Cyclosporin A increases expression of matrix metalloproteinase 9 and 2 and invasiveness in vitro of the first-trimester human trophoblast cells via the mitogen-activated protein kinase pathway

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BACKGROUND: Cyclosporin A (CsA) is an immunosuppressant which is used for preventing allograft rejection. Little is known, however, about the effect of CsA on the materno–fetal relationship. Our aim was to probe into the effect of CsA on the invasiveness of human first-trimester trophoblast cells and explore possible molecules involved, with a view to providing a new therapeutic approach for pregnancy complications with trophoblast disorder. METHODS: The effects of CsA on invasion of the first-trimester human trophoblasts were examined by matrigel invasion assay, and the transcription, translation and proteolytic activity of matrix metalloproteinase (MMP-9) and MMP-2 in these cells were estimated by RT-PCR, in-cell Western and zymography, respectively. The phosphorylation level of extracellular-signal related kinase (ERK) 1/2 in trophoblasts induced by CsA was also evaluated by in-cell Western. RESULTS: CsA increased the invasive index of first-trimester human trophoblasts (P < 0.01), as well as the messenger RNA, protein levels (both P < 0.01) and proteolytic activity (P < 0.05) of MMP-9 and MMP-U0126, a mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitor, inhibited the enhanced invasiveness and activity of MMP-9 and MMP-in these cells induced by CsA. In addition, CsA activated the ERK1/2 in a time-dependent manner. CONCLUSIONS: CsA improves the invasiveness and activity of MMP 9 and MMP 2 in vitro of the first-trimester human trophoblast cells through activation of mitogen-activated protein kinase/extracellular-signal related kinase (ERK) 1/2 signaling pathway, which suggests this drug has a favorable modulation on the function of human first-trimester trophoblast cells.

Keywords: cyclosporin A; invasiveness; MAPK; matrix metalloproteinase; trophoblast cell

Introduction

Normal fetal development depends on the ability of trophoblast cells to invade into the uterine decidual stroma and gain access to the maternal circulation. Only after the invading, trophoblast cells successfully degrade and migrate through the extracellular matrix (ECM), may they intimately interact with and further replace the uterine spiral arterial endothelial cells. The invasiveness of cytotrophoblast cells derived from anchoring villi is very crucial to this process. An insufficient trophoblast invasion of the spiral arteries has been confirmed not only associated with pre-eclampsia and fetal growth restriction but also human first-trimester spontaneous abortion and late miscarriage (Hustin et al., 1990; Ball et al., 2006; Kadyrov et al., 2006). Therefore, how to promote the physiological functions of trophoblast cells is of great interest for researchers on reproductive medicine. Several therapies have been put forward, but we still have a long way to improve the outcomes of pregnancy (Poston et al., 2006; Rumbold et al., 2006).

Trophoblast invasion is a series of tightly controlled program of intercellular signaling, that is, mediated by growth factors, cytokines and hormones which come from the embryo or the maternal uterine (Bischof and Campana, 2000; Meisser et al., 1999a, b). The penetrative ability of trophoblasts is facilitated by degradation of the ECM of the decidua via various proteinases, among which the matrix metalloproteinases (MMPs), such as MMP 9 and MMP 2, play an important role. Human first-trimester trophoblast cells have been shown to secrete MMP 9 and MMP 2, which can help to degrade the ECM and participate in trophoblast invasion (Shimonovitz et al., 2006).
The villous tissue was treated by repeated trypsin digestions according to the method of Wu et al. (2004). Briefly, the obtained placental tissues from 3–4 separate individuals were minced and digested with 0.25% trypsin (Bio Basic Inco, BBI, Ontario, Canada) and 0.02% DNase I (Sigma, Saint Louis, Missouri, USA) at 37°C with gentle agitation for 5 min. Then the digested suspension was discarded and the residual tissue was subjected to four cycles of 10-min digestion. The cell suspensions trypsinized in each time were pooled, and carefully layered over a discontinuous Percoll Gradient (65–20%, in 5% step), and centrifuged at 2000 rpm for 20 min. The cells sedimenting at densities between 1.048 and 1.062 g/ml were collected, and washed with DMEM-high glucose medium. These cells were then diluted to 5 × 10^5 cells/well, and maintained in DMEM-high glucose complete medium (2 mM glutamine, 25 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin), supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Gibco) and incubated in 5% CO_2 at 37°C. This method supplies a 95% purity of trophoblast cells.

**Matrigel invasion assay**

The invasion of trophoblast cells across Matrigel was evaluated objectively in invasion chamber based on our previous procedure (Huang et al., 2006). Briefly, the cell culture inserts (8 μm pore size, 6.5 mm diameter; Corning, Corning, NY, USA) coated with 5 μl pure Matrigel were placed in a 24-well plate. The purified trophoblast cells (2 × 10^5 in 200 μl DMEM with 1% FBS) were plated in the upper chamber. CsA at various final concentration of 0, 0.0001, 0.001, 0.01, 0.1, 1.0 and 10.0 μmol/l and CsA of 1.0 μmol/l combined with MEK1/2 inhibitor U0126 (30 μmol/l) or U0126 (30 μmol/l) alone were added, respectively. The lower chamber was filled with 800 μl DMEM with 10% FBS. The cells were then incubated at 37°C for 48 h. The inserts were removed, washed in phosphate-buffered saline (PBS) and the non-invading cells together with the Matrigel were removed from the upper surface of the filter by wiping with a cotton bud. The inserts were then fixed in methanol for 10 min at room temperature and stained with hematoxylin. The result was observed under Olympus BX51 + DP70 fluorescence microscope (Olympus, Tokyo, Japan). The cells migrated to the lower surface were counted in five predetermined fields at a magnification of 200 × . Each experiment was carried out in triplicate, repeated three times.

**Materials and Methods**

**Human placental tissue collection**

All procedures involving participants in the study were approved by the Obstetrics and Gynecology Hospital of Fudan University Human Research Ethics Committee, and all subjects completed an informed consent to collect tissue samples.

Placental tissues were from elective termination of the first-trimester pregnancies (gestational age, 6–9 weeks) for non-medical reasons. All the tissues were put immediately into ice-cold DMEM (Dulbecco’s modified Eagle medium high D-glucose; Gibco Grand Island, NY, USA), transported to the laboratory within 30 min after surgery and washed in calcium- and magnesium-free Hank’s balanced salt solution (HBSS) for trophoblast isolation.

**Isolation and primary culture of the first-trimester human trophoblast cells**

The villous tissue was treated by repeated trypsin digestions according to our previous method (Wu et al., 2004). Briefly, the obtained placental tissues from 3–4 separate individuals were pooled and digested by 0.25% trypsin (Bio Basic Inco, BBI, Ontario, Canada) and 0.02% DNase type I (Sigma, Saint Louis, Missouri, USA) at 37°C with gentle agitation for 5 min. Then the digested suspension was discarded and the residual tissue was subjected to cycles of 10-min digestion. The cell suspensions trypsinized in each time were pooled, and carefully layered over a discontinuous Percoll Gradient (65–20%, in 5% step), and centrifuged at 2000 rpm for 20 min. The cells sedimenting at densities between 1.048 and 1.062 g/ml were collected, and washed with DMEM-high glucose medium. These cells were then diluted to 5 × 10^5 cells/well, and maintained in DMEM-high glucose complete medium (2 mM glutamine, 25 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin), supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Gibco) and incubated in 5% CO_2 at 37°C. This method supplies a 95% purity of trophoblast cells.

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**Reverse transcription-polymerase chain reactions**

The isolated trophoblast cells were seeded at a density of 5 × 10^5 cells/well on 6-well plates pre-coated with Matrigel. After culture in DMEM with 1% FBS for 12 h the cells were treated with vehicle, CsA (1.0 μmol/l), 1.0 μmol/l of CsA combined with U0126 (30 μmol/l) or U0126 (30 μmol/l) alone for 24 h. Total cellular RNA of these cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA of 4 μg was used for first strand cDNA synthesis in 20 μl reaction volume with 200 units M-MLV reverse transcriptase. Then cDNA (10 μl) was amplified by PCR reaction in a final volume of 50 μl containing 2 mM dNTP, 0.8 μM specific primers, 1.25 u Tag DNA polymerase, 1 mM MgCl_2 and 1 × reaction buffer. In 15 min precycle at 95°C, the reaction was followed by 35 cycles of 1 min at 94°C, 45 s at 54°C, and 45 s at 72°C. When the final cycle was over, samples were kept at 72°C for 15 min to complete the synthesis. Primer pairs for cDNA amplification were as follows: 5'-GGA TGG GAA GTA CTG GCG ATT C-3' (forward) and 5'-CAC TTG GTC CAC CTG GTT CAA C-3' (reverse) for human MMP 9; 5'-TGA CAT CAA GGG CAT TTC AGG AGC-3' (forward) and 5'-GTC CGG CAA ATG AAC CCG TTC TTG-3' (reverse) for human MMP 2; 5'-GGG GAG CCA AAA GGG TCA -3' (forward) and 5'-GAG GGG CCA TCA ACA GTC TTC T-3' (reverse) for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ishii et al., 2000; Karmakar and Das, 2002; Qiu et al., 2004). The expected fragment lengths of each primer were 105 bp, respectively. The PCR reaction products of 10 μl were electrophoresed on 2% agarose gels and ethidium bromide-stained bands were photographed, and analysed by gel imaging systems. The relative intensity of MMP = absorbance value of MMP/that of GAPDH. The experiments were carried out in triplicate, repeated three times.
In-cell Western
According to the description by Egorina et al. (2006), we used a newly established assay called in-cell Western to determine the in-cell protein level of MMP 9 and MMP 2. The procedure was as follows: freshly isolated trophoblast cells (2 × 10^5/well) were seeded on 96-well plate. In incubation for 24 h, the trophoblast cells were starved in DMEM supplemented with 1% FBS for 12 h. Then these cells were incubated with vehicle, CsA (1.0 μmol/l), CsA of 1.0 μmol/l combined with U0126 (30 μmol/l) or U0126 (30 μmol/l) alone, respectively, for another 48 h. Then the cells were immediately fixed with 4% formaldehyde in 1 × PBS for 20 min at room temperature. After washing with 0.1% Triton, we blocked the cells by adding 100 μl of LI-COR Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, Nebraska, USA) for 90 min at room temperature. The cells were then incubated with mouse anti-human MMP 9 or MMP 2 (R&D Systems, Abingdon, UK) primary antibody, respectively. The housekeeping protein, rabbit anti-human actin, (Santa Cruz, CA, USA) was added to each well at the same time as control. After treated overnight at 4°C, the wells were then incubated with corresponding second IRDyeTM700DX conjugated affinity purified (red fluorescence) anti-mouse or IRDyeTM800DX conjugated affinity purified (green fluorescence) anti-rabbit fluorescence antibody recommended by the manufactures (Rockland, Inc, Gilbertsville, PA, USA). This procedure must be operated in dark place avoiding the exposure to light. Images of MMP 9 and MMP 2 were obtained using the Odyssey Infrared Imaging System (LI-COR Biosciences GmbH). The protein level of MMP was calculated as the ratio of the intensity of MMP 9 or MMP 2 to that of actin. The experiments were carried out in triplicate, repeated three times.

Gelatin zymography
Culture medium of the upper chamber in the invasion assay was harvested and the proteolytic activity of both MMP 9 and MMP 2 was measured by the technique of gelatin zymography described by Yoshizaki et al. (1997), with light modification. Briefly, collected culture supernatants containing 10 μg of total protein were mixed with sodium dodecyl sulfate (SDS) loading buffer, applied in each lane and electrophoresed on 10% SDS-polyacrylamide gels copolymerized with 0.2% gelatin. After electrophoresis, the gel was rinsed in 2.5% Triton-X 100 for 1 h to remove SDS. The gel was incubated for 12 h at 37°C in 50 mM Tris–HCl (pH 7.5), 200 mM NaCl and 10 mM CaCl2 and stained with 2.5% Coomassie blue R250 (Sigma Chemical Co, Saint Louis, Missouri, USA) dissolved in 40% (v/v) methanol and 10% acetic acid. The gels were then rinsed in three different decolorant solutions (A: 30% methanol, 10% acetic acid; B: 20% methanol, 10% acetic acid and C: 10% methanol, 5% acetic acid), respectively. The gel was photographed and assayed by the Odyssey Infrared Imaging System. The gelatinolytic activity was visualized as a clear white band against a dark background of stained gelatin. The proteolytic activity of MMP is equal to the intensity of MMP 9 or MMP 2 to that of total ERK. The experiments were carried out in triplicate, repeated three times.

Determination for phospho-ERK1/2 of human trophoblast cells
The cell culture and treatment was the same as described in in-cell Western. Then the trophoblast cells were fixed with 4% formaldehyde after management for 10, 20, 30, 60 or 90 min, respectively. The primary antibodies used here were mouse anti-human phospho-ERK and rabbit anti-human total ERK (Santa Cruz). The level of the phospho-ERK was showed by ratio of intensity of the phospho-ERK to that of total ERK. The experiments were carried out in triplicate, repeated three times.

Statistics
All values were shown in the mean ± SEM. One-way ANOVA was performed to detect the significance of invasion, expression of MMP 9 or MMP 2 and phosphorylation level of ERK1/2 in the first-trimester human trophoblast cells. Differences were accepted as significant at P < 0.05.

Results

CsA promoted invasion in vitro of the first-trimester human trophoblast cells through MAPK/ERK1/2 signaling pathway
To testify the effects of CsA on the invasion of human first-trimester trophoblast cells, a Matrigel-based transwell assay was carried out. The freshly isolated trophoblasts with different concentration of CsA were added to the upper chamber, and the number of cells migrating to the lower surface was counted in 48 h of incubation. As shown in Fig. 1A, the different concentration of CsA substantially increased invasion of human first-trimester trophoblast cells in a dose-responsive manner. The invasive index of human first-trimester trophoblast cells was significantly higher in each CsA group (0.0001, 0.001, 0.01, 0.1, 1.0 or 10.0 μmol/l, P < 0.05 or P < 0.01) when compared with the vehicle controls, and reached a peak in the CsA concentration of 1.0 μmol/l. Therefore, our following studies were all performed in the optimal concentration of 1.0 μmol/l of CsA.

MAPK/ERK1/2 signaling pathway is involved in modulation of migration and penetration of human trophoblasts (McKinnon et al., 2001; Qiu et al., 2004). CsA could activate this pathway in several cells and mediate different biological functions (Paslaru et al., 1997; Kiely et al., 2003; Yang et al., 2003). So, we wondered whether CsA improved the invasion of human first-trimester trophoblasts via MAPK/ERK1/2 signaling pathway or not. Figure 1D and F clearly showed that the U0126, a MEK inhibitor, significantly inhibited the enhanced invasion of trophoblast cells induced by CsA (when compared with the CsA-treated group, P < 0.01). But U0126 only partly suppressed the invasiveness of the trophoblasts up-regulated by CsA (when compared with the vehicle control, P < 0.05), suggesting besides MAPK signals, perhaps other signal pathways were also involved in the CsA-induced actions.

It could be concluded that the CsA stimulated invasiveness of human first-trimester trophoblast cells in an appropriate concentration range partly by activating the MAPK/ERK1/2 signaling pathway.

CsA modulated transcription of MMP 9 and MMP 2 in first-trimester human trophoblast
MMP 9 and MMP 2 are two important factors degrading the ECM and participating in human first-trimester trophoblast invasion (Shimonovitz et al., 1994; Staun-Ram et al., 2004). In the present study, the primary cultured human trophoblasts were administrated with vehicle, 1.0 μmol/l of CsA, CsA (1.0 μmol/l) combined with U0126 (30 μmol/l) or U0126 (30 μmol/l) alone, respectively. Then the transcriptional level of the two molecules in these cells was determined by RT-PCR. It was found that CsA (1.0 μmol/l) significantly
increased the mRNA expression of MMP 9 and MMP 2 in the first-trimester trophoblast cells when compared to the vehicle control, and U0126 remarkably abolished the transcriptional increase of MMP 9 and MMP 2 induced by CsA. Because U0126 alone could effectively inhibit the transcription of MMP 9 and MMP 2 in trophoblast cells, it is speculated that in physiological state, the mRNA expression of MMP 9 and MMP 2 in trophoblast cells could be mediated by the MAPK signaling pathway, and CsA has the potential to stimulate the MAPK/ERK1/2 signaling to increase the transcription of MMP 9 and MMP 2 (Fig. 2).

### CsA up-regulated the protein translation of MMP 9 and MMP 2 in the first-trimester human trophoblast

From Fig. 3, it was clearly shown that the protein level of both MMP 9 and MMP 2 in the first-trimester trophoblasts also increased after treatment with 1.0 μmol/l of CsA, and U0126 effectively abolished the enhanced protein expression of the two molecules induced by CsA. Since CsA could stimulate the transcription of MMP 9 and MMP 2 genes, whether the increased protein level of MMP 9 and MMP 2 is owing to the transcriptional activation of these two genes or the up-regulation of protein expression directly exerted by CsA remains unknown and deserves further research.

### CsA enhanced the proteolytic activity of MMP 9 and MMP 2 by the first-trimester human trophoblast

Both MMP 9 and MMP 2 are intracellularly synthesized. They exert their proteolytic functions through secretion into the ECM (Vu and Werb, 2000). Therefore, the conditioned medium from the upper chamber in the invasion assay were collected, and the gelatinolytic activity of MMP 9 and MMP 2.
2 was analysed by gelatin zymography. It was clearly shown in Fig. 4 that the activity of MMP 9 and MMP 2 to degrade gelatin significantly increased after treatment with 1.0 μmol/l of CsA, and U0126 remarkably inhibited the enhanced activity of MMP9 and MMP2 stimulated by CsA, which suggests that CsA promotes proteolytic activity of MMP 9 and MMP 2 of the first-trimester human trophoblasts through MAPK/ERK1/2 signaling pathway.

From the results above, it is proposed that CsA can up-regulate the expression and activity of MMP 9 and MMP 2 in the first-trimester human trophoblast cells. Furthermore, the MAPK/ERK1/2 signaling pathway plays a role in this CsA-mediated modulation.

CsA activated the MAPK/ERK1/2 signaling pathway in the first-trimester human trophoblast

The in-cell Western was used to determine the level of the phospho-ERK1/2 in the primary cultured human trophoblasts. The results in Fig. 5 suggested that CsA (1.0 μmol/l) caused the phosphorylation of ERK1/2 of the first-trimester human trophoblast cells in a time-dependent manner, and the activation of ERK1/2 stimulated by CsA could even sustain at least for 90 min. U0126, a MEK inhibitor, completely abolished the phosphorylation of ERK1/2 induced by CsA, which further confirms CsA can activate the MAPK/ERK1/2 signaling pathway in human trophoblast cells.

Discussion

As a distinct immunosuppressant, most effects of CsA are exerted through the formation of a specific complex with cyclophilins (CyPs) in the cytoplasm. The CyPs–CsA complex binds to calcineurin and suppresses its serine–threonine protein phosphatase activity. This inhibition represents a specific bottleneck of antigen-receptor signaling in immune-competent cells, which leads to inactivation of lymphokine genes essential for T cell proliferation and activation and ultimately results in immunosuppression (Johansson and Moller, 1990; Schreiber and Crabtree, 1992). However, different from the calcineurin/calmodulin/NF-AT pathway in immune cells, our study showed that CsA (1.0 μmol/l) could induce the activation of ERK1/2 of the first-trimester human trophoblast cells in a time-dependent manner and promote the invasiveness in vitro of these cells. U0126, a MEK inhibitor, could inhibit this enhanced phosphorylation of ERK1/2 and invasiveness of trophoblast cells induced by CsA, suggesting that the MAPK signaling pathway in trophoblasts is one of the target sites for CsA. Therefore, CsA may present different effect on different cells via different signaling pathway and the pharmacological action of this drug is far from being completely understood, and deserves further investigation.

The placentation starts from the invasion and migration of trophoblast cells into the maternal tissue to establish connection with the maternal circulation (Rinkenberger et al., 1997). These trophoblasts express high levels of MMP 9 and
MMP 2, and MMP inhibition in vitro decreases migration and degradation of ECM by these cells (Shimonovitz et al., 1994; Alexander et al., 1996; Staun-Ram et al., 2004). Yamamoto et al. (1998) even found that in Ets-2-deficient mice (Ets-2 is a member of the Ets family of transcription factors that regulate the transcription of diverse genes, particularly MMPs), an insufficient MMP 9 activity in ectoplacental trophoblasts was highly linked with abnormal placental phenotype and death of the embryos before 8.5 days of embryonic development. In the present study, it was found that CsA significantly promoted the proteolytic activity of MMP 9 and MMP 2 by the first-trimester human trophoblast cells and the invasiveness of these cells also increased correspondingly. U0126 could remarkably inhibit the elevated gelatinolytic activity and invasive ability of trophoblast cells induced by CsA. Thus, it could be inferred that CsA promotes the activity of MMP 9 and MMP 2 of the first-trimester human trophoblast cells via MAPK signaling pathway, leading to increased invasion of these cells. Aberrations in MMP 9 and MMP 2 are partly responsible for disturbing placentation and spiral arteries remodeling (Lim et al., 1997; Merchant et al., 2004). The up-regulation of CsA on MMP 9 and MMP 2 could contribute to the degradation of ECM and improvement of trophoblast invasion, which enlightens the treatment of some pregnancy complications suffered from an insufficient invasion of trophoblasts, including not only spontaneous abortion but also pre-eclampsia, fetal growth restriction and so on.

The discovery of MMPs was based on the observation that during amphibian metamorphosis, a collagenolytic activity has to be present to digest the collagen in tadpole tails (Gross and Lapiere, 1962). In fact, it has become clear in recent years that besides being responsible for the mass degradation of the ECM as bulldozers, MMPs play sophisticated roles in modulating normal cellular behavior and cell–cell communications, among which is the action of MMPs on cell growth through regulating several growth factors (Vu and Werb, 2000; McCawley and Matrisian, 2001). IGF-I and IGF-II (insulin-like growth factor), possess the ability to mediate growth and migration of human trophoblast cells through binding to their receptors (IGF receptors, IGFRs) (Maruo et al., 1995; McKinnon et al., 2001; Hills et al., 2004), while IGFBP (insulin-like growth factor-binding protein-1) produced by decidua could compete with the IGFRs for the ligands and limit the effects of IGFs (Han et al., 1996; Martina et al., 1997; Carter et al., 2005). MMP 9 and MMP 2 were shown to proteolyze IGFBP into a series of fragments which fail to bind IGF (Fowlkes et al., 1994; Coppock et al., 2004). It has been demonstrated, in our previous study that the CsA promotes growth of the first-trimester human cytotrophoblasts independent of its effect on the immune cells (Yan et al., 2002a), and in the present study.
we found that CsA enhanced the expression and activity of MMP 9 and MMP 2 by human trophoblast cells. Thus, it is speculated that the increased MMP 9 and MMP 2 expression, an action induced by CsA could accelerate the cleavage of IGFBP and release IGF from their inhibitory proteins. The rescued IGFs by MMP then promote growth of the trophoblast cells, which might be an indirect regulation of CsA on trophoblast functions.

In addition, it was found in the present study, U0126 alone could significantly inhibited the mRNA and protein expression of MMP 9 and MMP 2 in trophoblast cells (when compared with the vehicle control, \( P < 0.01 \)). Some other studies have also demonstrated that most receptor-associated kinases implicated in MMP transcriptional regulation are connected with the MAPK system (Leppa et al., 1998; Cho et al., 2000). Thus, in physiological state, the expression of MMP 9 and MMP 2 in trophoblast cells could be mediated via MAPK signaling pathway. Interestingly, U0126 completely inhibited the increased expression of MMP 9 and MMP 2 of the first-trimester human trophoblasts induced by CsA (when compared with the vehicle control, \( P > 0.05 \)), while only partly suppressed the enhanced invasive ability of these cells (when compared with the vehicle, \( P < 0.05 \)). These results indicated that CsA stimulated the expression of MMP 9 and MMP 2 mainly via the MAPK/ERK1/2 signaling pathway, but besides this signal, there might be other CsA-activated signals or molecules modulating the invasion of trophoblasts. It has been proofed in our previous study that the CsA could induce the expression of titin in first-trimester human trophoblast cells (Du et al., 2005). Titin, a giant muscle protein, exerts several crucial biological functions, especially migration and motility of cells (Werny et al., 2001). Therefore, the action of CsA on trophoblasts might be mediated through multiple signals rather than a singular molecule.

In conclusion, our study has demonstrated for the first time that CsA of 1.0 \( \mu \text{mol}/\text{l} \) could promote the expression of MMP 9 and MMP 2 in the first-trimester human trophoblast cells at both transcriptional and translational level through activating MAPK/ERK1/2 signaling pathway, which contributes to the improvement of invasiveness of trophoblasts. Our previous in vivo study also confirmed that the administration of CsA (5 mg/kg) only once at day 4 of gestation (the window of implantation), induced a Th2 bias and maternal tolerance to the allogeneic fetus, leading to a reduced fetal resorption rate in the abortion-prone CBA/J × DBA/2 matings (Du et al., 2007). It could be inferred that low dose of CsA has a favorable effect on the materno–fetal interface by promoting the functions of trophoblast cells and protecting the fetus from maternal attack. Thus, CsA might have the potential to be developed as a unique immunotherapy for pregnancy complications resulting from deficient trophoblast invasion or excessive activation of maternal immune system. It was reported that there was a moderate risk of fetal growth restriction when CsA was used throughout the pregnancy (Armenti et al., 2002). Therefore, although the dosage of CsA used in our research is much lower, the safety and long-term consequences of this drug application need more research.

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