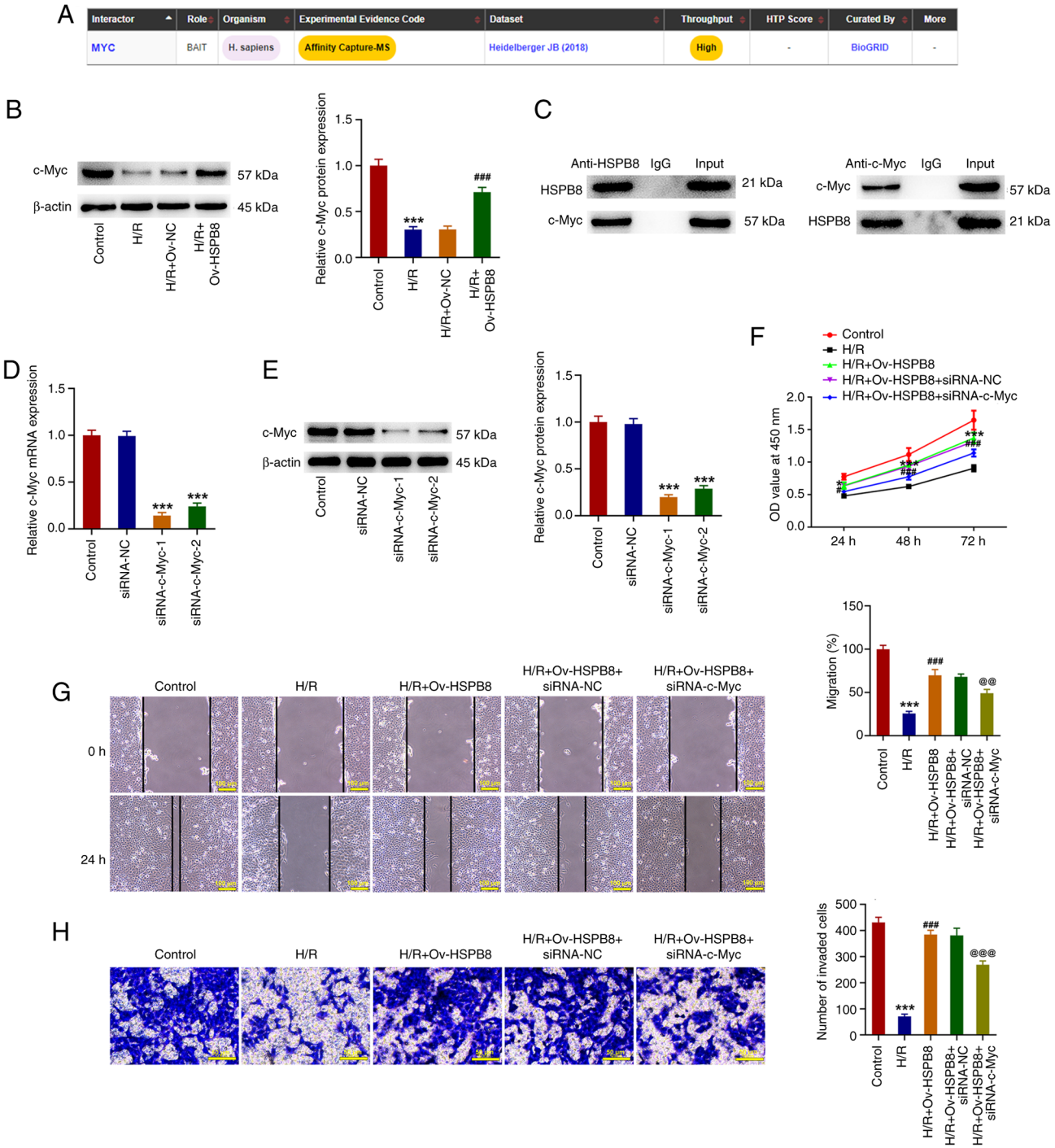

Figure 5. HSPB8 binding to c-Myc regulates the proliferation, migration and invasion of HTR-8/SVneo cells exposed to H/R conditions. (A) Biogrid database predicted that c-Myc interacted with HSPB8. (B) Western blotting was used to assess c-Myc expression following overexpression of HSPB8 in HTR-8/SVneo cells exposed to H/R conditions. ***P<0.001 vs. the control group; $^{\#\#\#}$P<0.001 vs. the H/R + Ov-NC group. (C) Interaction between HSPB8 and c-Myc was verified using the Co-IP assay. c-Myc expression following transfection of the cells with siRNA-c-Myc-1/2 in HTR-8/SVneo cells was assessed by (D) RT-qPCR and (E) western blotting. ***P<0.001 vs. siRNA-NC. (F) Cell viability was detected using the Cell Counting Kit-8 assay. The migration and invasion of HTR-8/SVneo cells was evaluated using (G) wound healing (scale bars, 100 μm) and (H) Transwell invasion assays (scale bars, 50 μm). *P<0.05, ***P<0.001 vs. the control group; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. the H/R group; $^{@@}$P<0.01 and $^{@@@}$P<0.001 vs. the H/R + Ov-HSPB8 + siRNA-NC group. HSPB8, heat shock protein B8; H/R, hypoxia/reoxygenation; Ov, overexpression; NC, negative control; si, short interference; Co-IP, co-immunoprecipitation; RT-qPCR, reverse transcription-quantitative PCR.

promotes the induction of apoptosis of melanoma cells (56). In a previous study examining PE, upregulation of c-Myc expression has been shown to enhance the proliferation, migration and invasion of trophoblast cells (27,57). The present study also demonstrated that H/R induced downregulation of c-Myc expression. Silencing of c-Myc expression reversed the effect of HSPB8 overexpression on trophoblast cell dysfunction and on the induction of oxidative stress.

The present study has a limitation. This study only discussed the role of HSPB8/c-Myc in the dysfunction

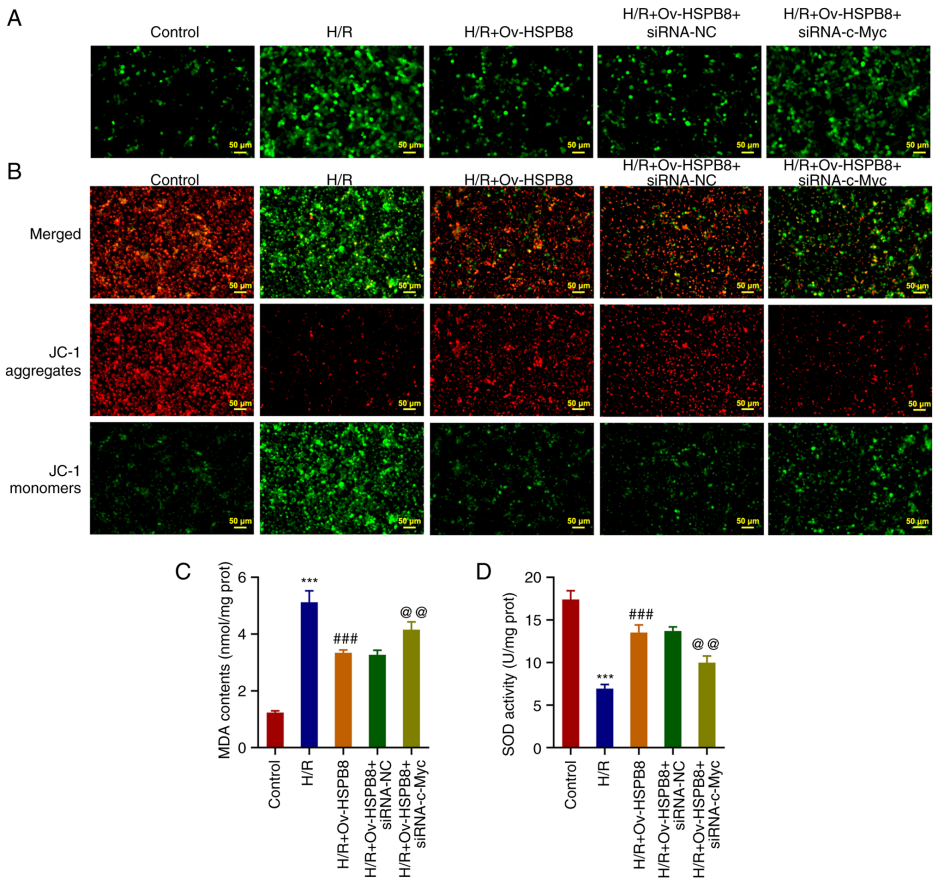

Figure 6. HSPB8 binding to c-Myc inhibits the oxidative stress of HTR-8/SVneo cells exposed to H/R conditions. (A) ROS levels were evaluated using DCFH-DA staining. (B) Mitochondrial membrane potential was assessed by JC-1 staining. Kits were employed for the measurement of (C) MDA and (D) SOD levels. Scale bars, 50 μm. ***P<0.001 vs. the control group; $^{###}$P<0.001 vs. the H/R group; $^{@@}$P<0.01 vs. the H/R + Ov-HSPB8 + siRNA-NC group. HSPB8, heat shock protein B8; H/R, hypoxia/reoxygenation; ROS, reactive oxygen species; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbo-cyanine iodide; MDA, malondialdehyde; SOD, superoxide dismutase; Ov, overexpression; NC, negative control; si, short interference.

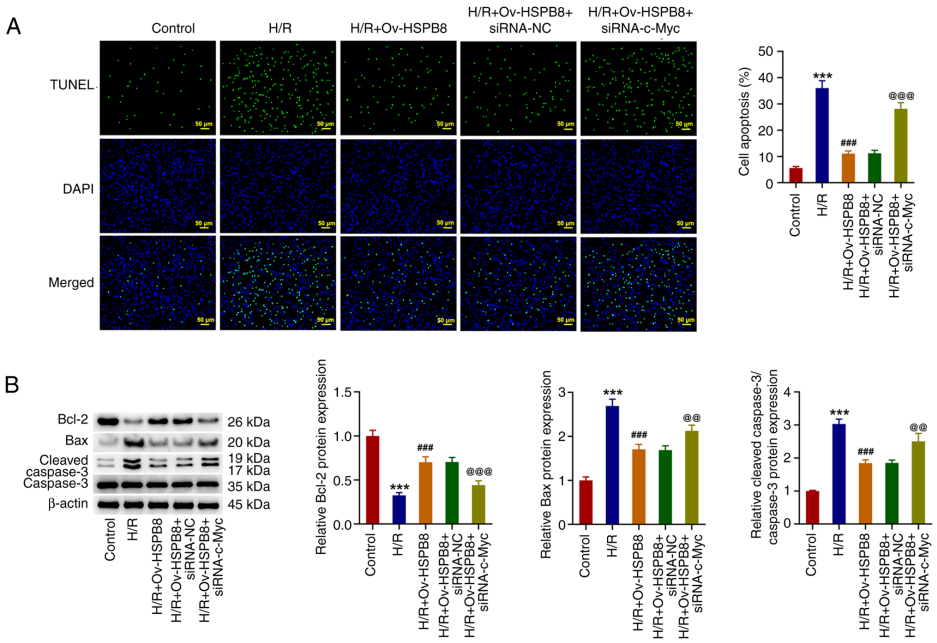

Figure 7. HSPB8 binding to c-Myc inhibits induction of apoptosis of HTR-8/SVneo cells exposed to H/R conditions. (A) TUNEL staining was used to evaluate the induction of apoptosis of HTR-8/SVneo cells. (B) Expression levels of apoptosis-related proteins were detected using western blotting. Scale bars, 50 μm. ***P<0.001 vs. the control group; $^{###}$P<0.001 vs. the H/R group; $^{@@}$P<0.01 and $^{@@@}$P<0.001 vs. the H/R + Ov-HSPB8 + siRNA-NC group. HSPB8, heat shock protein B8; H/R, hypoxia/reoxygenation; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; Ov, overexpression; NC, negative control; si, short interference.

of trophoblast cells exposed to H/R conditions using HTR-8/SVneo cells. Further *in vivo* animal studies will be performed in the future to obtain an in-depth understanding of their roles in PE pathogenesis.

In conclusion, to the best of our knowledge, the present study demonstrated for the first time that HSPB8 expression was significantly downregulated in PE tissues and H/R-stimulated trophoblast cells. Upregulation of HSPB8 expression improved the trophoblast cell dysfunction induced by H/R by suppressing oxidative stress by binding to c-Myc. The findings may highlight the role of HSPB8/c-Myc in the prevention and treatment of PE in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LC and MW were involved in the conceptualization and design of the study, acquisition and interpretation of data. LC and YZ interpreted the data and drafted the manuscript. MW revised the manuscript. All authors have read and approved the final manuscript. LC and MW confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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