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Transduction of Human Trophoblastic Cells by Replication-Deficient Recombinant Viral Vectors

Promoting Cellular Differentiation Affects Virus Entry

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We investigated the transfer of the *lacZ* reporter gene into human trophoblastic cells using herpes simplex virus and adeno-associated virus vectors. We used an established choriocarcinoma cell line (BeWo cells) that can be induced to terminally differentiate after treatment with cyclic-AMP. Our results demonstrate that transduction of trophoblastic cells by the herpes simplex virus vector, HSV.CMV*lac*, and the adeno-associated virus vector, AAV.CMV*lac*, is affected by cellular differentiation. Treatment of BeWo cells with cyclic-AMP reduced transduction by HSV.CMV*lac* but increased transduction by the AAV vector. In contrast, when BeWo cells were transfected with herpes simplex virus and adeno-associated virus plasmids, *lacZ* expression was not affected by treatment with cyclic-AMP. Southern blot analysis demonstrated 2.75 times less herpes simplex virus DNA in cyclic-AMP treated BeWo cells, but 2.0 to 7.4 times more adeno-associated virus DNA in treated cells. We conclude that inefficient transduction of differentiated trophoblastic cells with HSV.CMV*lac* is because of diminished viral entry, whereas cellular differentiation is associated with increased entry of AAV.CMV*lac*. These observations suggest that adeno-associated virus vectors may be used to modify trophoblast function and study placental physiology. Additionally, trophoblast differentiation leads to alterations in the mechanisms of virus uptake that may affect maternal-to-fetus transmission. ***(Am J Pathol 1998, 152:1521–1529)***

The syncytiotrophoblast layer of the human placenta is the interface between the maternal and fetal circulations. It is derived from mononucleated cytotrophoblastic cells, which are the replicating cells of the trophoblast lineage. Cytotrophoblastic cells differentiate into syncytiotrophoblast when they exit from the cell cycle and fuse to form multinucleated syncytia. As gestation progresses, cytotrophoblastic cells become relatively scarce and the syncytiotrophoblast predominates within placental villi. The terminally differentiated syncytiotrophoblast is responsible for the transport of nutrients from the mother to the fetus; it also performs multiple endocrine functions, including the production of sex steroid hormones, protein and glycoprotein hormones, and growth factors. As a continuous layer between the maternal and fetal circulations, the syncytiotrophoblast serves as a barrier against the transplacental passage of pathogens.[1]

We have been exploring the use of replication deficient recombinant viral vectors to transduce human trophoblast and transformed trophoblastic cell lines. Our ultimate goal is to selectively modify syncytiotrophoblast function *in vivo* to learn more about trophoblast physiology and to offer novel therapeutic options for pregnancy complications that result in part from trophoblast dysfunction. One example of placental dysfunction is intrauterine fetal growth restriction, the outcome of which may be ameliorated by transfer of nutrient transporter genes (such as glucose transporter-1) to the syncytiotrophoblast, resulting in augmented glucose transport to the fetus. The elucidation of mechanisms by which viral vectors infect and transduce trophoblastic cells also provides insight into the role that the placenta plays in facilitating or resisting congenital viral infections.

Here we report our results using two recombinant viral vectors, an amplicon herpes simplex virus-1 (HSV) vector and an adeno-associated virus (AAV) vector, to trans-

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duce human trophoblastic cells. These viral vectors were selected because existing evidence suggests that human placental tissue can be infected by the same wild-type viruses. HSV is known to undergo full replicative cycles in the trophoblast,[2] but transplacental infection with HSV has been infrequently reported.[3] AAV is a single-stranded DNA parvovirus that demonstrates no species or tissue specificity and is not associated with any known human diseases. Wild-type AAV stably integrates its genome into the host cell genome with a preference for chromosome 19q3.4–19ter but does not replicate without helper function provided by adenovirus (Ad), human papillomavirus, human cytomegalovirus (CMV), HSV, or chemical agents.[4,5] One of the helper functions promotes second strand synthesis of the single-stranded AAV DNA, which is essential for efficient expression of AAV vectors.[6,7] AAV DNA has been detected by Southern blot analysis in human placental tissue after first trimester abortion.[8]

Materials and Methods

Cell Culture

An established human choriocarcinoma cell line, which represents a facile system for exploring aspects of trophoblast physiology, was used for *in vitro* transduction experiments.[9] The b30 clone of BeWo choriocarcinoma cells was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/L Hepes, 50 μg/ml gentamicin, and 2 mmol/L L-glutamine and supplemented with 10% heat-inactivated fetal bovine serum. These cells have the characteristics of cytotrophoblastic cells but can be induced to terminally differentiate into syncytial structures in the presence of cyclic-AMP (cAMP) analogs.[9] After culture for 48 hours in DMEM containing 1.5 mmol/L 8-bromo-cAMP, BeWo cellular differentiation was detected by increased progesterone secretion and by phase contrast microscopy to identify syncytial structures.

Cytotrophoblastic cells were isolated from third trimester placentas as described by Kliman et al.[10] Briefly, minced placental villous tissue was serially digested with trypsin-DNase, followed by centrifugation through a Percoll gradient that yielded a highly purified preparation of cytotrophoblastic cells. After isolation, the cells were cultured in DMEM containing 25 mmol/L Hepes, 50 μg/ml gentamicin, and 2 mmol/L L-glutamine and supplemented with 10% heat-inactivated fetal bovine serum. Within 48 to 72 hours, the cytotrophoblastic cells spontaneously differentiate into syncytiotrophoblast.

Transformed human embryonic kidney (293) cells and small cell lung carcinoma (A549) cells were used as positive controls for detecting transduction by HSV and AAV vectors, respectively. 293 and A549 cells were cultured under identical conditions as for BeWo cells, either in the presence or absence of 1.5 mmol/L 8-bromo-cAMP.

Construction of Recombinant Viral Vectors

The amplicon HSV.CMV*lac* vector contained the HSV-1 origin of DNA replication (Ori_s), a packaging/cleavage sequence ("a"), the *Escherichia coli lacZ* reporter gene (which codes for intracellular β-galactosidase), and a human CMV promoter to drive the expression of *lacZ*.[11] The vector was propagated in RR1 baby hamster kidney cells that were stably transfected with HSV-1 immediate early-3 and *neomycin phosphotransferase* genes. The cells were transfected with HSV.CMV*lac* plasmid and then infected with D30EBA helper virus, an HSV-1 immediate early-3 deletion mutant. When more than 95% of cells demonstrated cytopathic changes (after approximately 36 to 48 hours), the cells were harvested and sonicated, and cellular debris was pelleted by centrifugation. The virus-containing supernatant was passaged once on RR1 cells, which yielded an HSV.CMV*lac* stock. To titrate the infectivity of the stock, an expression assay was performed using subconfluent NIH 3T3 mouse embryonic fibroblast cells that were infected with HSV.CMV*lac*. Twenty-four hours after infection, the cells were rinsed with cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 minutes. To detect expression of the *lacZ* transgene, fixed cells were incubated for 12 hours at 37°C with a staining solution containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), which is a substrate for the *lacZ* gene product, β-galactosidase. *LacZ*-expressing blue cells were counted, and the blue cell-forming units of the inoculating viral stocks were calculated. The blue cell-forming units of HSV.CMV*lac* stocks used in these studies ranged from 1×10^6/ml to 1×10^7/ml.

The recombinant AAV vector that we used contained the same transgene (*lacZ*) and promoter (human CMV) flanked by AAV inverted terminal repeat sequences that are required in *cis* for AAV DNA replication.[6] The vectors were constructed in permissive 293 cells that were transfected with recombinant AAV plasmids (containing inverted terminal repeat sequences flanking the *lacZ* gene and CMV promoter) and nonpackaging complementing plasmids (containing AAV *rep* and *cap* genes). The 293 cells were also infected with wild-type Ad to supply helper function for AAV replication and production of recombinant AAV progeny. Forty-eight hours after infection, the cells were scraped and pelleted by centrifugation at $1000 \times g$ for 20 minutes at 4°C. The supernatant contained both recombinant AAV and wild-type Ad, which was removed by density gradient (cesium chloride) centrifugation, yielding recombinant AAV at the 1.38 to 1.41 g/ml band. Fractions were used to transduce 293 cells that were assayed for β-galactosidase activity (X-gal staining) 24 hours after infection. Peak fractions were dialyzed in Hepes-buffered saline to remove the cesium chloride and heated at 55°C for 15 minutes to inactivate any contaminating Ad. Final recombinant viral titers were determined by counting blue cell-forming units of fixed 293 cells 24 hours after infection. The blue cell-forming units of AAV.CMV*lac* stock used in these studies measured 2.3×10^8/ml.

Transduction of Cultured Cells Using HSV.CMVlac and AAV.CMVlac

Cells were plated as a subconfluent monolayer (1 × 10^5 cells per well) in 24-well plates. Primary cultures of cytotrophoblastic cells spontaneously formed syncytia over 48 to 72 hours. Differentiation of BeWo cells was induced by adding 8-bromo-cAMP (1.5 mmol/L) to the culture medium at 0, 24, or 48 hours before viral infection. Undifferentiated BeWo cells were maintained in culture for 0 to 48 hours without adding 8-bromo-cAMP to the culture medium. In some experiments, BeWo cells were induced to differentiate by treatment with forskolin (20 μmol/L), which activates adenylate cyclase and increases intracellular cAMP levels. BeWo cell differentiation was monitored by phase contrast microscopy and radioimmunoassay of secreted progesterone in the culture medium.

Cells were infected with HSV.CMV*lac* (0.3 to 1.0 blue cell-forming units per cell) or AAV.CMV*lac* (0.1 to 1.0 blue cell-forming units per cell). We investigated the transduction efficiency of AAV.CMV*lac* in the presence or absence of wild-type Ad coinfection (1 × 10^4 viral particles per cell). Human embryonic kidney 293 cells and small cell lung carcinoma A549 cells were plated as subconfluent monolayers (1 × 10^5 cells per well) and were used as positive controls for detecting HSV.CMV*lac* and AAV.CMV*lac* transduction, respectively. Negative controls included infection of BeWo cells with wild-type Ad or no virus. BeWo cells were also infected with Ad.CMV*lac* (1 × 10^4 viral particles per cell). We previously reported that undifferentiated trophoblastic cells are readily transduced by Ad vectors, but after treatment with the cAMP analog transduction efficiency is dramatically reduced.[12] Thus, the markedly reduced transduction by Ad.CMV*lac* serves as a positive control for trophoblastic cell differentiation. All experiments were replicated on at least two separate occasions.

Measuring the Efficiency of Reporter Gene Transfer

Gene transfer and reporter protein expression were detected by staining for the β-galactosidase protein. Cells were rinsed with PBS 24 hours after viral infection and fixed with 0.5% glutaraldehyde for 10 minutes at room temperature. The fixed cells were washed twice with PBS for 10 minutes and stained with a solution containing X-gal. The staining solution contained 1 mmol/L magnesium chloride, 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, and 1 mg/ml X-gal in PBS, pH 7.4. The cells were incubated with the X-gal solution for 1 to 4 hours at 37°C. Transduction efficiency was determined by counting the number of blue-stained cells per 100 cells under phase contrast microscopy.

β-galactosidase enzyme activity was also assessed using a solution-based assay system (TROPIX, Inc., Bedford, MA). Twenty-four hours after gene delivery, cell monolayers were rinsed twice with PBS and covered with 150 μl of 1× reporter lysis buffer. After a 15 minutes incubation at room temperature, cell lysates were collected. The samples were sonicated for 10 seconds and centrifuged at top speed for 2 minutes at 4°C. Fifteen μl of lysate was added to 100 μl of reaction buffer and the reactions were incubated at room temperature for 50 minutes. After adding 150 μl of light emission accelerator, the samples were read for 5 seconds in a luminometer. Each experiment was performed at least three times. The mean β-galactosidase activities were reported as relative light units (RLU) ± SD and were tested for statistical significance by two-tailed *t*-tests.

Transfection of Cells Using pHSV.CMVlac and pAAV.CMVlac

To determine whether undifferentiated and differentiated BeWo cells were able to express the *E. coli lacZ* transgene in the context of the human CMV promoter and HSV or AAV origin of replication sequences (Ori_s and the AAV inverted terminal repeats), we transfected BeWo cells with pHSV.CMV*lac* or pAAV.CMV*lac* plasmids. BeWo cells (1 × 10^5 cells per well) were cultured in the presence or absence of 8-bromo-cAMP (1.5 mmol/L). At 0 or 48 hours after the addition of cAMP to the culture medium, the cells were rinsed twice with serum-free DMEM. The cells were then incubated in 1 ml of serum-free DMEM containing 2 μg of plasmid DNA (pHSV.CMV*lac* or pAAV.CMV*lac*) complexed with 10 μg of Lipofectamine (Gibco/BRL, Grand Island, NY). After a 5-hour incubation, 1 ml of DMEM supplemented with 20% fetal bovine serum was added to the cells and 8-bromo-cAMP (1.5 mmol/L) was added back to the cells that had been previously treated with the cAMP analog. The cells were cultured for another 48 hours before fixing and staining with X-gal or lysing with 1× reporter lysis buffer for assay of β-galactosidase enzyme activity.

Southern Blot Analysis of Hirt-Extracted DNA from BeWo Cells Transduced with HSV.CMVlac and AAV.CMVlac

To examine the ability of HSV and AAV vectors to infect undifferentiated and differentiated trophoblast, we performed Southern analyses to detect viral DNA in BeWo cells incubated with HSV.CMV*lac* and AAV.CMV*lac*. BeWo cells (1 × 10^6 cells in 35-mm plastic dishes) were cultured in the presence or absence of 8-bromo-cAMP (1.5 mmol/L) for 0 or 48 hours before infection with HSV.CMV*lac* (0.5 blue cell-forming units per cell) or AAV.CMV*lac* (1 blue cell-forming unit per cell).

Twenty-four hours after viral infection, the cells were rinsed with PBS and harvested with 0.05% trypsin. The enzymatic treatment also stripped virus associated with trypsin-sensitive cell surface proteins. The cells were pelleted by centrifugation at 3000 × *g* for 5 minutes. After removal of the supernatant, the cell pellets were resuspended in PBS and washed twice by centrifugation. The final supernatant was collected and analyzed for the presence of viral DNA (as described below) to ensure

that free (extracellular) viral vectors had been removed from the cell pellets.

Before beginning the Hirt DNA extractions, 10 μg of pSPORT containing a 1.6-kb StAR (steroidogenic acute regulatory protein) cDNA insert[13] was added to the cell pellets that had been transduced with HSV.CMV*lac* to ensure that equivalent amounts of DNA were extracted and transferred. Epichromosomal DNA was then extracted from the cell pellets (and supernatants) according to a modified protocol originally described by Hirt et al.[14] DNA concentrations were quantified by spectrophotometry.

Aliquots of DNA (10 μg for the HSV.CMV*lac* experiment (restricted with *Hin*dIII) and 15 μg for the AAV.CMV*lac* experiment (no restriction enzyme digestion)) were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide and transferred to nitrocellulose membranes by standard Southern blot techniques.[15] After transfer, the blots were probed with a 900-bp cDNA fragment of the *lacZ* gene and the 1.6-kb StAR cDNA insert. The membranes were sequentially washed with SSC buffer (sodium chloride, sodium citrate, pH 7.0) containing 1, 0.5, and 0.1% sodium dodecyl sulfate and placed against Kodak X-OMAT AR film.

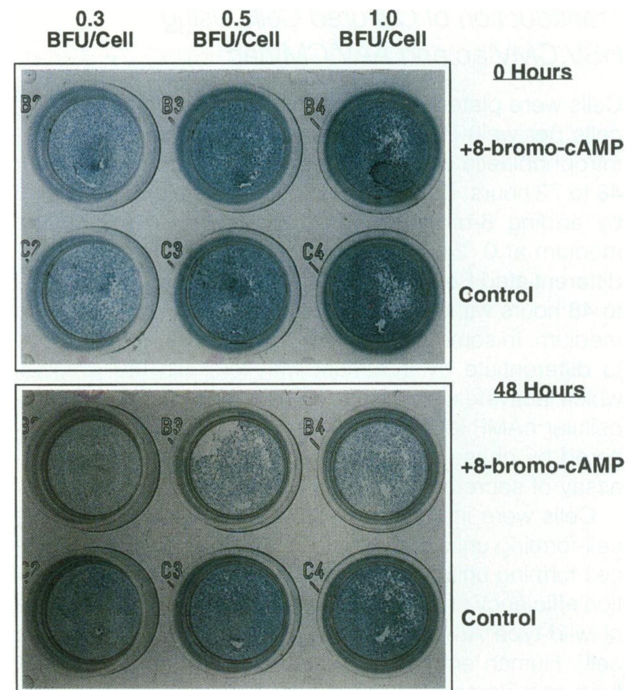

Figure 1. Expression of β-galactosidase in BeWo cells transduced by HSV.CMV*lac* (0.3 to 1.0 blue cell-forming units per cell) was diminished 48 hours after treatment with 8-bromo-cAMP. Cells were cultured in the presence (+8-bromo-cAMP) or absence (control) of 1.5 mmol/L 8-bromo-cAMP for 0 hours (**upper panel**) or 48 hours (**lower panel**) before infection with HSV.CMV*lac*. The cells were fixed and stained with X-gal 24 hours after infection.

Results

Transduction of Trophoblastic Cells by HSV.CMVlac is Diminished by Treatment with 8-bromo-cAMP

A dose-response relationship was established for the transduction of undifferentiated BeWo cells infected with HSV.CMV*lac* at 0.3, 0.5, and 1.0 blue cell-forming units per cell with maximum transduction efficiency demonstrated at 1.0 blue cell-forming unit per cell (80% blue-stained cells; RLU = 277 ± 23; $n = 3$). BeWo cells treated with 8-bromo-cAMP simultaneous to infection with HSV.CMV*lac* demonstrated an identical transduction efficiency (Figure 1; 80% blue-stained cells, RLU = 280 ± 35; $n = 3$; $P = 0.9$). No blue-stained cells were observed among uninfected BeWo cells and cells infected with wild-type Ad (1 × 10^4 viral particles per cell), demonstrating that staining for endogenous β-galactosidase was negligible. Cytopathic effect was not observed in BeWo cells infected with HSV.CMV*lac* at any of the vector concentrations used in these experiments.

Forty-eight hours after treatment with 8-bromo-cAMP, BeWo cells demonstrated reduced transduction efficiencies with HSV.CMV*lac* (25% blue-stained cells; RLU = 24 ± 12; $n = 4$). Untreated BeWo cells (undifferentiated cells) infected with HSV.CMV*lac* 48 hours after the cells were plated yielded 60% blue-stained cells (Figure 1; RLU = 86 ± 45; $n = 4$; $P = 0.03$). Transduction of 293 cells by HSV.CMV*lac* was not affected by treatment with cAMP analogs. Cellular differentiation of the cAMP-treated BeWo cells was documented by observing syncytialization and a doubling of progesterone secretion (10,408 ± 760 ng/ml *versus* 5538 ± 179 ng/ml, respectively) into the culture medium compared with control cells.

X-gal staining of freshly isolated human cytotrophoblastic cells infected with HSV.CMV*lac* (one blue cell-forming unit per cell) demonstrated 50% blue-stained cells with decreased transduction efficiencies as the trophoblastic cells spontaneously differentiated over a 48- to 72-hour culture period. Cells that were infected 36 hours after isolation yielded less than 10% blue-stained cells, whereas no blue-stained cells could be seen among trophoblastic cells infected with HSV.CMV*lac* 60 hours after isolation. The morphological changes associated with syncytialization were observed in infected and uninfected cells over 48 to 72 hours, and there was no evidence of cytopathic effect.

Exploration of Possible Mechanisms Underlying the Reduced Transduction of 8-bromo-cAMP-Treated Trophoblastic Cells by HSV.CMVlac

To examine the expression of the *lacZ* gene with respect to trophoblastic cellular differentiation, we transfected BeWo cells with pHSV.CMV*lac* after 0- and 48-hour incubations in the presence or absence of 8-bromo-cAMP. Treated and untreated BeWo cells demonstrated similar transfection efficiencies (30% blue-stained cells) and β-galactosidase enzyme activities (RLU = 75 ± 43, $n = 7$ *versus* RLU = 60 ± 47, $n = 5$; $P = 0.6$). These findings suggest that differentiated BeWo cells contain the tran-

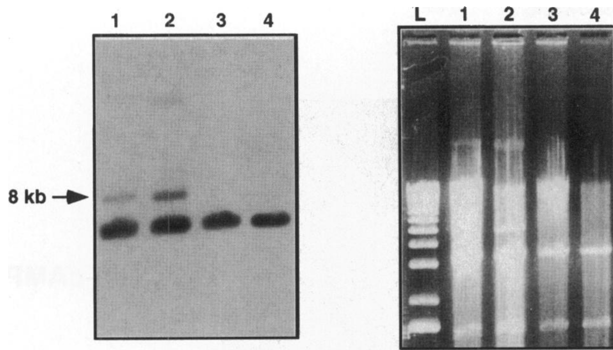

Figure 2. Southern blot analysis demonstrated reduced numbers of viral gene copies in differentiated BeWo cells infected with HSV.CMV*lac*. **Left:** Autoradiograph of a Southern blot containing aliquots of epichromosomal DNA extracted from BeWo cells (10 μg DNA/lane) cultured for 48 hours in the presence (**lane 1** and **3**) or absence (**lanes 2** and **4**) of 1.5 mmol/L 8-bromo-cAMP before infection with HSV.CMV*lac* (**lanes 1** and **2**, 0.5 blue cell-forming units per cell; **lanes 3** and **4**, control cells not infected with HSV.CMV*lac*). The cells were harvested 24 hours after infection with HSV.CMV*lac*, and 10 μg of pSPORT containing a StAR cDNA insert were added to each pellet.[13] The extracted DNA was digested with *Hin*dIII and the blot was probed with a cDNA fragment prepared from pAd.CMV*lac*. The 8-kb band corresponded to binding with HSV vector containing the *LacZ* transgene; the band at 6 kb demonstrated binding to a blue-white selection sequence within the StAR plasmid. **Right:** Photograph of the etidium bromide-stained agarose gel before Southern transfer.

scriptional/translational machinery necessary to express the *lacZ* gene driven by the CMV promoter in the HSV plasmid construct.

Southern blot analysis to detect delivery of the *lacZ* transgene to BeWo cells by our recombinant HSV vector demonstrated a band at 8 kb, corresponding to the *lacZ* gene contained within the HSV.CMV*lac* genome after digestion with *Hin*dIII. Decreased levels of the *lacZ* gene were detected in BeWo cells infected with HSV.CMV.*lac* 48 hours after treatment with 8-bromo-cAMP (Figure 2). By densitometric analysis, the 8-kb band was 2.75 times more intense in untreated BeWo cells than in BeWo cells infected with HSV.CMV*lac* 48 hours after treatment with 8-bromo-cAMP. Thus, delivery of the *lacZ* transgene was comparable with gene expression (60% *versus* 25% blue-stained cells). The 8-kb band was not detected in BeWo cells that were not infected with HSV.CMV*lac*, and no signal was detected in the BeWo cell washes, indicating that free vector was not contaminating the cell pellets. The 6-kb band was equivalent in all lanes and corresponded to hybridization of the *lacZ* probe with a blue-white selection sequence within the pSPORT plasmid. After removal of the *lacZ* probe, the membranes were rehybridized with radiolabeled StAR cDNA.[13] The intensity of the 6-kb band, corresponding to the StAR plasmid, was equal in all lanes additionally demonstrating equivalent loading of extrachromosomal DNA. Thus, Southern blot analysis demonstrates that entry of the HSV vector into differentiated BeWo cells is diminished.

*Cyclic-AMP Enhances Transduction of Trophoblastic Cells by AAV.CMV*lac

A dose-response relationship was established for the transduction efficiency of undifferentiated BeWo cells infected with AAV.CMV*lac* (± wild-type Ad coinfection) at 0.1, 0.5, and 1.0 blue cell-forming units per cell with maximum transduction efficiency (60% blue-stained cells) demonstrated at 1.0 blue cell-forming units per cell in cells coinfected with wild-type Ad. Consistent with findings previously reported by Fisher et al,[6] we established that the transduction efficiency of AAV.CMV*lac* in trophoblastic cells was increased when the cells were coinfected with wild-type Ad. Similar transduction efficiencies were observed by X-gal staining in A549 cells infected with AAV.CMV*lac* ± wild-type Ad (Figure 3). Cytopathic effect was not observed in BeWo cells infected with AAV.CMV*lac* at any of the concentrations used in these experiments.

BeWo cells that were treated with 8-bromo-cAMP either 48 hours before infection with AAV.CMV*lac* or simultaneous to infection with AAV.CMV*lac* yielded increased transduction efficiencies compared with untreated BeWo cells. Among BeWo cells that were infected with AAV.CMV*lac* alone (no wild-type Ad coinfection), we observed 30% blue-stained cells after simultaneous treatment with 8-bromo-cAMP (RLU = 10 ± 9, n = 5) *versus* fewer than 10% blue-stained cells among untreated BeWo cells (RLU = 0.6 ± 0.3, n = 6, P = 0.03) (Figure 3). BeWo cells that were infected with AAV.CMV*lac* 48 hours after treatment with 8-bromo-cAMP yielded 20% blue-stained cells (RLU = 4.6 ± 2.6, n = 5); untreated BeWo cells infected with AAV.CMV*lac* 48 hours after the cells were plated yielded fewer than 5% blue-stained cells (RLU = 0.9 ± 0.8, n = 6, P = 0.01). Cellular differentiation among 8-bromo-cAMP treated BeWo cells was documented by detecting histological changes and a tripling of progesterone secretion (5622 ± 74 ng/ml *versus* 1606 ± 93 ng/ml) into the culture medium of treated BeWo cells.

Primary cytotrophoblastic cells infected with AAV.CMV*lac* (one blue cell-forming unit per cell) demonstrated 10% blue-stained cells. Transduction efficiencies were not affected as the primary trophoblastic cells terminally differentiated over 48 to 72 hours.

The presence of 8-bromo-cAMP in the culture medium did not affect the transduction efficiencies of A549 cells. BeWo cells that were treated with forskolin 48 hours before infection with AAV.CMV*lac* alone (no wild-type Ad coinfection) demonstrated similar transduction frequencies (20 blue-stained cells per 100 cells; RLU = 3.3 ± 0.4; n = 5) as BeWo cells treated with 8-bromo-cAMP (Figure 4). These results demonstrate that transduction of BeWo cells by AAV vectors is enhanced by treatment with cAMP analogs and that this cAMP effect may be unique to trophoblastic cells.

*8-bromo-cAMP Increases the Uptake of AAV.CMV*lac *into BeWo Cells*

X-gal staining of BeWo cells transfected with the recombinant AAV plasmid pAAV.CMV*lac* yielded 30% blue-stained cells; the presence or absence of 8-bromo-cAMP did not affect transfection efficiencies. Assays for β-galactosidase activity demonstrated 135 ± 50 RLU (n = 3)

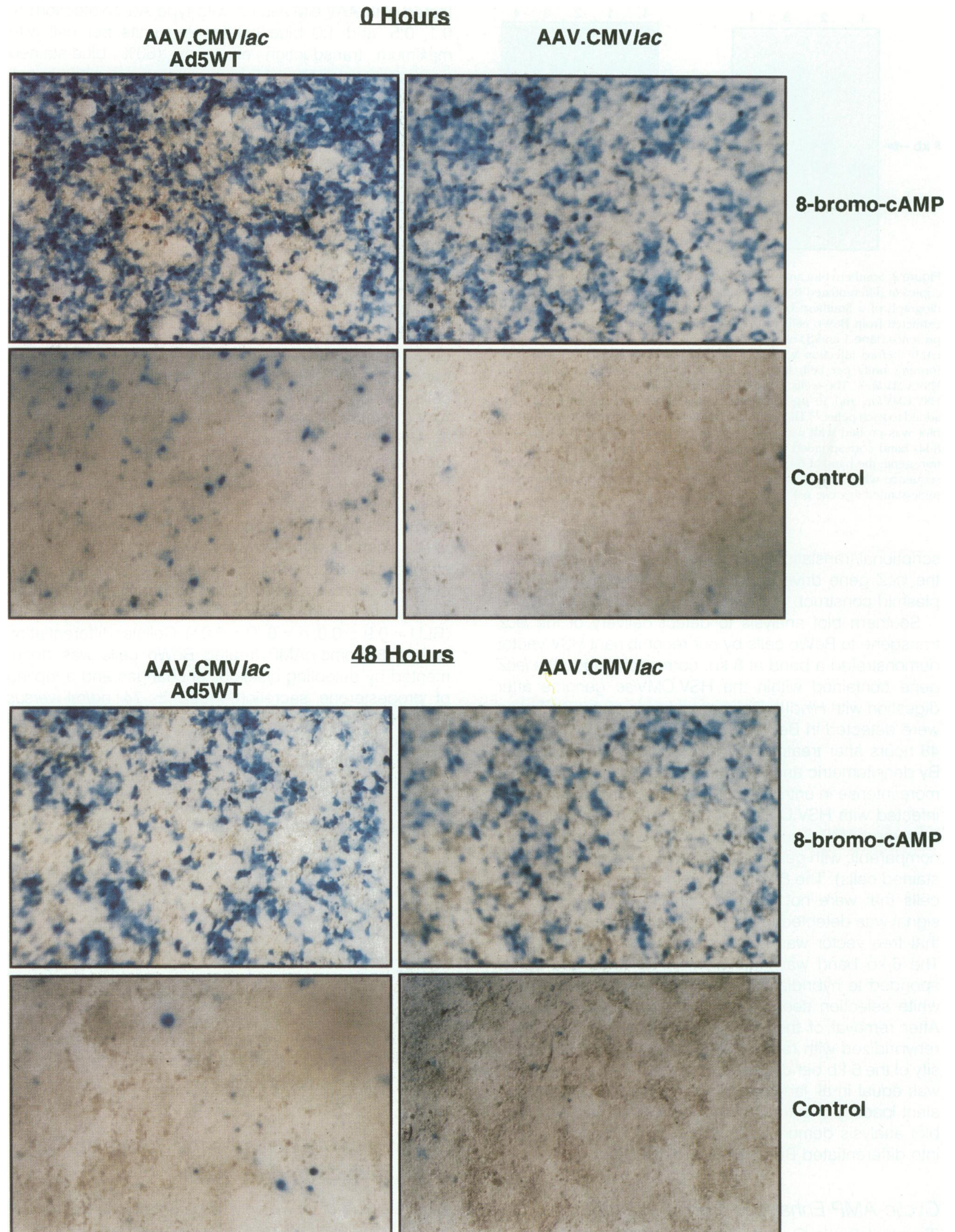

Figure 3. Expression of β-galactosidase in BeWo cells transduced by AAV.CMV*lac* (one blue cell-forming unit per cell) was increased by coinfection with wild-type Ad (Ad5WT) and after treatment with cAMP analogs. Cells were cultured in the presence (+8-bromo-cAMP) or absence (control) of 1.5 mmol/L 8-bromo-cAMP for 0 hours (**upper panel**) or 48 hours (**lower panel**) before infection with AAV.CMV*lac*. The cells in the left column were coinfected with Ad5WT (1×10^4 viral particles per cell). The cells were fixed and stained with X-gal 24 hours after infection.

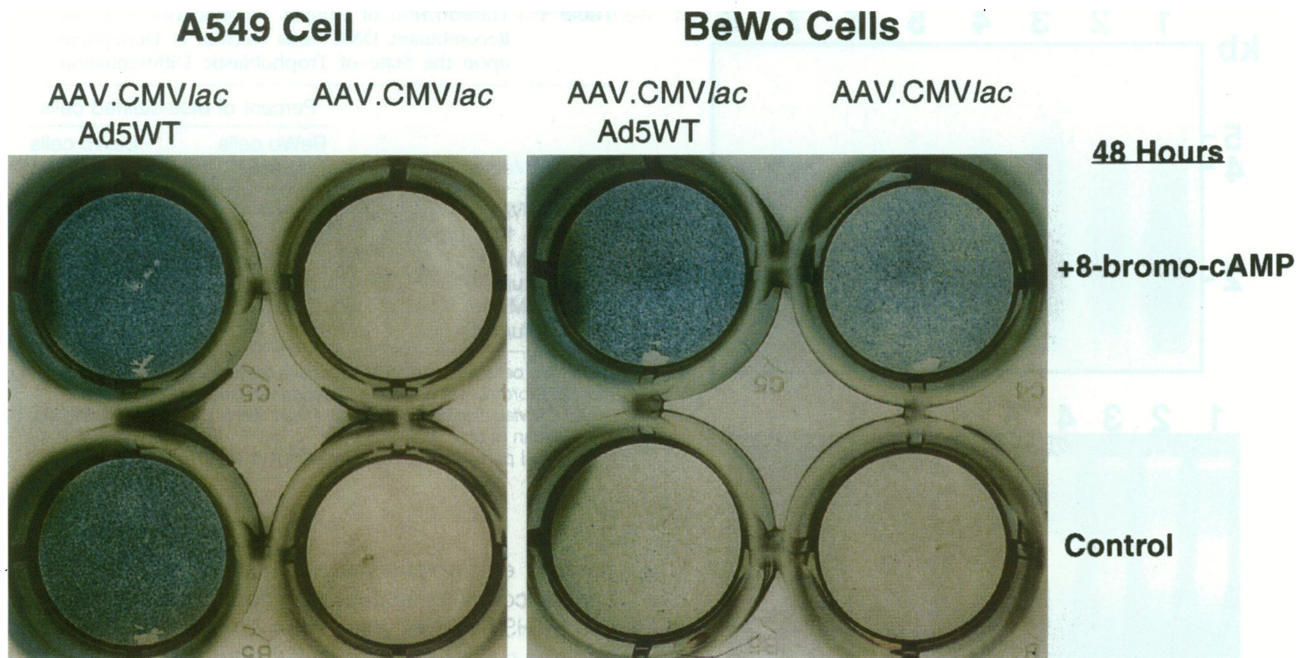

Figure 4. Increased expression of β-galactosidase after treatment with cAMP analogs was selective for BeWo cells transduced by AAV.CMV*lac* (one blue cell-forming unit per cell). BeWo and A549 cells were cultured in the presence (+8-bromo-cAMP) or absence (control) of 1.5 mmol/L 8-bromo-cAMP for 48 hours before infection with AAV.CMV*lac*. The cells in the left column of each plate were coinfected with wild-type Ad (1×10^4 viral particles per cell). The cells were fixed and stained with X-gal 24 hours after infection.

in BeWo cells treated with 8-bromo-cAMP *versus* 122 ± 40 RLU ($n = 5$) in untreated cells ($P = 0.7$). This suggests that the cAMP analog does not primarily affect the transcription or translation of the *lacZ* gene driven by the CMV promoter in trophoblastic cells.

Southern blot analysis of Hirt-extracted DNA from BeWo cells transduced with AAV.CMV*lac* demonstrated that 8-bromo-cAMP increased the entry of AAV.CMV*lac* into the trophoblastic cells. After infection for 12 or 24 hours with AAV.CMV*lac*, the single-stranded recombinant AAV genome was detected using the *lacZ* probe as 2 bands at 2 kb (Figure 5).[6] Efficient transduction by recombinant AAV vectors, however, requires second-strand synthesis of the single-stranded AAV genome. The double-stranded replicative form of the AAV.CMV*lac* genome was faintly detected as a single band at 4 kb. By densitometric analysis, the 2 kb-bands corresponding to single-stranded viral DNA were 2.0 to 7.4 times more intense in BeWo cells treated with 8-bromo-cAMP than in untreated BeWo cells. The bands at 4 kb were more difficult to visualize (secondary to the presence of relatively less replicative DNA) but were more intense in the 8-bromo-cAMP treated BeWo cells. Southern blot analysis is consistent with increased viral entry into cAMP-treated BeWo cells.

Discussion

Our observations demonstrate that trophoblastic cells display differentiation dependent susceptibility to recombinant viral vector mediated transduction. However, the factors that influence transgene expression in trophoblastic cells appear to vary among the different viral vectors (Table 1).

Recombinant HSV vectors efficiently transduce undifferentiated BeWo cells, but decreased transduction efficiencies are observed when differentiated BeWo cells are infected with HSV.CMV*lac*. Efficient transduction of target cells using viral based vectors is dependent on viral entry through specific receptors and transcription/translation of the viral transgene in target cells.[16] Transfection experiments using pHSV.CMV*lac* demonstrated that differentiated trophoblast maintains the cellular machinery that is necessary to express the *lacZ* transgene in context with the origin of replication and packaging sequence of HSV-1 (Table 2). Southern blot analysis revealed reduced numbers of viral gene copies in differentiated BeWo cells infected with HSV.CMV*lac*, indicating that reduced viral infectivity of differentiated trophoblast is responsible, at least in part, for the reduced transduction efficiency. This mechanism is similar to that which is responsible for the reduced transduction of 8-bromo-cAMP-treated BeWo cells by Ad.CMV*lac*, which is dramatically reduced because of restriction of viral entry.[12] We speculate that proteins that govern viral (Ad, HSV) entry into trophoblastic cells are not expressed as these cells terminally differentiate. Thus, additional investigation is required to determine why differentiated BeWo cells and syncytiotrophoblast are resistant to infection with recombinant Ad and HSV vectors *in vitro*.

Wild-type HSV initially interacts with a cell surface heparan sulfate receptor that has not been characterized in trophoblastic cells.[17] However, in no case has removal of heparan sulfate resulted in complete loss of attach-

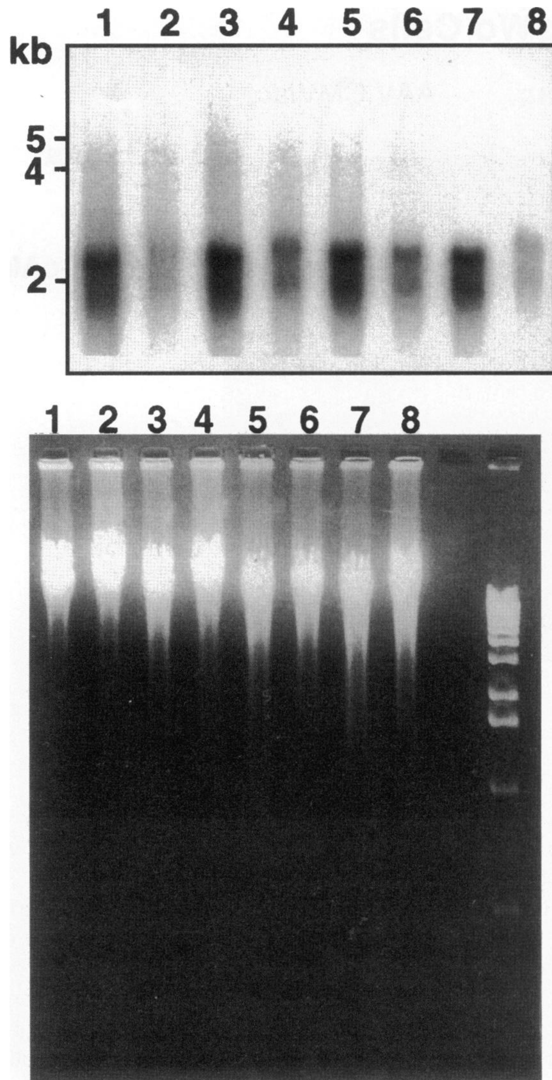

Figure 5. Southern blot analysis demonstrated increased numbers of viral gene copies in differentiated BeWo cells infected with AAV.CMV*lac*. **Top:** Autoradiograph of a Southern blot containing aliquots of epichromosomal DNA extracted from BeWo cells (15 μg DNA/lane) cultured in the presence (**lanes 1, 3, 5,** and **7**) or absence (**lanes 2, 4, 6,** and **8**) of 1.5 mmol/L 8-bromo-cAMP for 0 hours (**lanes 1** to **4**) or 48 hours (**lanes 5** to **8**) before infection with AAV.CMV*lac* (one blue cell-forming unit per cell). The cells were harvested 12 hours (**lanes 1, 2, 5,** and **6**) or 24 hours (**lanes 3, 4, 7,** and **8**) after infection with AAV.CMV*lac*. The blot was probed with a cDNA fragment prepared from pAd.CMV*lac*. The bands at 2 kb corresponded to binding with single-stranded AAV DNA containing the *LacZ* transgene. **Bottom:** Photograph of the ethidium bromide-stained agarose gel before Southern transfer.

ment or infectivity, suggesting that other factors (receptors) also mediate HSV entry into cells. Recently, hamster and swine cells resistant to HSV entry were rendered susceptible to HSV entry on expression of a human cDNA encoding a new member of the tumor necrosis factor/nerve growth factor receptor family.[18] It is not known whether this protein, designated herpesvirus entry mediator, is expressed by trophoblast cells. After entry into the host cell, recombinant HSV vector uses host RNA polymerase II to direct expression of its transgene.[19] Recombinant HSV does not possess specific mechanisms for integration; thus the viral genome most likely is expressed epichromosomally in host (target) cells.[19] The various components necessary for successful transduction by HSV vectors, including expression of cell surface heparan sulfate and herpesvirus entry mediator and transcription/translation of the viral genome, remain to be examined in relation to trophoblast cellular differentiation.

Table 1. Transduction of Human Trophoblastic Cells by Recombinant DNA Virus Vectors Is Dependent upon the State of Trophoblastic Differentiation

	Percent of blue-stained cells	
Vector	BeWo cells +cAMP*	BeWo cells −cAMP*
Ad.CMV*lac* (4 × 10^4 vp/cell)	0	80
HSV.CMV*lac* (1 bfu/cell)	25	60
AAV.CMV*lac* (1 bfu/cell)	20	2

*BeWo cells cultured for 48 hours in the presence or absence of 1.5 mmol/L 8-bromo-cAMP before infection with the viral vectors.
Ad.CMV*lac* transduction efficiencies were previously published by MacCalman et al.[12]
vp, viral particles; bfu, blue cell-forming units.

Recombinant AAV vectors have been reported to efficiently transduce various human cell types.[20,21] Our results are consistent with those of others who have demonstrated that transduction efficiencies are increased in cells coinfected with wild-type Ad, whose E4 gene provides helper function by increasing second strand synthesis of the single stranded AAV viral genome.[6,7] Second strand synthesis is reported to be the rate-limiting step for efficient transduction by recombinant AAV vectors. Our findings diverge from most previous observations, however, in that we demonstrated efficient transduction of terminally differentiated cells (BeWo cells stimulated to differentiate by treatment with cAMP analogs and syncytialized primary trophoblast cultures) using AAV.CMV*lac* in the absence of wild-type Ad coinfection.[22]

Cyclic-AMP analogs appear to promote the entry of AAV.CMV*lac* into BeWo cells. Enhanced transduction of trophoblastic cells treated with forskolin, which activates adenylate cyclase and increases intracellular cAMP, suggests that cAMP affects the BeWo cells and not the AAV

Table 2. Transfection of Human Trophoblastic Cells by Virus-Based Plasmid Vectors Is Not Dependent upon the State of Trophoblastic Differentiation

	Percent of blue-stained cells	
Plasmid Vector*	BeWo Cells +cAMP†	BeWo Cells −cAMP†
pAd.CMV*lac*[12]	50	50
pHSV.CMV*lac*	30	30
pAAV.CMV*lac*	30	30

*2 mg of plasmid DNA per 1 × 10^5 BeWo cells.
†BeWo cells cultured for 48 hours in the presence or absence of 1.5 mmol/L 8-bromo-cAMP before transfection with the plasmid vectors.

vector before entry into the cells. This effect is not seen in A549 cells and thus appears to be selective for trophoblastic cell lines. Finally, when compared with undifferentiated BeWo cells, Southern blot analysis of Hirt-extracted DNA from 8-bromo-cAMP-treated BeWo cells transduced with AAV.CMV*lac* revealed more intense bands at 2 and 4 kb, indicating the presence of greater amounts of single-stranded and double-stranded AAV DNA in the cAMP treated cells.

Large differences in the amount of gene product expressed per maximally transduced cells were observed in HSV.CMV*lac*- and AAV.CMV*lac*-infected cultures. For undifferentiated BeWo cells (1×10^5 cells) transduced with the HSV vector (one blue cell-forming unit per cell), the mean β-galactosidase activity was 277 RLU, whereas the mean β-galactosidase activity for differentiated BeWo cells transduced with the AAV vector (one blue cell-forming unit per cell) was 10 RLU. Because the transcription unit of both vectors was identical (CMV*lac*), it is probable that other factors contributed to this marked difference. The most likely explanation is that the HSV amplicon vector transfers approximately 15 copies of the transcription unit per infection count, owing to the concatenated nature of the HSV genome.[23] Meanwhile, recombinant AAV vectors transfer one copy of the transcription unit per infection count.[24]

Our experiments offer insights into the protective barrier provided by the syncytiotrophoblast layer against congenital viral infection. Primary maternal infection with numerous viruses is associated with transplacental infection of the fetus. Our results suggest that differentiated trophoblast displays characteristics that may protect the fetus from maternal viral infections, including resistance to Ad and HSV infection. Conversely, trophoblast differentiation is also associated with uptake of other viruses (AAV.CMV*lac*), possibly including various pathogens. Additional investigation is required to describe the expression of specific viral receptors, including heparan sulfate and the herpesvirus entry mediator, in trophoblast cells and to determine whether these cell surface receptors govern viral infection of the trophoblast lineage. Additionally, we must assess the ability of wild-type and recombinant viruses to express gene products and cross trophoblastic cells.

We conclude that viral infection of human trophoblastic cells can be affected by the state of differentiation of these cells. The mechanisms that modify transduction of BeWo cells with recombinant viral vectors appear to vary among Ad, HSV, and AAV vectors. Our experiments have the long term goal of developing novel strategies for the introduction of exogenous genetic material into trophoblastic cells to learn more about their physiology. The ability of AAV.CMV*lac* to efficiently transduce cAMP treated BeWo cells encourages us to continue to investigate AAV vectors as potential vehicles for trophoblast transduction in animal models. Ultimately placental gene therapy may provide new therapeutic options for complications of pregnancy that result in part from placental dysfunction.

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