Cytomegalovirus Infection of Human Syncytiotrophoblast Cells Strongly Interferes with Expression of Genes Involved in Placental Differentiation and Tissue Integrity

MARK R. SCHLEISS, BRUCE J. ARONOW, AND STUART HANDWERGER

Department of Pediatrics [M.R.S.], Division of Infectious Diseases and Immunology, Center for Infectious Diseases and Microbiology Translational Research, University of Minnesota, Minneapolis, Minnesota 55455; Cincinnati Children's Research Foundation [B.J.A., S.H.], Cincinnati, Ohio 45229

ABSTRACT: The principle route of acquisition of cytomegalovirus (CMV) for the fetus is believed to be *via* the placenta. We subjected purified cytotrophoblast cells obtained from full-term placentas to CMV infection and examined placental gene expression using microarray analyses. Cytotrophoblast cells purified from term placentas differentiated *in vitro* into a multinucleated syncytium that could be productively infected with CMV, with peak virus titers of approximately 10^4 plaque-forming units (PFU)/mL identified in supernatants at late time points postinoculation. Infected syncytiotrophoblast cells expressed CMV-specific transcripts and proteins, as demonstrated by Northern blot and immunofluorescence assays. Microarray analyses revealed that CMV infection strongly and reproducibly altered trophoblast gene expression, elevating expression of mitotic cell cycle genes, and repressing expression of genes associated with trophoblast differentiation, particularly those associated with formation and stabilization of the extracellular matrix. We conclude that purified, differentiated syncytiotrophoblasts are permissive for CMV replication. Infection of these cells induces significant perturbations in trophoblast transcription. An improved understanding of the molecular events that occur during CMV infection of trophoblasts could provide insights into interventions that might prevent or minimize congenital transmission. (*Pediatr Res* 61: 565–571, 2007)

Birth defects caused by congenital CMV infection represent a major public health problem. Congenital CMV transmission results in substantial long-term neurodevelopmental morbidity in newborns, including mental retardation and sensorineural hearing loss [reviewed in (1)]. While antiviral therapies may limit severity of SNHL, therapeutic intervention has limited ability to reverse other neurologic injuries (2). The most effective strategies for control of congenital CMV may require prevention of fetal infection by preconceptual vaccination or anti-CMV immunoglobulins (3,4). However, vaccines and immunoglobulins are not yet licensed for prevention of congenital CMV infection. Therefore, continued study of the molecular pathophysiology of CMV

infection in cell types relevant to congenital transmission is warranted, to help facilitate development of novel disease prevention strategies.

Although there is extensive evidence that CMV-related pathology is caused by direct infection of the fetus *in utero*, recent work has also highlighted the importance of the role of direct viral infection of the placenta in the pathogenesis of congenital CMV infection (5,6). There is evidence that the route of transmission of CMV to the fetus is *via* the placenta, which is seeded as a consequence of maternal viremia (5,6). In addition to serving as a gateway to the fetus, the placenta may be directly injured by CMV, conceivably leading to intrauterine growth retardation and fetal disease (7–10). Thus, study of CMV placental infection may facilitate improved understanding of these potentially diverse mechanisms of viral-mediated fetal injury.

A number of reports have identified CMV in placental tissue from clinical samples (11–14). Several *in vitro* models for studying CMV-placental interactions have also been developed (15–20). Explants of first-trimester placenta onto a matrigel matrix recapitulate *in vitro* many elements of placental differentiation and invasion, providing a useful system for studying CMV infection of placental cells (19,20). As we show in this report, culture and CMV infection of cytotrophoblast cells purified from term placentas also provides an *in vitro* model for the study of molecular and cellular events that occur during trophoblast differentiation. These cells aggregate spontaneously and fuse to form a multinucleated syncytium that expresses placental lactogen, human chorionic gonadotropin, and other proteins commonly expressed by differentiated syncytiotrophoblasts, and major genes and functional groups that exhibit dynamic expression changes during differentiation have been identified (21,22). These studies were undertaken to test the hypothesis that CMV infection of these cells would have pathologic effects on trophoblast gene expression in this system that could be identified by microarray analyses.

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Correspondence: Mark R. Schleiss, M.D., Center for Infectious Diseases and Microbiology Translational Research, University of Minnesota, 2001 6th Street SE, Minneapolis, MN 55455; e-mail: schleiss@umn.edu

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Abbreviations: CMV, cytomegalovirus; HFF, human foreskin fibroblast; MOI, multiplicity of infection; PFU, plaque-forming unit; SNHL, sensorineural hearing loss

METHODS

Viruses and cells. Trophoblast cells were purified from term placentas obtained from women undergoing elective cesarean section, following local institutional review board approval. ELISA (Accurate Scientific, Westbury, NY) confirmed that cord blood was negative for CMV antibodies (data not shown). Cytotrophoblast cells were isolated by enzymatic disaggregation and purified by negative CD9 selection, and cultured as previously described (21,22). The medium was changed at 12 h, when almost all cells were adherent to the bottom of the dish, and then at successive 24-h intervals until the cells exhibited morphologic evidence of syncytia formation (approximately 96 h after plating).

For CMV inoculations, stocks of the strains AD169, Towne, Davis (the gift of A.P. Geballe), and Toledo (the gift of E.S. Mocarski) were prepared in HFF culture (ATCC number SCRC-1041). Cell-free viral work pools were prepared by sonication and differential centrifugation of tissue culture supernatants after cells had achieved 95–100% cytopathic effect. Viral stock was prepared using the following methodology. Supernatants were centrifuged in a J2–21 centrifuge at 2500 *g* at 25°C for 30 min, to pellet cells. This supernatant was transferred to a new centrifuge bottle and centrifuged a second time, again at 2500 *g* for 30 min. The supernatant was next transferred to a new centrifuge bottle and centrifuged at 10,000 *g* for 90 min at 4°C, to pellet virus particles. These were resuspended in DMEM media with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) prepared in a sucrose-phosphate (0.2 M) cryogenic preservation buffer (23). Viral stocks were titrated and used to inoculate syncytiotrophoblast cultures at various MOI to assess whether these cells supported viral replication. For microarray experiments, cells were inoculated at a MOI of 5 PFU/cell, using the Toledo strain of CMV. As controls for all experiments, mock-infection was performed using media identical to that used for viral stock preparation (DMEM with 10% FBS and 0.2 M sucrose-phosphate).

One-step growth curve analyses. CMV stocks of the Toledo strain were used to inoculate syncytiotrophoblast cells and HFF for one-step growth curve analyses. Viruses were used to infect confluent cells at a MOI of 5 PFU/cell. After adsorption for 1 h at 37°C, the cells were washed with media and a zero time point was harvested. The remainder of the sample was incubated at 37°C with additional time points taken at indicated times (Fig. 1). The viral titer of each time point was then determined by plaque titration assay on HFF cells. All assays were done in triplicate, with three replicates obtained for each time point in each experiment. Data represent the mean $\pm$ SD of PFU recovered from each time point.

Immunofluorescence assays. CMV-infected and mock-infected syncytiotrophoblast cells were fixed, permeabilized, and subjected to immunofluorescence using CMV-specific MAb and standard techniques as described elsewhere (24). Cells were incubated with MAb specific for the CMV immediate early protein (IE72) and the structural tegument phosphoprotein, pp65 (UL83), according to the manufacturer's specifications (Argene Inc., North Massapequa, NY). An anti-mouse FITC-conjugated secondary anti-

body (Accurate Scientific) was used for visualization by fluorescence microscopy, as previously described (24) (Fig. 2).

RNA purification, Northern blot, and RT-PCR assays. Total RNA was purified from CMV-infected and mock-infected trophoblast cells using the Absolutely RNA purification system (Stratagene, La Jolla, CA), according to the manufacturer's specifications. For Northern blot, RNA was subjected to electrophoresis and transfer to Nytran membranes, following by hybridization with 32 P dCTP-labeled probes (Fig. 3A). To further examine transcription profiles in RNA recovered from mock-infected and CMV-infected cells, RT-PCR analyses for syncytin transcript were performed as described elsewhere (25) (Fig. 3B). Briefly, the relative amounts of mRNA for syncytin were determined by semiquantitative RT-PCR using GAPDH mRNA as an internal standard. Total RNA isolated from normal or CMV-infected trophoblast cells was subjected to first-strand cDNA synthesis performed using SuperScript II reverse transcriptase (Invitrogen) and oligo dT according to the manufacturer's protocol. Primer sequences used for detection of syncytin transcripts were 5' - TCT ATG GAG AAT GCA GCG TCC CG - 3' and 5' - TAG GCT TAC CAG GCG AGT ATG GG - 3', which resulted in a PCR product of 391 bp. Primers for human GAPDH were 5' - CCA TGG AGA AGG CTG GGG - 3' and 5' - CAA AGT TGT CAT GGA TGA CC - 3', which resulted in a PCR product of 192 bp. The PCR reactions were spiked with 0.1 μ L of [γ - 32 P] dCTP (3000 Ci/mM). Optimal PCR cycles required for linear amplification for each set of primers were determined, and amplification in each primer set was kept below saturation levels to permit products to remain within the exponential range. Radiolabeled PCR products were separated by 6% PAGE at 225 V for 3 h. The gel was transferred to 3M paper, dried, and quantified using a PhosphorImager and ImageQuant 1.2 software (Molecular Dynamics, Sunnyvale, CA).

Microarray analyses. DNA microarray analyses using Affymetrix human U133 GeneChips were performed as previously described (21,22) to compare RNA extracted from control trophoblast cells *versus* that extracted from cells infected with CMV (Toledo) at a late time point (96 h) postinfection. RNA was confirmed to have a RNA integrity number of ≥ 7 before hybridization. Expression data were analyzed using GENESPRING V.6.1 (Silicon Genetics, Redwood City, CA) to select genes that were differentially expressed between the Toledo strain of CMV and the mock-infected controls. Relative gene expression was determined from the Affymetrix cel files by using the Robust Multi-Array Analysis (RMA) algorithm, as developed and described by Irizarry *et al.* (26). The signal intensity of each of the probe sets was then transformed from a log base 2 to linear values. Each measurement was divided by the median of all measurements in that sample. The samples were normalized to the mean of the control sample(s) hybridized at the same time. Each measurement for each gene in those specific samples was divided by the median of that gene's measurements in the corresponding control samples. We analyzed the control *versus* CMV groups using a 2-fold expression level filter and Welch's approximate *t* test with multiple testing correction algorithm (Welch's approximate *t* test with Benjamini and Hochberg multiple testing correction algorithm; see Fig. 4).

Figure 1. Productive infection of trophoblasts with CMV. Differentiated syncytiotrophoblast cells or HFF were infected with the Toledo strain of CMV at a MOI of 5 PFU/cell. After adsorption of virus, tissue culture supernatants were cultured at the indicated time points to quantify viral recovery. Triangles represent HFF cells, and boxes represent syncytiotrophoblast cells. Data shown are mean $\pm$ SD of three independent titrating experiments.

Figure 2. Immunofluorescence analysis of CMV-infected trophoblast cells. Differentiated syncytiotrophoblast cells were infected with the Toledo strain of CMV. After infection, cells were incubated with an anti-CMV IE1 (IE72) MAb (A), or an anti-CMV pp65 (UL83) antibody (B). Cells were next incubated with a FITC-conjugated anti-mouse secondary antibody, and then visualized by fluorescence microscopy. The IE72 antibody exhibited the anticipated nuclear pattern of fluorescence (A, arrows) whereas the pp65 antibody (B) exhibited both nuclear (arrow) and cytoplasmic (arrowhead) patterns of fluorescence. No background fluorescence was observed using these antibodies with mock-infected cells (data not shown). Scale bar indicating degree of magnification is indicated.

Figure 3. Transcription from CMV-infected and control trophoblast cells. (A) Northern blot analysis of RNA purified from mock-infected (lanes 1, 3) or CMV-infected (lanes 2, 4) trophoblast cells probed either with a GAPDH probe (left blot) or a probe for the CMV TRL4 transcript (right blot). (B) Quantitative reverse-transcriptase PCR for GAPDH mRNA (left panel) and syncytin RNA (right panel) in mock-infected and CMV-infected trophoblast cells. Quantitative analysis (normalized to GAPDH expression, lanes 1 and 2) of the 391-bp syncytin product (arrow) indicates a reduction in syncytin expression levels of approximately 25% in CMV-infected cells (lane 4), compared with mock-infected control (lane 3). Results shown are representative of 3 independent experiments.

RESULTS

Syncytiotrophoblast cells support productive CMV infection in vitro. Cytotrophoblast cells were plated and allowed to differentiate into syncytiotrophoblast cells, as previously described (21,22). Replication kinetics were determined for the Toledo strain in syncytiotrophoblast cells (Fig. 1) and compared with replication in HFF. Tissue culture media from syncytiotrophoblast cells was found to contain on average a peak level of approximately $4 \log_{10}$ pfu/mL in the supernatant at late time points following viral inoculation (Fig. 1).

The permissiveness of syncytiotrophoblast for CMV replication was further explored using immunofluorescence assay (Fig. 2). These assays used MAbs that targeted IE72 protein, and a CMV structural protein, pp65 (ppUL83) (27). CMV-infected syncytiotrophoblasts expressed both proteins abundantly (Fig. 2). The localization of these proteins in infected syncytiotrophoblast cells was similar to that described in other CMV-permissive cells, with the IE72 protein localized exclusively in the nuclei of infected cells. In contrast, the pp65 protein localized to both nuclei and cytoplasm of infected cells (Fig. 2B, arrows and arrowheads).

Figure 4. Impact of HCMV infection on trophoblast cell gene expression. To examine perturbations in trophoblast cell gene expression induced by CMV infection, DNA microarray analyses using Affymetrix human U133 GeneChips were performed, comparing RNA extracted from control trophoblast cells vs that extracted from cells infected with the Toledo strain of CMV at a late time point (96 h) postinfection. (A) Expression data were analyzed using GENESPRING V.6.1 (Silicon Genetics) to select genes that were differentially expressed between the Toledo strain of CMV and the negative (mock-infected) trophoblast cell culture controls. Five hundred forty-four genes were identified with averaged expression intensity in CMV-infected samples higher or lower than the averaged expression intensity in the control samples by at least 2-fold, and expression in CMV-infected samples deviated from that of control samples with a significance cut-off of $p < 0.05$ (Welch's approximate t test with Benjamini and Hochberg multiple testing correction algorithm). (B) Profiles of normalized mock-infected trophoblast cells (yellow, control) and three independent experiments using RNA from CMV-infected trophoblast cells (CMV 1–3). The most down-regulated (blue) gene families are indicated in greater detail in Table 1, and the most up-regulated (red) are indicated in Table 2.

Transcriptional analyses of CMV-infected and control trophoblast cells. To examine the impact of CMV infection on transcription in cultured syncytiotrophoblast cells, RNA was purified from CMV-infected and mock-infected cells at 96 h postinfection. Northern blot analysis was then performed using a probe for the major early CMV transcript, 2.7 β (UL4) (28). This transcript was abundantly expressed in CMV-infected, but not mock-infected, trophoblast cells (Fig. 3B). To further compare transcription from mock-infected and infected cells, reverse-transcriptase PCR (RT-PCR) was per-

Table 1. Trophoblast genes identified as down-regulated by cytomegalovirus infection

Down-regulated trophoblast gene	Accession number	Fold decrease
Cell and tissue structural dynamics		
Matrix metalloproteinase 3 preproprotein	NM_002422	13.6
Cadherin 11, type 2 preproprotein	NM_001797	9.8
Junctional adhesion molecule 2 precursor	NM_021219	4.8
Laminin, alpha 4 precursor	NM_002290	4.7
Fibulin 1 isoform C precursor	NM_006487	3.1
UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase	NM_005476	3.0
Integrin beta chain, beta 3 precursor	NM_000212	2.6
Mannosidase, alpha, class 1A, member 1	NM_005907	2.4
Fibrillin 1	NM_000138	2.3
Cell cycle and apoptosis		
Cyclin-dependent kinase inhibitor 1C	NM_000076	3.6
PAR-6 beta	NM_032521	2.5
Epidermal growth factor receptor	AK_026818	2.2
Intracellular communication		
Junctional adhesion molecule 2 precursor	NM_021219	4.8
Stanniocalcin 1	NM_002195	3.1
Endothelial PAS domain protein 1	NM_006113	2.3
Epidermal growth factor receptor	NM_021005	2.2
Semaphorin 5A	NM_013402	2.1
Metabolism		
Fatty acid desaturase 1	NM_013402	2.1
Regulation of gene expression		
Ras protein-specific guanine nucleotide-releasing factor 2	NM_006909	3.2
Phospholipase C-like 2	NM_015184	3.2
Par-6 partitioning defective 6 homolog beta	NM_032521	2.5
Transcription factor AP-2 alpha	NM_001032280	2.5
GATA binding protein 3	NM_001002295	2.4
Zinc finger homeobox 1b	NM_014795	2.4
Nuclear receptor coactivator 3	NM_006534	2.4
Vav 3 oncogene	NM_006113	2.3
Nuclear receptor subfamily 2, group F, member 2	NM_003966	2.1

Total fold-change in genes is indicated in rank order, with GenBank accession number.

formed for the “housekeeping” gene, GAPDH, and the syncytin gene, which is abundantly expressed in trophoblast cells (25,29). These analyses indicated that CMV infection resulted in a 25% decrease in syncytin mRNA, normalized against expression of GAPDH (Fig. 3B).

Microarray analyses of RNA from CMV-infected and control trophoblasts reveal altered transcription patterns following infection. Microarrays were performed with RNA purified from mock-infected and CMV-infected syncytiotrophoblasts as described in “Methods.” Microarrays were compared by analysis of the control *versus* CMV groups. Data were analyzed from multiple, independently conducted infection experiments (a total of six experiments: three with mock-infected cells and three with CMV-infected cells). We used Welch’s approximate *t* test with Benjamini and Hochberg multiple testing correction algorithm and top-ranked differential expression criteria of RMA-normalized expression data (26). This resulted in the identification of 544 genes whose expression differed by more than 2-fold in CMV samples relative to the control sample, with a significance cut-off of

Table 2. Trophoblast genes identified as up-regulated by cytomegalovirus infection

Up-regulated trophoblast gene	Accession number	Fold increase
Cell and tissue structural dynamics		
Fibronectin leucine-rich transmembrane protein 3	NM_013281	4.5
Cadherin 3 type 1, P-cadherin	NM_001793	3.8
Tetrespanin 2	NM_005725	3.1
Laminin, alpha 3	NM_000227	2.5
Cell cycle and apoptosis		
TTK protein kinase	NM_003318	5.2
Cell division cycle 25A	NM_001789	4.3
DNA replication factor	NM_030928	2.8
CDC28 protein kinase regulatory subunit 2	NM_001827	2.6
RAD51 homolog	NM_002875	2.6
Stratifin	NM_006142	2.5
Transforming growth factor, alpha	NM_003236	2.4
Intracellular communication		
RAP1A, member of RAS oncogene family	NM_002824	44.8
Neuromedin U	NM_006681	14.5
Tachykinin, precursor 1	NM_003182	7.2
Natriuretic peptide precursor B	NM_002521	6.3
Glycine receptor, beta	NM_000824	5.8
Adrenergic, beta-2, receptor	NM_000024	5.8
Relaxin 2	NM_005059	5.0
Cholecystokinin	NM_000729	4.5
Fibronectin leucine rich transmembrane protein 3	NM_013281	4.5
Regulation of gene expression		
Zinc finger protein 85 (HPF4, HTF1)	NM_003429	4.7
Single-stranded DNA binding protein 2	NM_012446	3.5
Hairy and enhancer of split 4	NM_021170	3.5
Forkhead box Q1	NM_033260	3.2
Myelin expression factor 2	NM_016132	2.8
Homeobox A1	NM_005522	2.8

Total fold-change is indicated in rank order, along with the GenBank accession number.

$p < 0.05$ (Welch’s approximate *t* test with Benjamini and Hochberg multiple testing correction algorithm). Using a gene ontology analysis tool, DAVID 2.0 (30), we found that CMV infection strongly and reproducibly altered multiple trophoblast cell gene expression profiles. These effects are summarized in Tables 1 and 2. This analysis identified five gene families exhibiting noteworthy changes in the pattern of transcription: 1) cell and tissue structural dynamics; 2) cell cycle and apoptosis genes (in particular, elevation of the expression of mitotic cell cycle genes); 3) genes involved in intracellular communication; 4) metabolism genes; and 5) genes involved in regulation of gene expression (Fig. 4; Tables 1 and 2). Genes involved in the formation and stabilization of the extracellular matrix were significantly dysregulated, including matrix metalloproteinase 3 (13.6-fold down-regulation), cadherin (9.8-fold down-regulation), and laminin alpha-4 precursor (4.7-fold down-regulation). Other dysregulated genes included several in the intracellular communication gene family, including RAP1A, a member of the RAS oncogene family (up-regulated 44.8-fold), neuromedin (up-regulated 14.5-fold), and tachykinin (up-regulated 7.2-fold). It was of particular interest to note that transcription factor AP2 alpha was

decreased (2.5-fold down-regulation), inasmuch as previous studies identified a critical role for this gene in placental expression of lactogen, gonadotropin, and other genes involved in placental homeostasis (25,29; Table 1).

DISCUSSION

These studies of the impact of CMV infection on placental gene expression were undertaken to explore, using microarray analyses, potential modifications in trophoblast transcription, toward a long-term goal of understanding placental mechanisms of congenital virus transmission. Previous study of purified cytotrophoblast cells obtained from term placentas had identified genetic programs and pathways that underlie differentiation into syncytiotrophoblast cells (31). Thus, it was of interest to determine the permissivity of these cells for infection with CMV. We observed that these differentiated syncytiotrophoblast cells were fully permissive for CMV replication in our study, albeit at reduced levels compared with fibroblast cells (Fig. 1). It was of interest to compare these results to those described by other investigators who have explored CMV infection of placenta in cell and tissue culture models. Previous work with term placentas demonstrated the permissivity of purified trophoblasts for CMV infection, using the laboratory-adapted strain, AD169 (15). Syncytiotrophoblast infection was demonstrated *in vivo* in a study of third-trimester placenta, using *in situ* hybridization and PCR techniques (11). Another study of purified syncytiotrophoblast cultures demonstrated that these cells could be permissively infected with a number of laboratory-adapted strains of CMV, as with clinical isolates, although infection efficiencies differed amongst different strains (18). Similar to our observations, viral titers in tissue culture supernatant were relatively low in this study, presumably reflecting the cell-associated nature of CMV infection. Although our studies attest to the permissiveness of syncytiotrophoblast for productive infection, infection of these cells with CMV has not been as straightforward in the hands of other investigators. For example, in studies of first trimester placental explants, maintained either in primary tissue culture or following culture on a matrigel matrix, syncytiotrophoblasts are only rarely infected (17,19). Other regions, including the decidual basalis, have appeared to be more permissive for viral infection in studies of biopsies of primary tissue samples (32). Divergent findings from these various studies may be a result of different culturing procedures, or differences in the gestational age of placental tissue (6). Differences in expression of CMV receptors between syncytiotrophoblasts and cytotrophoblasts have also been proposed (16), and this factor could explain the variation in the permissiveness of placental cells for CMV infection described in previous reports.

We focused on term trophoblasts in this study, because of their availability, and because of the body of previously acquired data that carefully defined, by microarray analyses, the transcriptional profile of these cells (18). Our results confirm those of other investigators (18) regarding the permissivity of these cells for CMV infection *in vitro*. The Toledo strain of CMV was used for our microarray analyses because

of the fact that the Toledo genome is more similar to that of wild-type strains of CMV than other laboratory-adapted strains (33), and because the Toledo strain has been found to retain tropism for a wide range of cell types in other *in vitro* models (34). Future studies of primary clinical isolates of CMV would potentially be of significant translational interest in this model.

As noted, previous studies using microarrays had defined the normal transcriptional events that occur during the process of cytotrophoblast cell differentiation into syncytiotrophoblast cells *in vitro*, in the absence of CMV (21,22,31). In the present study, infection of syncytiotrophoblast cells with CMV resulted in modification of this previously defined program in several gene categories. Table 1 shows the most highly modified genes in each of the categories, along with the percent change in mRNA abundance from that of control, mock-infected cells cultured in the identical manner, except for infection with CMV. These analyses identified 544 genes with significant up- or down-regulation induced by CMV infection. Of note, CMV infection strongly and reproducibly altered trophoblast expression of mitotic cell cycle genes, and strongly repressed the expression of genes associated with trophoblast differentiation, particularly those associated with formation and stabilization of the extracellular matrix (Tables 1, 2; Fig. 4). It was of considerable interest that genes involved in the formation and stabilization of the extracellular matrix were significantly dysregulated, including matrix metalloproteinase 3 (13.6-fold down-regulation), cadherin (9.8-fold down-regulation), and laminin alpha-4 precursor (4.7-fold down-regulation). Down-regulation of the genes in the cell and tissue structural dynamics category may result in changes in the composition and function of the extracellular matrix of CMV-infected cells, an effect which in turn may impact the transmission of CMV to the fetus.

Other significantly dysregulated genes included several in the intracellular communication gene family, including RAP1A, a member of the RAS oncogene family (up-regulated 44.8-fold), neuromedin (up-regulated 14.5-fold), and tachykinin (up-regulated 7.2-fold). Transcription factor AP-2 alpha was also decreased, with a 2.5-fold down-regulation. Since AP-2 alpha has been shown to be critical for the expression of placental lactogen, chorionic gonadotropin, and many other proteins expressed by terminally differentiated syncytiotrophoblast cells, it is plausible that repression of AP-2 mRNA levels could impair the expression of these proteins in the infected cells and could result in decreased hormone production and alteration in other cellular functions (25,29), which in turn could have deleterious effects on placental development. Another dysregulated mRNA of interest within the family of genes involved in regulation of gene expression family was GATA binding protein 3 (Table 1; 2.4-fold decrease). Conceivably, decreased expression of this transcription factor could have an impact on placental structure, *via* down-regulation of syncytin expression (21,22). Consistent with this prediction, we observed a 25% decrease in syncytin expression, by RT-PCR, in RNA purified from CMV-infected trophoblast cells, compared with mock-infected cells (Fig. 3B).

Microarray analysis has been performed by other investigators to explore the effect of CMV infection on transcription in a variety of cell types, although it has not been previously described for CMV-infected trophoblasts. In a study of CMV-infected fibroblasts using an Affymetrix array containing 6,600 human mRNA, 258 mRNA were identified that changed by a factor of 4 or more within the first 24 h of viral infection (35). Another study of the impact of CMV on infected fibroblasts identified genes involved in transcriptional regulation, oncogenesis, cell cycle regulation, immune system regulation, signal transduction, and cell adhesion, as being significantly modified by CMV infection (36). Because of the importance of the placenta as a target for CMV infection before fetal transmission *in utero*, we chose to study syncytiotrophoblast cells, with the long-term goal of an improved understanding of viral factors that might be important in promoting congenital transmission. Our findings, although preliminary, provide support for further experimental evaluation of a hypothesis of fetal injury induced by CMV that is not necessarily due to the direct infection of fetal cells *per se*, but could be induced by CMV-mediated perturbations in placental structure and function that, in turn, could lead to fetal damage *via* placental insufficiency. The finding that CMV infection markedly perturbs expression of genes required for normal trophoblast differentiation is compatible with a model whereby viral infection leads to placental insufficiency and attendant fetal compromise through modification of expression of genes involved in structural integrity and differentiation. Future microarray-based analyses of recombinant CMVs, deleted in regulatory and immunomodulatory genes, could facilitate testing of hypotheses about the putative viral factor(s) responsible for mediating these effects, which in turn could have translational implications for the rational design of live, attenuated vaccines with optimal safety profiles for future clinical trials. In addition to future studies of clinical isolates of CMV on trophoblast transcription, additional studies are required of multiple time points postinfection, to better define kinetic classes of CMV genes involved in mediating these effects. Recent evidence suggests that the salutary effect of anti-CMV immune globulin in pregnant women with primary CMV infection is mediated through preservation of placental structure and function (37). Since optimizing the integrity and health of the placenta may be the key to preventing adverse fetal outcomes in the setting of maternal CMV infections (38), further study of the impact of CMV infection on placental gene expression may provide insights into novel therapeutic and preventative strategies for this important public health problem.

CONCLUSION

Syncytiotrophoblast cells purified from term placentas are fully permissive for CMV infection and inoculation of these cells results in a productive infection in which infectious virus is present in tissue culture supernatants. CMV-infected trophoblasts have dramatically altered patterns of transcription, compared with mock-infected cells. Microarray analyses indicate that CMV infection strongly and reproducibly alters

trophoblast expression of mitotic cell cycle genes, and strongly repressing the expression of genes associated with trophoblast differentiation, particularly those associated with formation and stabilization of the extracellular matrix. Whether these perturbations of trophoblast gene expression diminish integrity of the placental barrier at the cellular level, or modify CMV transmission to the fetus, requires further experimental analysis.

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