CTRP9 overexpression attenuates palmitic acid-induced inflammation, apoptosis and impaired migration in HTR8/SVneo cells through AMPK/SREBP1c signaling

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Abstract. Obesity in pregnant mothers often leads to a range of obstetric complications, including miscarriage, pre-eclampsia, gestational hypertension and diabetes. C1q/TNF-related protein 9 (CTRP9) is an adipokine with an anti-inflammatory effect. The aim of the present study was to identify the role of CTRP9 in the pathogenesis of maternal obesity during pregnancy. Following treatment with palmitic acid (PA), HTR8/SVneo cell viability and CTRP9 expression were analyzed using Cell Counting Kit-8 (CCK-8), reverse transcription-quantitative PCR (RT-qPCR) and western blot analyses. The effects of CTRP9 overexpression on cell viability, apoptosis, pro-inflammatory cytokine levels and migration were assessed using CCK-8, TUNEL, RT-qPCR and Transwell assays, respectively. Subsequently, sterol-regulatory element binding protein 1c (SREBP1c) overexpression efficiency was verified using RT-qPCR, and its effects on cell viability, apoptosis, pro-inflammatory cytokines and migration damage were then examined in HTR8/SVneo cells. The results showed that CTRP9 overexpression attenuated the inhibition of cell viability and apoptosis caused by PA in HTR8/SVneo cells, reduced pro-inflammatory cytokine release, improved cell migration and regulated the protein expression level of AMP-activated protein kinase (AMPK)/SREBP1c signaling. In addition, CTRP9 inhibited SREBP1c expression through AMPK signaling, thereby attenuating the inflammation, apoptosis and inhibited migration caused by PA in HTR8/SVneo cells. In brief, CTRP9 protected against inflammation, apoptosis and migration defects in HTR8/SVneo cells exposed to PA treatment through AMPK/SREBP1c signaling, which suggested the potential role of CTRP9 in alleviating the toxicity of PA.

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Introduction

Obesity poses a challenge to the health of pregnant women. Stubert *et al* (1) reported that the risk of pregnancy-related illness increases as the severity of obesity increases. Maternal obesity rates have already exceeded 30% in most European countries (2). Data from one study showed that obese pregnant women are more likely to suffer from gestational diabetes, pre-eclampsia, gestational hypertension, depression, caesarean sections and surgical site infections than women with a healthy weight (3). In addition to affecting maternal health, maternal obesity may also have long-term adverse effects on the health of the fetus and newborn. For example, the fetuses of obese women are also subject to premature births, stillbirths and fetal malformations (4). Maternal obesity has become a serious public health problem that needs to be addressed, and therefore exploring its pathogenesis is critical.

Growing evidence shows that the placenta plays an important role in regulating fetal growth (1). However, maternal obesity is a risk factor for placental dysfunction. According to a recent study, obese pregnant women are prone to placental inflammation (5). This is due to the fact that excess saturated fatty acid palmitic acid (PA) in the serum of obese pregnant women creates a lipotoxic environment in the placenta, which stimulates the production of pro-inflammatory cytokines, including TNF-α, IL-6 and IL-8 (6). The release of excessive pro-inflammatory cytokines can cause placental dysfunction and affect placental nutrient transport and adipose tissue metabolism (7). Recent studies have demonstrated that PA can cause inflammatory release, impaired invasion and migration, and apoptosis in trophoblasts (8,9).

C1q/TNF-related protein 9 (CTRP9) is the closest analogue of adiponectin, a classic anti-inflammatory agent, and plays a role in metabolic regulation, anti-atherosclerosis and anti-inflammation (10,11). A recent study demonstrated that CTRP9 expression level was decreased in the serum of obese patients with eclampsia during pregnancy (12). CTRP9 has been shown to reduce high fat diet-induced cardiac hypertrophy and cardiomyocyte lipotoxicity (13). It has also been shown that its expression is increased in the serum of patients with type 2 diabetes and is associated with insulin resistance (14). In addition, CTRP9 has been shown to be able to activate the expression of AMP-activated protein kinase (AMPK) signaling (15) and improve the anti-contractile effect

of diet-induced perivascular adipose tissue in obese mice through the AMPK-endothelial nitric oxide synthase pathway (eNOS) (16). AMPK activation has been shown to suppress inflammatory responses in various injury models, such as myocardial ischemia/reperfusion, acute lung and liver injury (17). Previous studies indicated that SREBP1c was the downstream gene of AMPK, and AMPK could inhibit downstream SREBP1c expression level (18-20).

SREBP1c is a key transcription factor for *de novo* lipogenesis (21). Studies have shown that SREBP1c expression level is increased in both human trophoblasts and porcine placental trophoblasts with lipid accumulation (22,23). Accordingly, the present study focused on exploring whether CTRP9 could inhibit SREBP1c expression through AMPK signaling, thereby reducing PA-induced inflammation, apoptosis and impaired migration in gestational trophoblasts.

Materials and methods

Kyoto encyclopedia of genes and genome (KEGG) analysis (24-26). The KEGG pathway database (https://www.genome.jp/kegg/pathway.html) was used to determine whether expression of SREBP1c, a downstream gene of AMPK, was suppressed by AMPK in AMPK signaling pathway (map04152).

Cell culture and treatment. A human trophoblast HTR8/SVneo cell line was obtained from the American Type Culture Collection (27). This cell line was originally generated using freshly isolated extravillous cytotrophoblast from first-trimester placenta and transfected with a plasmid containing the simian virus 40 large T antigen, which included two populations; one of epithelial and one of mesenchymal origin. HTR8/SVneo cells were grown in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 5% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified incubator with 5% CO_2 at 37˚C.

Prior to treatment, PA (ChemicalBook) was dissolved in 0.1 M NaOH at 70˚C to make 20 mM of stock solution and mixed with 30% fatty-acid-free BSA (Shanghai Weiao Biotechnology Co., Ltd.) for 2 h to produce 5 mM PA-BSA conjugate solution. HTR8/SVneo cells were treated with the PA-BSA conjugate solution at a concentration of 200, 400, 600 and 800 μM for 24 h at 37˚C. In each assay, 30% fatty-acid-free BSA was used as the vehicle. Compound C is a potent and selective AMPK inhibitor. HTR8/SVneo cells were pretreated with 10 μM Compound C (cat. no. S7840; Selleck Chemicals) for 2 h prior to being exposed to PA for 24 h at 37˚C.

Plasmid construction of CTRP9 and SREBP1c. The overexpression plasmid vectors (pcDNA 3.1) targeting CTRP9 (Ov-CTRP9) and SREBP1c (Ov-SREBP1c), as well as empty overexpression negative control vectors (Ov-NC), were constructed by Shanghai GenePharma Co., Ltd. HTR8/SVneo cells were seeded in 6-well culture plates ($2x10^5$ cells/well) and cultured for 24 h at 37˚C. Next, HTR8/SVneo cells were transfected with 200 nM Ov-CTRP9, 200 nM Ov-SREBP1c or 200 nM Ov-NC at 37˚C for 24 h using Lipofectamine® 2000 (Invitrogen; Thermo Fischer Scientific, Inc.), according to the manufacturer's instructions. The transfection efficiency was measured using reverse transcription-quantitative PCR (RT-qPCR), and cells were used for subsequent experiments 48 h after transfection.

Cell counting kit-8 (CCK-8). HTR8/SVneo cells ($2x10^4$ cells/well) were routinely seeded in 96-well plates for 24 h. Next, 10 μl CCK-8 solution (Glpbio) was added in each well and incubated for 2 h at 37˚C in an incubator. The optical density was measured at 450 nm with a microplate reader (M3000; China Med Device).

RT-qPCR. The mRNA expression of CTRP9, TNF-α, IL-1β, IL-6 and SREBP1c was examined using RT-qPCR. Total RNA extraction from HTR8/SVneo cells was performed using TRIzol® reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. cDNA was synthesized by reverse transcription of total RNA using a PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara Biotechnology Co., Ltd.), according to the manufacturer's instructions. qPCR was performed using a SYBR Green RT-qPCR Master Mix kit (MedChemExpress) on an ABI 7500 quantitative PCR instrument (PerkinElmer, Inc.). The thermocycling conditions were as follows: 95˚C for 25 sec, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 35 sec. The primer sequences used were as follows: CTRP9 forward, 5'-GAGGATCCCCAGGAAAACAT-3' and reverse, 5'-AGCTTCTCCTTTGGGACCAG-3'; TNF-α forward, 5'-GGCGTGGAGCTGAGAGATAA-3' and reverse, 5'-TTGATGGCAGAGAGGAGGTT-3'; IL-1β forward, 5'-GCATCCAGCTACGAATCTCC-3' and reverse, 5'-TGAAGGGAAAGAAGGTGCTC-3'; IL-6 forward, 5'-TTCGGTCCAGTTGCCTTCT-3' and reverse, 5'-GAGATGCCGTCGAGGATGTA-3'; SREBP1c forward, 5'-TGACTTCCCTGGCCTATTTG-3' and reverse, 5'-GCATGGACGGGTACATCTTC-3'; and GAPDH forward, 5'-CACCCATGGCAAATTCCATGGCA-3' and reverse, 5'-TCTAGACGGCAGGTCAGGTCCACC-3'. The relative mRNA expression of genes was calculated using the $2^{-\Delta\Delta Cq}$ method (28) following normalization to GAPDH.

Western blot analysis. Total protein was extracted from HTR8/SVneo cells using RIPA buffer (MilliporeSigma) on ice. Protein concentration was assessed using a BCA Protein Quantification Kit (Vazyme Biotech Co., Ltd.), according to the manufacturer's instructions. Equal amounts (20 μg) of protein samples were separated by SDS-PAGE on a 10% gel and transferred onto PVDF membranes (Roche Diagnostics). Blocking with 5% non-fat milk for 2 h at room temperature was performed, followed by incubation with primary antibodies against CTRP9 (dilution 1:400; cat. no. LS-C373857; LifeSpan Biosciences, Inc.), p-AMPK (dilution 1:1,000; cat. no. ab92701; Abcam), AMPK (dilution 1:1,000; cat. no. ab32047; Abcam), SREBP1c (dilution, 1:1,000; cat. no. ab28481; Abcam) and GAPDH (dilution 1:2,500; cat. no. ab9485; Abcam) at 4˚C overnight. Following three washes with PBS, an HRP-conjugated antibody (1:2,000; cat. no. ab6721; Abcam) was incubated together with the membranes at room temperature for a further 1 h. Finally, the protein bands were visualized using Bio-Rad ChemiDoc™ XRS+ System (Bio-Rad Laboratories, Inc.) and quantified using Image Lab 5.2.1 (Bio-Rad Laboratories, Inc.). The experiment was performed in triplicate.

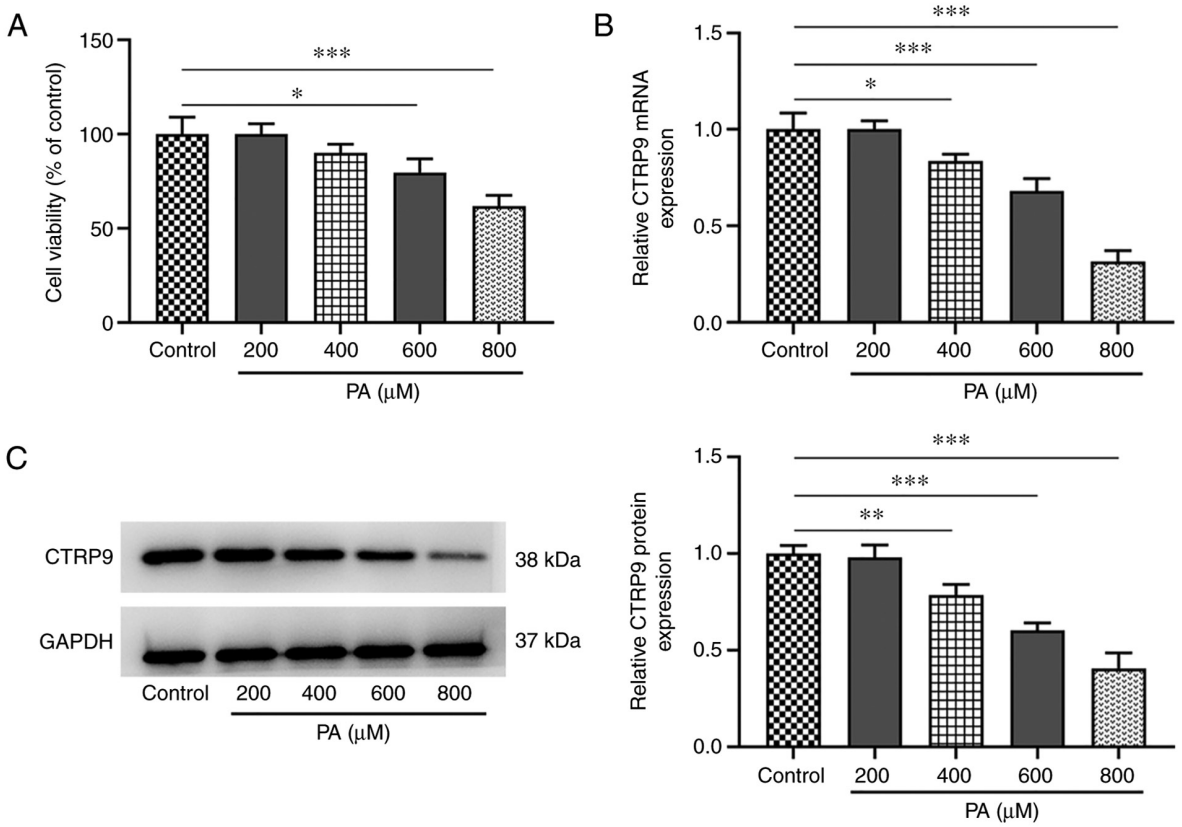

Figure 1. CTRP9 expression is reduced in PA-induced HTR8/SVneo cells. (A) Cell viability of HTR8/SVneo cells was detected by Cell Counting Kit-8 in the control group and the groups treated with PA at concentrations of 200, 400, 600 and 800 μM. (B) mRNA and (C) protein expression levels of CTRP9 were measured by western blotting and reverse transcription-quantitative PCR in the control group and the groups treated with PA at concentrations of 200, 400, 600 and 800 μM. *P<0.05, **P<0.01 and ***P<0.001. CTRP9, C1q/TNF-related protein 9; PA, palmitic acid.

Cell apoptosis detection using TUNEL assay. The apoptosis of HTR8/SVneo cells was assessed using a One Step TUNEL Apoptosis Assay Kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. HTR8/SVneo cells were fixed with 4% paraformaldehyde for 30 min at 4˚C. Following washing with PBS, these cells were incubated with PBS solution containing 0.3% Triton X-100 for 5 min at room temperature. A TUNEL Assay Kit was prepared as required by the configuration table. The samples were then incubated using 50 μl TUNEL reagent in the dark for 60 min at 37˚C, followed by staining of nuclear DNA with 10 μg/ml DAPI at 37˚C for 2-3 min. Following three washes with PBS, cells with green fluorescence were observed using fluorescence microscopy at a magnification of x200 in at least 5 fields of view after blocking with 30 μl anti-fluorescence quenching blocking solution (Beyotime Institute of Biotechnology) at room temperature for 5 min. The total cells were defined as blue dots and apoptotic cells were defined as green dots. The quantification of the apoptotic cells was determined by Image J 1.51 software (National Institutes of Health).

Transwell assay. HTR8/SVneo cells ($1x10^5$) were incubated in 200 μl serum-free RPMI-1640 medium for 24 h before plating them in the upper chamber of 24-well Transwell plates. Cells in the lower chamber were incubated for 24 h with 600 μl RPMI-1640 containing 20% FBS. After discarding the culture medium, the plates were washed twice with calcium-free PBS and then fixed in methanol for 30 min at room temperature. Subsequently, after cells were stained with 0.1% crystal violet for 20 min at room temperature, non-migrating cells in the upper layer were gently wiped off with a cotton swab and washed 3 more times with PBS. A total of five areas of cells were randomly selected and observed under a light microscope at a magnification of x100 (Shenzhen Boshida Instrument Co., Ltd.; https://www.cn-microscope.com/) for counting using EVOS M7000 Imaging System (Thermo Fisher Scientific, Inc.).

Statistical analysis. Statistical analysis was performed using SPSS version 18.0 (SPSS, Inc.). All data are presented as the mean ± SD. One-way ANOVA followed by Tukey's post hoc test was used for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference. All experiments were performed in triplicate.

Results

CTRP9 expression is reduced in PA-treated HTR8/SVneo cells. Cell viability and CTRP9 expression in HTR8/SVneo cells with or without PA treatment at concentrations of 200, 400, 600 and 800 μM was measured using CCK-8, western blotting and RT-qPCR analyses. HTR8/SVneo cell viability was gradually reduced in response to PA induction, compared with that of the control group. Higher PA concentrations resulted in lower cell viability, with the lowest viability at 800 μM PA (Fig. 1A). The mRNA and protein expression levels of CTRP9 were significantly downregulated in PA-treated HTR8/SVneo

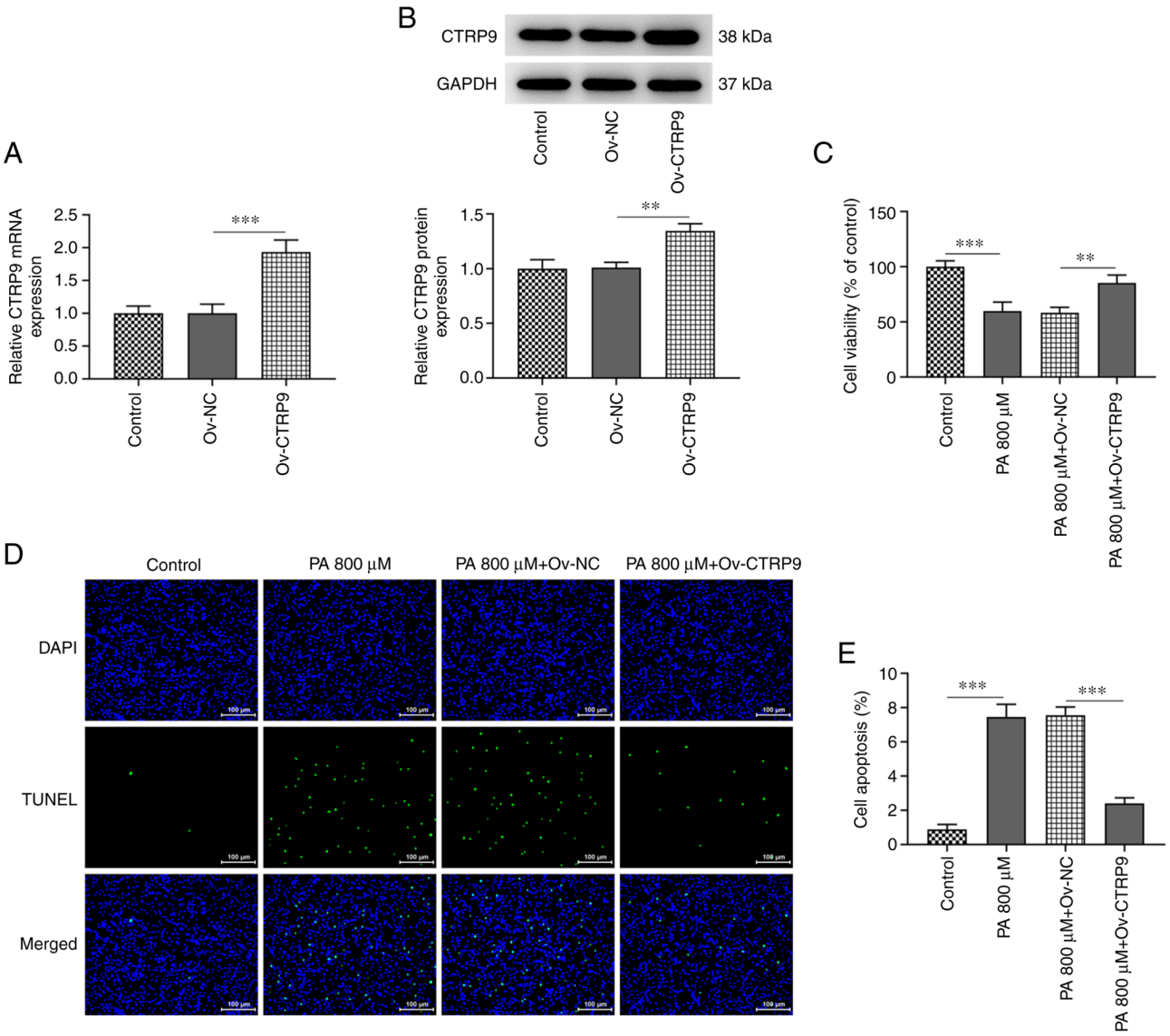

Figure 2. CTRP9 overexpression attenuates PA-induced cell viability impairment and apoptosis in HTR8/SVneo cells. CTRP9 overexpression in HTR8/SVneo cells, untransfected or transfected with Ov-NC or Ov-CTRP9, was assessed by (A) western blotting and (B) reverse transcription-quantitative PCR. (C) Viability of PA-induced HTR8/SVneo cells transfected with Ov-NC or Ov-CTRP9 was detected by Cell Counting Kit-8. (D) Apoptosis of PA-induced HTR8/SVneo cells transfected with Ov-NC or Ov-CTRP9 was detected by TUNEL assay and (E) quantified. Scale bar, 100 μm. **P<0.01 and ***P<0.001. CTRP9, C1q/TNF-related protein 9; PA, palmitic acid; Ov-, overexpression vector; NC, negative control.

cells (vs. control) in a dose-dependent manner, with the lowest expression level observed at 800 μM PA (Fig. 1B and C). Therefore, the group treated with 800 μM PA was selected for the next experiments.

CTRP9 overexpression attenuates PA-induced apoptosis and impaired cell viability in HTR8/SVneo cells. Following the transfection of HTR8/SVneo cells with Ov-CTRP9, the mRNA and protein expression levels of CTRP9 were significantly elevated compared with those in the Ov-NC group (Fig. 2A and B). As indicated in Fig. 2C, the viability of HTR8/SVneo cells increased in the PA+Ov-CTRP9 group, compared with that in the Ov-NC group. In addition, Fig. 2D and E revealed more TUNEL-positive HTR8/SVneo cells in the PA group (PA group vs. control) but notably less TUNEL-positive cells in the PA+Ov-CTRP9 group (PA+Ov-CTRP9 vs. PA+Ov-NC). Accordingly, the increased CTRP9 expression level in HTR8/SVneo cells could largely reduce the PA-induced impairment in cell viability and apoptosis.

CTRP9 overexpression attenuates inflammation and impaired migration in PA-treated HTR8/SVneo cells. RT-qPCR revealed that the mRNA levels of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β were increased in PA-treated HTR9/SVneo cells compared with the Control group and were reduced by CTRP9 overexpression compared with the PA 800 μM + Ov-NC group (Fig. 3A). The Transwell assay revealed decreased migratory ability in PA-treated HTR8/SVneo cells compared with that in the control group. However, CTRP9 overexpression increased the migration of PA-treated HTR8/SVneo cells compared with that in the Ov-NC group (Fig. 3B and C). Therefore, CTRP9 upregulation could reduce the release of inflammatory cytokines and protect against the impairment of PA-promoted migratory ability of HTR8/SVneo cells.

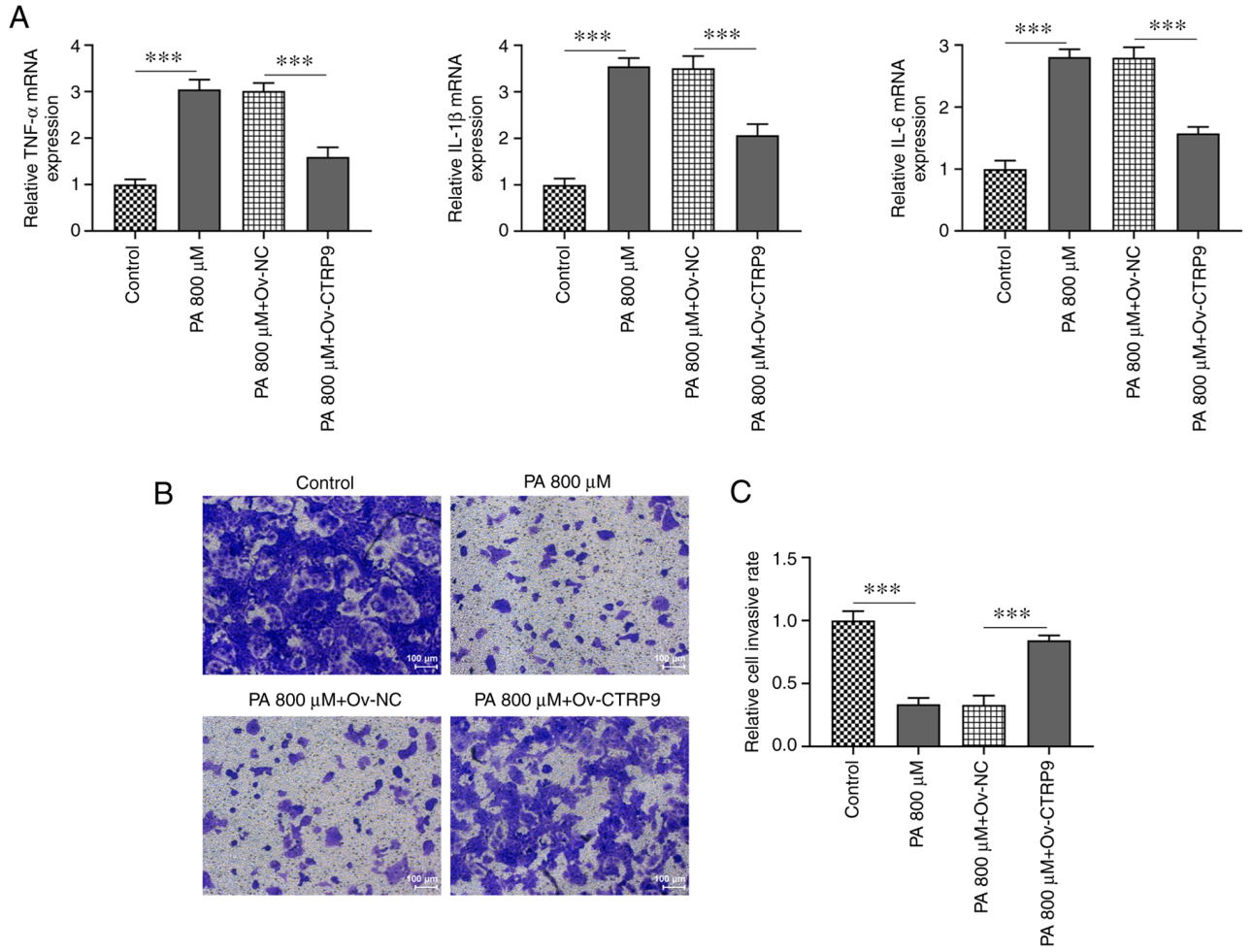

Figure 3. CTRP9 overexpression attenuates PA-induced inflammation and migration impairment in HTR8/SVneo cells. (A) mRNA expression levels of TNF-α, IL-6 and IL-1β in PA-induced HTR8/SVneo cells transfected with Ov-NC or Ov-CTRP9 were examined by reverse transcription-quantitative PCR. (B) Images of cell migration capacity of PA-induced HTR8/SVneo cells stained by crystal violet after transfection of Ov-NC or Ov-CTRP9 and (C) quantified. ***P<0.001. Scale bar, 100 μm. CTRP9, C1q/TNF-related protein 9; PA, palmitic acid; Ov-, overexpression vector; NC, negative control.

CTRP9 overexpression attenuates PA-induced cell viability impairment and apoptosis in HTR8/SVneo cells through AMPK/SREBP1c signaling. According to the KEGG pathway database, SREBP1c is a downstream gene of AMPK (Fig. 4). As shown in Fig. 5A, the protein expression level of p-AMPK was markedly decreased in the PA group compared with that in the control group, but was increased following CTRP9 overexpression in PA-induced HTR8/SVneo cells compared with the Ov-NC group. Treatment with the AMPK inhibitor caused a decrease in the protein level of p-AMPK compared with that in the PA+Ov-CTRP9 group. In addition, there were no obvious changes in AMPK protein level in the different groups. Furthermore, the protein expression level of SREBP1c increased in the PA group compared with the control group, and decreased in the PA+Ov-CTRP9 group compared with that in the PA+Ov-NC group. The protein expression level of SREBP1c was elevated following the addition of AMPK inhibitor, which indicated that CTRP9 overexpression played a regulatory role in AMPK/SREBP1c signaling in PA-induced HTR8/SVneo cells (Fig. 5A).

In the present study, HTR8/SVneo cells transfected with Ov-SREBP1c expressed a higher level of SREBP1c than those of the Ov-NC groups (Fig. 5B and C). In the aforementioned results, CTRP9 overexpression improved the viability of PA-induced HTR8/SVneo cells. However, the viability of these cells was decreased both in the Ov-CTRP9+AMPK inhibitor and Ov-CTRP9+Ov-SREBP1c groups (Fig. 5D). Similarly, Ov-CTRP9 reduced PA-induced apoptosis in the HTR8/SVneo cells. Nevertheless, apoptotic cells were increased in the Ov-CTRP9+AMPK inhibitor and Ov-CTRP9+Ov-SREBP1c groups (Fig. 5E and F). These results suggested that the increased CTRP9 expression level could prevent PA-induced impaired cell viability and reduce the apoptosis of HTR8/SVneo cells through AMPK/SREBP1c signaling.

CTRP9 overexpression attenuates inflammation-induced impairment in PA-induced HTR8/SVneo cell migration through AMPK/SREBP1c signaling. According to the aforementioned experimental findings, it was demonstrated that CTRP9 overexpression could reduce inflammatory cytokine release in PA-induced HTR8/SVneo cells. The mRNA levels of TNF-α, IL-6 and IL-1β were higher in both the Ov-CTRP9+AMPK inhibitor and Ov-CTRP9+Ov-SREBP1c groups compared with those in the PA+Ov-CTRP9 group (Fig. 6A). The migratory ability of PA-induced HTR8/SVneo

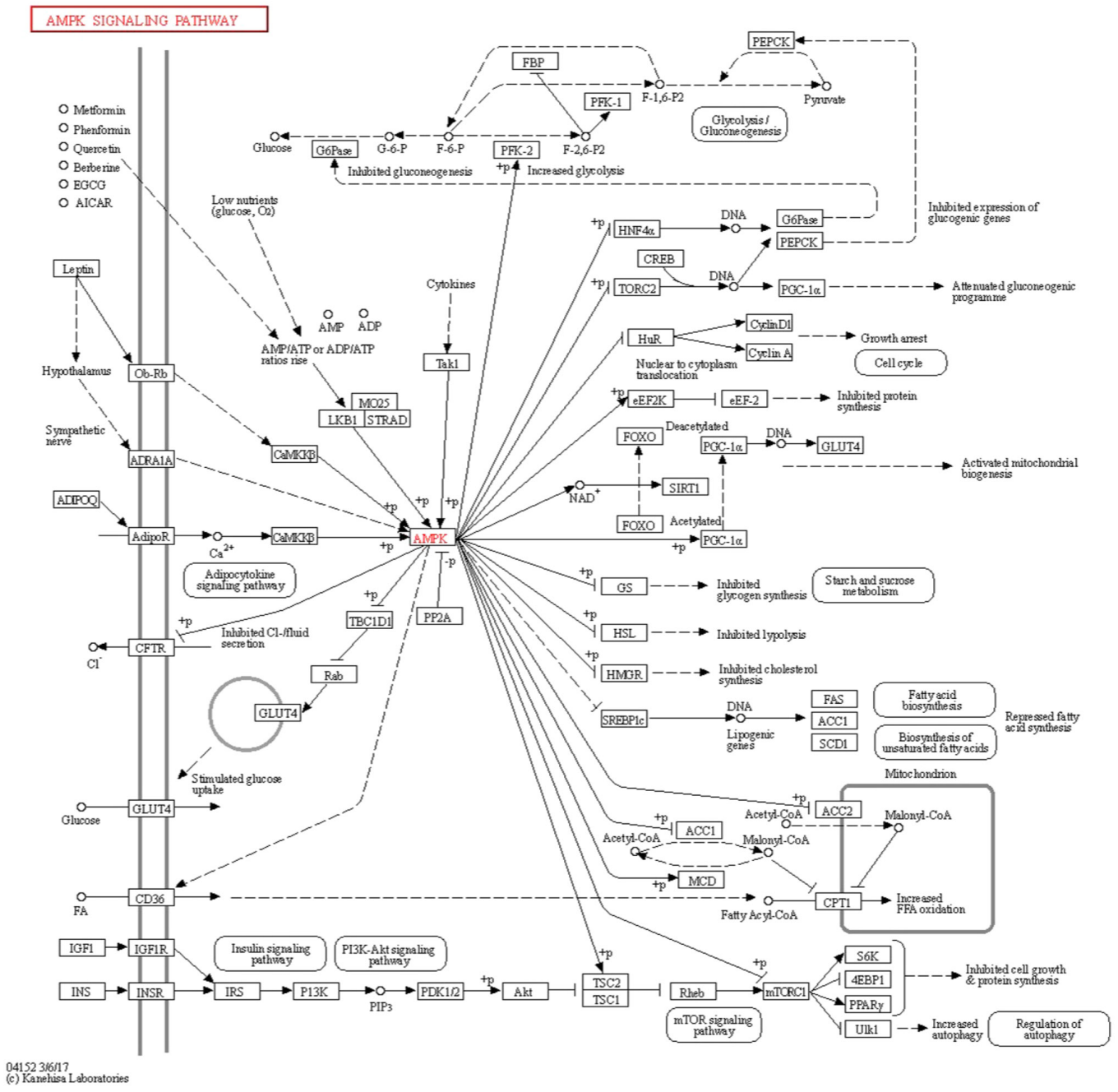

Figure 4. AMPK signaling pathway showed in KEGG pathway database. Permission for the use of this figure was obtained from KEGG. AMPK, AMP-activated protein kinase.

cells was promoted by CTRP9 overexpression, as aforementioned. However, according to the data presented in Fig. 6B and C, a notable decline in the migratory ability of PA-induced HTR8/SVneo cells was observed in the Ov-CTRP9+AMPK inhibitor and Ov-CTRP9+Ov-SREBP1c groups. These results suggested that CTRP9 overexpression could reduce PA-induced inflammation and migration impairment in HTR8/SVneo cells through AMPK/SREBP1c signaling.

Discussion

Maternal obesity has a serious impact on the health of both the mother and the fetus. Improving the metabolic environment of obese pregnant women and reducing the future economic, social and personal burden of maternal obesity is therefore of great significance. Studies have shown that placental cells from obese pregnant women contain high levels of PA, inducing an inflammatory response in trophoblasts and attenuating cell migration through a Pediocin PA-1-mediated mechanism, leading to placental dysfunction and apoptosis (8). CTRP9 has been reported to be downregulated in obese patients, and can affect the placental system and suppress pro-inflammatory genes (12,29). To explore the role of CTRP9 on placental inflammation in obese women, in the present study, trophoblasts were treated with high PA levels to mimic the lipotoxic environment of the placenta. Based on the experimental results, it was observed that CTRP9

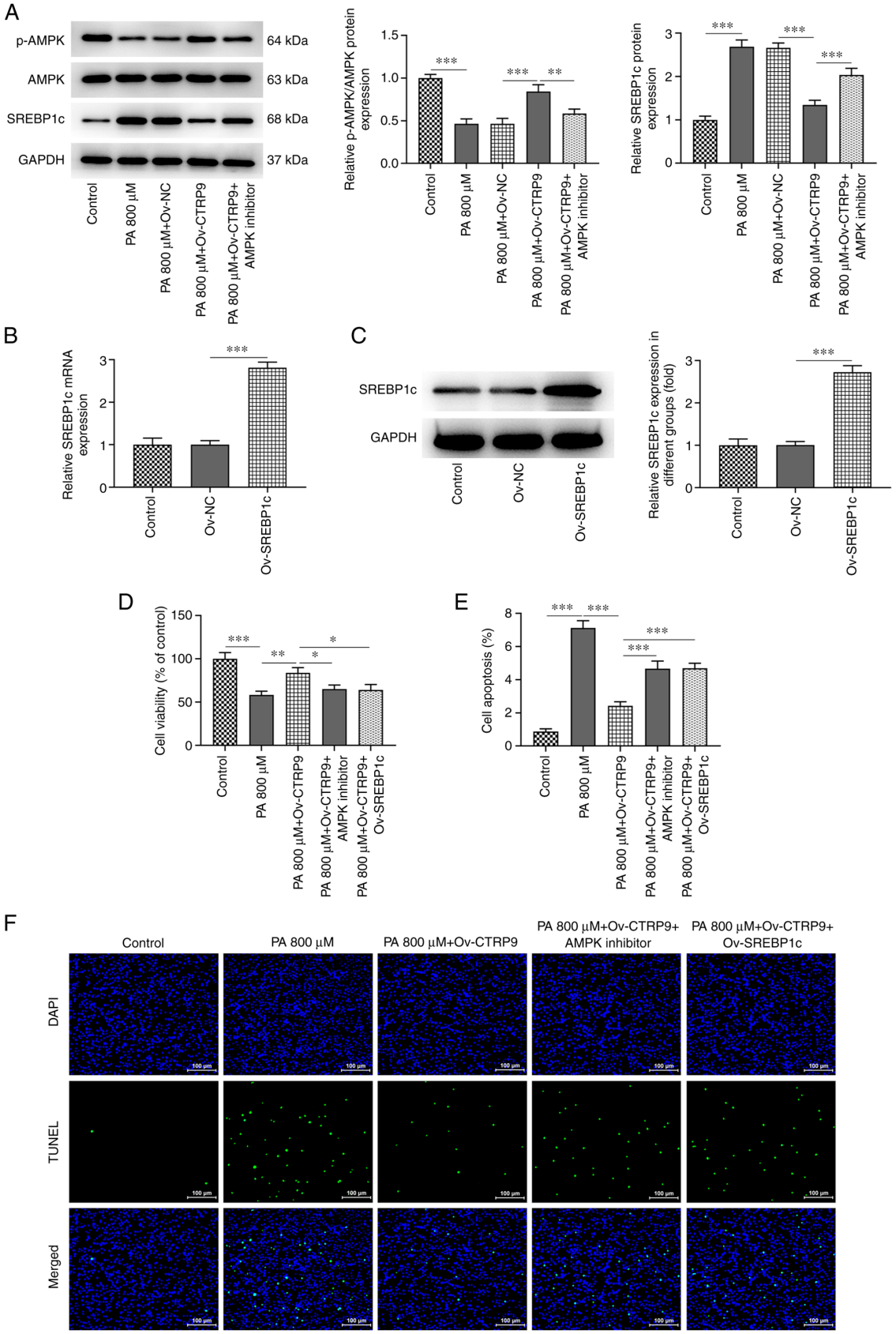

Figure 5. CTRP9 overexpression attenuates PA-induced cell viability impairment and apoptosis in HTR8/SVneo cells through AMPK/SREBP1c signaling. (A) Protein expression levels of p-AMPK, AMPK and SREBP1c in PA-induced HTR8/SVneo cells transfected with Ov-NC or Ov-CTRP9 were measured by western blotting after addition of AMPK inhibitor. SREBP1c overexpression in HTR8/SVneo cells transfected with none, Ov-NC, Ov-CTRP9 was detected by (B) reverse transcription-quantitative PCR and (C) western blotting. (D) Cell viability of PA-induced HTR8/SVneo cells was assessed by cell counting kit-8 in the groups of Ov-CTRP9+AMPK inhibitor and Ov-CTRP9+Ov-SREBP1c. (E) The quantification of (F) apoptosis of PA-induced HTR8/SVneo cells was evaluated by TUNEL assay in the Ov-CTRP9+AMPK inhibitor and Ov-CTRP9+Ov-SREBP1c groups. *P<0.05, **P<0.01 and ***P<0.001. SREBP1c, sterol-regulatory element binding protein 1c; AMPK, AMP-activated protein kinase; CTRP9, C1q/TNF-related protein 9; PA, palmitic acid; p-, phospho-; Ov-, overexpression vector; NC, negative control.

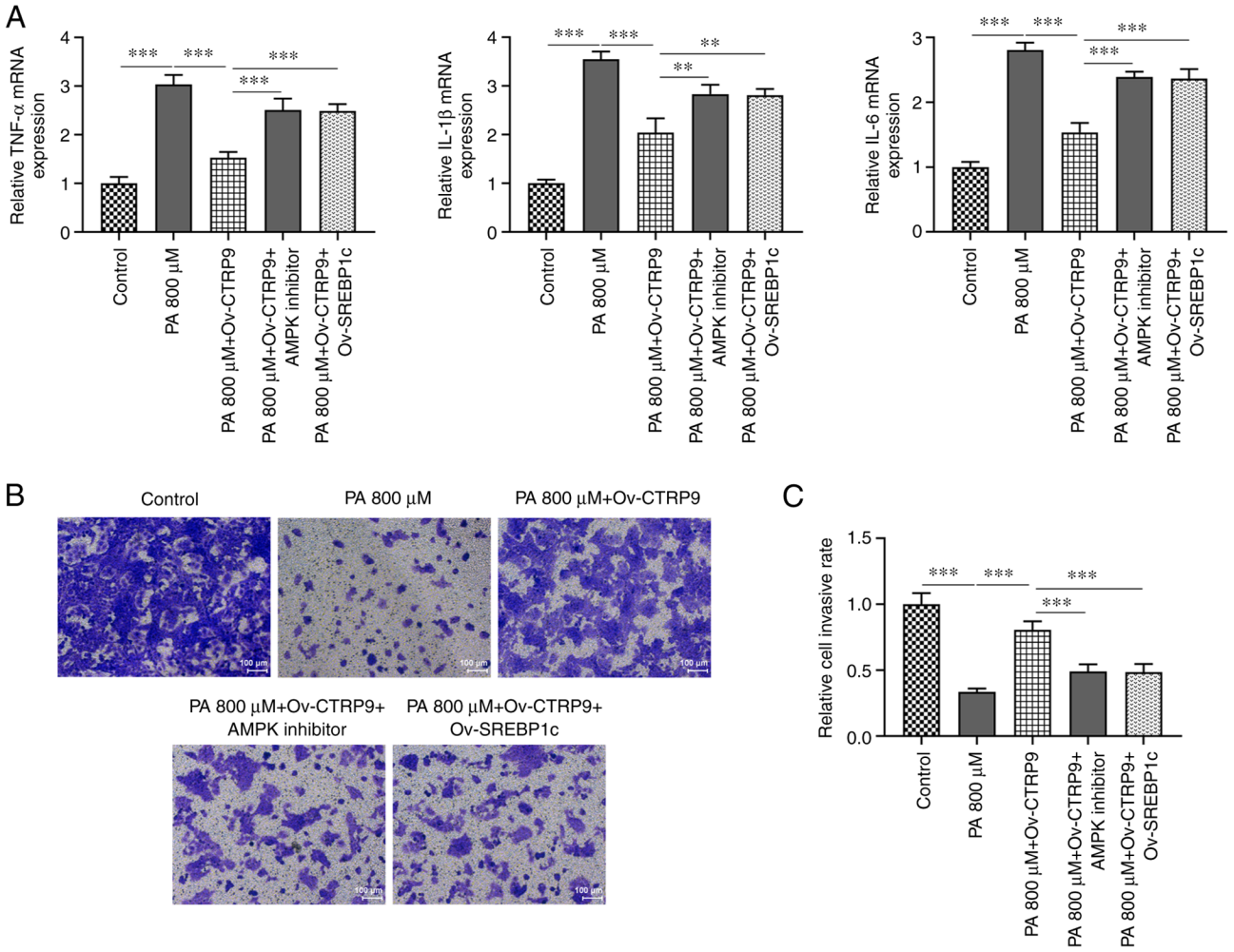

Figure 6. CTRP9 overexpression attenuates PA-induced inflammation and migration impairment in HTR8/SVneo cells through AMPK/SREBP1c signaling. (A) mRNA expression levels of TNF-α, IL-6 and IL-1β in PA-induced HTR8/SVneo cells were measured by reverse transcription-quantitative PCR in the groups of Ov-CTRP9+AMPK inhibitor and Ov-CTRP9+Ov-SREBP1c. (B) Images of cell migration capacity of PA-induced HTR8/SVneo cells stained by crystal violet was detected by Transwell assay in the groups of Ov-CTRP9+AMPK inhibitor and Ov-CTRP9+Ov-SREBP1c and (C) quantified. **P<0.01 and ***P<0.001. Scale bar, 100 μm. SREBP1c, sterol-regulatory element binding protein 1c; AMPK, AMP-activated protein kinase; CTRP9, C1q/TNF-related protein 9; PA, palmitic acid; Ov-, overexpression vector.

expression was reduced in PA-induced cells. By contrast, CTRP9 overexpression attenuated the impairment in cellular activity and apoptosis, and reduced inflammation and the impaired migratory ability of cells. In addition, CTRP9 was shown to activate AMPK to inhibit SREBP1c, and subsequent experiments also demonstrated that CTRP9 overexpression attenuated PA-induced impaired cell activity and apoptosis through AMPK/SREBP1c signaling by reducing the inflammatory and migration impairment.

CTRP9 belongs to the adipokine family and is considered an adipocytokine with cardioprotective properties. Sun (30) reported that CTRP9 could attenuate oxidized-low density lipoprotein (ox-LDL)-induced human umbilical vein endothelial cell (HUVEC) proliferation, apoptosis, migration and angiogenesis. In the present study, CTRP9 was poorly expressed in HTR8/SVneo cells treated with a high PA concentration. However, the elevated cell viability and decreased number of apoptotic cells following CTRP9 overexpression also indicated that CTRP9 overexpression could attenuate PA-induced HTR8/SVneo cell activity impairment and apoptosis.

By contrast, CTRP9 has been reported to inhibit pro-inflammatory factors and reduce inflammation (31). For instance, CTRP9 inhibits the expression of pro-inflammatory cytokines in macrophages (29). CTRP9 significantly reduces ox-LDL-induced TNF-α and monocyte chemoattractant protein-1 expression through the inhibition of the NF-κB signaling pathway in macrophages (32). In addition, IL-6 and TNF-α have been shown to be increased in the placenta of obese women (33,34), as well as to increase the activity of the amino acid transporter A system (35). IL-1β downregulates insulin-stimulated A system transport in primary trophoblasts (36), thereby affecting placental nutrient transport. In the present study, the pro-inflammatory factors TNF-α, IL-6 and IL-1β were upregulated in PA-treated HTR8/SVneo cells, but decreased following CTRP9 overexpression, suggesting that the increased CTRP9 expression level could alleviate inflammation in PA-induced HTR8/SVneo cells. In addition, TNF-α has also previously been shown to promote trophoblast apoptosis in the placenta (37). CTRP9 has previously been demonstrated to inhibit pro-inflammatory factors (31). Consequently, CTRP9 may also reduce apoptosis by inhibiting TNF-α release.

There is abundant evidence that CTRP9 activates AMPK signaling. For example, CTRP9 has been shown to increase AMPK phosphorylation in ischemic hearts (38). In addition, CTRP9 promotes AMPK, Akt and eNOS phosphorylation in HUVECs (39). In the present study, the elevated p-AMPK level in PA-induced HTR8/SVneo cells following CTRP9 overexpression suggested that CTRP9 overexpression did activate the expression of AMPK signaling. SREBP1c has been shown to be involved in pro-inflammatory processes. In a previous study, the activation of SREBP1c in *de novo* lipid synthesis was associated with the increased activation of Akt-mechanistic target of rapamycin (mTOR) signaling (40). The phosphorylation of Akt inhibits mTOR complex 1, leading to the downregulation of SREBP1c and the upregulation of peroxisome proliferator-activated receptor (PPARs), thereby preventing obesity and hepatic steatosis, and reducing the release of inflammatory factors (41). In the present study, SREBP1c expression was markedly decreased in PA-induced HTR8/SVneo cells following CTRP9 overexpression. However, the AMPK inhibitor rescued the decreased SREBP1c expression, which also confirmed that AMPK suppressed SREBP1c expression. In addition, SREBP1c overexpression, similar to AMPK inhibitors, reduced cell viability and promoted apoptosis in PA-induced HTR8/SVneo cells following CTRP9 overexpression, which suggested that CTRP9 overexpression attenuated PA-induced HTR8/SVneo cell viability impairment and apoptosis through AMPK/SREBP1c signaling. Similarly, increased TNF-α, IL-6 and IL-1β levels, and decreased migratory ability in PA-induced HTR8/SVneo cells transfected with Ov-CTRP9, also verified that CTRP9 overexpression mitigated inflammatory cytokine release and migration impairment in PA-treated HTR8/SVneo cells through AMPK/SREBP1c signaling.

In conclusion, in the present study, CTRP9 overexpression ameliorated inflammation, apoptosis and migration impairment in PA-induced HTR8/SVneo cells through AMPK/SREBP1c signaling. Therefore, CTRP9 is a key molecule in the study of the pathogenesis of obesity in pregnant women. However, the present study had certain limitations. The TUNEL assay was conducted to analyze apoptosis; however, the western blot analysis was missing for cleaved caspases 3/7 and/or PARP. Also, TNF-α, IL-1β and IL-6 RT-qPCR data have been presented, but ELISA data is missing. Clinical samples will be used in future to confirm the association between CTRP9, AMPK, SERBP1c and clinical features. Additional cell lines and animal experiments will also be conducted to strengthen the conclusion of this study based on these cell experiments. Since other cellular signals within the trophoblast, such as those of PPARs, STAT3, NF-κB and p38 MAPK, are also involved in regulating the expression of placental nutrient transport proteins, future studies will focus on the association between CTRP9 and the aforementioned cellular signals to further identify the mechanisms regulating the intrinsic cellular functions of obesity in pregnant women. In addition, the association between AMPK and autophagy and pyroptosis will be investigated 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

LL and JZ designed the study. LL performed the experiments with the help of ZG. LL made considerable contributions to the drafting of the manuscript. JZ revised the manuscript for important intellectual content. All authors have read and approved the final manuscript. LL and ZG 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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