**Original Article**

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

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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-β/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-β1, Smad1, Smad2, Smad3, Smad4, and Smad5 genes was significantly increased (P < 0.05), but that of TGF-β2, TGF-β3, TGFβRI, TGFβRII, 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-β1 protein level was significantly increased in infected EVT (all P < 0.05). Conclusions: HCMV may act on multiple steps of the TGF-β/Smad signaling pathway to impede EVT proliferation and invasion.

**Keywords:** Human cytomegalovirus, extravillous cytotrophoblast, TGF-β1, 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]. However, 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...
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-β (TGF-β) promotes endometrial decidualization, inhibits trophoblast cell proliferation, migration, and invasion and regulates the formation and function of placenta [6]. TGF-β1 plays an important role in trophoblast cell differentiation and EVT invasion. TGF-β 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-β/Smad signaling, in which it is a downstream target gene and its expression indicates TGF-β/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 10^5.0/0.1 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-β1 polyclonal antibody, rabbit anti-β-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 mm³ pieces and digested in enzyme solution (0.125% trypsin, 4.2 mM MgSO₄, 25 mM Hapes 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**

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>GGGGACCTCTGACGGAGGA</td>
<td>CCTCCCTTGGCCAAGA</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>GAGATGGTCCTGGCCATGG</td>
<td>TTGTCACATGATGAGG</td>
</tr>
<tr>
<td>TGFβRII</td>
<td>GGCTAGAATCTGTGGTTTG</td>
<td>GAGCTCTTGGCTGCA</td>
</tr>
<tr>
<td>Smad2</td>
<td>CAGAAATGGGTTGCAAAGG</td>
<td>CCAAGAAGCAGCAAATTC</td>
</tr>
<tr>
<td>Smad4</td>
<td>CGGCTACATGATGAGG</td>
<td>GTGGGAAATAACTGGCTGA</td>
</tr>
<tr>
<td>Smad6</td>
<td>TCCCTTCATGACGTTGCT</td>
<td>GTCAGGAGGTTGCTGGTG</td>
</tr>
<tr>
<td>MMP2</td>
<td>ATGACAGCTGACCTGGAG</td>
<td>ATGGTTCGCTGACCTGG</td>
</tr>
<tr>
<td>β-action</td>
<td>TTCCTGACATGAAAGTGTTC</td>
<td>TTCCTGACATGAAAGTGTTC</td>
</tr>
</tbody>
</table>

Ham’s F12 medium, supplemented with 10% FBS, seeded at a density of 50000 cells/cm² 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% CO₂ 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/cm² and cultured at 37°C and 5% CO₂ for 24 h. The EVT culture medium was replaced with the HCMV culture medium. The virus group was inoculated with 4 μL HCMV (TCID50: 10⁻⁵.46/0.1 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% CO₂ 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 10³/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
amplified length was 200 bp. β-actin was designed as the internal standard. Primer sequences are listed in **Table 1**. The PCR reaction system was prepared using 10 μL 2*SYBR Green general qPCR Master Mix (TaKaRa, Japan), 0.6 μL upstream/downstream primer, respectively (10 μmol•L⁻¹), and 8.8 μL 1:100 diluted cDNA. The total reaction volume was 20
μL. 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-β1, Smad7, and MMP-2 and MMP-9 expression levels. Mouse anti-human CK7, anti-Vim monoclonal antibody, rabbit anti-HCMVpp65, TGF-β1, Smad7, MMP-2, and MMP-9 polyclonal antibodies were diluted 1:100. TGF-β1, Smad7, MMP-2 and MMP-9 expression results were analyzed as mentioned before.

Western blot: TGF-β1, Smad7, MMP-2 and MMP-9 protein levels were determined by 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 μg 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-β1, Smad7, MMP-2 or MMP-9 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 β-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 μL medium containing conditioned medium and complete medium (1:1) was infused into well out of chamber. In the HCMV

![Figure 3. Effect of HCMV on EVT tested by MTT. Compared with the control group, infection with 10 μl-100 μL 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 μL and 100 μL virus solution for 24 h; 20, 30, 40 or 50 μL virus solution for 48 h; or 10 μL 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 μL, 30 μL or 40 μL virus solution (P > 0.05). These experiments above were performed in triplicate.](image1)

![Figure 4. EVT proliferation following HCMV infection with 20 μL, 30 μL and 40 μL virus solution. Compared with the control group, EVT proliferation was significantly inhibited (P < 0.05) after infection with 20 μL, 30 μL or 40 μL virus solution for 48 h and 72 h, without any significant difference in inhibition (P > 0.05). The experiments were performed in triplicate.](image2)
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A

B

C

D

E

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 1×10⁵/mL and 100 TCID50 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 statistical analysis and data were expressed as mean ± standard deviation (x±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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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 μL to 100 μL virus solution for 12 h had little effect on EVT proliferation (P > 0.05). However, EVT proliferation was significantly inhibited after infection with 70 μL to 100 μL virus solution for 24 h; 20 μL, 30 μL, 40 μL or 50 μL virus solution for 48 h; or 10 μL 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 μL, 30 μL or 40 μL virus solutions (P > 0.05). Based on these results, the 20 μL solution containing 100TCID50 HCMV, diluted in 120 μL medium, was selected for subsequent experiments (Figures 3 and 4).

Effect of HCMV on expression of major genes in TGF-β/Smad signaling pathways in EVT

The results of qRT-PCR showed that the gene expression of TGF-β1, Smad1, Smad2, Smad3, Smad4, and Smad5 was significantly increased (P < 0.05), but that of TGF-β2, TGF-β3, TGFβRI, TGFβRII, and Smad7 was significantly
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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-β1 and Smad7 protein. The TGF-β1 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 ± 3.61 in the control group and 49.2 ± 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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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 patho-
physiological mechanism in miscarriage, still-
birth, intrauterine growth retardation, prema-
ture 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 cytoskele-
tal protein of epithelial cells, c-erbB-2 is an epi-
dermal 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 chori-
onic villi and isolated primary EVTs were col-
lected. Immunocytochemistry staining showed
that the isolated primary EVTs were mononu-
clear, 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 occa-
asionally Vim positive. This suggests that a suf-
ficient number of highly pure primary EVTs were
isolated, thus laying the foundation for future
study. In this study, HCMV viremia during preg-
nancy was modeled by infecting primary EVTs
with HCMV. Immunofluorescence staining sh-
owed 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-β
promotes endometrial decidualization, inhibits
trophoblast cell proliferation, migration, and
invasion, stimulates trimester and later tropho-
blast cells to form multinucleated cells and
regulates the formation and function of the pla-
centa [26]. In early pregnancy, low TGF-β1 ex-
pression leads to inadequate maternal immune
tolerance and embryonic implantation disor-
ders, while high TGF-β1 expression affects the
normal invasion of trophoblast cells into endo-
metrial tissue, also resulting in embryonic im-
plantation disorders [27, 28]. TGF-β1 regulates
trophoblast cell differentiation and invasion as
well as EVT invasion in a dose-dependent man-
ner [29]. TGF-β1 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 con-
clusion of fetal growth at 35 weeks of pregnan-
cy [30, 31]. Results of the current study showed

that the primary EVT expressed TGF-β1, which
was significantly higher after HCMV infection.

The Smad proteins are the only confirmed sub-
strates of the TGF-β 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), in-
cluding Smad4 and Smad10, which are the
common mediators of TGF-β signaling; and (iii)
inhibitory Smads (I-Smads), including Smad6
and Smad7. Smad6 mainly inhibits the BMP
signaling and Smad7 mainly acts on the TGF-β
signaling pathway.

Smads are key mediators of the TGF-β signal
transduction pathway, as well as cell prolifera-
tion, differentiation, migration and apoptosis.
Binding of biologically active TGF-β to TGFβRII
activates TGFβRI, resulting in the phosphory-
lation of C-terminal serine residues and ac-
tivation 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 tran-
scription. Smad6 competes with Smad4 for
phosphorylated Smad1 or directly binds to acti-
vated TGFβRI to prevent phosphorylation of
R-Smads and Smad7 directly binds to activated
receptors, thus both function as negative regu-

ators of the TGF-β/Smad signaling pathway
[33]. Smad7 expression is a major indicator of
TGF-β pathway activation since TGF-β signaling
induces its expression [7, 8].

Smad7 induces apoptosis, reduces the expres-
sion and activity of MMP-2, MMP-9 and uPA,
inhibits cell invasion and metastasis [9-11] and
negatively regulates TGF-β1 receptors, thus
blocking TGF-β1 signal transduction and reduc-
ing ECM synthesis to inhibit fibrosis [34]. In
the placenta, Smad7 is mainly located in the
cytoplasm of villi CTs and STs. In the TGF-β/
Smad signaling pathway, TGF-β stimulates the
Smad7 transcription as a negative feedback to
decrease TGF-β1 signaling [35], thus regulat-
ing 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.
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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-β induces fibronectin, plasminogen activator inhibitor and TIMP-1 expression to inhibit MMPs activation [39]. Under normal circumstances, MMPs, TIMPs and TGF-β1 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-β1 levels. TGF-β1 significantly enhances the adhesion between the trophoblast cells, as well as inhibits MMP-2 and MMP-9 expression, indicating that TGF-β1 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-β1. 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-β1. Meanwhile, HCMV inhibits Smad7 expression, which suppresses the negative feedback, leading to a further increase in TGF-β1, which binds to receptors on trophoblast cells to excessively inhibit the secretion of MMP-2 and MMP-9. Therefore, abnormal TGF-β1 expression disrupts 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-β/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-β, transforming growth factor-β; MMP, Matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; CK7, cytokeratin 7; Vim, vimentin; uPA, urokinase plasminogen activator; ECM, extracellular matrix.

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HCMV in the proliferation and invasion of EVT


