Int J Clin Exp Pathol 2013;6(10):1999-2010
www.ijcep.com /ISSN:1936-2625/IJCEP1308019

Original Article

Cyclosporin A promotes proliferating cell nuclear antigen expression and migration of human cytotrophoblast cells via the mitgen-activated protein kinase-3/1-mediated nuclear factor-κB signaling pathways

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Received August 10, 2013; Accepted September 5, 2013; Epub September 15, 2013; Published October 1, 2013

Abstract: Our previous studies have demonstrated that cyclosporin A (CsA) promotes the proliferation and migration of human trophoblasts via the mitgen-activated protein kinase-3/1 (MAPK3/1) pathway. In the present study, we further investigated the role of nuclear factor (NF)-κB in the CsA-induced trophoblast proliferating cell nuclear antigen (PCNA) expression and migration, and its relationship to MAPK3/1 signal. Flow cytometry was used to analyze the expression of PCNA in trophoblasts. The migration of human primary trophoblasts was determined by wound-healing assay and transwell migration assay. Western blot analysis was performed to evaluate the activation of NF-κB p65 and NF-κB inhibitory protein I-κB in human trophoblasts. We found that treatment with CsA promotes PCNA expression and migration of human trophoblast in a dose-associated manner. Blocking of the MAPK3/1 signal abrogated the enhanced PCNA expression and migration in trophoblasts by CsA. In addition, CsA increased the phosphorylation of NF-κB p65 and the inhibitor I-κB in human trophoblasts in a time-related manner. Pretreatment with MAPK3/1 inhibitor U0126 abrogated the phosphorylation of NF-κB p65 and I-κB. Accordingly, the CsA-induced enhancement of PCNA expression and migration in trophoblasts was also decreased. This CsA-induced enhancement in the expression and migration of trophoblasts was abolished by pretreatment with pyrrolidine dithiocarbamate, a specific NF-κB inhibitor. Thus, our results suggest that CsA promotes PCNA expression and migration of human trophoblasts via MAPK-mediated NF-κB activation.

Keywords: Cyclosporine A, trophoblast, PCNA, migration, signal transduction pathway

Introduction

Adequate extravillous trophoblast (EVT) invasion is an essential step in placental formation and is important for fetal growth and well being. For uterine invasion by the trophoblast during placentation in humans, it is necessary that both proliferation and migration/invasion occur in a precisely coordinated manner such that a nutrition route is established between the embryo and the mother. Insufficient trophoblast migration and invasion can result in defective placentation, which is associated with some clinical pathological conditions of pregnancy, such as spontaneous miscarriage, fetal growth restriction, and preeclampsia [1-4]. Factors involved in the proliferation and migration of EVTs include various growth factors and their specific binding proteins, cell adhesion molecules, extracellular matrix proteins, as well as certain matrix metalloproteinases [5-8]. Numerous signaling cascades/proteins at the maternal–fetal interface, such as MAP3/1, focal adhesion kinase (FAK), and Rho-associated kinase (ROCK) signaling, are involved in the precise regulation of this process. Our previous study revealed that the transient activation of MAPK3/1 plays a pivotal role

in the proliferation and uterine invasion of trophoblasts [9]. This signal is also involved in the crosstalk between trophoblasts and decidual stromal cells, which contributes to trophoblast invasion and placentation [10, 11]. Interestingly, the selective immunosuppression cyclosporine A (CsA) can promote the proliferation and invasion of human first-trimester trophoblast cells through MAPK3/MAPK1, suggesting that CsA might be involved in the regulation of the biological behavior of trophoblasts [2, 12-14].

The most powerful immunosuppressant, CsA, has been widely used to prevent organ rejection and treat certain autoimmune diseases. CsA exerts its immunosuppressive effect mainly by binding to cyclophilin A, which inhibits calcium/calmodulin-dependent calcineurin activation and blocks the signaling pathway of the nuclear factor of activated T cells (NFAT) [15]. We have previously reported that CsA downregulates E-cadherin but upregulates titin, CXCL12, and MMP2/9 expression via MAPK3/1 signaling, thus promoting the proliferation and invasion of cytotrophoblasts [2, 16-18]. It remains to be determined whether this signal is also involved in the promotion of trophoblast cell proliferation and migration by CsA.

The transcriptional factor NF-κB was initially characterized as the central regulator of the response to pathogens and viruses. Subsequent studies revealed that NF-κB is activated in a range of human cancers, and that it promotes tumorigenesis via the regulation of target gene expression. NF-κB activation is induced by various inflammatory stimuli and is involved in immune response, cell proliferation, angiogenesis, cell survival, invasion, and epithelial-mesenchymal transition (EMT). Recent reports indicate that NF-κB is activated in embryo implantation and placental development [19-24].

The establishment of pregnancy requires uterine changes that allow for the attachment and implantation of a developing conceptus. Given that the uterine transcriptional profile during early conceptus development resembles a pro-inflammatory response, it is possible that the transcription factor NF-κB is involved in the establishment of pregnancy.

In the present study, we first determined the effects of CsA on the PCNA expression and migration of first-trimester human trophoblasts and then elucidated the involvement of MAPK3/1 signaling and transcription factor NF-κB activation in the CsA-mediated PCNA expression and migration of trophoblasts. These data may contribute to a better understanding of the underlying mechanisms by which CsA promotes cell proliferation and migration, and they may also suggest a potential for CsA therapy in recurrent miscarriage and other pregnancy complications with decreased proliferation and migration/invasiveness of trophoblast.

Materials and methods

Reagents and antibodies

Monoclonal antibodies to I-κBα, phospho-I-κBα (Ser32/36), NF-κB p65 antibody, phospho-NF-κB p65 (Ser536), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signalling Technology. PE-conjugated antibodies against PCNA and the corresponding isotype controls were purchased from (eBioscience, San Diego, CA, USA), and anti-human cytokeratin-7-FITC monoclonal antibody (Chemicon, Temecula, CA, USA). CsA, U0126, and PDTC were obtained from Sigma-Aldrich. Secondary antibodies conjugated with horseradish peroxidase (HRP) were purchased from Kang-Chen Biotech (Shanghai, China).

Tissue collection and isolation of human first-trimester trophoblast cells

Samples of human placental tissues were obtained from 104 healthy women who terminated pregnancy at an early stage (age, 26.53 ± 5.39 years; gestational age at sampling, 51.78 ± 5.89 days, mean ± SD) for non-medical reasons. All pregnancies were confirmed by ultrasound examination and blood test. The exclusion criteria included endocrine, anatomic, genetic abnormalities, infection, and smoking. Placentas with genetic abnormalities were excluded from the study by analyzing the chromosomal karyotype and content of the placental tissues. The tissue samples were placed immediately in ice-cold Dulbecco's modified Eagle's medium (DMEM high d-glucose; Gibco Grand Island, NY, USA), transported to the laboratory within 30 min after the surgery, and washed with Hank's balanced salt solution for the isolation of trophoblasts. All procedures involving participants in this study were

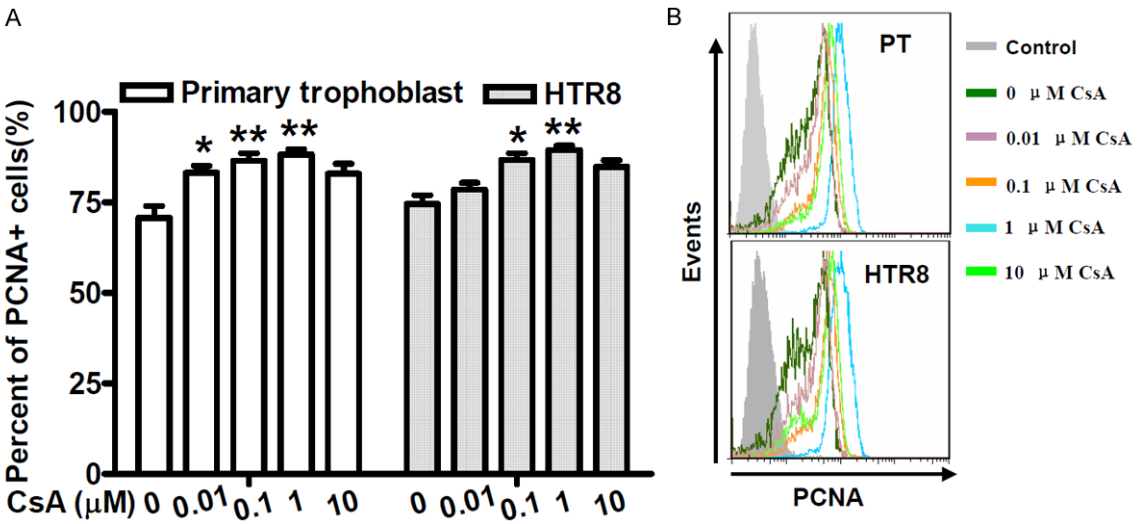

Figure 1. CsA promotes the PCNA expression of human trophoblast in dose-related manner. The freshly isolated primary trophoblasts from humans in early pregnancy and the HTR8 cell line were treated with an increasing dose of CsA for 48 h. The cells were stained with PCNA and cytokeratin-7 and then analyzed using flow cytometry for the level of PCNA. The results were highly reproducible in 4 independent experiments (A), and the picture is a representative one (B). Data represent the mean ± standard error (SE) of 4 independent experiments with a total of 16 villi, performed in triplicate wells with 4 different samples. *P < 0.05, **P < 0.01 compared to the corresponding controls. PT: primary trophoblasts.

approved by the Human Research Ethics Committee of Obstetrics and Gynecology Hospital, Fudan University, and all subjects provided informed consent to collect tissue samples.

The trophoblast cells were isolated by trypsin-DNase I digestion and discontinuous Percoll gradient centrifugation, as described in our previous study [18, 25, 26]. The primarily purified cells were then seeded in the tissue culture plates for further purification on the basis of the differential adherent velocity to eliminate the easily adhering fibroblast cells and unattached leukocytes. This method supplies a trophoblast cells (cytokeratin 7-positive, HLA-G-positive, and vimentin-negative cells, data not shown) of 95% purity. The freshly purified human primary trophoblast cells were seeded on culture plates in a high glucose DMEM containing 20% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY) and 2 mM glutamine, 25 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and incubated in 5% CO_2 at 37°C.

The HTR8/SVneo cell line is a human first-trimester extravillous placental trophoblast cell line characterized by a high ability for migration and invasion; it was cultured in RPMI 1640 complete media supplemented with 10% FBS in 5% CO_2 at 37°C. The HTR-8/SVneo cell line was developed from the explant culture of human first-trimester placenta and immortalized by transfection with a cDNA encoding the simian virus 40 large T antigen. These cells exhibit a high proliferation index and in vitro invasive ability. The HTR8/SVneo cells were cultured in RPMI 1640 complete media supplemented with 10% heat-inactivated FBS in 5% CO_2 at 37°C. Cells at passage 5–10 were used in this study.

Flow cytometry of PCNA expression in trophoblasts

PCNA is a protein of 36 kDa molecular weight that is synthesized in the early G1 and S phases of the cell cycle; thus, it can represent the proliferative ability of cells. In the present study, to determine the cell proliferation, pre-starved trophoblasts were treated with CsA (0–10 μM) for 48 h in the presence or absence of U0126 (30 μM) or/and PDTC (10 μM). To permeabilize the membranes, the digested cells were treated with 70% ethanol and 0.5% Triton X-100 for 20 min and centrifugation followed by blocking with 10% FBS. The collected cells were incubated with mouse anti-human PCNA-phycoerythrin (PE) monoclonal antibody or mouse IgG2a-PE isotype in the dark for 1 h and imme-

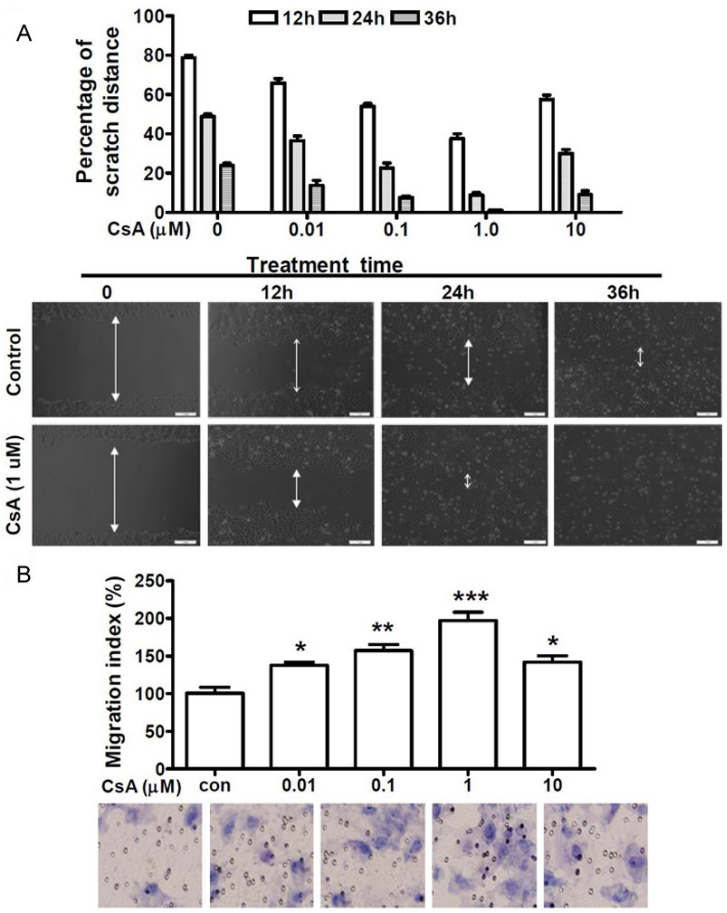

Figure 2. CsA promotes human trophoblast migration in a dose-related manner. A: The wound-healing assay in HTR8/SVneo cells treated with different concentrations of CsA. An open furrow was generated by scratching cells at 90% confluent by using a pipette tip. Treatment with different concentrations of CsA speeded up the confluency. The distance between the furrow edges in the control or CsA-treated cells in 3 independent experiments was measured and presented graphically as percentage of the initial distance (0 h). Data represent the mean ± standard error (SE) of 3 independent experiments with a total of 15 villi, performed in triplicate wells with 3 different samples. *P < 0.05, **P < 0.01, and ***P < 0.001 compared to the corresponding controls (upper). Representative images obtained along the wounds, at 0, 12, 24, and 36 h of stimulation with 1.0 μM of CsA and vehicle were presented (lower). B: First-trimester human trophoblast cells were cultured on transwell filters for 48 h in media with increasing concentrations of CsA. Cells that migrated to the lower surface of the insert were counted using light microscopy. In parallel experiments, the same numbers of cells were seeded in the 24-well plates with the same treatment protocol. The total cell numbers was counted after 48 h of the respective treatment. The migration index was calculated by the following formula: Migration Index = Mtest/Ntest ÷ Mcon/Ncon × 100, where Mtest represents the number of migrated cells under different CsA treatments, Ntest represents the total number of cells subjected to the respective CsA treatments, Mcon represents the number of migrated cells under control treatment, Ncon represents the number of total cells under the corresponding control treatment. Data represent the mean ± standard error (SE) of 3 independent experiments with a total of 16 villi, performed in triplicate wells with 3 different samples. *P < 0.05, **P < 0.01 compared to the corresponding controls (Upper). Representative images obtained in the transwell assay are presented (lower).

diately analyzed using a by flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA). The same sample was measured by flow cytometry twice, and the experiments were repeated at least 3 times.

Transwell cell migration assay

Cell migration was determined by their ability to cross the 8-μm pores of polycarbonate membranes (6.5-mm filters, 8-μm pore size; Corning, Corning, NY) fitted at the bottom of transwell chambers. In brief, the purified human first-trimester trophoblasts cells were plated in the upper chamber at a density of 1.0×10^5 cells/well in 200 μL DMEM with 2% FBS or various concentrations of CsA (0, 0.001, 0.01, 0.1, 1.0, and 10 μM), respectively. U0126 or PDTC was added to wells as indicated in **Figures 3B** and **5B**. The lower chamber was filled with 600 μL DMEM with 10% FBS. After 48 h of incubation at 37°C, cells from the upper surface of the filter were completely removed with gentle swabbing. The remaining migrated cells were fixed for 10 min at room temperature in methanol and stained with hematoxylin. The stained cells migrating to the reverse side of the membrane were counted in 10 randomly selected, non-overlapping fields at 200 × magnification, by using a light microscope. In the parallel experiments, we seeded the same number of trophoblasts with the same treatment in different plates. Cell migration ability was determined by calculating the ratio of migrated cell numbers to the total cell numbers. The migration index was calculated as the ratio of the percentage of cell migration from the various treatments to that of the vehicle.

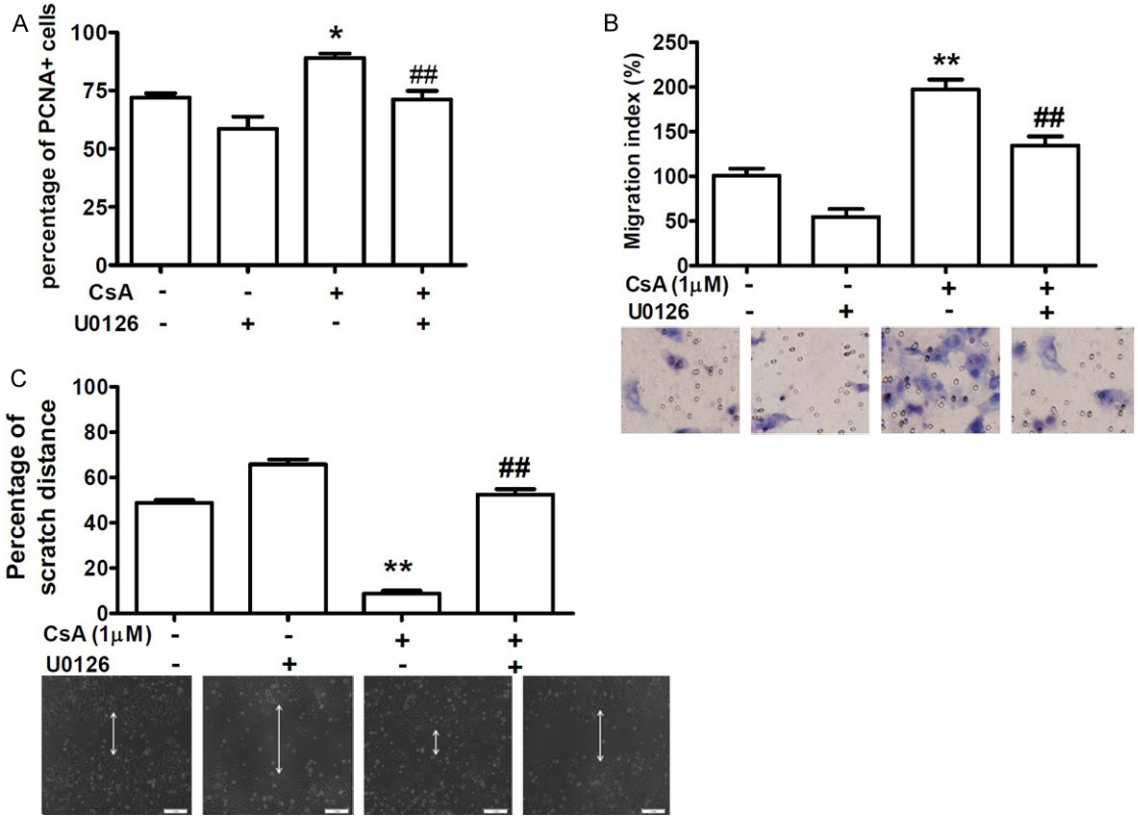

Figure 3. Blocking MAPK3/1 signaling inhibits CsA-induced PCNA expression and migration of trophoblasts. A: The expression of PCNA in trophoblasts was determined by flow cytometry. PCNA expression of human trophoblasts in CsA-treated group was significantly increased, and an addition of U0126 markedly inhibited the CsA-induced PCNA expression level. *P < 0.05, **P < 0.05, and ***P < 0.01 compared with the control; Error bars depict SEM of 4 independent experiments with 15 villi, performed in triplicate wells with 3 different samples. B: First-trimester human trophoblasts were cultured on transwell filters for 48 h in media containing vehicle or 1.0 μM CsA and/or 30 μM U0126. Cells that migrated to the lower surface of the insert were counted using light microscopy (lower). The total cell numbers were also determined in parallel experiments. The migration of cells under different conditions was normalized to control cells. Data represent the mean ± SE of 3 independent experiments with a total of 16 villi, performed in triplicate wells with 3 different samples (upper). *P < 0.05 compared to control; #P < 0.05 compared to the group treated with CsA. C: Wound-healing experiments were used to measure the vertical migration of human trophoblast cells. Values represent mean ± SE of 4 independent experiments, performed in triplicate wells. *P < 0.05 compared to control; #P < 0.05 compared to the group treated with CsA.

Wound-healing experiments

HTR-8 cell migration was measured by determining the ability of the cells to move into an acellular space, as described previously [27]. Briefly, the linear scratches were made when HTR-8/SVneo cells were grown to 90% confluence. The cells were allowed to grow, and the width of the wound was monitored at the indicated time of wound healing after treatment under the phase-contrast microscope. Photographs were taken and the relative distance traveled by the cells at the acellular front was measured at 5 different marked crossed locations per well.

Western blot analysis

Western blotting was performed as previously described (14). Membranes were incubated overnight at 4°C in primary antibodies (anti-p65 1:1000; anti-phospho-p65 (Ser536) 1:1,000; anti-I-κB 1:1,000; anti-phospho-I-κB (Ser32/36) 1:1,000; anti-GAPDH 1:20,000), followed by incubation (RT, 1 h) with HRP–conjugated anti-rabbit or anti-mouse secondary antibody (1:5,000). Peroxidase activity was visualized with ECL kit (Amersham Biosciences, Piscataway, NJ). Results were scanned and analyzed using Scion Image software (Scion, Inc., Frederick, MD).

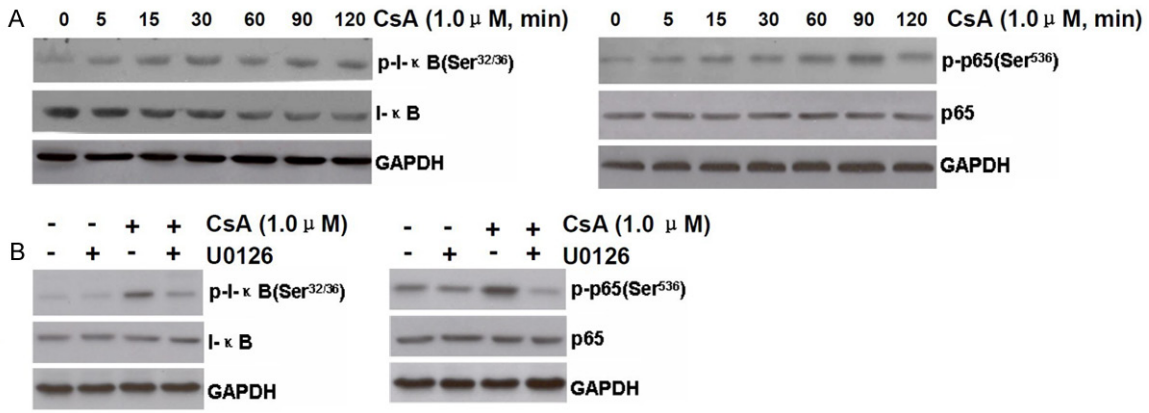

Figure 4. CsA activates NF-κB in human trophoblasts via MAPK3/1 signaling. A: Human trophoblast cells were serum starved overnight and then treated with 1.0 μM CsA at a different time. Western blot was used to analyze the total and phosphorylation levels of NF-κB p65 and I-κB. CsA induces NF-κB transactivation in human trophoblasts in time-related manner. B: Human trophoblast cells were serum starved overnight and then treated with vehicle, 1.0 μM CsA for 90 min. Some well were pre-added with 30 μM U0126 for 30 min. Protein expression were analyzed by Western blot. Blockade of MAPK3/1 inhibited the CsA-induced NF-κB transactivation. These pictures are representatives of 3 individual experiments with 16 villi.

Statistical analyses

The values are given as the mean ± SE of at least 3 independent experiments. Data were analyzed by analysis of variance and Newman-Keuls multiple comparison tests. The significance level was set at $P < 0.05$.

Results

Effects of CsA treatment on the PCNA expression of human trophoblasts

The ability of CsA to promote human trophoblast proliferation was investigated through PCNA analysis by flow cytometry. As shown in **Figure 1**, within the concentration range of 0.01-1.0 μM, CsA increases PCNA expression of trophoblasts in a dose-dependent manner, peaking at 1.0 μM. CsA also affected the PCNA expression of HTR8 in a similar manner.

CsA promotes the migration of human trophoblasts and the HTR8/SVneo cell line

To determine the effect of CsA on the migration of human trophoblast cells, human first-trimester extravillous placental trophoblast cell line HTR8/SVneo and primary trophoblast were used in the wound-healing assay and transwell migration assay, respectively. The results shown in **Figure 2A** indicate that the gap of the wound was gradually filled by migrating cells treated with different concentrations of CsA. Wound closure was significantly reduced when cells were incubated with 0.1 and 1 μM of CsA for 12 h, and the gap was almost completely closed after treatment with 1.0 μM of CsA for 24 h. In contrast, in the case of the control cells, the speed of wound closure was much slower and the gap remained widely open even after 36 h of culture. In addition, transwell assays with different concentrations of CsA-treated cells for 48 h showed a significant increase in the number of cells migrating through the polycarbonate membranes in a concentration-related manner, reaching a peak at 1.0 μM of CsA and declining at 10 μM of CsA (**Figure 2B**). These studies suggest that CsA can stimulate the migration of the first-trimester trophoblast cells in an appropriate concentration range by inducing alterations in both the horizontal and vertical directions. Because CsA increases both cell migration and proliferation, we counted the total cell number in each experiment to exclude the possibility that the improved migration of trophoblasts by CsA was due to enhanced proliferation. The data revealed that the migration index (migrated cell number normalized to total cell number) of human first-trimester trophoblasts was 1.39-fold, 1.57-fold, 1.97-fold, and 1.42-fold higher in the CsA concentrations of 0.01, 0.1, 1.0, and 10 μM, respectively, compared with the migration index for the vehicle control (**Figure 2B** upper). Hence, CsA treatment appeared to predominantly promote cell migration rather than the proliferation of trophoblasts.

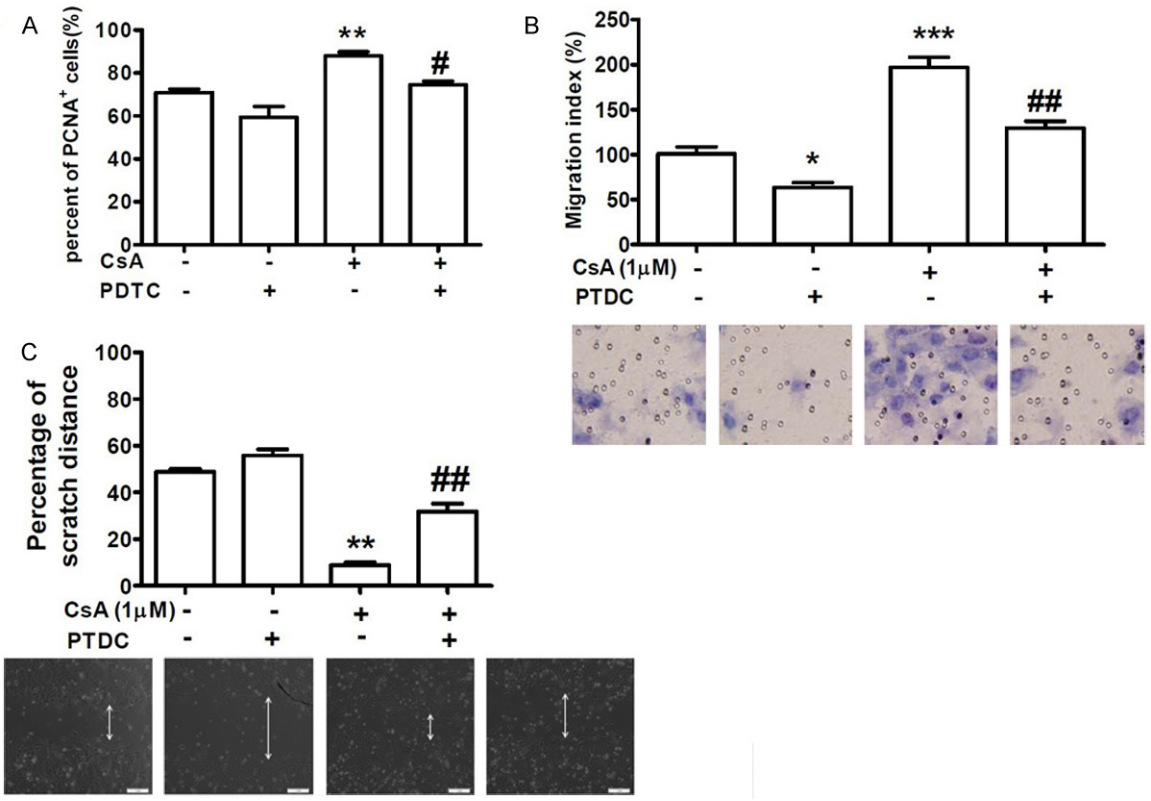

Figure 5. CsA-mediated PCNA expression and migration of human trophoblasts is dependent on NF-κB activation. A: The expression of PCNA in trophoblasts was determined by flow cytometry. PCNA expression of human trophoblasts in CsA-treated group was significantly increased in vitro, and an addition of PTDC markedly inhibited the CsA-induced PCNA expression level (A). **P < 0.01 compared with the control; #P < 0.05 and ##P < 0.01 compared with the CsA group. Values represent mean ± SE of 4 independent experiments with a total of 16 villi, performed in triplicate wells with 4 different samples. B: First-trimester human trophoblasts were cultured for 48 h on transwell filters in media with 1.0 μM of CsA and/or 10 μM of PTDC. Cells that migrated to the lower surface of the insert were counted using light microscopy. The migration of cells under different conditions was normalized to that of the control cells. PDTC inhibited the CsA-induced migration of trophoblasts. Data represent the mean ± SE of 3 independent experiments with a total of 14 villi, performed in triplicate wells with 3 different samples. *P < 0.05, **P < 0.05 compared to control; #P < 0.05, ##P < 0.01 compared to the group treated with CsA. C: Wound-healing experiments were used to measure the vertical migration of human trophoblast cells. Data represent the mean ± SE of 3 independent experiments, performed in triplicate wells with 3 different samples. *P < 0.05, **P < 0.05 compared to the control; #P < 0.05, ##P < 0.01 compared to the group treated with CsA.

Blocking MAPK3/1 signaling inhibited CsA-induced PCNA expression and migration of human trophoblast cells

Our previous studies have shown that CsA could activate the MAPK3/1 signaling in human trophoblasts [2, 12-14, 16-18, 28]; therefore, we used the MAPK3/1-specific inhibitor U0126 to directly analyze the role of MAPK3/1 in CsA-regulated PCNA expression and migration of human trophoblasts. The results shown in **Figure 3A** indicate that U0126 significantly inhibited the CsA-induced increase in PCNA expression of human trophoblast, suggesting that CsA promotes PCNA expression of human trophoblast cells via MAPK3/1 signaling. Similarly, we observed the effect of MAPK3/1 signaling on the CsA-induced migration. The transwell migration assay and wound-healing assay were used. The results shown in **Figure 3B** and **3C** suggest that blocking MAPK3/1 with U0126 inhibited the CsA-induced trophoblast cell migration in both the horizontal and vertical directions.

CsA activates NF-κB through MAPK3/1 signaling

NF-κB has been reported to be activated in embryo implantation and placental develop-

ment [21, 24]. Thus, we sought to investigate whether CsA could activate NF-κB in trophoblast cells. Western blot analysis was performed to determine the amount of total and phosphorylated NF-κB p65 and I-κB of trophoblasts after treatment with 1 μM CsA for different time periods. The results shown in **Figure 4A** indicate that CsA stimulated I-κB phosphorylation in a time-dependent manner, which was accompanied by the subsequent degradation and NF-κB p65 phosphorylation in human trophoblasts. The maximal phosphorylation level of p65 was observed at 90 min at a CsA concentration of 1.0 μM. We further sought to determine whether the CsA-induced activation of I-κB and NF-κB occurred via MAPK3/1 signaling. As shown in **Figure 4B**, pretreatment with U0126 eliminated I-κBα phosphorylation and CsA-induced NF-κB activation. These data indicate that activation of MAPK3/MAPK1 signaling was important for CsA-induced NF-κB transactivation of human trophoblasts.

Blocking NF-κB inhibited CsA-promoted PCNA expression and migration of trophoblasts

Having established the elevation of NF-κB activity in trophoblast cells by CsA treatment, we next investigated the potential for the inhibition of NF-κB to attenuate the PCNA expression and migration of trophoblast cells. Toward this end, we used a commercially available NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC), to pharmacologically block NF-κB activity. Inhibition of NF-κB activity by PDTC after CsA treatment for 48 h resulted in a change in PCNA expression, as determined by flow cytometry analysis (**Figure 5A**). Importantly, the inhibition of NF-κB by PDTC led to a marked decrease in the migration of trophoblast cells compared with that of control-treated cells in the transwell migration assay and wound-healing assay under 48 h of CsA treatment (**Figure 5B**). When the cells were treated with PDTC alone, they also showed significantly lower PCNA expression and migration ability than their respective controls (**Figure 5A** and **5B**). Importantly, despite these changes in PCNA expression and cell migration attributable to NF-κB blockade, we did not observe complete inhibition by PDTC, which suggests the existence of NF-κB-independent biochemical events that also contribute to the CsA-induced PCNA expression and migration in trophoblast cells.

Discussion

The migration and invasion of placental trophoblast cells into the maternal decidua is crucial for a successful pregnancy. Trophoblast migration and invasion are dependent on proliferating EVT cells, which are located proximal to the placental villous cell column, for the continuous generation of invasive cells during placental development. Insufficient migration and shallow invasion of cytotrophoblasts into the maternal deciduas is linked to recurrent spontaneous abortion, fetal intrauterine growth restriction, and preeclampsia [4]. The proliferation, invasion, and differentiation of trophoblast cells during implantation is a tightly controlled process of intercellular signaling that is mediated by cytokines, growth factors, and hormones. However, the relevant mechanisms are not well understood. The improvement of the pregnancy outcomes related to cytotrophoblast function disorders remains a challenge to researchers and doctors practicing reproductive medicine. Unfortunately, few effective treatments have been developed thus far.

The calcineurin inhibitor CsA is a potent immunosuppressive agent that is the pharmacologic cornerstone for solid organ transplantation. CsA prevents the activation of lymphokine genes essential for T-cell proliferation by disrupting calcium-dependent signal transduction pathways in leukocytes [15]. Emerging evidence indicates that CsA may exert potent effects on a wide range of cell types, including endothelial cells [25], vascular smooth muscle cells [26], cardiac myocytes [29], epithelial cells [30], and tumor cells [31], by regulating disparate biological functions depending on the cell type and dosage of CsA. Our previous studies have demonstrated that administration of low-dose CsA in the early stages of pregnancy successfully increases the fetal survival rate of abortion-prone CBA/J × DBA/2 matings [2]. Further studies have suggested that CsA might exert its protective effect on pregnancy by promoting maternal–fetal tolerance and improving the biological function of human first-trimester trophoblasts. CsA administration at low concentrations (0, 0.0001, 0.001, 0.01, 0.1, and 1.0 μM) has been reported to increase the proliferation and invasion of human first-trimester cytotrophoblasts and inhibit apoptosis of these cells [14, 17]. Although potential mechanisms have been intensely investigated, the exact

regulation of CsA on trophoblasts is not fully elucidated.

PCNA, which is also known as cyclin, is a 36-kDa and multifunctional protein, and it is highly expressed in the G1 and S phases of the dividing cells. PCNA is an essential component of the DNA replication mechanism and is considered as a marker for the actively replicated cells. Analysis of PCNA provides valuable information on the state of cell proliferation. As an extension of our previous studies, the present data showed that CsA promoted PCNA expression and migration of human trophoblasts. Blocking MAPK3/1 signal abrogated the CsA-induced enhancement in PCNA expression and migration in trophoblasts. In addition, CsA activated p65NF-κB in human trophoblasts, which was accompanied by the phosphorylation and subsequent degradation of I-κB. Pretreatment with the MAPK3/1 inhibitor U0126 abrogates the phosphorylation of p65NF-κB and I-κB. Accordingly, the enhanced PCNA expression and migration in trophoblasts induced by CsA were also decreased. Furthermore, pretreatment with PDTC, a specific NF-κB inhibitor, abolished the CsA-induced enhancement in PCNA expression and migration in trophoblasts. These results suggest that CsA promotes PCNA expression and migration of human trophoblasts via the MAPK3/1-mediated NF-κB activation. These results may help provide a rationale to develop a novel therapeutic strategy for pregnancy disorders from insufficient trophoblast function.

MAPK pathways are evolutionarily conserved signaling modules through which cells transduce extracellular signals into intracellular response. MAPK3 and MAPK1 are widely expressed and markedly activated in the villous cytotrophoblasts of early-stage embryos, thereby suggesting that the activated MAPK3/1 cascade might be involved in the proliferation and invasion of these cells [32]. A previous study confirmed that the disruption of the MAPK1 locus leads to early embryonic lethality in mouse development after implantation [33]. Consistent with these studies, our data indicated that the activation of the MAPK3/1 cascade was involved in the CsA-induced improvement in trophoblast cell growth and migration. Our previous studies have demonstrated that CsA can promote proliferation and invasion in human trophoblasts through MAPK3/1 signaling [12-14, 28]. Similarly, blocking MAPK3/1 signaling using U0126 attenuated the CsA-increased PCNA expression and cell migration, which suggests that MAPK3/1 signaling is involved in the CsA-induced proliferation and migration of human trophoblasts.

Initially, NF-κB was characterized as the central regulator in inflammatory and immune responses. Subsequent studies revealed that NF-κB modulates the expression of genes with diverse functions. The activity of NF-κB is regulated by the NF-κB inhibitory protein I-κB, which binds to and sequesters NF-κB family members in the cytoplasm. NF-κB binds to its target gene promoters as homodimers or heterodimers composed of 5 subunits: RELA (p65), RELB, c-REL, NF-κB1 (p105/p50), and NF-κB2 (p100/p52). NF-κB activation is exclusively regulated by 2 independent pathways. When the NF-κB pathway is activated, I-κB is phosphorylated by I-κB kinase (IKK), which phosphorylates I-κB. Phosphorylated I-κB is subjected to ubiquitination and proteasome-mediated degradation, which results in the translocation of NF-κB to the nucleus. NF-κB is closely involved in the progression of EMT and tumor metastasis [34-36]. NF-κB is a ubiquitous transcription factor regulated by many stimuli, including hypoxia, cytokines, and chemotherapeutic drugs, and has recently emerged as a target for cancer treatment. Establishment of pregnancy requires uterine changes to allow for the attachment and implantation of a developing conceptus; these changes display inflammatory characteristics [37, 38]. A typical feature of placental development is the trophoblast function similar to tumor cells, and the tumor-like properties of trophoblasts are critical for appropriate materno-fetal interactions [39].

Since CsA can activate MAPK3/1 and NF-κB signaling, both of which might be involved in the proliferation and migration of human trophoblasts, we further determined the relationship between MAPK3/1 activation and the transactivation of NF-κB by CsA. This study showed that CsA-activated NF-κB transcription can be abrogated by the MAPK inhibitor U0126, suggesting that MAPK3/1 signaling is the upstream event in CsA-induced NF-κB activation.

In summary, the present data showed that CsA promotes PCNA expression and migration of

human cytotrophoblast cells via the MAPK3/1-mediated NF-κB signaling pathways. Considering the importance of trophoblast functions for placentation and normal pregnancy, the results of this study indicated that CsA has potential for use as a drug for treating complications of pregnancy resulting from deficient trophoblast function. We are currently investigating the safety and long-term consequences of CsA treatment on pregnancy outcome. Further research is required to determine the clinical application of CsA in pregnancy complications.

Acknowledgements

This work was supported by National Nature Science Foundation of China (NSFC) 81070537 (to MRD), NSFC 31171437 (to MRD), and the Shanghai PuJiang Talent Program 10PJ1401600 (to MRD); Shanghai Shenkang Foundation SHDC12010122 (to DJL), NSFC30910103909 and NSFC 31270969 (to DJL), the Key Project of Shanghai Basic Research 12JC1401600 (to DJL); The Foundations from Shanghai Science and Technology Committee 134119a4300 (to LPJ).

Disclosure of conflict of interest

None.

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