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Limited replication of human cytomegalovirus in a trophoblast cell line

Kadeem Hyde[1], Nowshin Sultana[1], Andy C. Tran[1], Narina Bileckaja[1], Claire L. Donald[2], Alain Kohl[2], Richard J. Stanton[3] and Blair L. Strang[1,*]

ABSTRACT

Several viruses, including human cytomegalovirus (HCMV), are thought to replicate in the placenta. However, there is little understanding of the molecular mechanisms involved in HCMV replication in this tissue. We investigated replication of HCMV in the extravillous trophoblast cell line SGHPL-4, a commonly used model of HCMV replication in the placenta. We found limited HCMV protein expression and virus replication in SGHPL-4 cells. This was associated with a lack of trophoblast progenitor cell protein markers in SGHPL-4 cells, suggesting a relationship between trophoblast differentiation and limited HCMV replication. We proposed that limited HCMV replication in trophoblast cells is advantageous to vertical transmission of HCMV, as there is a greater opportunity for vertical transmission when the placenta is intact and functional. Furthermore, when we investigated the replication of other vertically transmitted viruses in SGHPL-4 cells we found some limitation to replication of Zika virus, but not herpes simplex virus. Thus, limited replication of some, but not all, vertically transmitted viruses may be a feature of trophoblast cells.

INTRODUCTION

TORCH [toxoplasma, other (including Zika virus), rubella, cytomegalovirus, herpes (herpes simplex 1 and herpes simplex 2)] pathogens and viruses such as human immunodeficiency virus, parvovirus B19, hepatitis E virus and varicella zoster virus are widely studied, as vertical transmission is an important route of their dissemination in human populations and infection *in utero* can have significant consequences for the health of the mother, foetus and/or child [1–5].

TORCH pathogens and the viruses mentioned above can reach the placenta via ascending or descending infections. Transvaginal ascending infections can reach the foetus via infection of the amniotic membranes [1], whereas in descending infection, interaction of pathogens with trophoblast cells at the foetal–maternal interface can be key to vertical transmission [1]. In the placenta the key features of the maternal–foetal interface are the branch-like villous protrusions from the placenta that invade the maternal decidual tissue, anchoring the placenta to the decidual tissue, and the floating villous protrusions that are in direct contact with maternal blood [1]. Covering these protrusions are a layer of syncytiotrophoblasts and a layer of cytotrophoblasts. At the tip of the protrusions are extravillious trophoblasts (EVTs). Maternal to foetal transmission of pathogens across the placenta can occur via several routes, including infection of invasive EVTs, paracellular or transcellular transport, transfer of infected cells across the maternal-foetal interface and infection of the amniotic membranes [1, 2]. TORCH pathogens are not restricted to using a single method to cross the maternal to foetal interface [1].

Virus infection of the placenta and subsequent infection of the foetus can cause a number of pathologies, including stillbirth and congenital disease [1–3]. Human cytomegalovirus (HCMV) infection can cause placental dysfunction

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Author affiliations: [1]Institute for Infection and Immunity, St George's, University of London, London, UK; [2]MRC-University of Glasgow Centre for Virus Research, Glasgow, UK; [3]Division of Infection and Immunity, Cardiff University School of Medicine, Cardiff, UK.
***Correspondence:** Blair L. Strang, bstrang@sgul.ac.uk
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Abbreviations: DMEM, dulbecco's modified eagle's medium; EVTs, extravillious trophoblasts; FACS, florescence-activated cell sorting; GFP, green florescent protein; GPCMV, Guinea pig cytomegalovirus; HCMV, human cytomegalovirus; HFF, human foreskin fibroblast; HSV, herpes simplex virus; IRF3, interferon regulatory factor 3; MTT, 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide; NPro, N-terminal protease; PBS, phosphate-buffered saline; PDGFRα, platelet-derived growth factor receptor alpha; RPMI, Rosslyn Park Memorial Institute; SV40, simian virus 40; TBPCs, trophoblast progenitor cells; TORCH, Toxoplasma, Other, Rubella, Cytomegalovirus, Herpes; ZIKV, Zika virus.
Three supplementary figures are available with the online version of this article.

that leads to preterm birth, foetal growth restriction and direct injury to the foetus, leading to miscarriage [1–3]. Notably, HCMV is the most common congenital disease and is a major cause of blindness, deafness, cognitive impairment/neuronal disability (for example, cerebral palsy) and neonatal mortality [2, 3]. HCMV seroprevalence in women of reproductive age can reach 100% in some populations and in most populations HCMV infection of pregnant women results in congenital HCMV infection in up to 2% of all live births [6–9]. As multiple strains of HCMV exist, convalescent immunity from previous HCMV infection is not likely to be sufficient to protect against future HCMV infections [10]. Thus, seropositive mothers remain at risk of acquiring and vertically transmitting HCMV [10]. Congenital infection can arise from primary infection of the mother or from reactivation of latent HCMV during pregnancy [1, 3]. The impact of acquiring HCMV disease *in utero* can have long-term consequences, especially as HCMV can establish a lifelong latent infection in an infected host that can be reactivated to cause further disease throughout their lifetime [11]. However, it is interesting and important to note that acquisition of HCMV post-partum, for example via breast milk, may not lead to severe HCMV-related disease [12].

There are different mechanisms by which HCMV can cross the maternal–foetal interface in the placenta and ultimately cause congenital disease. Maternal IgG antibodies can facilitate immunoglobulin-meditated transcytosis of HCMV in EVT cells by binding HCMV and the neonatal Fc receptor for IgG [13]. Alternatively, HCMV can infect EVT cells [13]. Presently, infection of Guinea pigs with Guinea pig cytomegalovirus (GPCMV) is the only robust model of cytomegalovirus vertical transmission, as GPCMV can infect Guinea pig trophoblasts and the amniotic sac, plus infection of Guinea pigs can recapitulate hearing loss found in congenital infection of humans [2, 14–17]. New models of congenital rhesus cytomegalovirus infection are developing rapidly [18] and it will soon be possible to understand how useful they will be in understanding vertical transmission of HCMV.

To understand vertical transmission of HCMV across the placenta it is largely necessary to rely upon studies of HCMV infection of human explant tissue and human EVT cell lines. A common EVT cell line used to study HCMV replication in the placenta is SGHPL-4 cells. Studies using these cells have suggested many pathogenic mechanisms in which HCMV can perturb EVT function and cause placental and foetal damage *in utero* [3]. These include inhibiting invasion and migration of EVT and destruction of EVT via apoptosis [3]. However, there is very little understanding of the molecular basis of HCMV replication in SGHPL-4 cells. Therefore, we set out to study HCMV replication in SGHPL-4 cells. We also asked if HCMV infection of SGHPL-4 cells was similar to infection by other viruses that can be acquired via ascending or descending infection, such as herpes simplex virus (HSV) and Zika virus (ZIKV). Like HCMV, these viruses are a major cause of complications in pregnancy and foetal infection by these viruses results in serious congenital disease [1].

METHODS

Cells

Human foreskin fibroblast (HFF) cells (CRL-1684, clone Hs29) were obtained from the American Type Culture Collection (ATCC, USA). Vero cells were a kind gift from Donald Coen (Harvard Medical School). Human adenocarcinomic alveolar basal epithelial cell line A549 and A549-N^{pro} [expressing the bovine diarrhoea virus N-terminal protease (N^{pro})] cells were kindly provided by Steve Goodbourn (St George's, University of London). The aforementioned cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% foetal bovine serum (FBS) (Gibco), plus 1% penicillin/streptomycin (Invitrogen). SGHPL-4 and SGHPL-5 [19] cells were kind gifts from Guy Whitley (St George's, University of London). All trophoblast cell lines were maintained in Rosslyn Park Memorial Institute (RPMI) media (Gibco) or HAM F-12 media (Gibco) containing 10% FBS (Gibco), plus 1% penicillin/streptomycin (Invitrogen). HepG2 cells were generously provided by Joe Grove (UCL) and incubated in DMEM containing 10% FBS, plus 1% penicillin/streptomycin.

Human cytomegalovirus strains and mutants

HCMV strain Merlin(RCMV1111), which contains deletions in open reading frames encoding RL13 and UL128, has been reported elsewhere [20]. Merlin(R1111)UL36GFP, a virus that expresses a fusion peptide of HCMV UL36 and green florescent protein (GFP) separated by a self-cleaving P2A protein sequence, has been described elsewhere [21]. All virus stocks were generated by low-multiplicity infections of HFF cells. Viral titre was determined by virus titration on HFF cells. HCMV strains TB40/E (generated from a bacterial artificial chromosome encoding the TB40/E genome [22]) and AD169 were generously provided by Matthew Reeves (UCL) and Donald Coen (Harvard Medical School), respectively.

FACS analysis

Uninfected or infected cells were infected as described in the figure legends. At the time points indicated in the text, cells were trypsinized, washed once in phosphate-buffered saline (PBS), and then resuspended in PBS. Green florescent protein expression in cells was analysed using florescence-activated cell sorting (FACS). In each case, 10, 000 cells were acquired using a BD FACSCalibur cytometer. Data were analysed using FlowJo V10.

Herpes simplex virus strains

HSV-1 strain KOS was a kind gift from Donald Coen (Harvard Medical School), while HSV-1 strains 17+, MG1 and SG16 were all gifts from Stacey Efstathiou and Mike Nicholl (NIBSC). All HSV-2 strains were generously

donated by David Knipe (Harvard Medical School). All virus stocks were generated by low-multiplicity infections of Vero cells. Viral titre was determined by virus titration on Vero cells.

Zika virus strains

Zika virus (ZIKV) *ZIKV/H. sapiens/Brazil/PE243/2015* (abbreviated to PE243, isolated from an infected human patient, Brazil, 2015), has been described previously [23]. Strains VR-84 (strain MR766, isolated from experimental forest sentinel rhesus monkey, Uganda, 1947) and VR-1845 (strain P6-740, isolated from *Aedes aegypti*, Malaysia, 1966) were obtained from the ATCC (USA). All virus stocks were generated by low-multiplicity infections of A549-N^{pro} cells. Viral titre was determined by virus titration on Vero cells.

Determination of viral titre by virus titration

Titres were determined by serial dilution of viral supernatant onto HFF (for HCMV) or Vero (for HSV and ZIKV) cell monolayers, which were then overlaid with DMEM containing 5% FBS (Gibco), 0.6% (w/v) methylcellulose and 1% penicillin/streptomycin (Invitrogen). After incubation for 3 days (HSV), 5 days (ZIKA) or 14 days (HCMV), cells were stained with crystal violet and plaques in the infected cell monolayers were counted to determine plaque-forming units (p.f.u. ml^{-1})

MTT cytotoxicity assays

In this colorimetric assay the ability of cellular NAD(P)H-dependent cellular oxidoreductase enzymes to reduce the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) to formazan was measured. HFF cells were seeded at 1×10^4 per well into 96-well plates. After overnight incubation to allow cell attachment, cells were treated as indicated in the figure legend and text. At 72 h post-infection (p.i.) MTT assays were then performed according to the manufacturer's instructions (GE Healthcare).

Western blotting

Lysates of uninfected or infected HFF cells (see text and figure legends) were prepared for Western blotting by washing cells once in PBS and then suspending them directly in 2× Laemmli buffer containing 5% β-mercaptoethanol before incubation at 95 °C for 5 min. Western blotting of proteins separated on 8 or 10% polyacrylamide gels was carried out as described elsewhere [24], using antibodies recognizing IE1/2, UL44, pp28, (all Virusys, 1:1000 dilution), β-actin (SIGMA, 1:5000 dilution), PDGFRa (ABCAM ab203491, 1:1000 dilution), Hand1 (ABCAM, 1:1000 dilution), GATA-3, GATA-4 and HMGA2 (all Cell Signaling Technology, all 1:1000 dilution). All primary antibodies were incubated overnight at 4 °C and detected using anti-mouse- or anti-rabbit-horseradish peroxidase (HRP)-conjugated antibodies (Cell Signaling Technology). Chemiluminescence solution (GE Healthcare) was used to detect secondary antibodies on film. Where necessary, blots were stripped and reprobed.

RESULTS

HCMV protein expression in SGHPL-4 cells

SGHPL-4 cells are EVT trophoblast cell lines taken from placental tissue of a first trimester pregnancy termination [19]. SGHPL-4 cells had previously been used in several studies of placental trophoblast function upon infection with HCMV [3, 25–32] and were known to support replication of an HCMV GFP reporter mutant [26]. However, how well HCMV replicates in other cells compared to replication in SGHPL-4 was not assessed [26]. Indeed, to our knowledge, there had been no rigorous examination of the ability of HCMV to replicate in SGHPL-4 cells and no examination of HCMV protein expression in SGHPL-4 cells.

We noted that SGHPL-4 cells expressed the simian virus 40 (SV40) middle T antigen, whose presence is required to maintain SGHPL-4 cell viability [19]. As this protein promotes progression through the cell cycle, we hypothesized that HCMV replication in SGHPL-4 cells would be inefficient. It has been observed elsewhere that serum starvation of cells can arrest the cell cycle at a stage (G0/G1) that is advantageous for efficient HCMV immediate-early gene expression [33]. Therefore, we incubated HFF and SGHPL-4 cells in a high concentration of serum [10% volume/volume of the tissue culture media (10% (v/v)] and then either continued incubation under these conditions and infected cells with HCMV in 10% (v/v) media, or incubated cells in a low concentration of serum [0.5% volume/volume of the tissue culture media (0.5% (v/v)] for 24 h and then infected cells in 0.5% (v/v) media (Fig. 1a). All infections were carried out using the HCMV virus Merlin(R1111) [20, 31]. The genome of this virus is similar to wild-type HCMV genomes, but does not express HCMV proteins RL13 (which promotes cell to cell spread of HCMV) or UL128 (part of the viral glycoprotein gH/gL/UL128-UL131 pentamer complex, which mediates virus entry into certain cell types) [20]. We prepared cell lysate for Western blotting at 24 h intervals p.i. (Fig. 1a) and used Western blotting to assay the production of the immediate-early proteins IE1 and IE2 over time in infected cells (Fig. 1b(i)). We observed robust expression of IE1 and IE2 proteins in HFF cells over time in the presence of both high and low concentrations of serum (Fig. 1b(i), lanes 2–4 and 10–12, respectively). Compared to protein expression in HFF cells, we found limited IE1 and IE2 protein expression in SGHPL-4 cells incubated in 10% (v/v) media (Fig. 1b(i), lanes 6–8). However, compared to protein expression in HFF cells, we observed only a modest defect in IE1 and IE2 protein expression in SGHPL-4 cells incubated in 0.5% (v/v) media (Fig. 1b(i), lanes 14–16). Although IE1 and IE2 protein expression was similar in HFF and SGHPL-4 cells at 24 h p.i. (Fig. 1b(i), compare lanes 10 and 14), we observed that while protein expression increased in HFF over time (Fig. 1b(i), lanes 10–12), protein expression in SGHPL-4

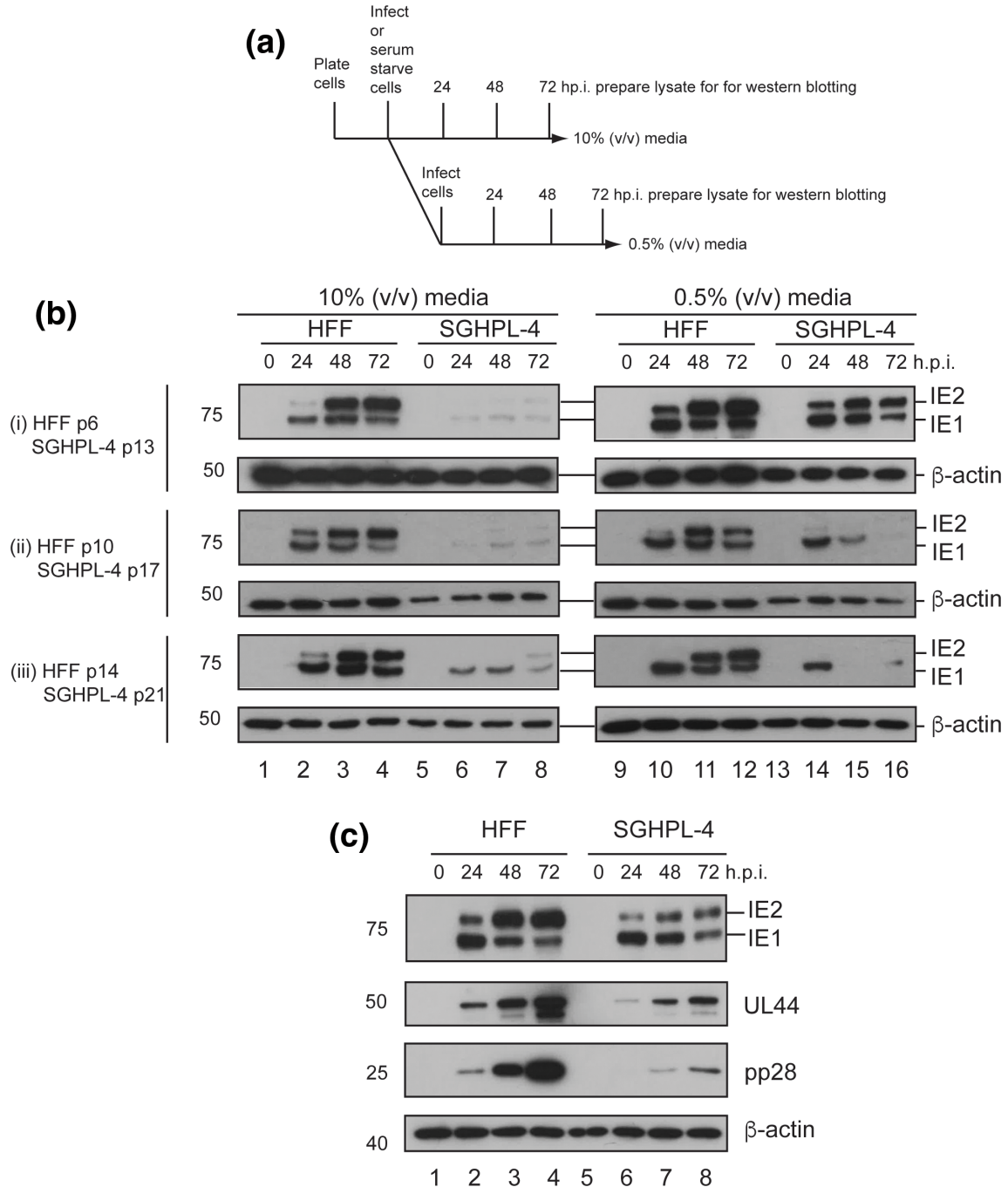

Fig. 1. HCMV protein expression in SGHPL-4 cells. (a) Timeline of cell preparation, cell infection and sample collection. (b) Lysate from uninfected or infected HFF and SGHPL-4 cells [HCMV strain Merlin(R1111), multiplicity of infection of 1] were prepared for Western blotting at the time points [h post-infection (p.i.] indicated above the figure [also, see panel (a)]. The passage number (p) of the cells used is indicated at the far left of the figure. Figures (i)-(iii) are increasing passages of the same HFF or SGHPL-4 cells. (c) Lysates from uninfected or infected HFF and SGHPL-4 cells (p8 and p14, respectively) [HCMV strain Merlin(R1111), multiplicity of infection of 1] incubated in 0.5% (v/v) media 24 h prior to infection and during infection were prepared for Western blotting at the time points indicated above the figure. In both (b) and (c) uninfected cells harvested at the time of infection are shown as 0 h p.i. Proteins recognized by the antibodies used in the experiment are indicated to the right of each Western blot panel. In each experiment the presence of β-actin was assayed to assess the amount of cell lysate assayed in each lane. The positions of molecular weight markers (kDa) are indicated to the left of each the figure.

cells did not (Fig. 1b(i), lanes 13–16). Furthermore, with increased passage of cells the aforementioned phenotype became more prominent [compare panels in Fig. 1b(i) (early passage) with panels in Fig. 1b(ii) and 1b(iii) (later passages of the same cells)], to the extent that IE1 or IE2 protein expression after 24 h p.i. could not be observed in SGHPL-4 cells (Fig. 1b(iii)). Therefore, immediate-early protein expression in SGHPL-4 cells was possible under serum starvation conditions. However, this protein expression in SGHPL-4 cells was not comparable with protein expression in HFF cells either over the time of infection or with increasing passage of cells.

Comparable expression of IE1 or IE2 in HCMV-infected HFF and SGHPL-4 cells at 24 h p.i. in cells of low and high passage incubated in 0.5% (v/v) media [Fig. 1b(i)–(iii)] suggested that there was no obvious barrier to HCMV entry in SGHPL-4 cells compared to HCMV entry in HFF cells. To confirm this we incubated low- and high-passage cells in 0.5% (v/v) media and infected cells with a derivative of Merlin(R1111), Merlin(R1111)UL36GFP, which expresses GFP early in HCMV infection [21]. After 24 h, uninfected and infected cells were analysed by FACS (Fig. S1). We found no obvious difference in the number of low- or high-passage HFF and SGHPL-4 cells expressing GFP, suggesting that there was no obvious barrier to HCMV entry in SGHPL-4 cells and the HCMV pentamer glycoprotein complex was not required for viral entry.

As we had seen a decrease in IE1 and IE2 protein expression over time in early-passage SGHPL-4 cells compared to HFF cells, we then investigated whether this defect in immediate-early protein expression was associated with defects in early and late HCMV protein expression. Therefore, we prepared cell lysates for Western blotting from early-passage HFF and SGHPL-4 cells, both of which were incubated in 0.5% (v/v) media for 24 h before infection. Western blotting was carried out using antibodies recognizing immediate-early (IE1 and IE2), early (UL44) and late (pp28) viral proteins (Fig. 1c). We observed similar IE1 and IE2 protein expression in HFF and SGHPL-4 cells to that seen in Fig. 1b (Fig. 1c lanes 2–4 and 6–8, respectively). Compared to protein expression in HFF cells (Fig. 1c, lanes 2–4), we found limited UL44 and pp28 protein expression in SGHPL-4 cells (Fig. 1c, lanes 6–8). Therefore, the defect in immediate-early protein expression in SGHPL-4 cells was associated with limited early and late HCMV protein expression in those cells.

Replication of HCMV in SGHPL-4 cells

As we had observed limited HCMV protein expression in SGHPL-4 cells incubated in low-serum-concentration media (Fig. 1), we hypothesized that limited protein expression would be associated with limited production of HCMV virus. Therefore, we tested the ability of HCMV to replicate in HFF and SGHPL-4 cells incubated in 10 and 0.5% (v/v) media in both low- and high-passage cells. The experimental plan is shown in Fig. 2a. Robust HCMV replication was found in low-passage HFF cells incubated in both 10 and 0.5% (v/v) media (Fig. 2b). Compared to HCMV replication in HFF cells, limited replication of HCMV was found in low-passage SGHPL-4 cells incubated in 10% (v/v) media, but replication of HCMV was greater in low-passage SGHPL-4 cells incubated in 0.5% (v/v) media (Fig. 2b). Continued passage of cells had no obvious effect on HCMV replication in HFF cells (Fig. 2c). Similarly, compared to HCMV replication in HFF cells, limited replication of HCMV was found in high-passage SGHPL-4 cells incubated in either 10% or 5% (v/v) media (Fig. 2c). Therefore, HCMV replication was limited in SGHPL-4 cells compared to HFF cells and differed with passage of cells.

Although we did not find any data suggesting a barrier to HCMV Merlin(R1111) entry into SGHPL-4 cells (Figs 1 and S1), we speculated that different HCMV strains may have different abilities to replicate in SGHPL-4 cells. We incubated low-passage HFF and SGHPL-4 cells in 0.5% (v/v) media and infected them with Merlin(R1111), AD169 (an HCMV strain that does not express the HCMV glycoprotein pentamer and a large number of proteins that influence HCMV pathogenesis [31]) or TB40/E (an HCMV strain similar to wild-type Merlin that does express the HCMV glycoprotein pentamer [31]) (Fig. S2a). We found no obvious difference in the ability of any HCMV strain to replicate in SGHPL-4 cells. Similar data were observed using high-passage SGHPL-4 cells (data not shown). Using Western blotting, we also examined the expression of the HCMV receptor platelet-derived growth factor receptor alpha (PDGFRα), which is required for the entry of HCMV virus lacking the glycoprotein pentamer (Fig. S2b). Consistent with data described elsewhere [28], SGHPL-4 cells expressed PDGFRα, though not to the same extent as human fibroblast cells. Interestingly, we observed that serum starvation of HFF and SGHPL-4 cells increased PDGFRα expression in both cell lines, suggesting that serum starvation may aid virus entry into both cell lines.

We also considered that HCMV replication in SGHPL-4 may be delayed compared to HFF. Therefore, we analysed HCMV Merlin(R1111) replication over a time course of infection in low-passage HFF and SGHPL-4 cells incubated in 0.5% (v/v) media (Fig. S2c). HCMV replication in HFF cells increased over time, until after 5 days p.i. when cell death was evident in the infected HFF cell monolayer and no more time points were examined. HCMV replication in SGHPL-4 cells was limited compared to HCMV replication in HFF cells, peaking at 5 days p.i. and then declining after that time point. Therefore, a delay in HCMV replication in SGHPL-4 cells was unlikely to reflect the limited HCMV replication we observed.

Cell viability of uninfected and infected trophoblast cell lines

We observed poor protein production and virus replication in SGHPL-4 cells infected with HCMV (Figs 1 and 2). It was speculated that this may have been due to poor cell viability in the presence or absence of high concentrations of serum and/or HCMV. Therefore, we assayed cell viability by measuring the

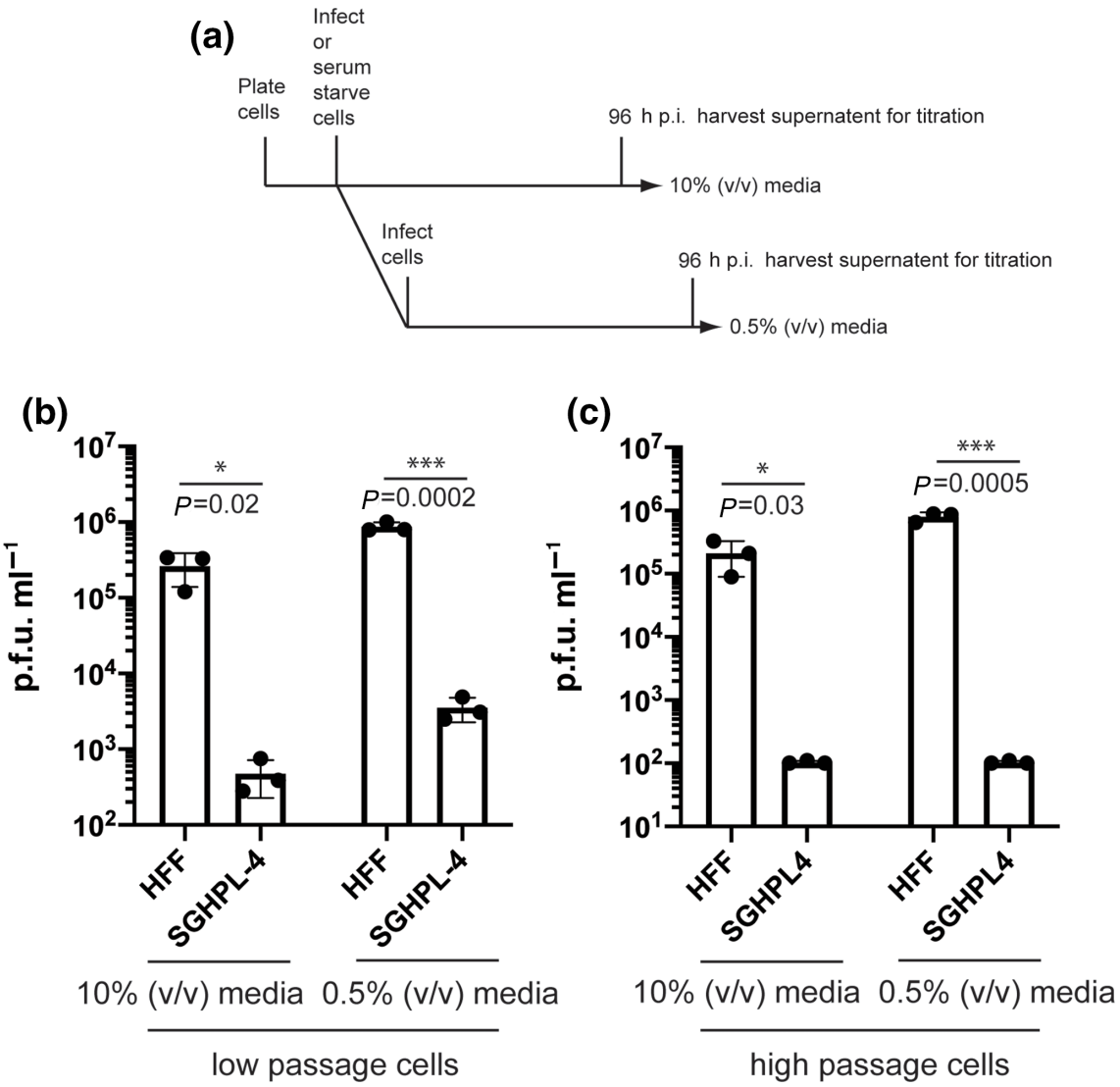

Fig. 2. HCMV replication in SGHPL-4 cells. (a) Timeline of cell preparation, cell infection and sample collection. (b) Low- and (c) high-passage HFF or SGHPL-4 cells (passages 6–10 and 13–15, respectively) were treated as shown in panel (a) and infected with HCMV strain Merlin(R1111) at a multiplicity of infection of 1. Viruses were harvested at 96 h post-infection and viral titre [plaque-forming units (p.f.u.) ml^{-1}] was determined by titration of viral supernatant on HFF cells. Data from three independent experiments are presented in each figure. The bar chart and error bars represent the mean and standard deviation of that data, respectively. The statistical difference between the indicated conditions was measured using an unpaired *t*-test (two-tailed) and is indicated above each figure. A statistically relevant difference was where $P<0.05$.

ability of cellular mitochondrial enzymes lost during apoptosis to reduce the compound MTT (Fig. 3). Low- or high-passage HFF and SGHPL-4 cells were incubated in either 10% or 0.5% (v/v) media and in the presence or absence of HCMV, and then exposed to MTT (Fig. 3a). When we analysed MTT reduction under the aforementioned conditions, we found that there was no obvious difference in the viability of HFF cells in high- or low-concentration-serum media, and nor was cell viability affected by the presence of virus or the passage of the cells (compare Fig. 3b, d). However, there was a modest difference in the viability of high-passage SGHPL-4 cells in the presence of 0.5% (v/v) media and/or HCMV (compare Fig. 3c, e). Therefore, a defect in cell viability may have contributed to the limited HCMV protein expression and replication seen in high-passage SGHPL-4 cells (Figs 1 and 2). However, this reduction in cell viability was modest and there were likely other factors that contributed to limited HCMV protein expression and replication in SGHPL-4 cells.

Analysis of TBPC proteins in trophoblast cell lines

We gave further consideration as to why HCMV replication in SGHPL-4 cells was limited. It had been reported that robust replication of HCMV was possible in first trimester

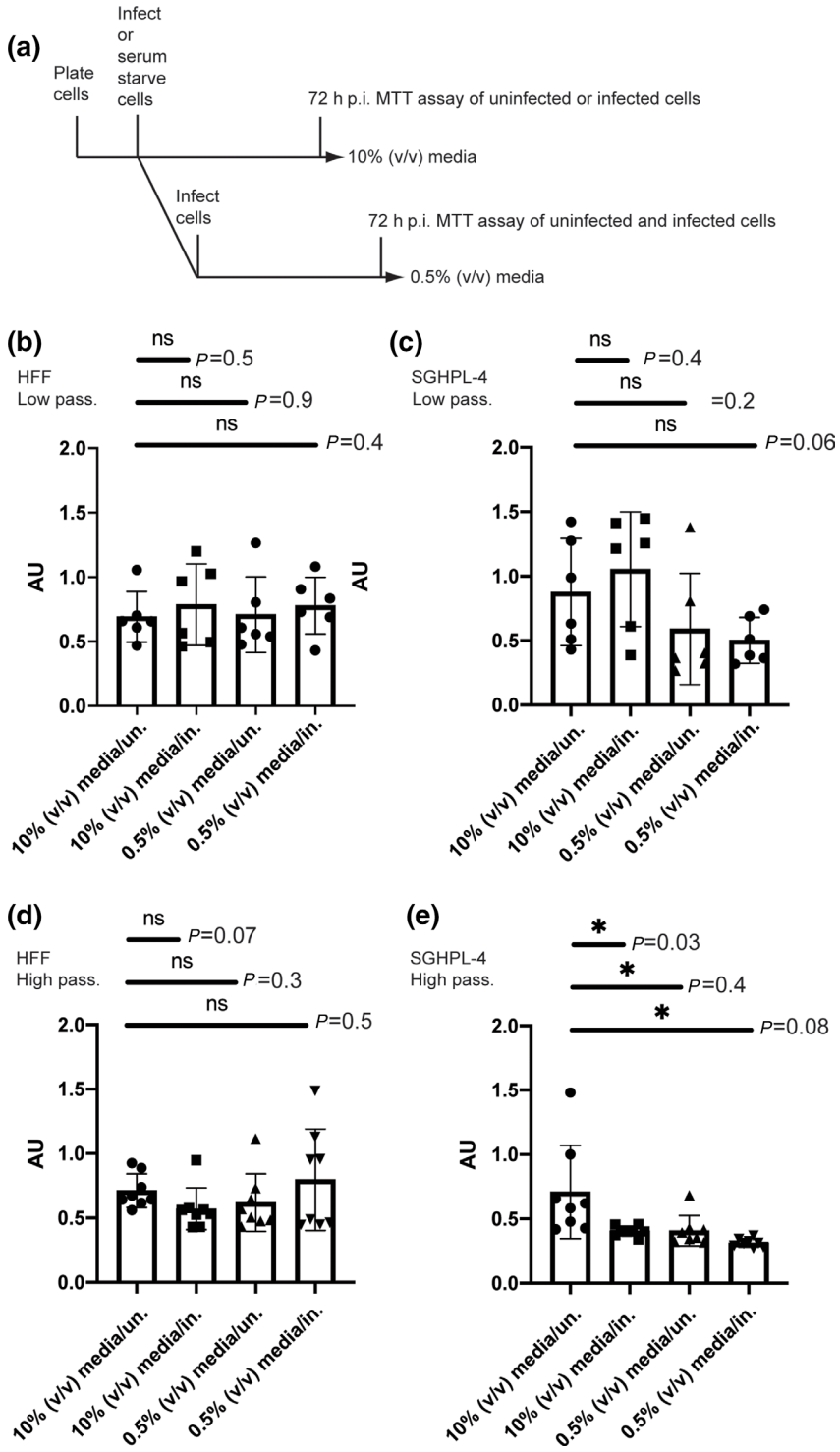

Fig. 3. Cell viability in the presence and absence of HCMV and serum. (a) Timeline of cell preparation, cell infection and sample collection. (b–e) Low- and high-passage HFF and SGHPL cells (HFF passage 6–9 and 15–18, SGHPL-4 passage 13–15 and 19–21) were treated as shown in (a) and infected with HCMV strain Merlin(R1111) at a multiplicity of infection of 1. At 72 h p.i. cell viability was determined using an MTT assay [arbitrary units (AU)]. Each data point represents the data from eight biological replicates in each condition. The bar and error bars represent the mean and standard deviation of those data, respectively. The data in this figure are representative of two independent experiments measured at 72 h p.i. The statistical difference between the indicated conditions was measured using an unpaired *t*-test (two-tailed) and is indicated above each figure. A statistically relevant difference was where $P<0.05$. Not significant, ns. Uninfected, un. Infected, in.

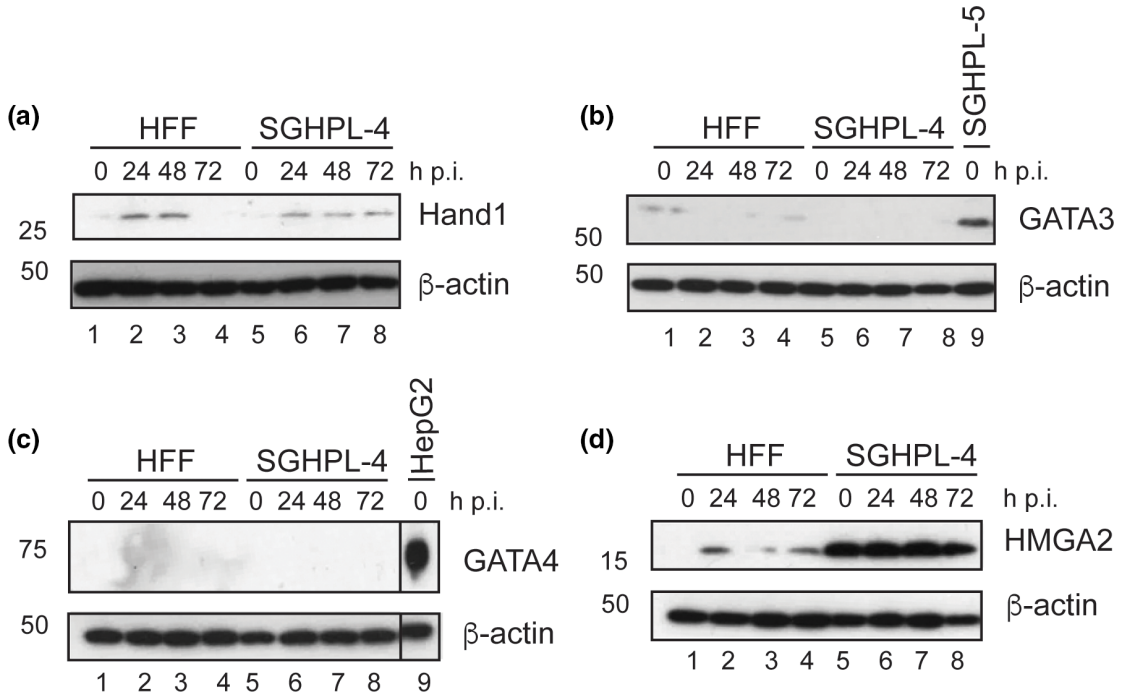

Fig. 4. Trophoblast protein expression in uninfected and HCMV-infected cells. Lysates from uninfected or infected HFF or SGHPL-4 cells [HCMV strain Merlin(R1111), multiplicity of infection of 1] (HFF passage 7, SGHPL-4 passage 14) incubated in 0.5% (v/v) media 24 h prior to infection and during infection were prepared for Western blotting at the time points (h p.i.) indicated above the figure. Uninfected cells harvested at the time of infection are shown as 0 h p.i. Where indicated, lysate from the control cell lines SGHPL-5 and HepG2 were analysed. Proteins recognized by the antibodies used in the experiment are indicated to the right of each Western blot panel. In each experiment the presence of *β*-actin was assayed to assess the amount of cell lysate analysed in each lane. The positions of molecular weight markers (kDa) are indicated to the left of the figure. In (c) figure C, each panel shows data from the same exposure of film.

trophoblast progenitor cells (TBPCs) [34] and that HCMV infection could alter the expression of TBPC regulatory proteins (Hand1, GATA3, GATA4 and HMGA2) involved in the self-renewal and differentiation that define TBPCs [35]. Other reports had indicated that HCMV replication in cells differentiated into cytotrophoblasts or in trophoblasts from full-term placental tissue was limited [36–39]. As all trophoblasts differentiate from progenitor cells, this suggested that HCMV replication in trophoblast cells that had differentiated from progenitor cells may have been inefficient. We hypothesized that SGHPL-4 cells may have moved beyond the trophoblast progenitor stage of their differentiation and this may have been associated with the limited HCMV replication we had observed.

To investigate this, we prepared cell lysate from uninfected and HCMV-infected low-passage serum-starved HFF and SGHPL-4 cells. Antibodies recognizing the aforementioned TBPC regulatory proteins were used in Western blotting of cell lysates (Fig. 4). We found that uninfected SGHPL-4 cells did not express all four TBPC regulatory proteins at levels detectable in our assay (Fig. 4a–d), indicating that SGHPL-4 cells were not TBPCs. Hand1 and HGMA2 were detected in the presence or absence of HCMV, but to different extents, in both cell lines (Fig. 4a, d). GATA3 and GATA4 expression was detected in control cell lines, SGHPL-5 and HepG2 cells (Fig. 4b, c, respectively), but not in either HFF or SGHPL-4 cells (Fig. 4b, c).

We also noted that HCMV infection of SGHPL-4 cells did not result in differences in TBPC regulatory protein expression that had previously been reported in HCMV infection of TBPCs [35]. For example, we did not observe loss of HMGA2 expression in SGHPL-4 cells (Fig. 4d), which had been reported in HCMV-infected TBPCs [35]. These data further indicated that SGHPL-4 cells did not have TBPC-like properties.

Therefore, the inability to detect all TBPC regulatory proteins in uninfected or HCMV-infected SGHPL-4 cells was associated with limited HCMV protein expression and replication in those cells (Figs 1 and 2). This suggested that once trophoblast cells had left their progenitor state, HCMV replication became limited.

HSV and ZIKV replication in trophoblast cell lines

We then investigated replication of other TORCH pathogen viruses in SGHPL-4 cells to understand if, like HCMV, replication of those viruses was limited in those cells. Therefore, we tested the ability of viruses related and unrelated to HCMV (the herpesvirus HSV and the flavivirus ZIKA, respectively) to replicate in SGHPL-4 cells.

It has been reported that there are differences in the genome content of laboratory and wild-type strains of HSV-1 and HSV-2, although how these changes reflect virus replication and pathogenesis is largely unclear or unknown [40–47]. It was, therefore, unknown if there would be differences in the ability of HSV-1 and HSV-2 to replicate in SGHPL-4 cells. Thus, we tested the ability of both laboratory and wild-type HSV-1 and HSV-2 strains to replicate in HFF and SGHPL-4 cells.

We found no obvious difference in the ability of HSV-1 laboratory strain virus 17+ to replicate in low-passage cells incubated in either high or low concentrations of serum [10 and 0.5% (v/v) media, respectively] (Fig. 5a, b), indicating that the serum concentration of the media had no obvious effect on HSV replication. When we tested the ability of laboratory and wild-type HSV-1 and HSV-2 strains to replicate in HFF and low-passage SGHPL-4 cells incubated in 10% (v/v) media, we found that there was similar replication of each strain in HFF and SGHPL-4 cells (Fig. 5c). However, we observed a trend wherein replication of virus in SGHPL-4 cells was moderately limited compared to HSV replication in HFF cells, but not to any degree of statistical relevance. We also tested replication of each strain in high-passage HFF and SGHPL-4 cells. We found that there was no statistical difference in the ability of any HSV strain to replicate in either high-passage HFF and SGHPL-4 cells compared to virus replication in early-passage cells (Fig. 5c, d).

Therefore, HSV could replicate in SGHPL-4 cells, regardless of cell culture conditions. There was no statistical difference in the ability of HSV-1 or HSV-2 laboratory or wild-type strains to replicate in SGHPL-4 cells.

We tested the ability of ZIKV to replicate in SGHPL-4. In the first instance, we wished to compare ZIKV replication in SGHPL-4 cells to replication in HFF cells. ZIKV has been reported in human dermal fibroblast cells [48]. However, in preliminary experiments we found very poor replication of ZIKV in HFF cells, regardless of multiplicity of infection used or time allowed for ZIKV to replicate (data not shown). It was unknown if the differences between our observations and those made elsewhere were due to the cell line or strain of ZIKV used or another experimental difference. Therefore, we decided to test ZIKV replication in SGHPL-4 cells compared to replication in Vero cells, a cell line known to allow robust replication of ZIKV. We did not know if there would be differences in the ability of different ZIKV strains to replicate in trophoblast cell lines. Therefore, we tested the ability of ZIKV laboratory strains VR-84 and VR-1845 to replicate in SGHPL-4 cells, as well as ZIKV strain PE243, a recently described strain that has not been extensively passaged in culture [23]. There was no obvious difference in the ability of ZIKV strain VR-1845 to replicate in Vero or SGHPL-4 cells at high or low serum concentrations [10 and 0.5% (v/v) media, respectively] (Fig. 6a, b). We tested the ability of all ZIKV strains to replicate in Vero or low-passage SGHPL-4 cells incubated in 10% (v/v) media (Fig. 6c). We found that all ZIKV showed robust replication in Vero cells, but considerably less replication in SGHPL-4 cells, regardless of what strain was used (Fig. 6c). We observed similar results when the experiments were repeated using high-passage cells (Fig. 6d).

We considered that ZIKV replication in Vero cells may be more efficient than replication in SGHPL-4 cells, as Vero cells do not produce type I interferons [49, 50]. Therefore, we compared the ability of ZIKV to replicate in either Vero cells, human A549 cells or human A549-N^{pro} cells, which do not have a functional type I interferon signalling system due to the presence of the N^{pro} protein that promotes proteasomal degradation of interferon regulatory factor 3 (IRF3) (Fig. S3) [51]. Consistent with observations made elsewhere [52], we found that replication of ZIKV was similar in all three cell types. This was likely due to the ability of ZIKV to inhibit the type I interferon response to infection [23]. This indicates that robust replication of ZIKV in Vero was not due to the absence of a type I interferon response to the virus.

Therefore, replication of Zika virus strains in SGHPL-4 cells was poor compared to replication in Vero cells. There was no obvious difference in replication of different Zika virus strains in any cell line tested. However, like HCMV, Zika virus replication in our trophoblast cell lines was limited compared to the control cell line used in our experiments.

DISCUSSION

As yet, we have little understanding of the molecular basis of HCMV replication in SGHPL-4 cells. We found that HCMV protein expression and HCMV replication in SGHPL-4 cells was limited. Limited viral protein expression may have been, in part, due to the presence of the SV40 T antigen in SGHPL-4 cells. However, circumventing that issue, we found that limitation of HCMV protein expression may have been a feature of HCMV replication in EVT cells. We argue that limited viral protein expression and replication may be of advantage to HCMV during vertical transmission. Limiting virus replication in placental cells would allow HCMV to generate sufficient progeny for the virus to be transmitted from mother to foetus, but not cause pathologies associated with HCMV infection that would damage the placenta and inhibit vertical transmission. It is also interesting to note that transmission of HCMV from mother to foetus in the first trimester of pregnancy may not be as efficient as transmission in the third trimester [53, 54]. This may tally with our observations that there is limited HCMV replication in SGHPL-4 cell lines, which derived from placentas taken from pregnancies in the first trimester [19].

The molecular basis of the limited HCMV protein expression that we have observed is unknown. It remains unknown why IE protein expression in SGHPL-4 cells decreases over time after 24 h p.i. and why this should be associated with passage of cells in culture. To our knowledge, this has not been observed before in any other setting of HCMV infection. Microscopy experiments examining SGHPL-4 cells infected with HCMV-expressing UL36GFP

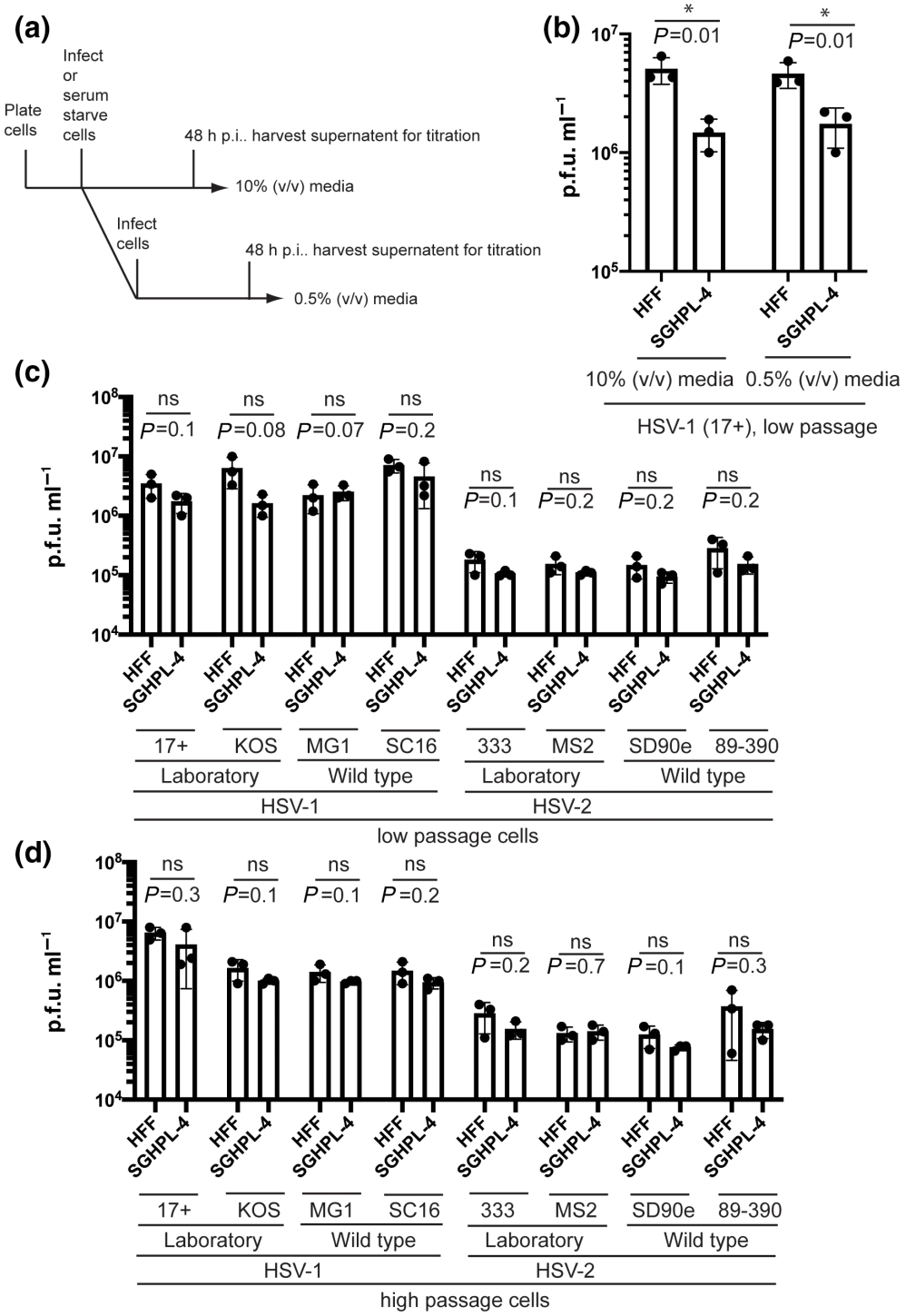

Fig. 5. HSV replication in HFF and SGHPL-4 cell lines. (a) Timeline of cell preparation, cell infection and sample collection. (b) Low-passage HFF and SGHPL-4 cells (HFF passage 6–9, SGHPL-4 passage 13–15) were incubated in either 10% or 5% (v/v) media and infected with the HSV-1 strain 17+. (c) Low-passage or (d) high-passage HFF and SGHPL-4 cells (HFF passage 6–9 and 15–18, SGHPL-4 passage 13–15 and 19–21) were incubated in 10% (v/v) media and infected with the HSV-1 and HSV-2 strains indicated at the top of the figures (multiplicity of infection 1). In all experiments viruses were harvested at 48 h p.i. and viral titre (p.f.u. ml^{-1}) was determined by titration of viral supernatant on Vero cells. The data from three independent experiments are presented in each figure. The bar chart and error bars represent the mean and standard deviation of those data, respectively. The statistical difference between the indicated conditions was measured using an unpaired *t*-test (two-tailed) and is indicated above each figure. A statistically relevant difference was where $P<0.05$. Not significant, ns.

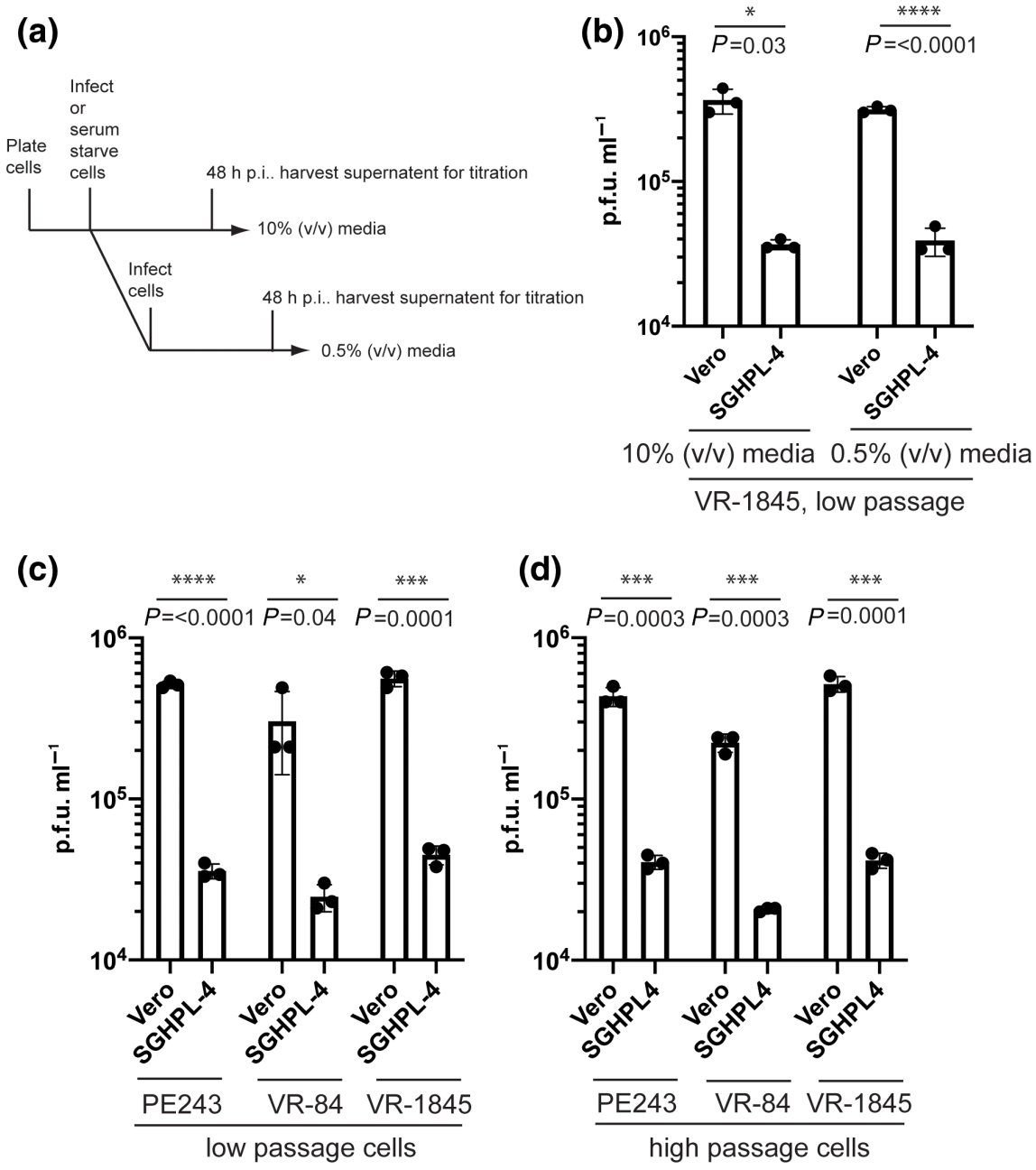

Fig. 6. ZIKV replication in HFF and SGHPL-4 cell lines. (a) Timeline of cell preparation, cell infection and sample collection. (b) Low-passage HFF and SGHPL-4 cells (HFF passage 7–9, SGHPL-4 passage 14–16) were incubated in either 10% or 5% (v/v) media and infected with the Zika strain VR-1845. (c) Low-passage or (d) high-passage HFF and SGHPL-4 cells (HFF passage 6–9 and 15–18, SGHPL-4 passage 13–15 and 19–21) were incubated in 10% (v/v) media and infected with the ZIKV strains indicated at the top of the figures (multiplicity of infection 0.1). In all experiments viruses were harvested at 48 h p.i and viral titre (p.f.u. ml^{-1}) was determined by titration of viral supernatant on Vero cells. The data from three independent experiments are presented in each figure. The bar chart and error bars represent the mean and standard deviation of that data, respectively. The statistical difference between the indicated conditions was measured using an unpaired *t*-test (two-tailed) and is indicated above each figure. A statistically relevant difference was where $P<0.05$.

suggest that all infected cells lose GFP expression over time (data not shown). This suggested a model where loss of protein expression occurs in all infected SGHPL-4 cells over time, rather than a model where only a subset of infected SGHPL-4 cells are permissive for HCMV replication. We speculated that the reduction in IE protein expression was associated either with repression of transcription from the HCMV major immediate-early promoter or was associated

with proteolysis of IE protein in infected cells. However, treatment of HCMV-infected SGHPL-4 cells with either histone deacetylase inhibitors or the ubiquitin-mediated proteasome inhibitor MG132 had no obvious effect on IE protein expression in SGHPL-4 cells under any condition tested so far (data not shown). Therefore, IE transcriptional repression or protein proteolysis was unlikely to be associated with the HCMV IE protein expression phenotype we observed in SGHPL-4 cells. Rather, there may be an issue with IE RNA metabolism and/or protein translation in HCMV-infected SGHPL-4 cells that resulted in the loss of IE protein expression. Our data were consistent with a previous report of poor HCMV GFP reporter virus replication in SGHPL-4 cells [26]. However, it was not possible to directly compare our data with this previous report as it was not stated what cell culture conditions were used in the aforementioned study [26].

We also considered if the presence of the SV40 T antigen was limiting HCMV replication in SGHPL-4 cells. Expression of the SV40 T antigen in fibroblasts is a barrier to HCMV entry into cells, via the loss of the viral receptor platelet-derived growth factor receptor α (PDGFRα), and decreased expression of IE2, but not IE1, over the course of HCMV replication [55]. As we observe no obvious barrier to HCMV entry into SGHPL-4 cells and decreases in both IE1 and IE2 expression over time in HCMV-infected SGHPL-4 cells, it is unlikely that the presence of the SV40 T antigen was directly responsible for the limited HCMV replication we observed in those cells.

We argue that the difference in the ability of HCMV to replicate in SGHPL-4 cells compared to HFF cells was not due to the ability of HCMV to enter the SGHPL-4 cells. The Merlin(R1111) virus that we used throughout our studies does not express the HCMV pentamer glycoprotein complex that allows virus entry into a range of cell types other than fibroblasts. It has been demonstrated that pentamer expression on Merlin virions only results in a very modest increase in the ability of Merlin to enter SGHPL-4 cells (less than twofold compared to Merlin virions not expressing pentamer) [28]. However, the pentamer glycoprotein complex was essential for Merlin to enter another human trophoblast cell line, HTR [28]. This is likely due to the presence of PDGFRα on SGHPL-4 cells, but not HTR cells [28]. HCMV can regulate the amount of glycoprotein pentamer complexes that are displayed on HCMV virions [56]. Therefore, HCMV entry into different trophoblast cells may require different viral glycoprotein complexes and assessing where PDGFRα is expressed in placental tissue may be of importance in understanding HCMV replication. It is possible that the glycoprotein pentamer is not displayed on all HCMV virions and that the presence of glycoprotein pentamer on HCMV virions is not essential for HCMV replication in the placenta. However, the pentamer complexes of Guinea pig cytomegalovirus and rhesus cytomegaloviruses must be present for those viruses to be efficiently vertically transmitted and cause congenital infection [16, 18]. Therefore, the presence of the HCMV pentamer may also be important in vertical transmission and development of congenital disease in humans.

Further investigation is required to understand what limitations there are on HCMV replication in primary trophoblasts. We noted that differences in the ability of HCMV to replicate in different EVT cell lines was associated with the differentiation state of the cell line. In future experiments it will be important to understand the differentiation state of primary trophoblast cells or trophoblast cell lines used and how that impacts upon the ability of HCMV to replicate in those cells. To our knowledge, it is not yet possible to alter the differentiation of SGHPL-4 cells to address the relationship between progenitor cell status and ability of HCMV to replicate in those cells.

SGHPL-4 cells are commonly used to study HCMV replication [3, 25–30]. Based on the data we present here, future studies using SGHPL-4 cells to investigate HCMV replication may need to consider the cell culture conditions used and how that might influence HCMV protein expression.

While our studies highlighted how HCMV replication in EVT cells was limited, we also noted that there was limited replication of ZIKV in SGHPL-4 cells. Again, we argue that limited viral replication may be advantageous during vertical transmission of ZIKV, as it would allow ZIKV replication and maximize the opportunity for virus transmission across a healthy maternal–foetal tissue interface. In future it will be important to understand the molecular basis of limited ZIKV replication.

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Author contributions
K.H.: investigation. N.S.: investigation. N.B.: methodology, investigation. A.C.T.: investigation C.L.D.: methodology, resources, writing – review and editing. A.K.: methodology, resources, writing – review and editing, funding. R.J.S.: methodology, resources, writing – review and editing, funding. B.L.S.: conceptualization, methodology, investigation, data curation, writing – original draft preparation, writing – review and editing, supervision, project administration, funding.

Conflicts of interest
The authors declare there are no conflicts of interest.

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