# $p57^{KIP2}$-mediated inhibition of human trophoblast apoptosis and promotion of invasion *in vitro*

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**Abstract.** Placental hypoxia serves a role in the early stages of normal pregnancy and is involved in the pathophysiology of preeclampsia. Previously, it was suggested that $p57^{kinase\ inhibitory\ protein\ (KIP)2}$ regulates the cell cycle during embryogenesis and apoptosis. Recent evidence has indicated that $p57^{KIP2}$ is increased in preeclamptic placentas and absence of $p57^{KIP2}$ induces preeclampsia-type symptoms in rats. However, effects of $p57^{KIP2}$ on apoptosis under hypoxic conditions remain to be elucidated. In the present study, HTR-8/SVneo trophoblasts were cultured under hypoxic conditions (2% $O_2$). Knockdown using small interfering (si)RNA and overexpression of $p57^{KIP2}$ were utilized to explore the biological function of $p57^{KIP2}$ in apoptosis and cell function *in vitro*. Furthermore, expression of $p57^{KIP2}$ and apoptosis were evaluated by western blotting, flow cytometry and TUNEL assays, and the response of trophoblasts to hypoxia and the role of $p57^{KIP2}$ in trophoblast migration and invasion was assessed. The role of $p57^{KIP2}$ in the JNK signaling pathway in HTR-8/SVneo trophoblasts was further studies. *In vitro*, protein expression of $p57^{KIP2}$ was increased in HTR-8/SVneo cells exposed to 2% $O_2$. Exogenous $p57^{KIP2}$ overexpression significantly decreased the expression of pro-apoptosis proteins, including p53, Bax and cleaved caspase3, under hypoxic conditions for 24 h. In addition, knockdown of $p57^{KIP2}$ increased the response to apoptosis following hypoxia for 24 h. The present study revealed that overexpression of $p57^{KIP2}$ decreased the levels of phosphorylated-JNK. JNK inhibitor treatment combined with the overexpression of $p57^{KIP2}$ significantly decreased the levels of apoptosis and increased cell invasion and migration. Taken together, $p57^{KIP2}$ knockdown significantly increased apoptosis in HTR-8/SVneo cells exposed to 2% $O_2$, whereas overexpression of $p57^{KIP2}$ had opposite effects, mediated by the JNK/stress activated protein kinase (SAPK) signaling pathway. The results indicated that hypoxia-induced expression of $p57^{KIP2}$ promoted trophoblast migration and invasion by mediating the JNK/SAPK signaling pathway, which is crucial during placentation. These results may provide a novel molecular mechanism to understand the involvement of $p57^{KIP2}$ in the pathogenesis of preeclampsia.

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## Introduction

In the early stages of pregnancy (<10 weeks), trophoblasts exist in a hypoxic environment (1-2% $O_2$) and hypoxia serves an important role in normal trophoblast differentiation and development (1-4). In addition, hypoxia has been shown to directly induce trophoblast apoptosis and represents a significant pathological factor that contributes to the deficient placentation observed in the early onset of preeclampsia (PE) (5-8). PE is one of the most common pathological complications of pregnancy and is a leading cause of maternal and perinatal morbidity and mortality (9). As for the pathogenesis of PE, it is commonly believed that inadequate migration and the reduced invasive ability of placental extravillous trophoblasts (EVTs) may contribute to PE development (10). A recent study with PE placental explants reported that increased apoptosis and lack of proliferation stimulation were observed in trophoblasts (11). These studies suggest that placental hypoxia is critical for trophoblast physiology and pathology, and apoptosis may be involved in diseases of placental origin. Gaining greater understanding of these mechanisms and pathophysiology in the first trimester of pregnancy will be beneficial for PE treatment and therapy approaches.

p57 [also known as cyclin-dependent kinase inhibitor 1C or kinase inhibitory protein (KIP)-2; $p57^{KIP2}$] is ubiquitously expressed in human placental cells (12,13). $p57^{KIP2}$ is considered to be a master regulator of the cell cycle during embryogenesis (14-17). Pregnant mice with $p57^{KIP2}$ deficiency presented with symptoms similar to PE (10). Several studies have shown the proapoptotic role of $p57^{KIP2}$ in the pathogenesis of different cancer cell lines (18-21). An antiapoptotic activity of $p57^{KIP2}$

has also been observed during embryogenesis (19,22-24). A previous study reported that p57$^{KIP2}$ was significantly increased in all cell types of preeclamptic human placentas with the exception of syncytiotrophoblasts (12). A decrease in p57$^{KIP2}$ expression was implicated with abnormal trophoblast proliferation (25). However, the role of p57$^{KIP2}$ in trophoblast function and apoptosis remains unclear. In the present study, p57$^{KIP2}$ expression in trophoblasts (HTR-8/SVneo) under hypoxic conditions was evaluated and attempts were made to further investigate the effect of p57$^{KIP2}$ on migration, invasion and apoptosis in response to hypoxia. The results showed that overexpression of p57$^{KIP2}$ decreased apoptosis and increased cell invasion and migration in HTR-8/SVneo cells exposed to 2% $O_2$. This may be mediated by the JNK/stress activated protein kinase (SAPK) signaling pathway. The present study may provide a novel molecular mechanism to understand the involvement of p57$^{KIP2}$ in the pathogenesis of preeclampsia.

## Materials and methods

*Cell culture and treatment*. The human cell line HTR-8/SVneo was provided by Professor Wenming Xu (Sichuan University, Chengdu, China) (26), derived from short-lived primary EVTs, was used in the present study. Cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 $\mu$g/ml streptomycin. Cells were plated in 6-wells plates ($2x10^5$ cells/well). After 24 h in non-hypoxic atmosphere (37˚C; 5% $CO_2$; 21% $O_2$), cells were transferred to an incubator with 2% $O_2$ and 5% $CO_2$ atmosphere for 0, 12, 24, 48 or 72 h at 37˚C.

*Plasmid constructs and transfection*. Small interfering (si)RNA that mediated the knockdown of human p57$^{KIP2}$ (si-p57) and the nonspecific siRNA control (si-ctrl) were obtained from Thermo Fisher Scientific, Inc. The present study used the following oligos: si-p57, forward, 5'-GGACCUUCCCAGUACUAG UTT-3' and reverse, 5'-ACUAGUACUGGGAAGGUCCTT-3'; and si-ctrl, forward, 5'-AAGGGACTTCCTGTAACAA AT GCA-3' and reverse, 5'-CTGGAACGGTGAAGGTGACA-3'. To ectopically express p57$^{KIP2}$, the synthetic human p57$^{KIP2}$ sequence (950 bp) was cloned into the plasmid vector. The overexpression plasmids of p57$^{KIP2}$ (pcDNA3.1-p57) and empty vector plasmid as the control (pcDNA3.1) were purchased from ProteinTech Group, Inc. Cells ($2x10^5$) were cultured in 6-well plates overnight prior to transfection. At 70% confluence, cells were transiently transfected with pcDNA3.1 empty vector, pcDNA3.1-p57, si-p57 or si-ctrl at 50 nmol/l. Cell transfection was performed using Lipofectamine 3000™ (Thermo Fisher Scientific, Inc.) according to the manufacturer's instruction. Following transfection for 24 h, cells were incubated under hypoxic conditions for 24 h and then harvested to test the knockdown or overexpression efficiency via RT-qPCR and western blotting, respectively.

*Drug treatment*. The JNK inhibitor SP600125 was purchased from Sigma-Aldrich (Merck KGaA) and dissolved in DMSO (final concentration, 10 $\mu$M). Transfected HTR-8/SVneo cells ($2x10^5$) were incubated with SP600125 for 30 min prior to and throughout hypoxia culturing for 24 h. Cells were then harvested for subsequent experiments. All cells were compared with a control group, which received an equal volume of DMSO.

*Cell migration and invasion assays*. Cell migration and invasion was determined by Transwell assay as described previously (27). For the migration assay, cells were resuspended in RPMI-1640 medium containing 1% FBS and placed into the upper well of a Transwell chamber (pore diameter, 8 $\mu$m) at $2.5x10^5$ cells/well. Medium containing 10% FBS was placed into the lower wells as a chemoattractant. Cells were incubated under hypoxic conditions for 24 h. Invasion assays were performed as the migration assays, but the filters were coated with growth factor-induced Matrigel (BD Biosciences). Following incubation, cells on the upper surface of the membrane were collected with a sterile cotton swab. Cells that migrated to the lower surface were fixed and stained with 0.1% crystal violet for 10 min at room temperature. The number of migrated and invaded cells was examined via digital light microscopy (magnification, x20). A total of ten visual fields were counted for each sample.

*Flow cytometry*. HTR-8/SVneo cells were harvested after 24 h hypoxia using trypsin without EDTA, washed with PBS, resuspended in 1 ml binding buffer (eBioscience™ Annexin V-FITC Apoptosis kit; #BMS500FI-100; Invitrogen; Thermo Fisher Scientific, Inc.) and stained for 15 min with FITC-Annexin V and propidium iodide in the dark at room temperature. The analysis of the cells was conducted via flow cytometry (FACScan; BD Biosciences) equipped with CellQuest V6.0 (BD Biosciences). Cells were sorted into living, necrotic, early apoptotic and late apoptotic cells. The relative ratio of early to late apoptotic cells was counted for further comparison. This assay was repeated >3 times.

*TUNEL assays*. Apoptosis was validated using the *In Situ* Cell Death Detection kit (Roche Diagnostics). Cells ($5x10^4$) on coverslips were fixed with 4% PFA for 30 min at room temperature and washed twice with PBS (pH 7.0). Cells were then treated with 3% $H_2O_2$ for 10 min at room temperature, 0.1% Triton X-100 for 2 min at 4˚C, incubated with TUNEL reaction mixture at 37˚C for 1 h and then stained with DAPI (1:100) at room temperature for 10 min. Images were taken using a fluorescent inverted microscope (magnification, x40; Nikon Corporation). The percentage of TUNEL-positive cells to apoptotic nuclei was determined in three independent samples and quantified using ImageJ 1.8.0 (National Instituted of Health). The apoptotic index (AI) was calculated as follows: AI = (number of apoptotic cells/total number of cells) x100%.

*Western blot analysis*. Cells were lysed in cold radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) containing protease inhibitor cocktails. Bicinchoninic acid assays were used to quantify protein concentrations. Equal amounts of protein (80 $\mu$g) were separated on 10% SDS-PAGE gels and transferred to PVDF membranes. Membranes were incubated with rabbit monoclonal antibodies, including anti-p57$^{KIP2}$ (1:700; #2557; Cell Signaling Technology, Inc.), anti-p53 (1:1,000; #ab183544; Abcam), anti-Bax (1:2,000; #50599-2-Ig; ProteinTech Group, Inc.),

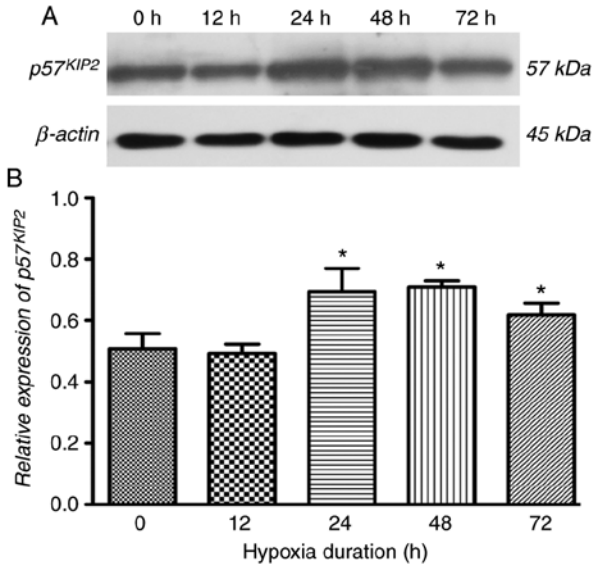


Figure 1. Hypoxia induces expression of $p57^{KIP2}$. HTR-8/SVneo cells were subjected to hypoxia for 0, 12, 24, 48 and 72 h. Protein expression was assessed by western blot and (A) images and (B) quantification data are presented for $p57^{KIP2}$; β-actin was used as internal control. $^{*}$P<0.05 vs. 0 h. KIP, kinase inhibitory protein 2.

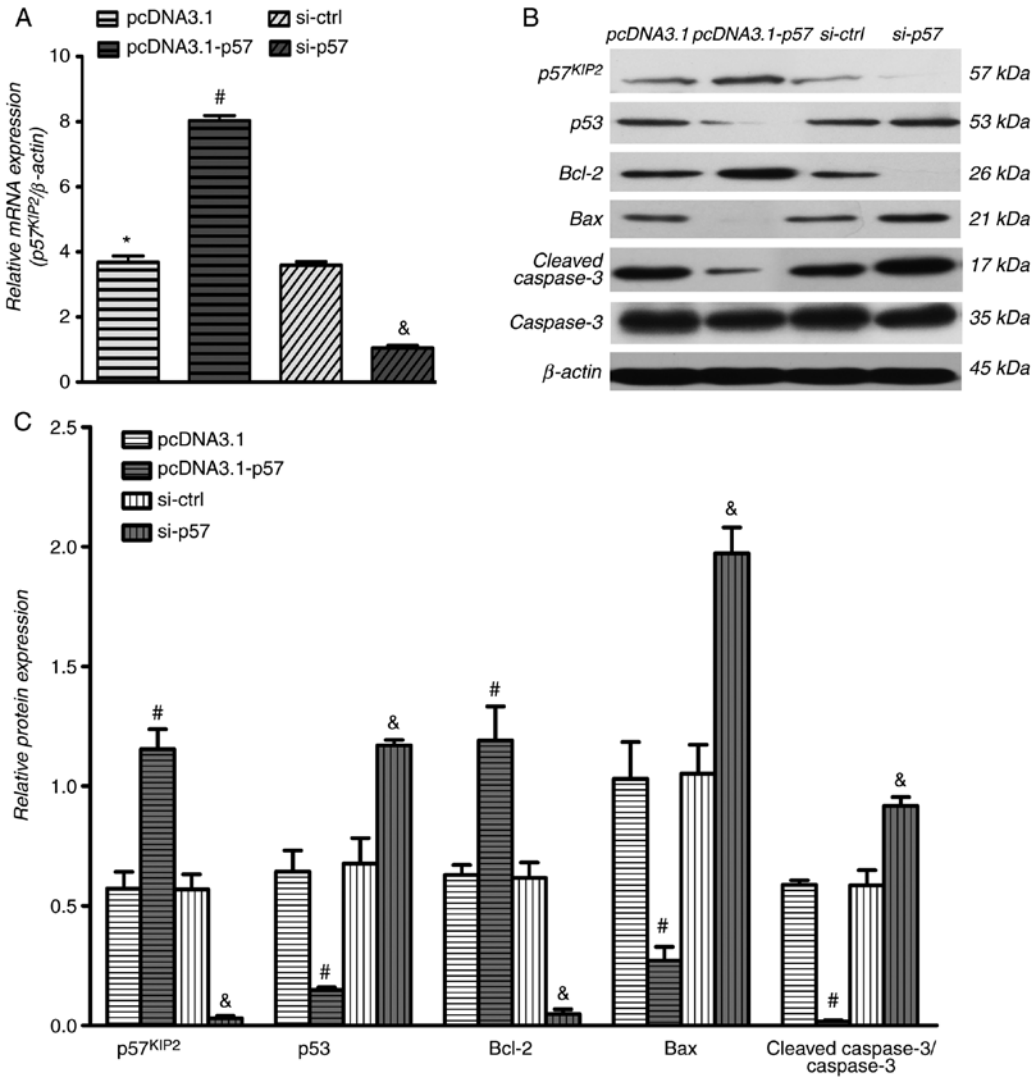


Figure 2. $p57^{KIP2}$ affects the expression of apoptosis-associated proteins at hypoxia for 24 h. HTR-8/SVneo cells were transfected with pcDNA3.1-p57, si-p57 or respective controls, followed by hypoxia treatment for 24 h. (A) mRNA expression of $p57^{KIP2}$ in the transfected cells, determined by RT-qPCR. Western blot (B) images and (C) quantification determined in the transfected cells for $p57^{KIP2}$, p53, Bax, cleaved caspase3 and Bcl-2; β-actin was used as internal control. $^{\#}$P<0.05 vs. pcDNA3.1; $^{\&}$P<0.05 vs. si-ctrl. si-ctrl, siRNA control; si-p57, siRNA targeting $p57^{KIP2}$; KIP, kinase inhibitory protein 2.

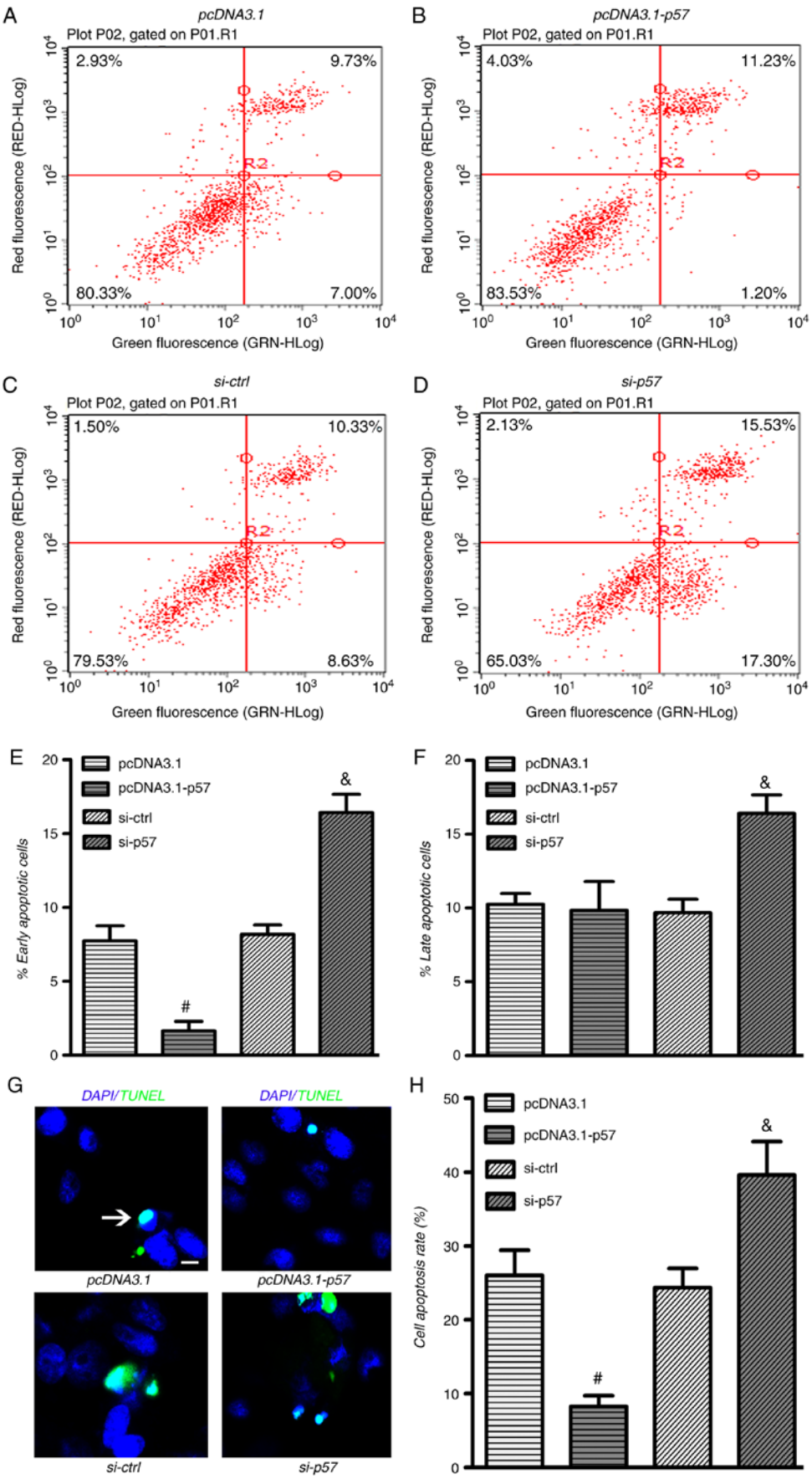


Figure 3. p57$^{KIP2}$ affects cell apoptosis as determined by flow cytometry and TUNEL assays. HTR-8/SVneo cells were transfected with pcDNA3.1-p57, si-p57 or respective controls, followed by hypoxia treatment for 24 h. Flow cytometry data for the (A) pcDNA3.1, (B) pcDNA3.1-p57, (C) si-ctrl and (D) si-p57 transfected cells exposed to 2% $O_2$ for 24 h. Quantitative analysis of (E) early and (F) late apoptosis rates determined by flow cytometry. (G) TUNEL-positive cells (white arrow) following hypoxia 24 h; scale bar, 10 $\mu$m. (H) Quantitative analysis of the TUNEL data. $^{\#}$P<0.05 vs. pcDNA3.1; $^{\&}$P<0.05 vs. si-ctrl. si-ctrl, siRNA control; si-p57, siRNA targeting p57$^{KIP2}$; KIP, kinase inhibitory protein 2.

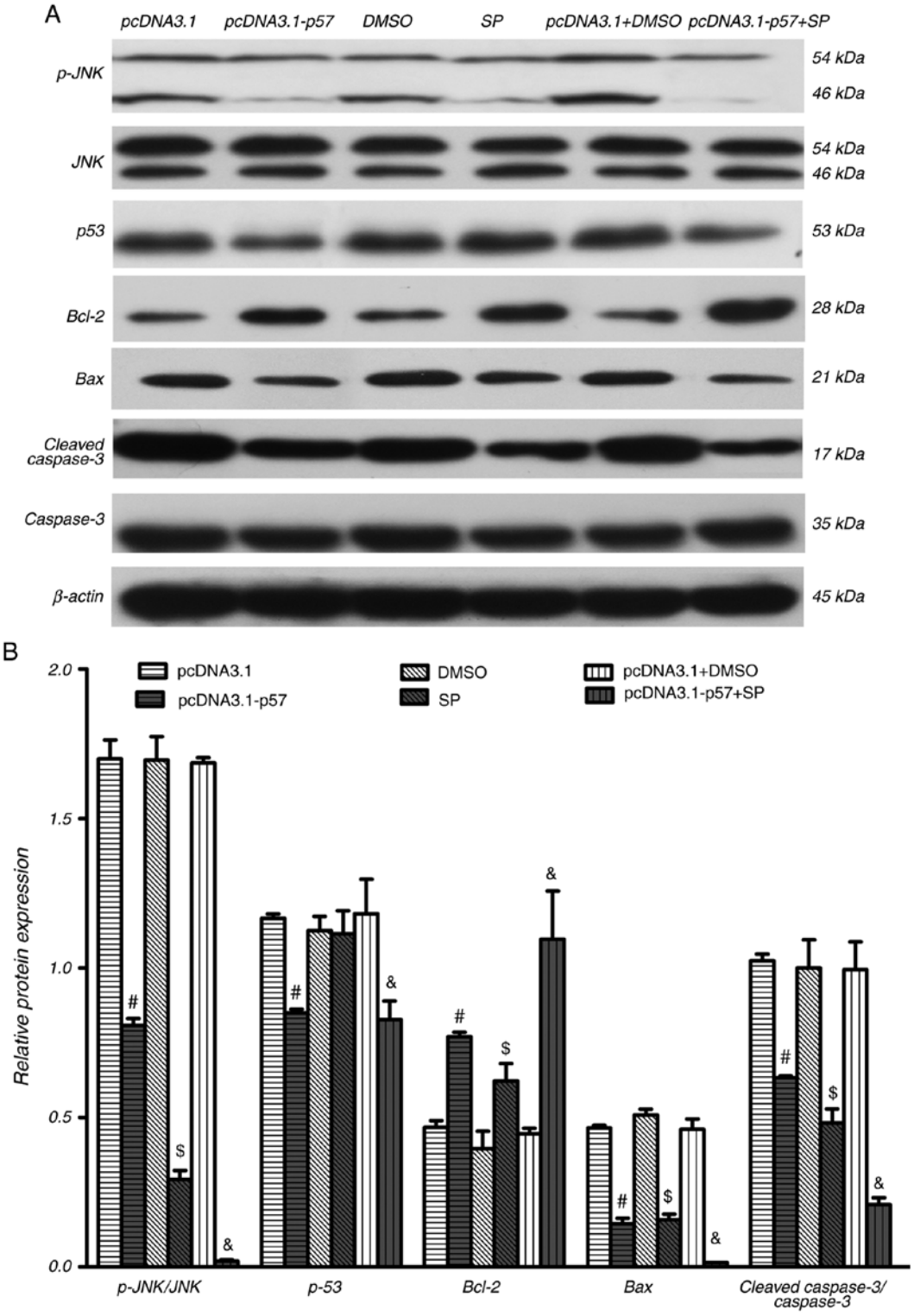


Figure 4. JNK signaling pathway inhibitor and overexpression of $p57^{KIP2}$ decrease apoptosis-associated proteins under hypoxic conditions. HTR-8/SVneo cells were transfected with pcDNA3.1-p57 or pcDNA3.1, treated with JNK inhibitor or DMSO, or a combination of both, followed by hypoxia for 24 h. Western blot (A) images and (B) quantification p-JNK, JNK, p53, Bcl-2, Bax and cleaved caspase3 levels; β-actin was used as internal control. $^{\#}$P<0.05 vs. pcDNA3.1; $^{\$}$P<0.05 vs. DMSO; $^{\&}$P<0.05 vs. pcDNA3.1+DMSO. KIP, kinase inhibitory protein 2; SP, JNK inhibitor SP600125; p-, phosphorylated.

anti-Bcl-2 (1:1,000; #12789-1-AP; ProteinTech Group, Inc.), anti-caspase3 (1:1,000; #9662; Cell Signaling Technology, Inc.), anti-cleaved caspase3 (1:1,000; #9664P; Cell Signaling Technology, Inc.), anti-JNK (1:1,000; #9252; Cell Signaling Technology, Inc.), anti-phosphorylated (p-)Thr183/Tyr18-JNK (p-JNK; 1:1,000; #4671; Cell Signaling Technology, Inc.) and anti-β-actin (1:10,000; #AF0003; Zhengneng Biotechnology, Inc.) overnight at 4˚C. Membranes were washed with TBST and incubated with a secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (1:10,000; #A0208 or #A0208; Zhengneng Biotechnology, Inc.) at room temperature for 1 h followed by enhanced chemiluminescence assay for

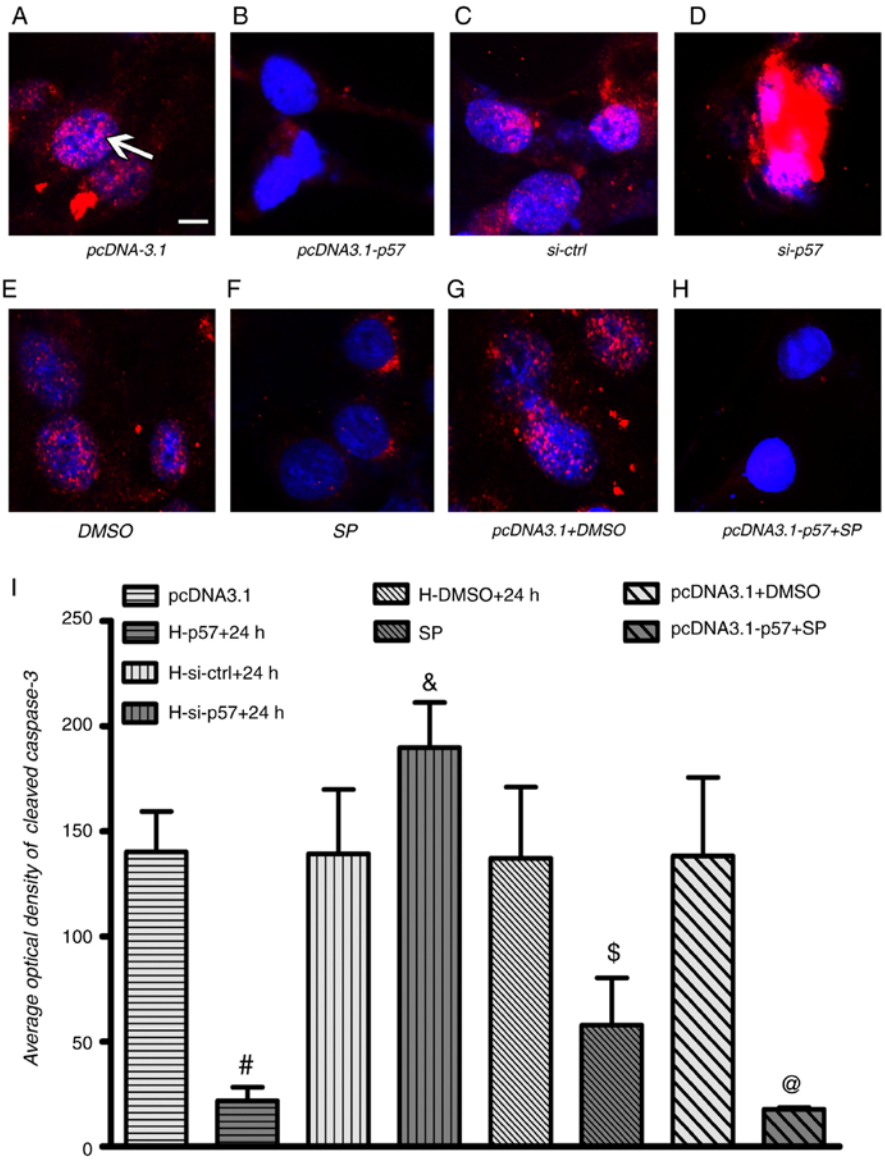


Figure 5. Cleaved caspase3 is affected by p57$^{KIP2}$ under hypoxia. HTR-8/SVneo cells were transfected with pcDNA3.1-p57 or pcDNA3.1, treated with JNK inhibitor or DMSO, or a combination of both and p57$^{KIP2}$ knockdown was achieved by transfection with si-p57; all procedures were followed by hypoxia for 24 h. Immunofluorescence images with cleaved caspase3 (red; white arrow) and nuclear stain (DAPI; blue) for (A) pcDNA3.1, (B) pcDNA3.1-p57, (C) si-ctrl and (D) si-p57 transfected cell, for (E) DMSO and (F) SP treated cells, and for (G) pcDNA3.1 transfected and DMSO and (H) pcDNA3.1-p57 transfected and SP treated cells; scale bar, 10 $\mu$m. (I) Quantified optical density results for cleaved caspase3 analysis. $^{\#}$P<0.05 vs. pcDNA3.1; $^{\&}$P<0.05 vs. si-ctrl; $^{\$}$P<0.05 vs. DMSO; $^{@}$P<0.05 vs. pcDNA3.1+DMSO. si-ctrl, siRNA control; si-p58, siRNA targeting p57$^{KIP2}$; KIP, kinase inhibitory protein 2; SP, JNK inhibitor SP600125.

visualization (EMD Millipore). β-actin was used as a loading control. The optical density of the bands was quantitatively analyzed using ImageJ 1.8.0 (National Institutes of Health).

*RT-qPCR*. SYBR-Green-based RT-qPCR (Invitrogen; Thermo Fisher Scientific, Inc.) was used to examine changes in mRNA levels of p57$^{KIP2}$. Total RNA was isolated from the cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). PCR was performed with the ABI Prism 7500 Sequence Detection system (Thermo Fisher Scientific, Inc.). The reaction was subjected to 95˚C for 60 sec followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. Primers for p57$^{KIP2}$ were obtained from Thermo Fisher Scientific, Inc. β-actin was used as the internal control. All reactions were performed in triplicate. The primer sequences were used as follows: p57$^{KIP2}$, forward, 5'-TTCTCAGGCGCTGATCTCTT-3' and reverse, 5'-AGCTGCACTCGGGGATTT-3'; and β-actin, forward, 5'-AAGGGACTTCCTGTAACAATGCA-3' and reverse, 5'-CTGGAACGGTGAAGGTGACA-3'. All results were normalized to β-actin. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (27).

*Immunofluorescence*. Cells (5x10$^4$) were grown on a coverslip and fixed with 4% PFA at room temperature for 30 min. Cells were probed with rabbit anti-cleaved caspase3 at 4˚C overnight. Alexa Fluor 594 goat anti-rabbit IgG-FITC secondary antibody (1:500; Invitrogen; Thermo Fisher Scientific, Inc.) was added for 2 h at room temperature. DAPI (1:100) was used to stain the cell nuclei for 10 min at room temperature. Cells were mounted with ProLong® Gold antifade reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Images were obtained by an inverted fluorescence microscope (magnification, x60; Nikon

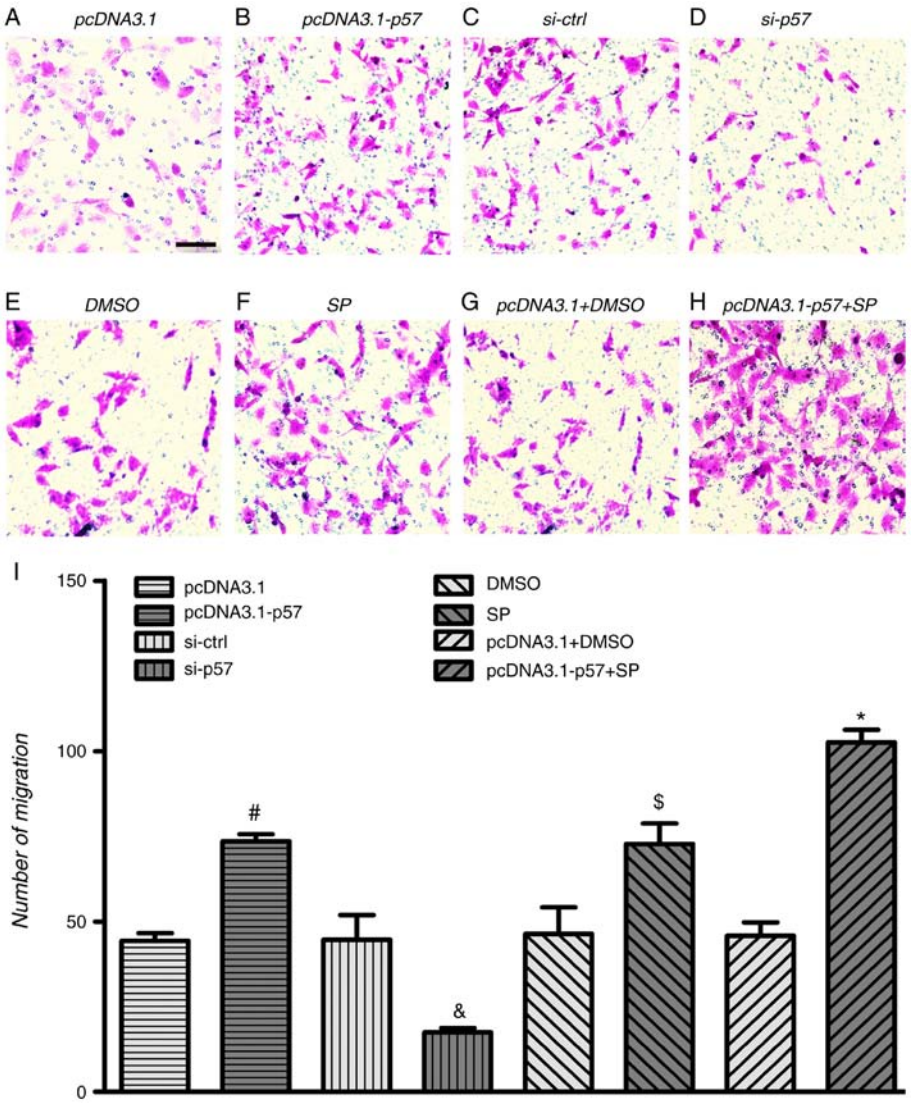


Figure 6. p57$^{KIP2}$ and JNK inhibitor affect cell migration under hypoxic conditions. HTR-8/SVneo cells were transfected with pcDNA3.1-p57 or pcDNA3.1, treated with JNK inhibitor or DMSO, or a combination of both and p57$^{KIP2}$ knockdown was achieved by transfection with si-p57; all procedures were followed by hypoxia for 24 h. Microscopy images if the Transwell migration assay for (A) pcDNA3.1, (B) pcDNA3.1-p57, (C) si-ctrl and (D) si-p57 transfected cell, for (E) DMSO and (F) SP treated cells, and for (G) pcDNA3.1 transfected and DMSO and (H) pcDNA3.1-p57 transfected and SP treated cells; magnification, x20; scale bar, 10 $\mu$m. (I) Number of migrated cells. $^{\#}$P<0.05 vs. pcDNA3.1; $^{\&}$P<0.05 vs. si-ctrl; $^{\$}$P<0.05 vs. DMSO; $^{*}$P<0.05 vs. pcDNA3.1+DMSO. si-ctrl, siRNA control; si-p58, siRNA targeting p57$^{KIP2}$; KIP, kinase inhibitory protein 2; SP, JNK inhibitor SP600125.

Corporation) and analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Inc.) and Adobe Photoshop CS5 (Adobe Systems, Inc.).

*Statistical analysis*. All data are expressed as the mean ± standard deviation. Comparisons between two groups were analyzed by unpaired Student's t-tests. One-way ANOVA followed by Tukey's test was used to assess differences among >2 groups. Data were analyzed using GraphPad Prism 5 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

## Results

*Hypoxia treatment induces p75$^{KIP2}$ expression*. To investigate the effect of hypoxia on the expression of p57$^{KIP2}$ in trophoblasts, HTR-8/SVneo cells were incubated with 2% $O_2$. It was observed that the expression of p57$^{KIP2}$ was significantly increased when exposed to hypoxia for 24-72 h compared with the cells at 0 h (Fig. 1). In the following experiments, hypoxia treatment for 24 h was chosen to elucidate the mechanism.

*Overexpression and downregulation of p57$^{KIP2}$ in human trophoblasts*. HTR-8/SVneo cells were transfected with the pcDNA3.1-p57, si-p57 or respective controls for 24 h. Following transfection, cells were exposed to hypoxia for further 24 h. RT-qPCR results demonstrated that p57$^{KIP2}$ was significantly overexpressed in the pcDNA3.1-p57 transfected cells compared with the empty vector control and p57$^{KIP2}$ was significantly downregulated in the presence of si-p57 compared with si-ctrl (Fig. 2A). In addition, the western blotting results of p57$^{KIP2}$ were consistent with the RT-qPCR findings (Fig. 2B).

*p57$^{KIP2}$ expression affects cell apoptosis under hypoxic conditions*. Western blot analysis, flow cytometry and TUNEL assays

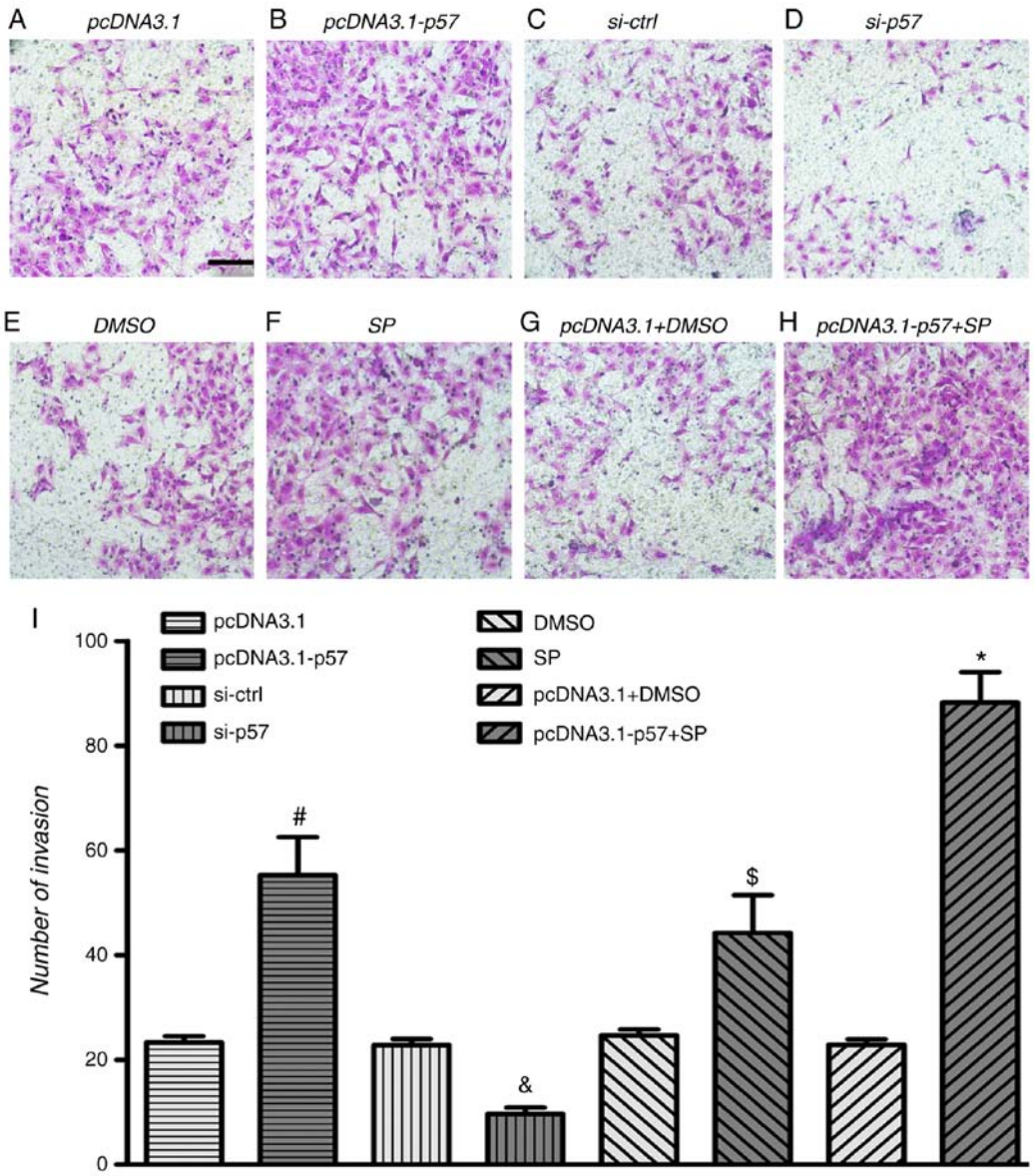


Figure 7. p57$^{KIP2}$ and JNK inhibitor affect cell invasion under hypoxic conditions. HTR-8/SVneo cells were transfected with pcDNA3.1-p57 or pcDNA3.1, treated with JNK inhibitor or DMSO, or a combination of both and p57$^{KIP2}$ knockdown was achieved by transfection with si-p57; all procedures were followed by hypoxia for 24 h. Microscopy images if the Transwell invasion assay for (A) pcDNA3.1, (B) pcDNA3.1-p57, (C) si-ctrl and (D) si-p57 transfected cell, for (E) DMSO and (F) SP treated cells, and for (G) pcDNA3.1 transfected and DMSO and (H) pcDNA3.1-p57 transfected and SP treated cells; magnification, x20; scale bar, 10 $\mu$m. (I) Number of invaded cells. $^{\#}$P<0.05 vs. pcDNA3.1; $^{\&}$P<0.05 vs. si-ctrl; $^{\$}$P<0.05 vs. DMSO; $^{*}$P<0.05 vs. pcDNA3.1+DMSO. si-ctrl, siRNA control; si-p58, siRNA targeting p57$^{KIP2}$; KIP, kinase inhibitory protein 2; SP, JNK inhibitor SP600125.

were used to obtain comprehensive information regarding the impact of p57$^{KIP2}$ on cell apoptosis. As shown in Fig. 2B, overexpression of p57$^{KIP2}$ increased the protein levels of the antiapoptosis protein Bcl-2 and inhibited expression of the proapoptosis proteins p53, Bax and cleaved caspase3 compared with the empty vector control. The analysis further indicated that expression levels of Bcl-2 decreased and expression of p53, Bax and cleaved caspase3 increased in cells with p57$^{KIP2}$ knockdown compared with the si-ctrl transfected cells. In HTR-8/SVneo cells transfected with si-p57 a significant increase in apoptosis was further observed compared with the si-ctrl cells and a decrease in apoptosis was observed in the p57$^{KIP2}$ overexpressing cells compared with the empty vector control (Fig. 3A-D). Using TUNEL, a similar rate of cell apoptosis was determined for the different samples (Fig. 3E and F).

*p57$^{KIP2}$ inhibits the JNK signaling pathway to affect apoptosis under hypoxia*. The present study next explored the potential mechanisms underlying the regulation of p57$^{KIP2}$ on apoptosis under hypoxic conditions. It has been reported that p57$^{KIP2}$ interacts with JNK/SAPK through its QT domain, mediating a variety of cellular activities, including cell differentiation and survival (28). As shown in Fig. 4, there was a decreasing trend in p-JNK levels after 24 h of hypoxia in p57$^{KIP2}$ overexpressing cells compared with the empty vector control. SP600125, a JNK inhibitor, decreased the ratio of p-JNK/JNK at 24 h compared with the DMSO-treated control cells. Inhibition of JNK further decreased the expression of Bax and cleaved caspase3, and increased the expression of Bcl-2 compared with the DMSO control. Overexpression of p57$^{KIP2}$ in combination with JNK inhibitor treatment markedly decreased

the ratio of p-JNK/JNK and expression of Bax and cleaved caspase3, but increased Bcl-2 expression compared with the empty vector plus DMSO-treated control. The effect of overexpression of $p57^{KIP2}$ in combination with JNK inhibitor treatment on Bax and Bcl-2 appeared stronger compared with the overexpression of $p57^{KIP2}$ or JNK inhibitor treatment alone. The inhibition of JNK had no effect on p53 expression levels. Cleaved caspase3 proteolytically cleaves and activates other caspases and is thought to serve an important role in apoptosis (29). As shown in Fig. 5, immunofluorescence results suggested that cleaved caspase3 was mainly located in the cytoplasm and cell nucleus following 24 h of hypoxia. Quantification of the data showed that cleaved caspase3 was significantly increased following si-p57 transfection and 24 h hypoxia compared with the si-ctrl (Fig. 5I). Overexpression of $p57^{KIP2}$ and inhibition of the JNK pathway significantly decreased the optical density of cleaved caspase3 at 24 h hypoxia compared with the respective controls. It is suggested that $p57^{KIP2}$ may regulate the JNK signaling pathway to inhibit apoptosis during hypoxic injury.

*$p57^{KIP2}$ expression affects cell migration and invasion under hypoxic conditions*. HTR-8/SVneo cells have been extensively used to study trophoblast migration and invasion (30). Hypoxia markedly affects trophoblast biological processes, including cell migration and invasion (27). To determine the function of $p57^{KIP2}$ in trophoblast migration, HTR8/SVneo cells were transfected with pcDNA3.1-p57, si-p57, JNK inhibitor or respective controls and exposed to 2% $O_2$ for 24 h. It was revealed that HTR-8/SVneo cells overexpressing $p57^{KIP2}$ exhibited significantly increased migration and invasion abilities compared with the empty vector control (Figs. 6 and 7). $p57^{KIP2}$ knockdown significantly decreased cell migration and invasion following 24 h of hypoxia compared with the si-ctrl cells. This indicated that $p57^{KIP2}$ enhanced migration and invasion abilities of trophoblasts under hypoxia.

As shown in Fig. 6F, the JNK inhibitor significantly increased HTR-8/SVneo cell migration and invasion at 24 h of hypoxia compared with the DMSO treated control. Overexpression of $p57^{KIP2}$ combined with JNK inhibitor treatment significantly increased HTR-8/SVneo cell migration and invasion compared with the empty vector transfected and DMSO treated cells. The results indicated that under hypoxic conditions $p57^{KIP2}$ affected HTR-8/SVneo cell migration and invasion through the JNK signaling pathway.

## Discussion

As a member of the CIP/KIP family, $p57^{KIP2}$ is considered to be a master regulator of the cell cycle during embryogenesis, serving a role in cell cycle control and regulating the induction of apoptosis (31,32). It has been shown that $p57^{KIP2}$ has a minor proapoptotic effect on its own, sensitizing cells to apoptosis (17). A previous study reported that $p57^{KIP2}$ expression enhanced apoptosis in HeLa cells and showed that $p57^{KIP2}$ was a target of caspase activity (21). Other studies have reported an increase in apoptosis and altered differentiation during mouse development in a $p57^{KIP2}$ knockout model (17,33). Previously, it has been shown that $p57^{KIP2}$ serves a role in antiapoptosis regulation, suggesting that whether it promotes or inhibits apoptosis is mainly cellular context-dependent (24). In agreement with these previous studies, the present study revealed that overexpression of $p57^{KIP2}$ increased expression of the antiapoptotic protein Bcl-2 following hypoxia and silencing $p57^{KIP2}$ induced the expression of the proapoptosis-associated proteins p53, Bax and cleaved caspase3. It appeared that in the HTR-8/SVneo hypoxia model, $p57^{KIP2}$ acted as an antiapoptotic molecule.

The family of mammalian mitogen-activated protein kinases includes several subgroups, such as extracellular signal-regulated kinase and JNK/SAPK (34). Accordingly, the JNK/SAPK signaling pathway is implicated in the control of cell growth, transformation, survival and death. It has been shown that $p57^{KIP2}$ interacts with and inhibits the kinase activity of JNK/SAPK through its QT domain (29,34). The present study provided evidence showing that $p57^{KIP2}$ affected the phosphorylation of JNK under hypoxic conditions. Furthermore, $p57^{KIP2}$ modulated apoptosis by negatively regulating the JNK signaling pathway. In addition, overexpression of $p57^{KIP2}$ or treatment with the JNK inhibitor increased cell migration and invasion under hypoxic conditions. Treatment of the $p57^{KIP2}$ overexpressing cells with the JNK inhibitor further affected cell apoptosis, migration and invasion. These results showed that $p57^{KIP2}$ functioned as an inhibitory protein of JNK and affected HTR-8/SVneo migration and invasion.

In conclusion, the results of the present study indicated that $p57^{KIP2}$ served a protective role in apoptosis and increased cell invasion and migration through the JNK signaling pathway under hypoxic conditions. These results suggested that at hypoxia, the $p57^{KIP2}$-JNK network may serve an important role in regulating HTR-8/SVneo apoptosis and function.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

GQH, WMX and GLH conceived and designed the study. GQH, GYL and HJL performed the experiments. XHL performed the analysis of data. GHQ and GLH wrote the manuscript. GLH revised the article. All authors read and approved the final manuscript.

## Ethics approval and consent for participation

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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