

## Original Article

# Role of human cytomegalovirus in the proliferation and invasion of extravillous cytotrophoblasts isolated from early placentae

Tao Liu<sup>1,2</sup>, Xiaofei Zheng<sup>3</sup>, Qin Li<sup>3</sup>, Juanjuan Chen<sup>4</sup>, Zongzhi Yin<sup>5</sup>, Juan Xiao<sup>6</sup>, Dandan Zhang<sup>7</sup>, Wei Li<sup>6</sup>, Yuan Qiao<sup>6</sup>, Suhua Chen<sup>6</sup>

<sup>1</sup>Center for Reproductive Medicine, Shandong Provincial Hospital, Shandong University, Jinan 250021, China; <sup>2</sup>Department of Obstetrics and Gynecology, The Central Hospital of Taian, Taian 271000, Shandong, China; <sup>3</sup>The Information Center, The Central Hospital of Taian, Taian 271000, Shandong, China; <sup>4</sup>Department of Obstetrics and Gynecology, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou 510120, Guangdong, China; <sup>5</sup>Department of Obstetrics and Gynecology, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui, China; <sup>6</sup>Department of Obstetrics and Gynecology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China; <sup>7</sup>Department of Obstetrics and Gynecology, The International Peace Maternity and Child Health Hospital of China Welfare Institute, Shanghai 20030, China

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**Abstract:** Aim: We investigated the role of human cytomegalovirus (HCMV) and its mechanism in extravillous cytotrophoblast (EVT) proliferation and invasion *in vitro*. Methods: Differential enzymatic digestion combined with gradient centrifugation, was used to isolate primary EVT from human chorionic villi collected from early placentae of healthy pregnant women. HCMV infection was determined by immunofluorescence staining of HCMVpp65 antigen expression. An MTT assay was used to examine the role of HCMV in the proliferation of EVT. Quantitative real-time polymerase chain reaction (qRT-PCR), immunocytochemical staining and Western blots were carried out in a control group (EVT) and a virus group (EVT+HCMV) to examine the expression of major genes and protein in TGF- $\beta$ /Smad signaling pathways in EVT 48 h after inoculation with HCMV. An *in vitro* cell invasion assay was performed to analyze the influence of HCMV on EVT invasion. Results: HCMV significantly inhibited the proliferation of EVT 48 h after viral infection ( $P < 0.05$ ). The expression of TGF- $\beta$ 1, Smad1, Smad2, Smad3, Smad4, and Smad5 genes was significantly increased ( $P < 0.05$ ), but that of TGF- $\beta$ 2, TGF- $\beta$ 3, TGF $\beta$ RI, TGF $\beta$ RII, Smad7, MMP2, and MMP9 was significantly decreased in the virus group 48 h after HCMV infection ( $P < 0.05$ ). Smad7, MMP-2 and MMP-9 protein levels were significantly decreased and the TGF- $\beta$ 1 protein level was significantly increased in infected EVT (all  $P < 0.05$ ). Conclusions: HCMV may act on multiple steps of the TGF- $\beta$ /Smad signaling pathway to impede EVT proliferation and invasion.

**Keywords:** Human cytomegalovirus, extravillous cytotrophoblast, TGF- $\beta$ 1, Smad7, invasion, proliferation

## Introduction

Human cytomegalovirus (HCMV) is the most common pathogen that causes intrauterine infection. In the United States and Europe, the neonatal HCMV infection rate is about 2% [1]. In large cities of China, the active HCMV infection rate in pregnant women is about 6.97%, and newborn babies are diagnosed with HCMV in 37.32% cases [2]. HCMV infection during pregnancy can lead to miscarriage, stillbirth, retardation of fetal growth and brain [3]. How-

ever, the underlying mechanism(s) have not been fully elucidated.

During normal pregnancy, cytotrophoblasts (CTs) differentiate into villous trophoblasts (VTs) and extravillous cytotrophoblasts (EVTs) after implantation of the late blastocyst. VTs fuse to form syncytiotrophoblasts (STs), which are involved in endocrine, immune, defense and placental exchange. EVT proliferate to form multilayered columns of cells and then invade the endometrium and the uterine spiral arteries

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in order to anchor chorionic villi in the uterus and remodel the uterine arterioles. EVTs are formed about three weeks after fertilization and then gradually invade the decidua and superficial muscle, replacing the endothelial cells of the uterine spiral arteries, and remodel the spiral arteries for successful pregnancy [4]. Therefore, dysfunction of EVT invasion often leads to poor remodeling of spiral arteries and insufficient blood supply to the placenta, which are important factors in the pathology of pregnancy-related complications such as miscarriage, stillbirth, fetal growth retardation and pregnancy-induced hypertension [5]. In our previous study, we found that HCMV reduced the invasion of EVT [2].

During pregnancy, transforming growth factor- $\beta$  (TGF- $\beta$ ) promotes endometrial decidualization, inhibits trophoblast cell proliferation, migration, and invasion and regulates the formation and function of placenta [6]. TGF- $\beta$ 1 plays an important role in trophoblast cell differentiation and EVT invasion. TGF- $\beta$  acts via Smad transcriptional factors to regulate cell proliferation, differentiation, migration and apoptosis. *Smad7* is expressed in human chorionic villi CTs and STs and antagonizes TGF- $\beta$ /Smad signaling, in which it is a downstream target gene and its expression indicates TGF- $\beta$ /Smad signaling pathway activation [7, 8]. *Smad7* inhibits cell invasion and metastasis by inducing apoptosis and reducing the expression and activity of tissue matrix metalloproteinases-2 (MMP-2) and -9 (MMP-9) [9-11]. Matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, are the rate-limiting enzymes, which mediate EVT invasion and placenta formation [12].

In the current study, an *in vitro* culture of primary EVTs was generated and successfully infected with HCMV. This model was used to investigate the effect of HCMV on EVT proliferation and invasion. The possible molecular mechanisms underlying HCMV-induced miscarriage, stillbirth, and fetal growth retardation were also briefly examined.

### Materials and methods

#### Materials

**Tissue samples and virus:** Placenta samples were obtained from healthy pregnant women (peripheral HCMV IgM negative) who voluntarily

chose to terminate their pregnancies during the first trimester (5-10 weeks). Samples were collected between July 2012 and July 2014 in the Obstetrics and Gynecology department of the Central Hospital of Taian. The Medical Ethics Committee of the Central Hospital of Taian approved all experimental procedures and patients signed informed consent. The virus strain, HCMV AD169, was provided by the Hubei Provincial Institute of Virology and the TCID50 was  $10^{-5}$ /0.1 ml.

**Reagents:** The following materials were used: DMEM/Ham's F 12 medium (Gibco, Life Technologies, Carlsbad, CA, USA); fetal bovine serum (FBS, Hyclone, GE Healthcare, Little Chalfont, UK); DNase I and type I rat tail collagen (Sigma-Aldrich, St. Louis, MO, USA); Percoll (Amersham Pharmacia, GE Healthcare, Little Chalfont, UK); mouse anti-human cytokeratin 7 (CK7) monoclonal antibody, anti-vimentin (Vim) monoclonal antibody, rabbit anti-HCMV pp65 polyclonal antibody, rabbit anti-TGF- $\beta$ 1 polyclonal antibody, rabbit anti- $\beta$ -actin polyclonal antibody, rabbit anti-MMP-2 polyclonal antibody, rabbit anti-MMP-9 polyclonal antibody, rabbit anti-Smad7 polyclonal antibody (Santa Cruz Biotech, Dallas, Texas, USA); and total protein extraction kit (BestBio, Shanghai, China).

#### Methods

**Isolation and culture of primary EVTs:** EVT isolation and primary culture was carried out according to a modified protocol of Handschuh et al [13]. The human chorionic villi were thoroughly washed in sterile D-Hank's to remove the decidua and matrix. The remaining tissue was cut into 1 mm<sup>3</sup> pieces and digested in enzyme solution (0.125% trypsin, 4.2 mM MgSO<sub>4</sub>, 25 mM Hepes and 20 U/ml DNase I) at 37°C, with gentle shaking, for 50 min. The supernatant was collected and filtered through an 80 mesh (177 micron) and 300 metal mesh. The filtrate was centrifuged at 500× g for 10 min and the supernatant was discarded. The pellets were suspended in DMEM/Ham's F-12 medium and the cell suspension was supplemented with 35%, 40%, 45% or 50% Percoll gradient solution and centrifuged at 1000× g for 25 min. The cell suspension in 40~45% Percoll gradient was transferred to another centrifuge tube and washed twice in DMEM/Ham's F-12 medium, supplemented with 10% FBS. The cells were suspended in DMEM/

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**Table 1.** Primer sequences information

mRNA		Primers (5'-3')
TGF-β1	Forward	GGGACTATCCACCTGCAAGA
	Reverse	CCTCCTGGCGTAGTAGTCA
TGF-β2	Forward	CGCCAAGGAGGTTTACAAA
	Reverse	CTCCATTGCTGAGACGTCAA
TGF-β3	Forward	GAGATGTTGTGTGGCAATGG
	Reverse	TTGCCCTTAATCCCAGACAG
TGFβRI	Forward	GATGGGCTCTGCTTTGTCTC
	Reverse	CAAGGCCAGGTGATGACTTT
TGFβRII	Forward	GGGGAACAATACTGGCTGA
	Reverse	GAGCTCTTGAGTCCCTGTG
Smad1	Forward	CTACCCTCACTCTCCACCA
	Reverse	GCACCAGTGTGTTGGTTCCT
Smad2	Forward	CGAAATGCCACGGTAGAAAT
	Reverse	CCAGAAGAGCAGCAAATTCC
Smad3	Forward	CCCCAGAGCAATATCCAGA
	Reverse	GGCTCGCAGTAGGTAACCTGG
Smad4	Forward	CCATTTCCAATCATCCTGCT
	Reverse	ACCTTTGCCTATGTGCAACC
Smad5	Forward	AACCTGAGCCACAATGAACC
	Reverse	GTGGCATATAGGCAGGAGGA
Smad6	Forward	ACGGTGACCTGCTGTCTCTT
	Reverse	ACGTGACGGTTTTGAGTCC
Smad7	Forward	TCCTGCTGTGCAAAGTGTTT
	Reverse	TCTGGACAGTCTGCAGTTGG
MMP2	Forward	ATGACAGCTGCACCACTGAG
	Reverse	ATTTGTTGCCAGGAAAGTG
MMP9	Forward	TTGACAGCGACAAGAAGTGG
	Reverse	GCCATTCACGTGTCCTTAT
β-action	Forward	CATCCGTAAGACCTCTATGCCAAC
	Reverse	ATGGAGCCACCGATCCACA

Ham's F12 medium, supplemented with 10% FBS, seeded at a density of 50000 cells/cm<sup>2</sup> in 48 well-culture plates coated with type I rat tail collagen (0.006 mmol/L acetic acid solution of rat tail collagen) and cultured in a 37°C, 5% CO<sub>2</sub> incubator for 24 h. The cultures were then washed 3 times with sterile D-Hank's and fresh medium was added.

**EVT infection by HCMV:** EVTs were seeded in 6-well plates at a density of 50 000/cm<sup>2</sup> and cultured at 37°C and 5% CO<sub>2</sub> for 24 h. The EVT culture medium was replaced with the HCMV culture medium. The virus group was inoculated with 4 μL HCMV (TCID50:10<sup>-5.46</sup>/0.1 mL) and the normal group was treated with PBS. Two hours later, the HCMV medium was discarded,

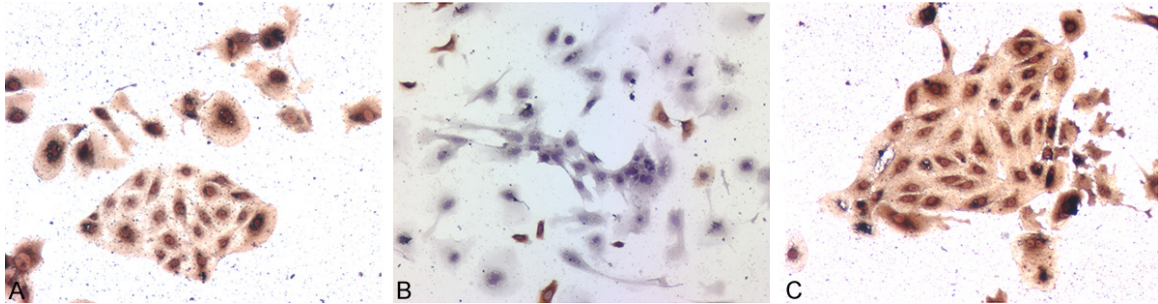
and EVTs were washed with sterile D-Hank's 3 times. The EVT culture medium was added, and the EVTs were cultured at 37°C and 5% CO<sub>2</sub> for 12, 24, 48, and 72 h. The HCMVpp65 antigen was detected with immunofluorescence staining, HCMVpp65 expression results were analyzed using a HMIAS-2000 high-resolution color medical image analysis system. Ten high magnification photomicrographs were randomly selected. The mean optical density of positive cells was determined and used for semi-quantitative statistical analysis.

**MTT assay:** Cells were seeded in 96-well plates at a density of 10<sup>3</sup>/ml. Each well contained 200 μL of cell solution and a blank control was included in each plate. The cells were divided into control and virus groups. The viral group was further divided into 7 subgroups and 10, 20, 30, 40, 50, 70 or 100 μL of HCMV solution was added to each subgroup. A 20 μL volume of MTT solution was added to each well at 12, 24, 48 and 72 h and the plate was incubated for another 4 h. The plate was removed and the medium was discarded. After addition of 150 μL DMSO to each well the plate was shaken for 10 min. The OD value at 490 nm was measured by enzyme-linked immunosorbent assay (ELISA) and the results were used to analyze the effects of HCMV on EVT proliferation. The minimum viral titer with a significant effect on cell proliferation was considered to be the optimal viral titer for inoculation.

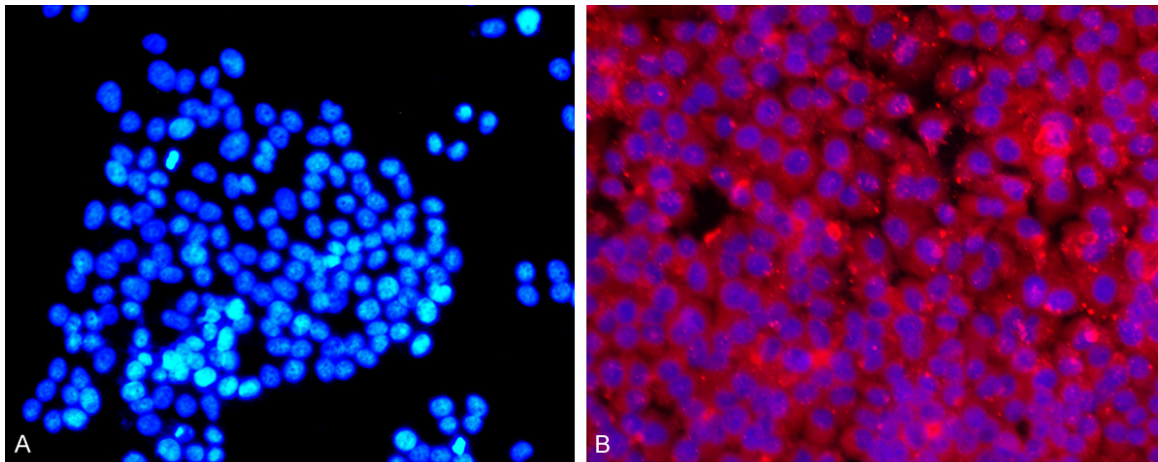
**Grouping:** The virus group was inoculated with HCMV (by volume, 100 TCID50 HCMV: DMEM/ Ham's F-12 containing 3% FBS = 1:6); the control group received the same volume of PBS. After 2 h, the supernatant was removed. The cells were washed 3 times with PBS and then cultured in DMEM/ Ham's F-12 medium containing 10% FBS, for 48 h. Cells were collected for immunocytochemistry and Western blotting analysis.

**Quantitative real-time polymerase chain reaction (qRT-PCR):** Two groups of EVTs were treated for 48 h and EVT was used for extracting total RNA with an RNA extraction kit (Takara, Japan). RNA was transcribed into cDNA using a reverse transcription kit (Takara, Japan) for the following experiments. According to the sequences of major genes in TGF-β/Smad signaling pathways, real-time fluorescent quantitative PCR primers were designed, and the

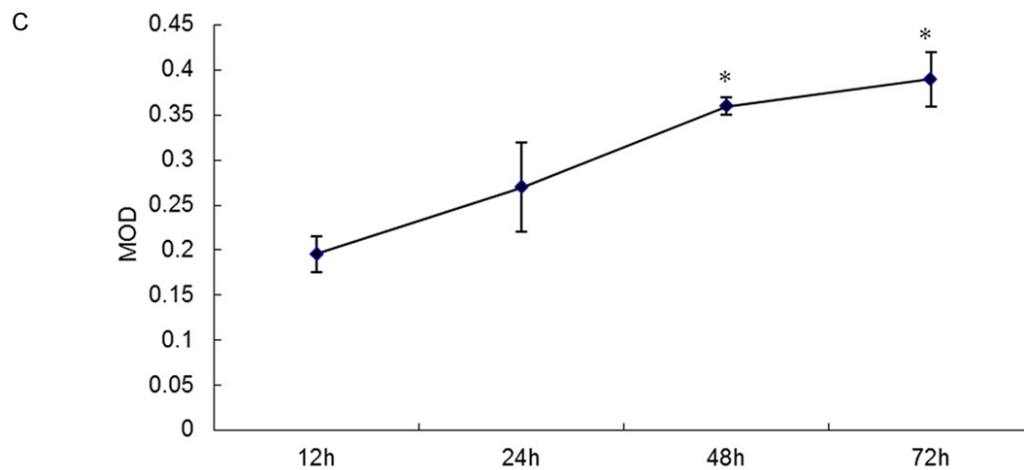
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**Figure 1.** Identification of primary EVT by CK7, Vim and c-erbB-2 antigens. A-C. CK7, Vim and c-erbB-2 antigens were detected, respectively, by immunocytochemistry  $\times 100$ .



The proliferation of HCMV in EVT



**Figure 2.** Immunofluorescence staining of EVT. A. Control group (Nucleus stained blue; no red signal); B. Virus group (HCMV pp65 antigen stained red; nucleus stained blue)  $\times 200$ ; C. Immunofluorescence staining quantitative analysis: HCMVpp65 expression in EVT at 48 h and 72 h was significantly higher than at 12 h and 24 h ( $P < 0.05$ ), but was significantly different between 48 h and 72 h ( $P > 0.05$ ).

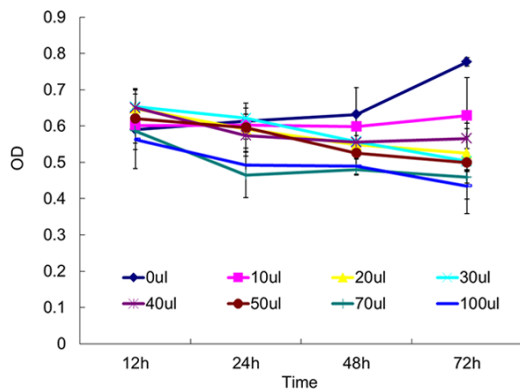
amplified length was 200 bp.  $\beta$ -actin was designed as the internal standard. Primer sequences are listed in **Table 1**. The PCR reaction system was prepared using 10  $\mu$ L 2\*SYBR

Green general qPCR Master Mix (TaKaRa, Japan), 0.6  $\mu$ L upstream/downstream primer, respectively (10  $\mu$ mmol $\cdot$ L $^{-1}$ ), and 8.8  $\mu$ L 1:100 diluted cDNA. The total reaction volume was 20



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The effect on EVT growth of HCMV infection to be tested by MTT assay



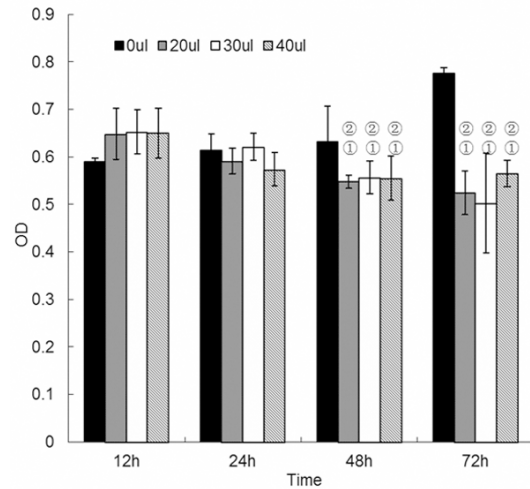
**Figure 3.** Effect of HCMV on EVT tested by MTT. Compared with the control group, infection with 10  $\mu$ L-100  $\mu$ L virus solution for 12 h had little effect on EVT proliferation ( $P > 0.05$ ). However, EVT proliferation was significantly inhibited ( $P < 0.05$ ) after infection with 70  $\mu$ L and 100  $\mu$ L virus solution for 24 h; 20, 30, 40 or 50  $\mu$ L virus solution for 48 h; or 10  $\mu$ L virus solution for 72 h. There was no significant difference in the inhibition of EVT cell proliferation at 48 h or 72 h after infection with 20  $\mu$ L, 30  $\mu$ L or 40  $\mu$ L virus solution ( $P > 0.05$ ). These experiments above were performed in triplicate.

$\mu$ L. The reaction mixture was precipitated following centrifugation at 1500 rpm/min. The PCR was conducted under the following reaction conditions: pre-degenerated for 30 s at 95°C; degeneration for 3 s at 95°C; and annealing and extension for 30 s at 60°C. The dissolution curve was constructed. Finally, the data were directly read off the real-time fluorescence quantitative PCR system (Applied Biosystems, Foster City, CA, USA).

**Immunocytochemistry (fluorescence) staining:** Forty-eight h after HCMV infection, the cells were fixed for 30 min in a methanol-acetone (1:1) solution at room temperature. SP immunocytochemical staining was used to detect CK7, Vim and c-erbB-2 in order to determine purity. HCMV infection was determined by HCMV pp65 antigen staining and EVT cell invasion was indirectly estimated by analysis of TGF- $\beta$ 1, Smad7, and MMP-2 and MMP-9 expression levels. Mouse anti-human CK7, anti-Vim monoclonal antibody, rabbit anti-HCMVpp65, TGF- $\beta$ 1, Smad7, MMP-2, and MMP-9 polyclonal antibodies were diluted 1:100. TGF- $\beta$ 1, Smad7, MMP-2 and MMP-9 expression results were analyzed as mentioned before.

**Western blot:** TGF- $\beta$ 1, Smad7, MMP-2 and MMP-9 protein levels were determined by

The effect on proliferation of EVT infected by HCMV

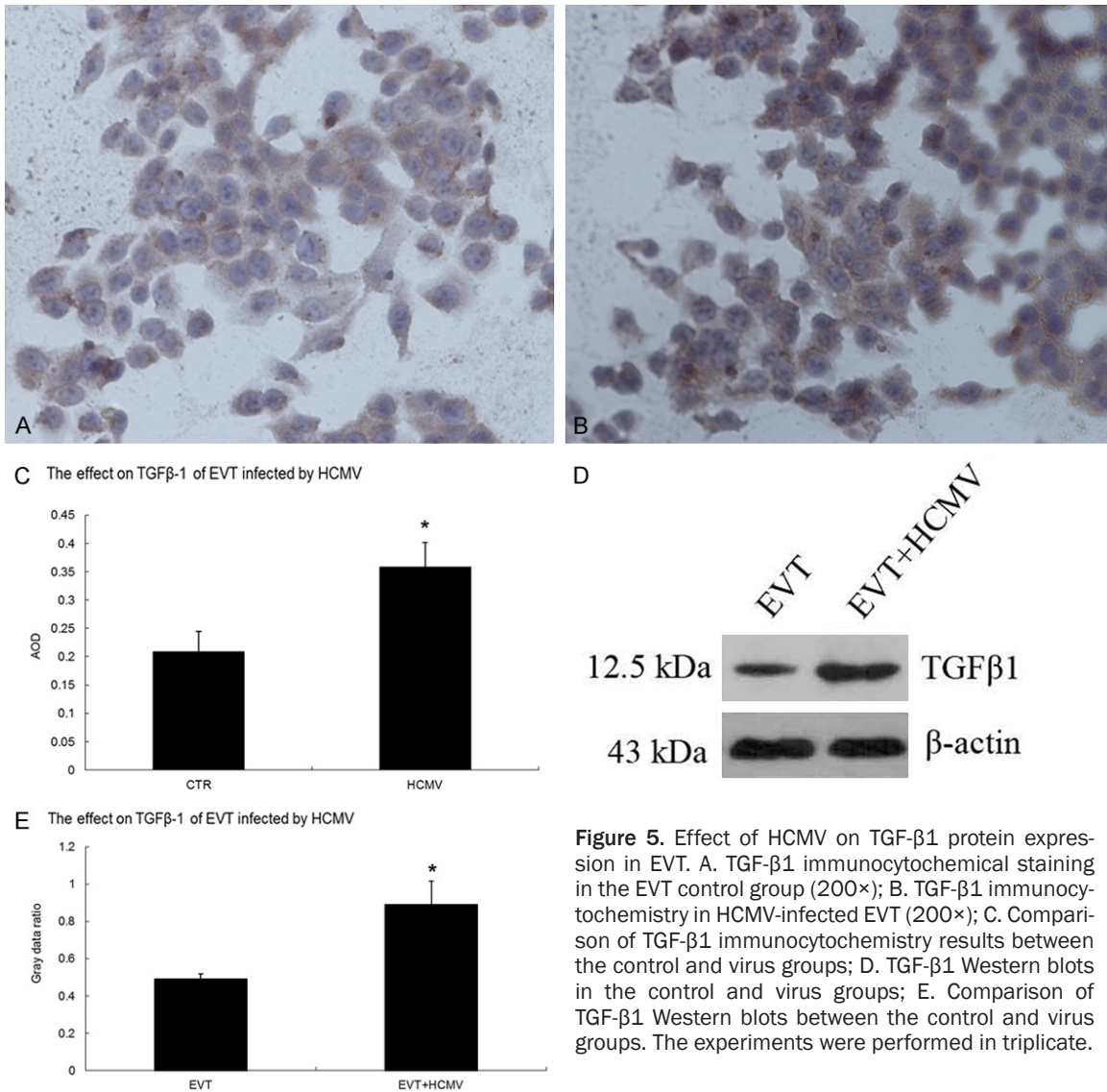


**Figure 4.** EVT proliferation following HCMV infection with 20  $\mu$ L, 30  $\mu$ L and 40  $\mu$ L virus solution. Compared with the control group, EVT proliferation was significantly inhibited ( $P < 0.05$ ) after infection with 20  $\mu$ L, 30  $\mu$ L or 40  $\mu$ L virus solution for 48 h and 72 h, without any significant difference in inhibition ( $P > 0.05$ ). The experiments were performed in triplicate.

Western blot. The total protein was extracted and the concentration was measured using a total protein extraction kit (BestBio), according to the manufacturer's instructions. Fifty  $\mu$ g of total protein from each sample was separated on a 10% SDS-PAGE gel by electrophoresis and transferred to a PVDF membrane. The membrane was blocked in 5% skim milk at room temperature for 2 h and then incubated with rabbit anti-TGF- $\beta$ 1, Smad7, MMP-2 or MM-P9 polyclonal antibody (1:500 dilution) overnight at 4°C. The membrane was then washed in TBST and incubated with the secondary antibody at room temperature for 2 h. The membrane was washed again in TBST, incubated with ECL reagent and protein bands were visualized under a gel imager. Gel image Quantity One analysis software was used to measure the absorbance values of the target protein and the internal control  $\beta$ -actin protein. The mean absorbance of the target protein over that of the internal control was used as the relative expression level of the target protein.

**In vitro invasion assay:** *In vitro* invasion assay was used for determining the invasive potential of EVTs. The Transwell chamber coated with Matrigel was placed in 24-well culture plates and 400  $\mu$ L medium containing conditioned medium and complete medium (1:1) was infused into well out of chamber. In the HCMV

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**Figure 5.** Effect of HCMV on TGF-β1 protein expression in EVT. A. TGF-β1 immunocytochemical staining in the EVT control group (200×); B. TGF-β1 immunocytochemistry in HCMV-infected EVT (200×); C. Comparison of TGF-β1 immunocytochemistry results between the control and virus groups; D. TGF-β1 Western blots in the control and virus groups; E. Comparison of TGF-β1 Western blots between the control and virus groups. The experiments were performed in triplicate.

group, EVT at  $1 \times 10^5$ /mL and 100 TCID<sub>50</sub> HCMV 14.29 μL (with a total amount of 100 μL) was used in the Transwell chamber. In the control group, an identical volume of PBS was used instead of HCMV solution. Each group had 4 duplicate samples. After incubation for 24 h, the sample was fixed with formaldehyde, hematoxylin-stained and observed under an inverted microscope to count the number of cells migrating through the micropore membrane. For each sample, 10 randomly selected high power fields were counted.

### Statistical analysis

All experiments were performed 3 times. The SPSS 18.0 software package was used for sta-

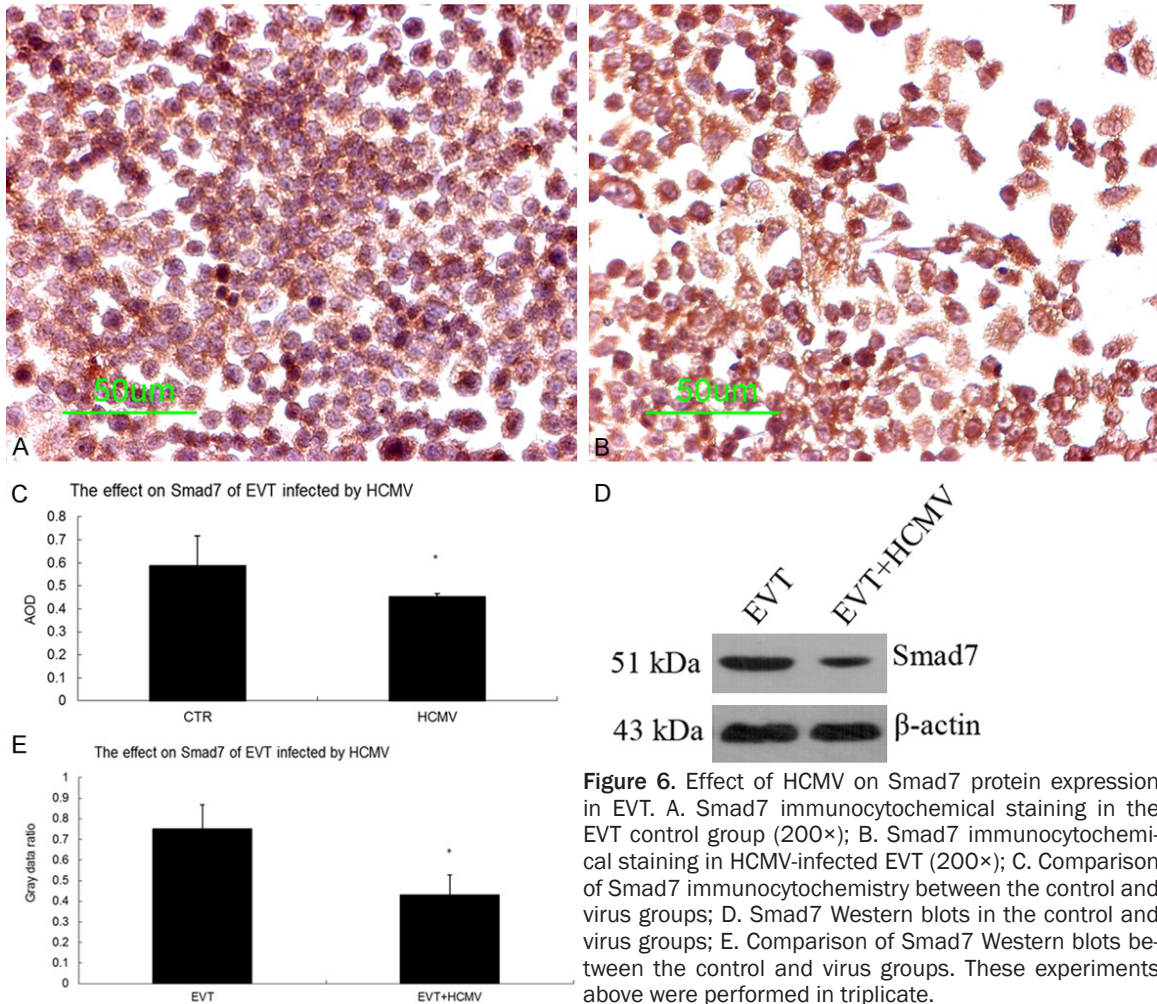
tistical analysis and data were expressed as mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). The significance was assessed by ANOVA. A  $P < 0.05$  was considered to be statistically significant.

## Results

### Purity of isolated EVTs

Immunocytochemistry showed that the majority of the isolated cells contained a single nucleus and were triangular and irregular in shape. More than 96% of the cells were positively stained for CK7 and c-erbB-2, and occasionally for Vim, suggesting that the primary EVT isolated from human chorionic villi were of high purity (**Figure 1**).

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**Figure 6.** Effect of HCMV on Smad7 protein expression in EVT. A. Smad7 immunocytochemical staining in the EVT control group (200×); B. Smad7 immunocytochemical staining in HCMV-infected EVT (200×); C. Comparison of Smad7 immunocytochemistry between the control and virus groups; D. Smad7 Western blots in the control and virus groups; E. Comparison of Smad7 Western blots between the control and virus groups. These experiments above were performed in triplicate.

### *In vitro* HCMV infection of EVT

As shown by **Figure 2**, HCMV-infected EVT showed strong HCMVpp65 staining (red signal), while non-infected EVT showed no detectable HCMVpp65 expression, suggesting that HCMV infected and proliferated in host EVT. Using immunofluorescence staining quantitative analysis, we found that HCMVpp65 expression in EVT at 48 h and 72 h was significantly higher than that of 12 h and 24 h ( $P < 0.05$ ), but was significantly different between 48 h and 72 h ( $P > 0.05$ ). This phenomenon may be caused by excessive proliferation of the virus resulting in a higher number of EVT deaths.

### Effect of HCMV on EVT proliferation

Compared with the control group, infection with 10  $\mu$ L to 100  $\mu$ L virus solution for 12 h had little effect on EVT proliferation ( $P > 0.05$ ). However,

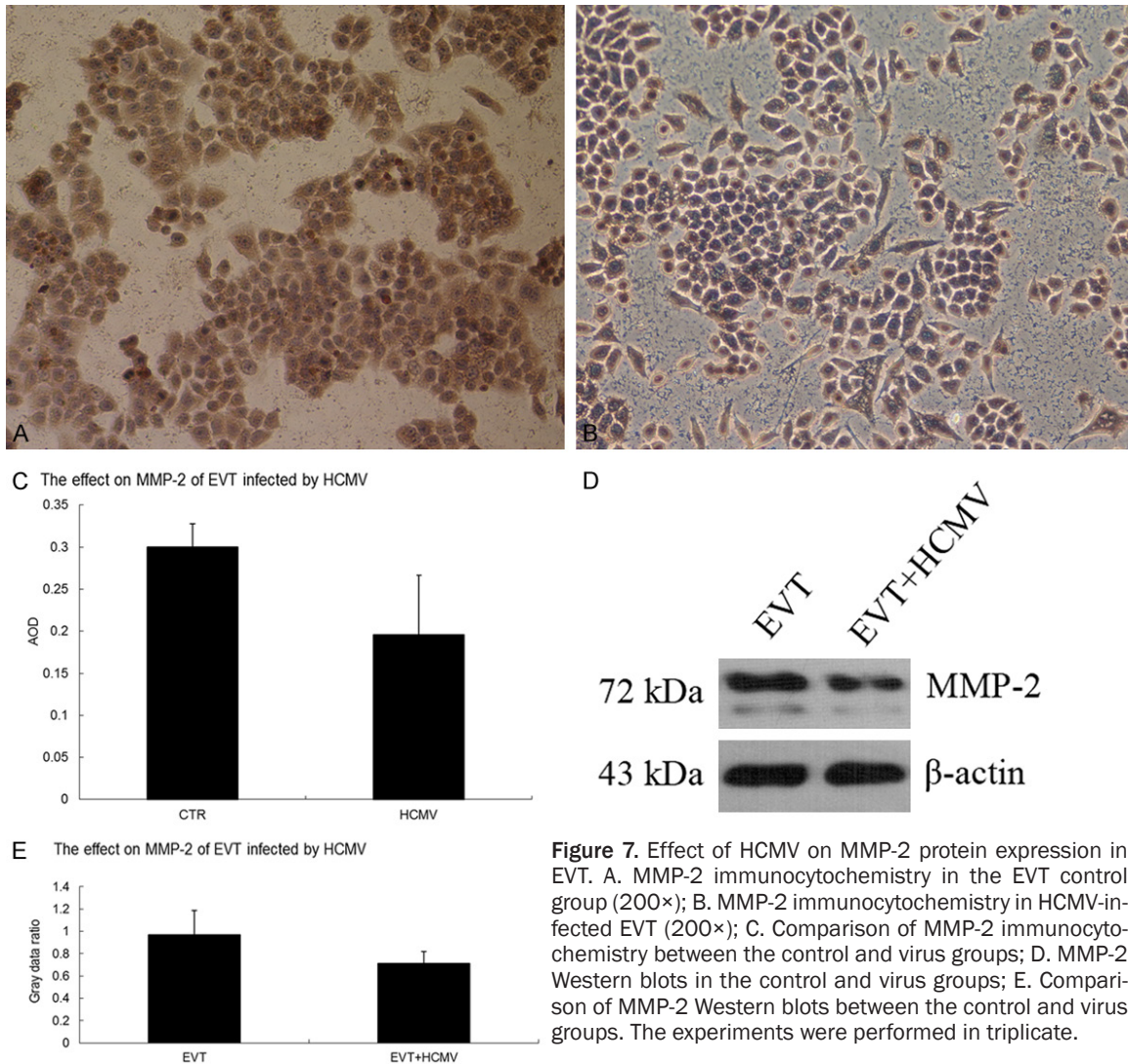
EVT proliferation was significantly inhibited after infection with 70  $\mu$ L to 100  $\mu$ L virus solution for 24 h; 20  $\mu$ L, 30  $\mu$ L, 40  $\mu$ L or 50  $\mu$ L virus solution for 48 h; or 10  $\mu$ L virus solution for 72 h ( $P < 0.05$ ). No significant difference was observed in the inhibition of EVT cell proliferation at 48 h or 72 h after infection between the two groups of 20  $\mu$ L, 30  $\mu$ L or 40  $\mu$ L virus solutions ( $P > 0.05$ ). Based on these results, the 20  $\mu$ L solution containing 100TCID<sub>50</sub> HCMV, diluted in 120  $\mu$ L medium, was selected for subsequent experiments (**Figures 3 and 4**).

### Effect of HCMV on expression of major genes in TGF- $\beta$ /Smad signaling pathways in EVT

The results of qRT-PCR showed that the gene expression of TGF- $\beta$ 1, Smad1, Smad2, Smad3, Smad4, and Smad5 was significantly increased ( $P < 0.05$ ), but that of TGF- $\beta$ 2, TGF- $\beta$ 3, TGF $\beta$ RI, TGF $\beta$ RII, and Smad7 was significantly



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**Figure 7.** Effect of HCMV on MMP-2 protein expression in EVT. A. MMP-2 immunocytochemistry in the EVT control group (200 $\times$ ); B. MMP-2 immunocytochemistry in HCMV-infected EVT (200 $\times$ ); C. Comparison of MMP-2 immunocytochemistry between the control and virus groups; D. MMP-2 Western blots in the control and virus groups; E. Comparison of MMP-2 Western blots between the control and virus groups. The experiments were performed in triplicate.

decreased in the virus group 48 h after HCMV infection ( $P < 0.05$ ). Immunocytochemistry and Western blot results showed that the control and virus groups expressed the TGF- $\beta$ 1 and Smad7 protein. The TGF- $\beta$ 1 protein level was significantly elevated in the virus group 48 h after HCMV infection ( $P < 0.05$ ) (Figure 5) whereas the Smad7 protein level was significantly decreased in the virus group 48 h after HCMV infection ( $P < 0.05$ ) (Figure 6).

### Effect of HCMV on expression of MMP-2 and MMP-9 in EVT

The results of qRT-PCR showed that the gene expression of *MMP2* and *MMP9* was significantly decreased in the virus group 48 h after HCMV infection ( $P < 0.05$ ). Immunocytochemistry and Western blot results showed that both

the control and virus groups expressed the MMP-2 and MMP-9 proteins. The MMP-2 and MMP-9 protein levels were decreased in the virus group 48 h after HCMV infection ( $P < 0.05$ ) (Figures 7 and 8).

### Effect of HCMV on EVT invasion

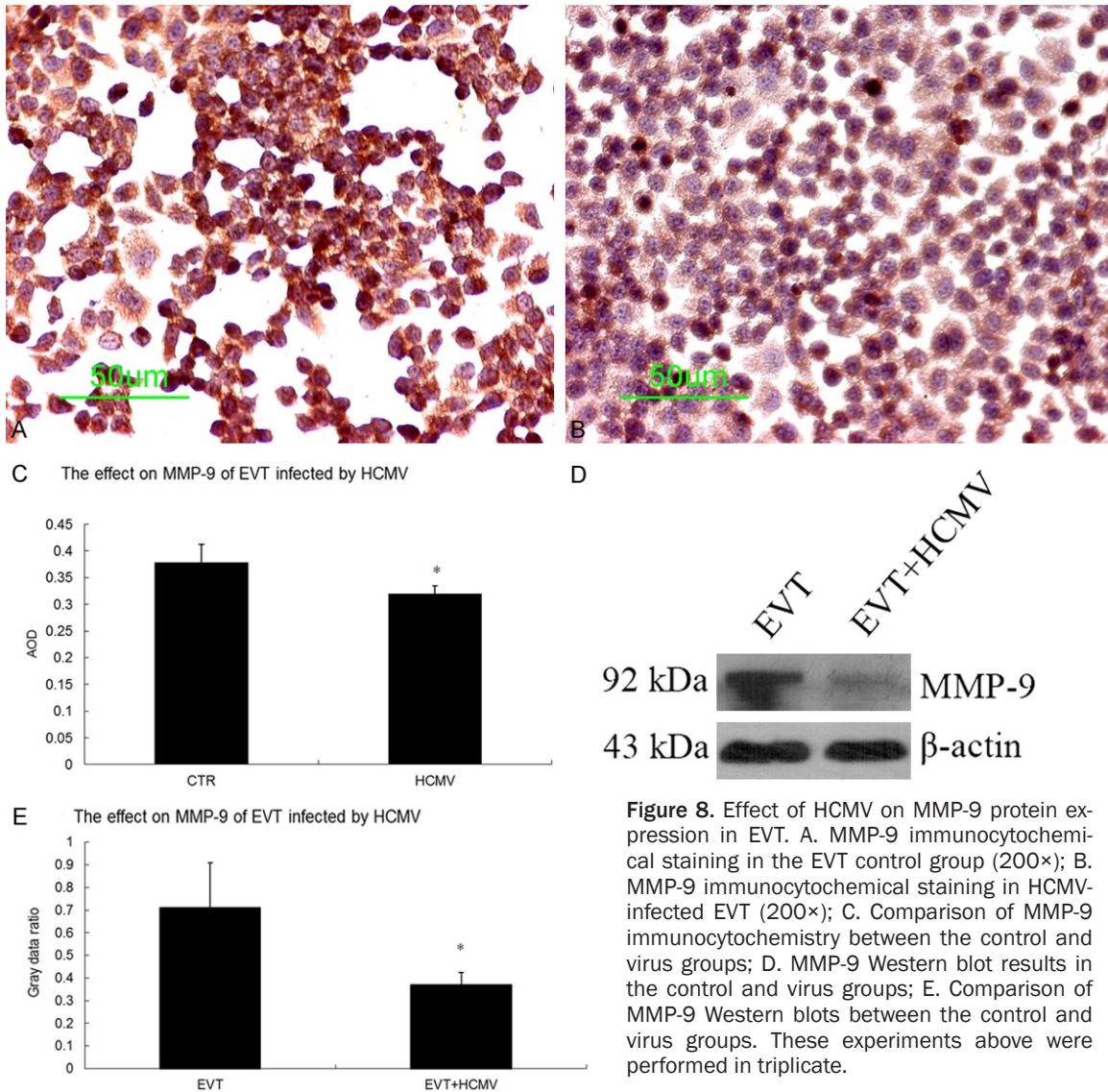
EVT invaded the Matrigel. The number of EVT penetrating the Matrigel was  $57.0 \pm 3.61$  in the control group and  $49.2 \pm 3.27$  in the virus group ( $P < 0.05$ ), suggesting that HCMV reduced EVT invasion.

### Discussion

HCMV is distributed worldwide and humans are the only host. Several HCMV strains have been identified based on differences in antigen



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**Figure 8.** Effect of HCMV on MMP-9 protein expression in EVT. A. MMP-9 immunocytochemical staining in the EVT control group (200×); B. MMP-9 immunocytochemical staining in HCMV-infected EVT (200×); C. Comparison of MMP-9 immunocytochemistry between the control and virus groups; D. MMP-9 Western blot results in the control and virus groups; E. Comparison of MMP-9 Western blots between the control and virus groups. These experiments above were performed in triplicate.

expression. The HCMV AD169 strain is the standard laboratory strain. The HCMVpp65 antigen is an early indicator of active HCMV infection since it is expressed in vascular endothelial cells, peripheral blood lymphocytes, monocytes and polymorphonuclear leukocytes within 6-24 h of infection [14, 15]. Therefore, the HCMV pp65 antigen was selected as an infection marker in this study.

Currently the mechanism(s) underlying intrauterine HCMV infection is not clear, though trophoblast cells are considered the primary site of intrauterine HCMV infection. HCMV infects different types of trophoblast cells [1, 16-20], including CTs, STs, VTs and EVT, leading to cell damage and death, poor differentiation of CTs,

impaired VT fusion, diminished numbers of and immune secretion dysfunction in STs, and reduced EVT proliferation and invasion [1].

EVT invasion mainly depends on two pathways: MMPs and the urokinase plasminogen activator (uPA) system [21]. EVT infiltration occurs along the spiral arteries at around 10 weeks, reaching the decidua at around 12 weeks and invading a third of the myometrium within 20 weeks of pregnancy. Therefore, EVT invasion of the uterine decidua and superficial myometrium, in addition to physiological remodeling of uterine spiral arteries, are prerequisites for a successful pregnancy. EVT proliferation and invasion dysfunction can result in poor remodeling of spiral arteries and poor placental sub-

stance exchange, which is an important pathophysiological mechanism in miscarriage, stillbirth, intrauterine growth retardation, premature birth and pregnancy-induced hypertension [22, 23].

Primary EVT culture provides an appropriate tool to study the mechanisms related to the aforementioned conditions. CK is a cytoskeletal protein of epithelial cells, c-erbB-2 is an epidermal growth factor receptor with tyrosine kinase activity and can be used as a specific EVT marker and Vim is a marker of endothelial and stromal cells but is not expressed in EVTs [24]. In the current study, healthy early chorionic villi and isolated primary EVTs were collected. Immunocytochemistry staining showed that the isolated primary EVTs were mononuclear, that the majority of cells were triangular or irregular in shape and that almost all of the cells were CK7 and c-erbB-2 positive, and occasionally Vim positive. This suggests that a sufficient number of highly pure primary EVTs were isolated, thus laying the foundation for future study. In this study, HCMV viremia during pregnancy was modeled by infecting primary EVTs with HCMV. Immunofluorescence staining showed that the HCMVpp65 signal appeared within the infected primary EVTs, but not in the control group, indicating that HCMV infected and replicated within primary cultured EVTs that served as HCMV host cells [25].

During the normal course of pregnancy, TGF- $\beta$  promotes endometrial decidualization, inhibits trophoblast cell proliferation, migration, and invasion, stimulates trimester and later trophoblast cells to form multinucleated cells and regulates the formation and function of the placenta [26]. In early pregnancy, low TGF- $\beta$ 1 expression leads to inadequate maternal immune tolerance and embryonic implantation disorders, while high TGF- $\beta$ 1 expression affects the normal invasion of trophoblast cells into endometrial tissue, also resulting in embryonic implantation disorders [27, 28]. TGF- $\beta$ 1 regulates trophoblast cell differentiation and invasion as well as EVT invasion in a dose-dependent manner [29]. TGF- $\beta$ 1 expression peaks at 17 and 34 weeks of pregnancy, which is consistent with the completion of EVT invasion into the decidua and placenta at 18 weeks and the conclusion of fetal growth at 35 weeks of pregnancy [30, 31]. Results of the current study showed

that the primary EVT expressed TGF- $\beta$ 1, which was significantly higher after HCMV infection.

The Smad proteins are the only confirmed substrates of the TGF- $\beta$  receptor [32] thus far. They are functionally divided into three categories: (i) receptor-regulated Smads (R-Smads), including Smad1, Smad2, Smad3, Smad5 and Smad8; (ii) common mediator SMAD (Co-Smads), including Smad4 and Smad10, which are the common mediators of TGF- $\beta$  signaling; and (iii) inhibitory Smads (I-Smads), including Smad6 and Smad7. Smad6 mainly inhibits the BMP signaling and Smad7 mainly acts on the TGF- $\beta$  signaling pathway.

Smads are key mediators of the TGF- $\beta$  signal transduction pathway, as well as cell proliferation, differentiation, migration and apoptosis. Binding of biologically active TGF- $\beta$  to TGF $\beta$ RII activates TGF $\beta$ RI, resulting in the phosphorylation of C-terminal serine residues and activation of receptor-specific Smad proteins (Smad1/Smad5 or Smad2/Smad3). Activated R-Smads associate with the Co-Smads (Smad4) to form heterodimers and translocate to the nucleus, where they directly bind to the DNA sequence in conjunction with other positive or negative transcription factors to stimulate transcription. Smad6 competes with Smad4 for phosphorylated Smad1 or directly binds to activated TGF $\beta$ RI to prevent phosphorylation of R-Smads and Smad7 directly binds to activated receptors, thus both function as negative regulators of the TGF- $\beta$ /Smad signaling pathway [33]. Smad7 expression is a major indicator of TGF- $\beta$  pathway activation since TGF- $\beta$  signaling induces its expression [7, 8].

Smad7 induces apoptosis, reduces the expression and activity of MMP-2, MMP-9 and uPA, inhibits cell invasion and metastasis [9-11] and negatively regulates TGF- $\beta$ 1 receptors, thus blocking TGF- $\beta$ 1 signal transduction and reducing ECM synthesis to inhibit fibrosis [34]. In the placenta, Smad7 is mainly located in the cytoplasm of villi CTs and STs. In the TGF- $\beta$ /Smad signaling pathway, TGF- $\beta$  stimulates the Smad7 transcription as a negative feedback to decrease TGF- $\beta$  signaling [35], thus regulating EVT invasion temporally and spatially. Our results suggest that the primary EVT expressed Smad7 and after HCMV infection, and Smad7 expression in primary EVT was significantly reduced.

## HCMV in the proliferation and invasion of EVT

MMPs are closely related to blastocyst implantation, placental formation and vascular remodeling [36] and function as a rate-limiting biochemical mediator and marker of trophoblast invasion [37]. MMP-2 and MMP-9 are considered to be the rate-limiting enzymes involved in EVT invasion and placenta formation [12]. During placenta formation, the physical environment of the maternal-fetal interface changes, including the ECM components. A variety of hormones, growth factors and cytokines are also produced by the placenta or maternal decidua, all of which directly or indirectly affect the MMPs/tissue inhibitor of metalloproteinases (TIMPs) system to regulate EVT invasion [38]. TGF- $\beta$  induces fibronectin, plasminogen activator inhibitor and TIMP-1 expression to inhibit MMPs activation [39]. Under normal circumstances, MMPs, TIMPs and TGF- $\beta$ 1 are temporally and spatially coordinated to regulate the synthesis and degradation of collagen type IV in ECM. MMPs/TIMPs dysregulation and abnormal EVT invasion cause miscarriage, intrauterine growth restriction, hypertensive disorders in pregnancy and trophoblastic disease [40, 41]. MMP-9 secretion by trophoblast cells is negatively correlated with exogenous TGF- $\beta$ 1 levels. TGF- $\beta$ 1 significantly enhances the adhesion between the trophoblast cells, as well as inhibits MMP-2 and MMP-9 expression, indicating that TGF- $\beta$ 1 may promote adhesion between trophoblasts, inhibit the secretion of MMP-2 and MMP-9 and regulate EVT invasion during normal pregnancy [42, 43].

Results of the current study confirmed that primary EVTs express MMP-2 and MMP-9 with enzymatic activity and that HCMV infection reduced the expression level and activity of both enzymes. In addition, it was found that HCMV infection reduced EVT invasion.

In short, in EVTs and villi, HCMV infection boosted the expression of TGF- $\beta$ 1. It also reduced the expression of Smad7, the expression and activity of MMP-2 and MMP-9 and the invasion of EVT. This suggests that HCMV damages EVT, resulting in increased secretion of TGF- $\beta$ 1. Meanwhile, HCMV inhibits Smad7 expression, which suppresses the negative feedback, leading to a further increase in TGF- $\beta$ 1, which binds to receptors on trophoblast cells to excessively inhibit the secretion of MMP-2 and MMP-9. Therefore, abnormal TGF- $\beta$ 1 expression dis-

rupts the balance between the inhibition and stimulation of trophoblast cell differentiation and invasion, leading to excessive suppression of invasion and differentiation of trophoblast cells, leading to shallow the EVT implantation that prevents the formation of uterine spiral artery endothelial cells.

HCMV acts on multiple steps of the TGF- $\beta$ /Smad signaling pathway to impede EVT proliferation and invasion, leading to narrow spiral arteries, high blood impedance, decreased placental blood perfusion and substance exchange, resulting in poor pregnancy outcomes. Additional studies will be conducted using whole animal experiments.

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### Disclosure of conflict of interest

None.

### Abbreviations

HCMV, Human cytomegalovirus; CT, cytotrophoblast; VT, villous trophoblast; EVT, extravillous cytotrophoblast; ST, syncytiotrophoblast; TGF- $\beta$ , transforming growth factor- $\beta$ ; MMP, Matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; CK7, cytokeratin 7; Vim, vimentin; uPA, urokinase plasminogen activator; ECM, extracellular matrix.

**Address correspondence to:** Dr. Suhua Chen, Department of Obstetrics and Gynecology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China. Tel: +8613375388585; E-mail: tj\_sch@163.com

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