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E-Cadherin, as a Negative Regulator of Invasive Behavior of Human Trophoblast Cells, Is Down-Regulated by Cyclosporin A Via Epidermal Growth Factor/Extracellular Signal-Regulated Protein Kinase Signaling Pathway[1]

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ABSTRACT

Our previous study has demonstrated cyclosporin A (CsA) promotes the invasiveness of human first-trimester trophoblast cells. In the present study, we further investigated the intracellular signaling pathway responsible for the improvements in CsA-induced invasiveness of human trophoblast cells. We showed that CsA down-regulated E-cadherin transcription and translation in human primary cultured trophoblast cells and choriocarcinoma cell line JEG-3. U0126, an inhibitor of extracellular signal-regulated protein kinase (ERK), attenuated the CsA-induced transcriptional repressor SNAI2 (also called Slug) expression and restored E-cadherin expression inhibited by CsA in JEG-3 cells. We further demonstrated that CsA amplified epidermal growth factor (EGF)-stimulated EGF receptor (EGFR) tyrosine phosphorylation in JEG-3 cells, and inhibition of EGFR tyrosine phosphorylation by AG1478, an EGFR tyrosine kinase inhibitor, abolished the down-regulation of E-cadherin by CsA through ERK signaling pathway. Moreover, our data showed that E-cadherin expression was negatively correlated to the invasiveness of JEG-3 cells, and CsA could reverse the decreased invasiveness of JEG-3 cells that resulted from E-cadherin overexpression. In conclusion, these observations indicate that CsA may decrease E-cadherin expression via EGFR/ERK signaling pathway and, ultimately, contribute to the invasiveness improvement of human trophoblast cells.

cyclosporin A, E-cadherin, EGFR, ERK, invasion, signal transduction, trophoblast, trophoblast cells

INTRODUCTION

Cyclosporin A (CsA) is a potent immunosuppressive agent that has been widely used to prevent organ rejection and to treat various autoimmune diseases. CsA exerts its immunosuppressive effect mainly by binding to cyclophilin A, which inhibits calcium/calmodulin-dependent calcineurin activation and blocks the nuclear factor of activated T cells (NFAT) signaling pathway [1–3]. The blockage of NFAT by CsA leads to the inactivation of lymphokine genes essential for T-cell proliferation and activation, resulting in immunosuppression. Recent studies have shown that in addition to leukocytes, CsA also exerts potent effects on many distinct types of cells and, thus, regulates disparate biological functions [4–8]. Evidence is emerging that CsA regulates cell proliferation and invasion not only through calcium/calcineurin/NFAT cascade but also through other signal pathways, such as extracellular signal-regulated protein kinase (ERK) [7, 9] and transforming growth factor β [10]. A more comprehensive signal transduction network of CsA needs to be elucidated.

Our previous study in vivo has demonstrated that administration of CsA at the early stage of pregnancy in mice successfully decreases the fetal resorption rate in the abortion-prone CBA/J×DBA/2 matings [11]. Further data in vivo have shown that administration of CsA induces maternal hyporesponsiveness to paternal antigen and expands the maternal peripheral regulatory T cells, which are required for the maternal immune tolerance of the fetus [12]. Meanwhile, the in vitro studies have provided evidence that CsA at low concentrations promotes the invasion of human first-trimester trophoblast cells [12–14]. During early pregnancy, the invasion of human trophoblast cells into the uterus is one of the essential events in the establishment of a successful pregnancy. Aberrant invasion of human trophoblast cells is thought to play a role in the pathogenesis of a variety of pregnancy-related disorders, including preeclampsia, spontaneous abortion, and trophoblastic disease [15–17]. Therefore, CsA may improve the outcome of pregnancy, in part, through increasing the invasiveness of human trophoblast cells. Our previous study has shown that the ERK signaling pathway is involved in the CsA-induced improvement of the invasiveness of trophoblast cells. However, the molecular mechanisms by which CsA activates the ERK cascade and modulates the downstream target molecule remain largely unknown.

E-cadherin, an important member of the cadherin family, is usually expressed in epithelial cells and is involved in calcium-dependent cell-cell adhesion. The highly conserved cytoplasmic domain of E-cadherin interacts with α-, β-, and γ-catenins, mediating connections between E-cadherin and cytoskeleton

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TABLE 1. Sequences of the primers.

Name	Sequence	Size (bp)
E-cadherin	5′-AGTGACGAATGTGGTACCTTTTGA-3′ (sense) 5′-TACTGAATGGTCCATTGGGGCACTCGCC-3′ (antisense)	507
SNAI2	5′-GTGATTATTTCCCCGTATCTCTAT-3′ (sense) 5′-CAATGGCATGGGGGTCTGAAAG-3′ (antisense)	292
GAPDH	5′-ACCACAGTCCATGCCATCAC-3′ (sense) 5′-TCCACCACCCTGTTGCTGTA-3′ (antisense)	452

[18, 19]. E-cadherin is correlated to the invasion and metastasis of many types of tumors. Repression of E-cadherin gene expression is a crucial step in enabling tumor cells to migrate and invade the surrounding tissues [20, 21]. In the placenta, E-cadherin mediates a strong intercellular interaction between adjacent trophoblast cells, as it does in all other normal epithelia [22, 23]. E-cadherin expression is moderate in the trophoblast cells in the superficial portion of the placental site, whereas those cells deep in the myometrium exhibit weak or undetectable E-cadherin expression [24, 25]. During the first trimester of pregnancy, trophoblastic E-cadherin expression is temporarily down-regulated so that the trophoblast cells possess a potential for migration and invasiveness [26]. These findings suggest that E-cadherin may act as a key modulator for the invasive behavior of human trophoblast cells.

In the present study, we show that CsA down-regulates E-cadherin expression via the epidermal growth factor receptor (EGFR)/ERK signaling pathway and contributes to the improvement in invasiveness of JEG-3 cells. These data describe a detailed signaling pathway responsible for the CsA-induced invasiveness improvement of JEG-3 cells, which may provide a clue for a better understanding of the intracellular signaling pathway network of CsA in human trophoblast cells and contribute to the therapeutic applications of CsA in miscarriage and other complications of pregnancy with decreased invasiveness of trophoblast cells.

MATERIALS AND METHODS

Reagents and Antibodies

Monoclonal antibodies to ERK, phosphorylated-ERK, SNAI2 (Slug), EGFR, PY20, cytokeratin 7 (CK-7; official symbol KRT7), vimentin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology. Monoclonal antibody to E-cadherin was purchased from BD Transduction Laboratories. Epidermal growth factor (EGF), U0126, ionomycin, and AG1478 were obtained from Sigma-Aldrich. Secondary antibodies conjugated with horseradish peroxidase (HRP) were purchased from Kang-Chen Biotech.

Isolation and Primary Culture of Human First-Trimester Trophoblast Cells

The first-trimester human placentas (6–9 wk of gestation) were obtained from normal pregnancies that were terminated for nonmedical reasons. The present study was approved by the Human Research Ethics Committee of the Obstetrics and Gynecology Hospital, Fudan University, and each patient completed a signed, written consent form. The trophoblast cells were isolated by the trypsin-DNase I digestion and discontinuous Percoll gradient centrifugation, as described previously [27]. In this way, 95% purity of trophoblast cells was obtained. The isolated human primary trophoblast cells were cultured in Dulbecco modified Eagle medium/high-glucose complete medium (2 mM glutamine, 25 mM Hepes, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin) supplemented with 15% fetal bovine serum (FBS; Gibco) and incubated in 5% CO_2 at 37°C. Vimentin and KRT7 were employed as markers to identify the purity of trophoblast cells. KRT7 is currently regarded as the best marker for trophoblast cells [28]. The trophoblast cells were positive for KRT7 and negative for vimentin. The purity of the isolated trophoblast cells was greater than 95%. The human choriocarcinoma cell line JEG-3 was cultured in 1640 complete medium supplemented with 10% FBS in 5% CO_2 at 37°C.

RNA Isolation and RT-PCR

Total RNA was isolated using the Trizol system (Watson Biotechnologies) according to the manufacturer's guidelines. RT-PCR was performed to quantify the mRNA level of E-cadherin. Oligo dT primer and Moloney murine leukemia virus reverse transcriptase (MMLV-RTase; Takara) were used for the first-strand synthesis. The cDNA products (2 μl) were mixed with Taq DNA polymerase (SABC), 50 pmol/L of each appropriate primer, 200 μmol/L of each dNTP in a reaction buffer containing 10 mmol/L of Tris-HCl (pH 8.3), 50 mmol/L of KCl, 0.01% (w/v) bovine serum albumin (BSA), and 2 mmol/L of $MgCl_2$ in a final volume of 100 μl. The primers used for the detection of E-cadherin, SNAI2, and GAPDH were indicated in Table 1. The samples were amplified for 30 cycles at cyclic temperatures of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min. PCR products were analyzed through 2% agarose gel electrophoresis and ethidium bromide staining. The band area of E-cadherin was measured and normalized to that of GAPDH, and then the specific E-cadherin mRNA level was estimated.

Transfection of Small Interfering RNA and Plasmid

E-cadherin small interfering RNA (siRNA) transfection was performed as described previously [29] with slight modification. JEG-3 cells were seeded in 24-well plates at 1×10^5 cells/well and transfected with siRNA with a specific target sequence for human E-cadherin, GCAGAAUUGCUCACAUUUC, or with nontargeting control (Eurogentec) in a final concentration of 20 μM using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's recommendations. At 48 h posttransfection, these cells were subjected to Western blotting and cell-cell adhesion assay and cell invasion assay.

The plasmid pcDNA3.0-wt E-cad containing human full-length E-cadherin cDNA was kindly supplied by Dr. Cara J. Gottardi (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). The plasmid pcDNA3.0-wt E-cad was purified and transfected into 3×10^5 JEG-3 cells using Lipofectamine 2000 reagent according to the manufacturer's recommendations.

Western Blot Analysis

JEG-3 cells were lysed in 1× SDS lysis buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 1 mM PMSF, and 1 mM Na_3VO_4), as described previously [30]. Equal amounts of total protein were loaded on SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Millipore). After blocking with 5% BSA in PBS (containing 0.05% Tween 20), the membrane was incubated with specific primary antibodies, followed by incubation with HRP-conjugated secondary antibodies (Kang-Chen Biotech). The protein bands of interest were visualized by fluorography using an enhanced chemiluminescence system (Perfect Biotech).

Immunofluorescence

Human primary cultured trophoblast cells were grown on glass coverslips, fixed in 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked with 3% BSA in PBS for 30 min. Specimen was incubated with monoclonal E-cadherin antibody (1:50 dilution in blocking solution) and monoclonal KRT7 antibody (1:50 dilution in blocking solution) at 37°C for 3 h, followed by fluorescein isothiocyanate-conjugated secondary antibody (1:50 dilution in blocking solution; Sigma) and Rhodamine-conjugated secondary antibody (1:50 dilution in blocking solution; Santa Cruz Biotechnology) at 37°C for 1 h. After washing with PBS, the coverslips were mounted upside down on object slides using fluorescent-mounting medium. Immunofluorescence was visualized using an immunoflu-

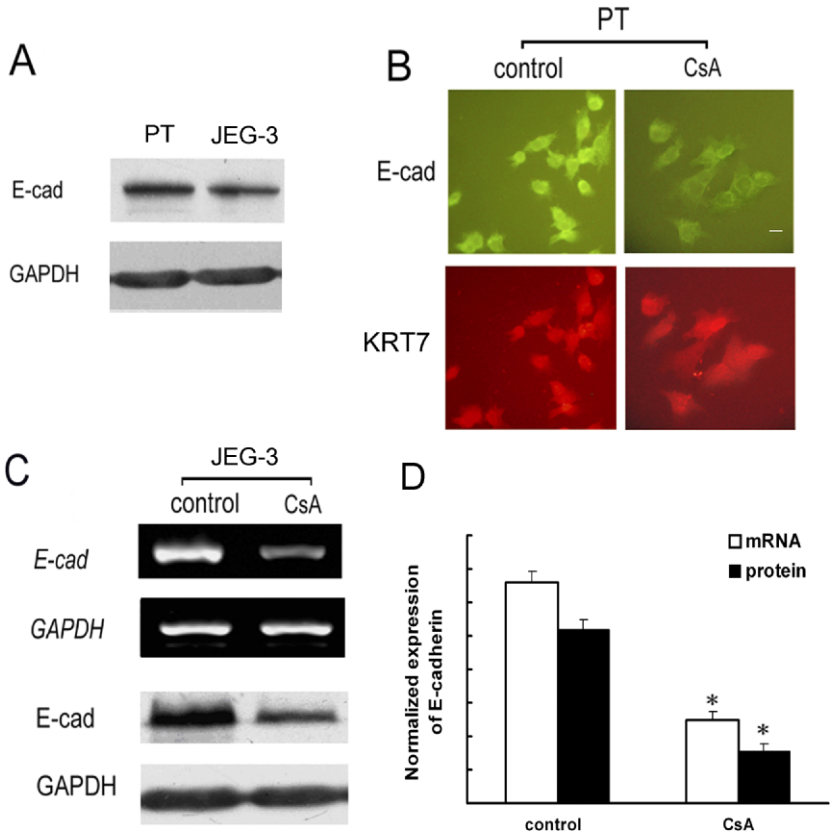

FIG. 1. CsA down-regulates E-cadherin expression in human primary cultured trophoblast cells and JEG-3 cells. **A**) Western blot analysis of E-cadherin (E-cad) expression in human primary cultured trophoblast cells (PT)and JEG-3 cells. **B**) CsA down-regulates E-cadherin expression in human primary cultured trophoblast cells. Human primary cultured trophoblast cells were treated with 0.1 μM CsA in 1640 complete medium supplemented with 10% FBS for 48 h and then subjected to fluorescent analysis. KRT7 was used as the marker of human trophoblast cells. Bar = 10 μm. **C** and **D**) CsA down-regulates E-cadherin transcription and translation in JEG-3 cells. JEG-3 cells were treated with 0.1 μM CsA in 1640 complete medium supplemented with 10% FBS for 48 h, and total mRNA and protein lysate were subsequently harvested and subjected to RT-PCR (upper panels) and Western blot analysis (lower panels), respectively. GAPDH was used as a loading control. A typical blot (**C**) and densitometric scans of triplicate blots (**D**) are shown. Data are presented as the mean ± SEM. $*P < 0.05$ compared to the control.

orescence microscope (Olympus BX51), and images were recorded by using a DP70 digital camera (Olympus Optical Co., Ltd.).

Immunoprecipitation

JEG-3 cells were washed three times with ice-cold PBS and solubilized with 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Nonidet P-40, 5 mM ethylenediaminetetra-acetic acid, 5 mM ethylene glycol tetraacetic acid (EGTA), 15 mM $MgCl_2$, 60 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM NaF, 0.1 mM benzamide, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, and 1 mM PMSF). Detergent-insoluble materials were removed by a centrifugation at 12 000 × *g* for 10 min at 4°C. After protein concentrations were determined by the Lowry assay, 500 μg of total cell lysates were incubated with primary antibody at 4°C for 2 h. Pre-equilibrated protein A/G-agarose beads (Santa Cruz Biotechnology) with lysis buffer were then added and incubated overnight. They were collected by a centrifugation at 12 000 × *g* and then gently washed three times with the lysis buffer. The bound proteins were eluted by boiling in 2× SDS sample buffer, and then Western blot analysis was performed according to the standard protocols.

Cell-Cell Adhesion Assay

Cell-cell adhesion assay was performed as described previously [31] with slight modifications. JEG-3 cells were detached with HCMF buffer (150 mM NaCl, 0.6 mM Na_2HPO_4, 10 mM glucose, and 10 mM Hepes; [pH 7.4]) containing 0.02% trypsin and 2 mM $CaCl_2$. Calcium-dependent aggregation was calculated by subtracting values obtained from aggregation assays in the presence of 2 mM EGTA from the values of the total number of aggregating cells. The levels of aggregating cells in EGTA were typically less than 5% of the total number of aggregating cells.

Cell Invasion Assay

Cell invasion was determined using transwell chamber assay as described previously [32] with minor modifications. JEG-3 cells were resuspended in medium containing 0.1% BSA at a concentration of 10^6 cells/ml, and 2×10^5 cells were added to the top well of transwell chambers (Costar Corporation). The cell suspensions were then placed into the upper compartment, and medium containing 5% fetal bovine serum was placed into the lower compartment as an inducer. After 12 h of incubation, those cells that had not migrated were removed from the upper surface of the filters using cotton swabs, and the cells that had migrated to the lower surface of the filters were fixed in methanol and stained by crystal violet. Cell invasion was determined by counting the number of cells that had migrated to the lower surface. Six visual fields were counted for each assay.

Statistical Analysis

Data are expressed as the mean ± SEM, and statistical evaluation was performed using one-way ANOVA followed by a Dunnett test. Differences were accepted as significant at $P < 0.05$.

RESULTS

Effect of CsA on E-Cadherin Expression in Human Primary Cultured Trophoblast Cells and Choriocarcinoma Cell Line JEG-3

As shown in Figure 1A, E-cadherin is moderately expressed in human primary cultured trophoblast cells and JEG-3 cells. To examine the effect of CsA on E-cadherin expression, human primary cultured trophoblast cells were treated with 0.1 μM CsA for 48 h and then subjected to immunofluorescence analysis. Herein, KRT7 was used as the marker of human trophoblast cells. As shown in Figure 1B, immunofluorescence staining of E-cadherin (green) in human primary cultured trophoblast cells treated with CsA was weaker than that of the control, indicating that CsA down-regulates E-cadherin expression in human primary cultured trophoblast cells. Similarly, E-cadherin expression in JEG-3 cells was decreased after CsA treatment (Fig. 1, C and D). RT-PCR analysis revealed that CsA attenuated the mRNA expression of E-cadherin in JEG-3 cells, which provides further evidence that

CsA down-regulates E-cadherin expression at the transcriptional level.

Role of ERK and SNAI2 in CsA-Induced Down-Regulation of E-Cadherin in JEG-3 Cells

As shown in Figure 2, CsA induced a rapid ERK phosphorylation in JEG-3 cells. U0126, an inhibitor of the ERK signaling pathway, decreased the CsA-induced ERK phosphorylation and, meanwhile, restored the E-cadherin expression, implying that CsA may inhibit E-cadherin expression via the ERK signaling pathway.

It has been shown that ERK activation increases the protein levels of the transcriptional repressors SNAI2 and SNAIL, which in turn down-regulates E-cadherin expression [33, 34]. Our present data showed that CsA increased the expression of SNAI2, which could be inhibited by U0126 treatment (Fig. 2). By contrast, SNAIL expression was not influenced by CsA and U0126 treatment (data not shown). These results indicate that CsA may down-regulate E-cadherin expression through ERK and its downstream SNAI2 in JEG-3 cells.

As mentioned before, CsA inhibits calcium/calmodulin-induced calcineurin activation and blocks the NFAT signaling pathway. To testify whether CsA down-regulates E-cadherin expression by blocking the calcium/calcineurin/NFAT cascade, we used ionomycin (an activator of calcineurin) to activate calcineurin and rescue the NFAT signaling pathway after CsA treatment in JEG-3 cells. As shown in Figure 2, ionomycin did not recover the CsA-induced down-regulation of E-cadherin, excluding the possibility that CsA decreases E-cadherin expression through blocking the calcium/calcineurin/NFAT signaling pathway. In addition, ionomycin appeared not to affect the ERK phosphorylation induced by CsA, which implies that the CsA-induced ERK activation is independent of the calcium/calcineurin/NFAT signaling pathway.

Role of EGFR in CsA-Induced Down-Regulation of E-Cadherin in JEG-3 Cells

We next investigated the effect of CsA on EGFR tyrosine phosphorylation in JEG-3 cells. CsA was added to JEG-3 cell culture in the absence or presence of EGF (50 ng/ml); tyrosine phosphorylation of EGFR was then detected by immunoprecipitation analysis. As shown in Figure 3, A and B, no difference in EGFR tyrosine phosphorylation was observed between treatment with and without 0.1 μM CsA in the absence of EGF, indicating that CsA alone is insufficient to induce EGFR activation. However, after JEG-3 cells were pretreated with 0.1 μM CsA for 48 h and then stimulated by EGF, the EGFR tyrosine phosphorylation was increased markedly compared with that of those cells only treated with EGF. These data suggest that CsA may amplify the EGF-induced EGFR activation in JEG-3 cells.

To assess the role of the EGFR/ERK cascade in the CsA-induced down-regulation of E-cadherin, we treated JEG-3 cells with AG1478 (an inhibitor of EGFR kinase) and then examined ERK phosphorylation and E-cadherin expression. As shown in Figure 3, C and D, CsA failed to activate ERK phosphorylation and decrease E-cadherin expression after AG1478 treatment, implying that CsA may down-regulate E-cadherin expression through EGFR/ERK signaling pathway. Different from the data shown in Figures 1 and 2, CsA alone failed to decrease E-cadherin expression of JEG-3 cells in serum-free culture medium (Fig. 3, C and D), indicating that CsA may down-regulate E-cadherin expression in a serum- or EGF-dependent manner.

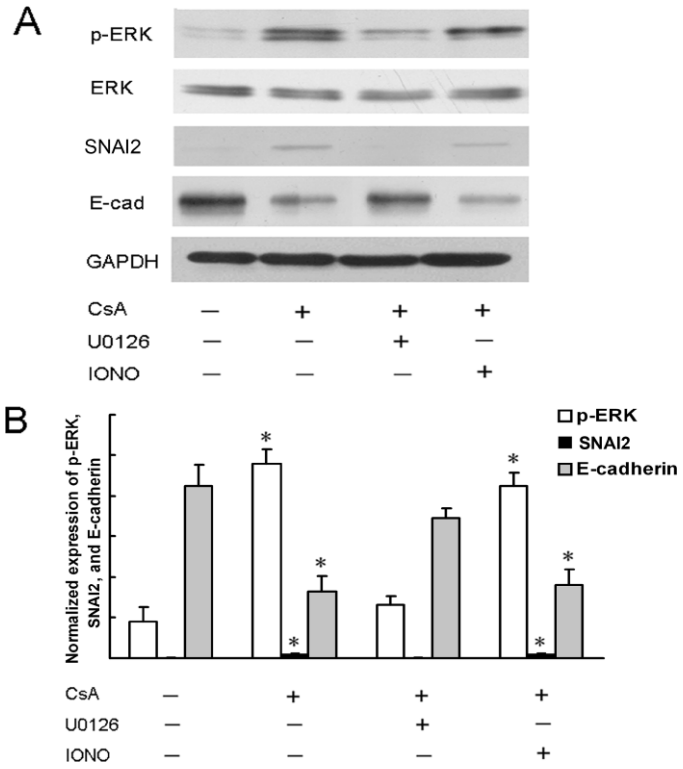

FIG. 2. CsA down-regulates E-cadherin expression through the ERK/SNAI2 signaling pathway in JEG-3 cells. Western blot analysis of phosphorylated-ERK (p-ERK), ERK, E-cadherin (E-cad), and SNAI2 in JEG-3 cells treated in the presence of U0126 or ionomycin (IONO). JEG-3 cells were treated with 0.1 μM CsA in 1640 complete medium supplemented with 10% FBS for 48 h and/or 20 μM U0126 and/or 2 μM IONO as indicated. To detect p-ERK and ERK, total cell lysates were harvested after 30 min of treatment. To analyze SNAI2, E-cadherin and GAPDH, total cell lysates were harvested after 48 h of treatment. GAPDH was used as a loading control. U0126 is an inhibitor of the ERK signaling pathway; IONO is an activator of calcineurin. A typical blot (**A**) and densitometric scans of triplicate blots (**B**) are shown. Data are presented as the mean ± SEM. $*P < 0.05$ compared to the control.

CsA Promotes Invasiveness of JEG-3 Cells Through Down-Regulating E-Cadherin Expression

As shown in Figure 4, inhibition of E-cadherin expression with siRNA significantly impaired the cell-cell adhesion of JEG-3 cells but increased the invasiveness compared to that of those transfected with nontargeting control siRNA. In contrast, overexpression of E-cadherin by transient transfection of the plasmid pcDNA3.0-wt E-cad significantly enhanced the cell-cell adhesion of JEG-3 cells but decreased the invasiveness compared to those transfected with pcDNA3.0 (control). These data suggest that E-cadherin may play a regulatory role in the invasive behavior of human trophoblast cells. Furthermore, CsA reversed the decreased invasiveness of JEG-3 that resulted from E-cadherin overexpression (Fig. 4), indicating that CsA may promote the invasiveness of human trophoblast cells through down-regulating E-cadherin expression.

DISCUSSION

The immunosuppressant drug of CsA is widely used to prevent organ rejection and to treat certain autoimmune diseases. Previous data from our laboratory have shown that CsA promotes the invasiveness of human first-trimester trophoblast cells through the ERK signaling pathway [12–

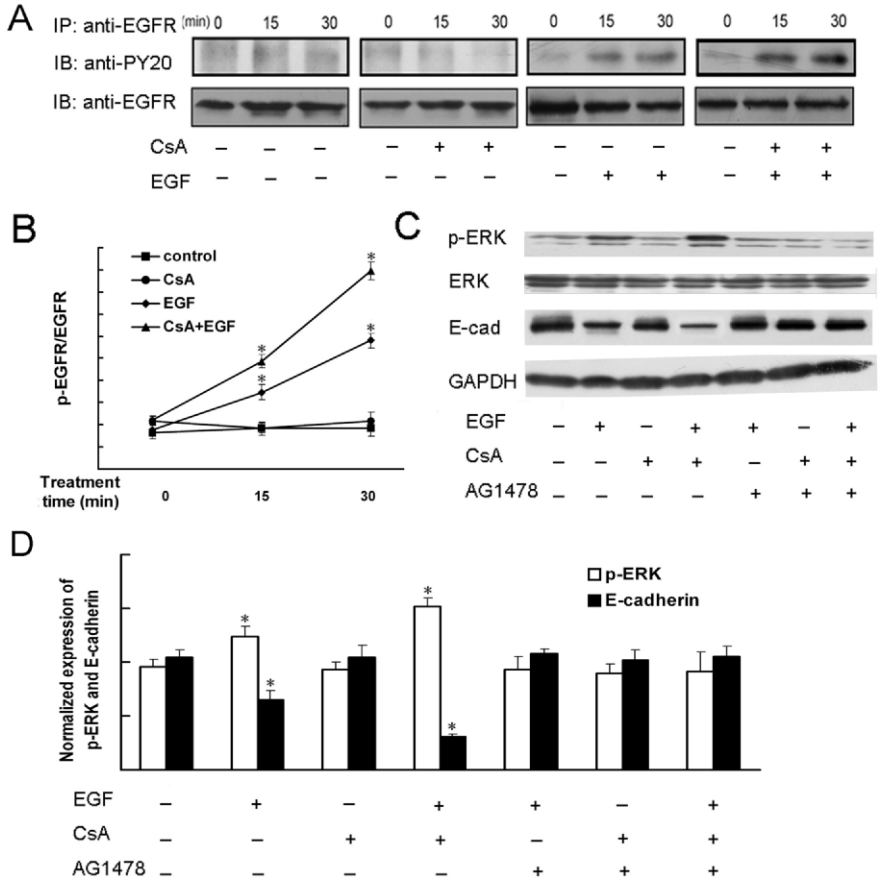

FIG. 3. CsA down-regulates E-cadherin expression through the EGFR/ERK signaling pathway in JEG-3 cells. **A** and **B**) CsA amplifies EGF-stimulated EGFR tyrosine phosphorylation in JEG-3 cells. JEG-3 cells were treated with 0.1 μM CsA in serum-free 1640 complete medium for 48 h and then stimulated with 50 ng/ml of EGF for the indicated times. The cells were then collected, and EGFR was separated by immunoprecipitation with an antibody against EGFR. Tyrosine phosphorylation levels of EGFR were analyzed by Western blotting using an antiphosphotyrosine antibody, PY20. The polyvinylidene fluoride membrane was reprobed with the antibody against EGFR to verify that an equal amount of precipitated protein was obtained. A typical blot (**A**) and densitometric scans of triplicate blots (**B**) are shown. Data are presented as the mean ± SEM. $^*P < 0.05$ compared to the control. **C** and **D**) AG1478 blocks the CsA-induced ERK activation and down-regulation of E-cadherin (E-cad). JEG-3 cells were treated with 0.1 μM CsA in serum-free 1640 complete medium for 48 h and/or 50 ng/ml of EGF for 30 min and/or 200 nM AG1478 for 30 min as indicated. To detect p-ERK and ERK, total cell lysates were harvested after 30 min of treatment. To analyze E-cadherin and GAPDH, total cell lysates were harvested after 48 h of treatment. GAPDH was used as a loading control. A typical blot (**C**) and densitometric scans of triplicate blots (**D**) are shown. Data are presented as the mean ± SEM. $^*P < 0.05$ compared to the control.

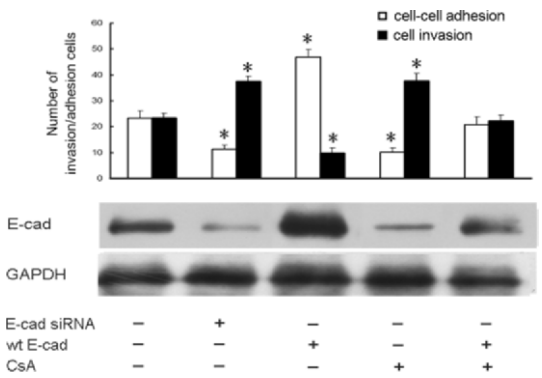

FIG. 4. CsA promotes the invasive behavior of JEG-3 cells through down-regulating E-cadherin (E-cad) expression. JEG-3 cells were transiently transfected with the plasmid pcDNA3.0-wt E-cad or E-cadherin siRNA and treated with 0.1 μM CsA in 1640 complete medium supplemented with 10% FBS for 48 h. The cell-cell adhesion and cell invasion was detected (upper panels). The results are presented as the mean ± SEM of three independent experiments. $^*P < 0.05$ compared to the control. Western blot analysis of E-cadherin and GAPDH expression (lower panels) is also shown, as is a typical blot of triplicate blots.

14]. ERK has been implicated in the regulation of cellular proliferation and differentiation, angiogenesis, embryo development, and tumor invasion [35–37]. It is also activated in human trophoblast cells and involved in the development of placenta. ERK is frequently activated by EGF-induced EGFR phosphorylation, which leads us to postulate that CsA may promote ERK activation via EGFR. EGFR signaling pathway plays a key role in tumor metastasis by facilitating the invasion of tumor cells [38, 39]. In placenta, EGFR is frequently highly activated and controls the proliferation and invasion of human trophoblast cells [40]. In the present study, CsA alone could not stimulate EGFR activation as a stimulus; however, it effectively amplified EGF-induced EGFR tyrosine phosphorylation.

It is intriguing how CsA promotes EGF-induced EGFR activation. As mentioned, CsA binds to cyclophilin A, inhibits calcium/calmodulin-dependent calcineurin activation, and blocks the NFAT signaling pathway. Calmodulin is a ubiquitous, calcium-binding protein that can bind to and regulate a multitude of different protein targets, thereby affecting different cellular functions, such as inflammation, metabolism, apoptosis, and the immune response. In fact, the

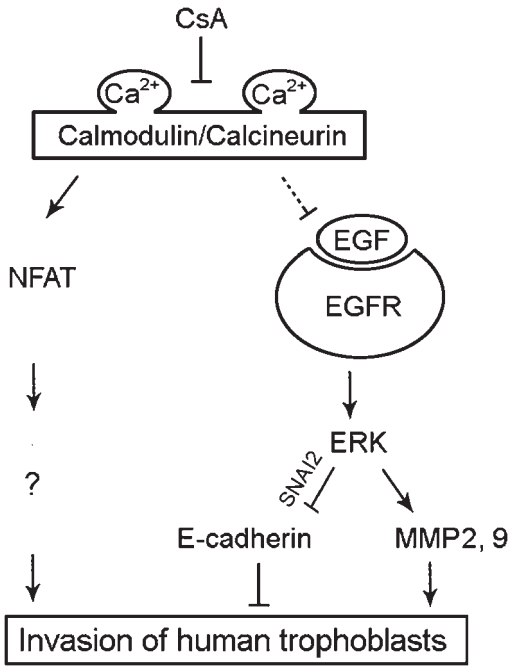

FIG. 5. Schematic representation of intracellular signaling pathway responsible for the CsA-induced invasiveness improvement of JEG-3 cells. CsA may promote the invasiveness of human trophoblast cells via two independent signaling pathways, calcium/calcineurin/NFAT and EGFR/ERK. E-cadherin, MMP2, and MMP9 may be the downstream effective molecules of the CsA/EGFR/ERK signaling pathway.

role of calmodulin in the regulation of EGFR activity has been described at various levels [41]. San Jose et al. [42] showed that calmodulin bound to EGFR and negatively regulated its tyrosine kinase activity. Feinmesser et al. [43] revealed that calmodulin kinase II likely was responsible for the inhibition of EGFR tyrosine kinase activity by calmodulin. These findings indicate the possible involvement of calmodulin in the CsA-induced EGFR activation.

Calcineurin, a serine/threonine phosphatase controlled by calcium and calmodulin, has been implicated in a wide variety of biological responses, including lymphocyte activation as well as neuronal and muscle development. In fact, calcineurin has also been reported by Sullivan and Rubin [44] to cooperate with other factors and restrain EGFR activation in *Drosophila* development. Thus, we hypothesize that CsA may relieve the inhibition of EGFR phosphorylation induced by calmodulin or calcineurin and, ultimately, promote the activation of EGFR. Further investigation is in process to identify the role of calmodulin or calcineurin in the CsA-induced EGFR activation.

In our present study, we have demonstrated that CsA at low concentrations down-regulates E-cadherin expression through the EGFR/ERK signaling pathway, which suggests E-cadherin may act as a novel regulatory target of CsA. E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent cell-cell adhesion, which contributes to the maintenance of tissue integrity. Evidence is emerging that invasiveness of trophoblast cells may be attributable, in part, to the loss of their adhesive properties mediated by E-cadherin. In our study, inhibition of E-cadherin expression with siRNA improved the invasiveness of JEG-3 cells; conversely, the overexpression of E-cadherin resulted in the decreased invasiveness of JEG-3 cells. These data indicate the important role of E-cadherin in the regulation of invasive behavior of human trophoblast cells.

In addition to E-cadherin, our previous study has shown that matrix metalloproteinase (MMP) 2 and MMP9 are also involved in the CsA-induced trophoblast invasion [13]. Presumably, MMP2, MMP9, and E-cadherin are target molecules of the EGFR/ERK signaling pathway. These data suggest that CsA may modulate several target molecules through EGFR/ERK signaling pathway, which collectively contribute to the improvement of the invasive behavior of human trophoblast (Fig. 5).

During pregnancy, human trophoblastic cells invade the uterus and its vasculature and remodel the maternal spiral arteries. As a result, the spiral arteries are transformed into large-caliber, low-resistance vascular channels that direct blood into the intervillous space, which is necessary for the higher blood requirement of the fetus. Therefore, the invasion of human trophoblast cells is a critical step in establishment and maintenance of a normal pregnancy. Although the safety and long-term consequences of CsA need more research, CsA may be developed as a potential drug for complications of pregnancy resulting from deficient trophoblast invasion.

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