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The BeWo cell line derived from a human placental choriocarcinoma is permissive for respiratory syncytial virus infection

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Abstract
The respiratory syncytial virus (RSV) is the main pathogen associated with upper respiratory tract infections during early childhood. Vertical transmission of this virus has been suggested in humans, based on observations recorded during animal studies that revealed an association of RSV with persistent structural and functional changes in the developing lungs of the offspring. However, human placentas have not yet been evaluated for susceptibility to RSV infection. In this study, we examined the capacity of RSV to infect a human trophoblast model, the BeWo cell line. Our results suggest that BeWo cells are susceptible to RSV infection since they allow RNA viral replication, viral protein translation, leading to the production of infectious RSV particles. In this report, we demonstrate that a human placenta model system, consisting of BeWo cells, is permissive to RSV infection. Thus, the BeWo cell line may represent a useful model for studies that aim to characterize the events of a possible RSV infection at the human maternal–fetal interface.

Keywords Respiratory syncytial virus · BeWo cells · Trophoblast · Placenta · Viral cycle replication

Introduction

The respiratory syncytial virus (RSV) is considered to be the most frequent causative pathogen in neonatal and infant pneumonia and bronchiolitis worldwide [1]. While the main tissue tropism of RSV in airway epithelial cells is well-documented, other extra-pulmonary cells, such as neural, endothelial, and immunological cells, may also be susceptible to RSV infection [2–4]. The susceptibility of extra-pulmonary cells to RSV has been suggested by the expression of cellular proteins such as nucleolin, glycosaminoglycan, and TLR4 that function as potential receptors and attachment factors [5]. Interestingly, clinical symptoms such as apnea, seizures, and increased cardiac and liver enzyme activities are common in children with severe RSV infections [6, 7]. These symptoms could be linked to extra-pulmonary tropism of RSV, suggesting the possibility of hematogenous dissemination of RSV from the lungs to other targets in the host.

Previous reports had suggested that some viruses can infect highly vascularized or immune-privileged tissues, such as placenta, potentially causing vertical RSV transmission to the fetus [8]. Vertical RSV transmission has been suggested for humans and demonstrated in an animal pregnancy model. In the animal studies, detection of viral particles was associated with functional consequences in fetuses exposed to RSV in utero; for example, RSV-positive lungs were seen in newborns animals that persisted into adulthood [9, 10]. In addition, RSV RNA has been detected in blood and bone marrow of infected infants and adults, respectively [11, 12]. A recent study detected low levels of RSV

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in human cord blood samples, and release of infectious RSV from cultured mononuclear cord blood cells, thus suggesting the harbor of active virus in these cells and supporting the possibility of RSV transmission in utero [13].

Human choriocarcinoma cell lines are common models for evaluating viral infections in human placenta, since they maintain many structural and functional characteristics of placental trophoblasts in early gestation [14–17]. The current study aimed to evaluate the capacity of RSV to infect a human trophoblast model, the BeWo cell line.

Results and discussion

To identify the susceptibility of BeWo cells (ATCC CCL98) to infection, we used RSV strain Long (ATCC-VR26) for all experiments, propagated and titrated in cell line HEp-2 (ATCC CCL23). Immunofluorescence assay was performed to evaluate the presence of viral antigens in BeWo and HEp-2 cells, the latter being positive controls. Both cell lines were infected at MOI of 5 for 24 h, and subsequently fixed. Immunofluorescence analysis was performed using primary Anti-F antibody (Novus, USA), which was applied for 1 h at 37 °C. Anti-mouse FITC (Thermo Fisher, USA) was used as secondary antibody, and DAPI (Vector, USA) was used as a nuclear stain. Immunofluorescence signals were captured by confocal microscopy (Leica, USA). We detected a positive signal for the virus F antigen in RSV-infected BeWo cells, and no signal in mock-infected cells (Fig. 1a). Interestingly, the staining pattern of F protein in BeWo cells was very similar to that in HEp-2 cells, a cell line permissible to RSV infection. These data showed the expression of RSV proteins in BeWo cells, thereby suggesting the entry of RSV into BeWo cells. To evaluate RSV replication and translation processes in BeWo cells, monolayers of BeWo cells and control HEp-2 cells were infected with RSV at MOI of 1 and 5 for up to 36 h. At different times post-infection, RNA was isolated using TRIzol (Invitrogen, USA) and cDNA was synthetized (Thermo Scientific, USA). Finally, the relative expression of viral gene was determined by q-PCR using LC Fastart DNA Master SYBR Green I, using specific primers detectingthe L gene of RSV (TGCAACAAGCCAGATCAA GA, TGGAGAAGCTGATTCCAAGC) and β-actin (GCA TGGAGTCCTGTGGCAT, AGGTCTTTGCGGATGTCC ACGT) and results analyzed using the comparative cycle threshold method.

Our results showed that in BeWo cells, viral RNA was more evident at 24 h and at MOI of 5. Consistently, in HEp-2 cells, we observed a time-dependent L-gene expression during infection (Fig. 1b). These data supported the idea that BeWo cells produce viral RNA; however, permissiveness of BeWo cells was different in comparison to that of HEp-2 cells. Additionally, we performed viral infection kinetics at MOI of 5 in BeWo cells and evaluated the accumulation of M2-1 viral protein by western blot. We identified a high level of M2-1 protein in RSV-infected BeWo cells, at 36 h post-infection, in comparison to that in early time-point or mock-infected cells (Fig. 1c). These data together suggested that RSV-infected BeWo cells permit events of viral genome replication and translation.

A characteristic of RSV infection is the presence of cytopathic effect associated with syncytium formation. To evaluate the cytopathic effect in BeWo cells, the cell line was infected at MOI of 5 and syncytium formation was monitored by light microscopy at different time points post-infection. Infected BeWo cells showed syncytium formation and additional morphological changes (cell rounding, membrane shrinkage, and monolayer detachment), mainly at 36 h post-infection (Fig. 1d). Consistently, supernatants of BeWo cells infected at MOI of 5 for 36 h retained the ability to generate syncytium in a time-dependent manner in Hep-2 cells (Fig. 1e). These data together suggested RSV infection in BeWo cells, and that viral particles obtained from this infection are able to induce cytopathic effect in both cell lines. Since cellular permissiveness depends on molecular factors, such as receptors for viral entry, we evaluated protein levels of putative RSV receptors in BeWo cells. Proteins were detected using anti-Nucleolin (Abcam, UK), incubated for 1 h at room temperature, and TLR-4 (Santa Cruz, USA) incubated overnight at 4 °C. We detected both TLR-4 and Nucleolin in BeWo cells (Fig. 1e), supporting their possible role in facilitating RSV entry into BeWo cells.

The final event of a viral replication cycle is the formation of infectious virus particles. To evaluate this, supernatants of RSV-infected BeWo cells (at MOI of 1, 5, and 10; after 24-h incubation), were used to infect HEp-2 cells in an assay that determines the number of plaque forming units (PFU). Our results showed that supernatants from BeWo cells infected at MOI of 1 were sufficient for the detection of infective viruses (3×10^3 PFU). An accumulative effect of viral-particle production was observed when BeWo cells were infected with higher MOIs (Fig. 2a). In addition, to determine the infective capacity of the viral particles, we tested them in a typical cellular model of RSV infection. Alveolar epithelial cells A549 (ATCC CCL185) were exposed for 16, 24, and 36 h to supernatants of RSV-infected BeWo cells (infected with RSV at MOI of 5; 24-h incubation). After different infection times, RSV F protein levels were evaluated by immunofluorescence. We detected F protein signals in A549 cells after a 24-h exposure to BeWo supernatants; the strongest F protein signal was detected at 36 h post-infection. In agreement with the infectious capacity of BeWo supernatants, no signal was detected in A549 cells exposed to mock-supernatants (Fig. 2b). These results together suggested that RSV-infected BeWo cells produce viral particles that are able to infect only permissive cellular models of RSV infection.

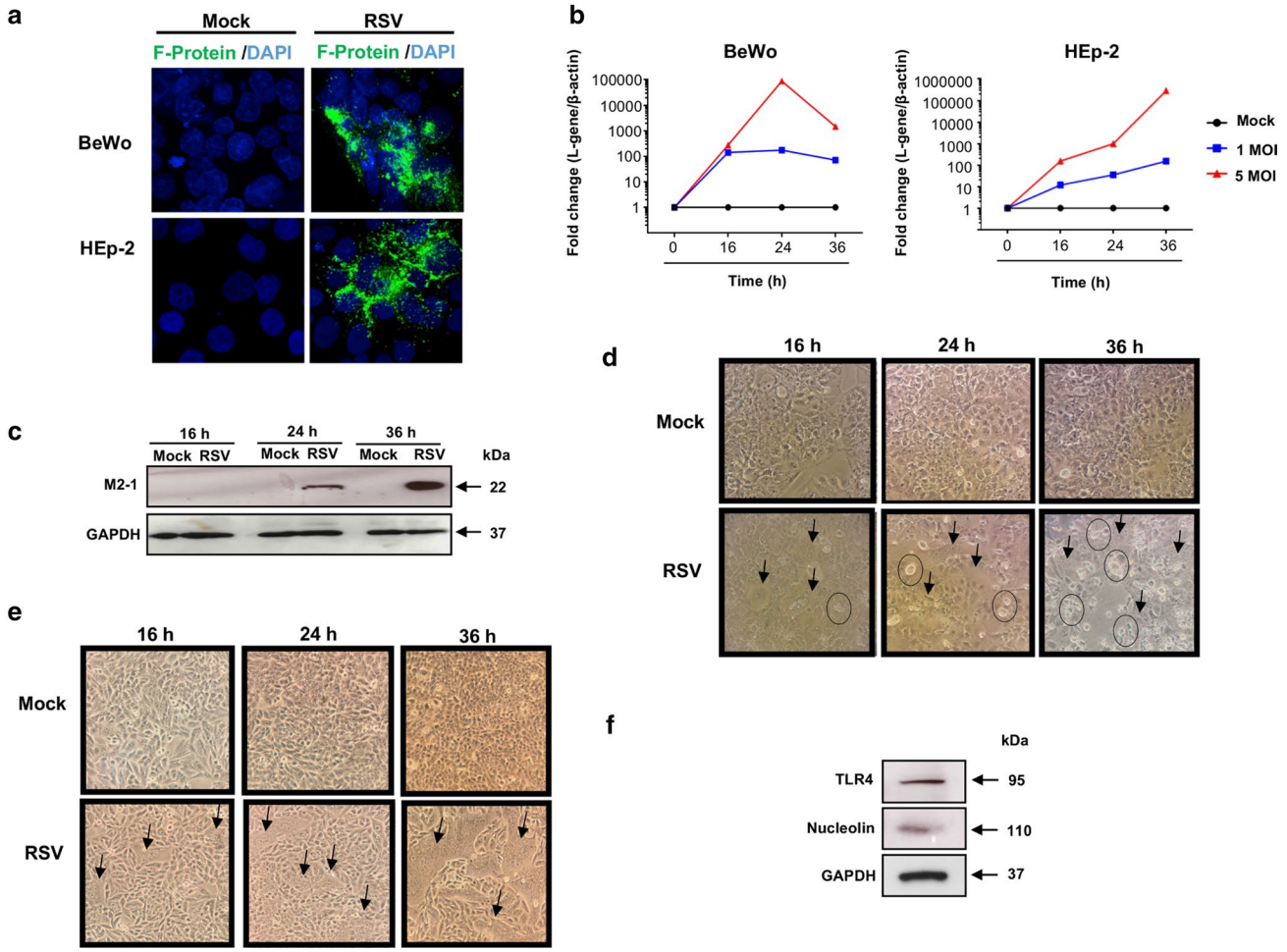

Fig. 1 Respiratory syncytial virus (RSV) presence in BeWo cells **a** RSV and mock-infected BeWo and HEp-2 (positive control) cells were fixed after 24 h post-infection and incubated with anti-F protein (green) and DAPI (blue) for DNA stain; **b** L-gene expression differences between BeWo and Hep-2 cells infected with RSV (1(-), 5(-) MOI) in comparison with Mock groups were evaluated by RT-qPCR. L-gene and β-actin expression were determined in infected and Mock conditions, then ΔCt, ΔΔCt, and $2^{\Delta\Delta Ct}$ were calculated according to comparative threshold method. $2^{\Delta\Delta Ct}$ was graphed and expressed as fold induction changes. **c** BeWo cells were Mock- or 5 MOI-infected at 16, 24, and 36 h. At discrete time points, infection cell lysates of infected BeWo cells were prepared and analyzed by western blotting for evaluation of M2-1 viral protein (22 kDa) levels. **d** RSV induced cytytopathic effects were monitored during kinetic of infection, syncytia (arrows), cell rounding and monolayer cells detachment (circles) were evident to prolonged time of infection. **e** Supernatants of BeWo cell infected with RSV conserved the cytopathic capacity when was evaluated in Hep-2 cells, the syncytia are presents in cell (arrows) and this effect was more evident in 36 h post-infection. (f) Receptors for RSV entry, TLR-4 (95 kDa) and Nucleolin (110 kDa) were detected in BeWo cells by immunoblotting. In all western blots GAPDH (36 kDa) was used as a loading control

Vertical infections during pregnancy are associated with impaired fetal health that results in complications such as fetal abnormalities, post-natal infections, and even fetal death [18–20]. Several studies have validated the transmission of different viral agents from the mother to her offspring [21, 22]. Despite the clinical and experimental evidence [23–25], RSV infections have not yet been studied in a human model of placenta. BeWo cells can be an important model to study the physiological and infectious processes in the placenta, and have been employed to evaluate the effect of viral infections at the maternal–fetal interface [26–28]. Based on these observations, and considering the clinical importance of investigating a possible vertical transmission route for RSV in humans, we evaluated the capacity of RSV to infect a human trophoblast model. Toward that, BeWo cells were infected with RSV, viral RNA was detected, and viral antigens expressed; viral proteins accumulated depending on the MOI factor and time of infection. A typical cytopathic effect of RSV infection, evident in our model, involved whole syncytium formation and drastic cell damage. The cytopathic capacity and infective ability of viral particles derived from BeWo cells were maintained in two permissive models (HEp-2 and

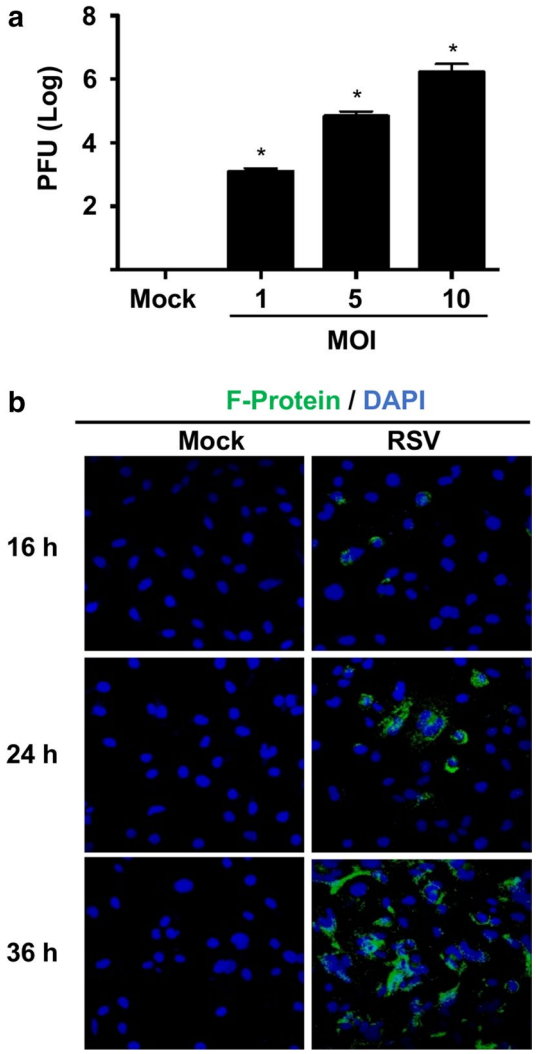

Fig. 2 BeWo cells produce infectious respiratory syncytial virus (RSV) particles **a** HEp-2 cells were treated with supernatants of 1, 5, and 10 MOI-infected BeWo cells for 2 h. After 5 days of incubation, HEp-2 cells were fixed, stained with crystal violet, and subjected to viral titers determination by PFU calculation. The graph shows PFU values (mean ± SD) in response to BeWo inoculum with different MOI. Data are representative of at least four independent experiments, $*p < 0.05$ vs. mock-infected cells; **b** Supernatants of five MOI RSV-infected cells were harvested 24 h post-infection, centrifuged, and used as inoculum to infect A549 cells at 16, 24, and 36 h time points. After completion of infection, A549 cells were stained with F viral protein (green) and nuclei were visualized with DAPI stain (blue). Representative immunofluorescence images are shown

A549), suggesting that infective properties do not change during infection cycle in BeWo cells.

The permissiveness of several trophoblast cell lines to viral infection appears to depend on cellular properties, such as expression levels of receptors, immunological factors, and intrinsic cell characteristics [29, 30]. In agreement with this observation, we detected basal expression of TLR-4 and nucleolin in BeWo cells, suggesting that they might facilitate RSV entry in this cell, as reported in other permissive RSV models [31].

Our current study suggests the possibility of a human model of placental cells being permissible to RSV infection, adding appropriate evidence that RSV infection might occur in human placenta. Interestingly, in this study, RSV-infected trophoblast cell line was able to produce infective viral particles, thereby suggesting that transmission of RSV might occur between different cell types of fetal–placental tissue, hence contributing to establish a productive infection similar to that of Zika or Cytomegalovirus [32, 33]. Future studies, focused on evaluating RSV infection in placental primary cell cultures or placental tissues of infected mother, should be considered to evaluate the possible occurrence of RSV infection in human maternal–fetal interface.

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Compliance with ethical standards

Conflict of interest All authors declare that no conflicts of interest exist.

Research involving human participants or animals This article does not contain any study with human participants or animals, performed by any of the authors.

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