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Generation and characterization of a porcine endometrial endothelial cell line susceptible to porcine reproductive and respiratory syndrome virus

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ABSTRACT

Previous studies on the underlying mechanism for porcine reproductive and respiratory syndrome virus (PRRSV)-induced reproductive failure have been focused on the viral replication in the endothelial macrophages, and the susceptibility of porcine endometrial endothelial (PEE) cells to PRRSV has not yet been investigated. Therefore, in the present study we generated a PEE cell line by transfection of the primary cells with a SV40 large T antigen expression vector. The PEE cell line maintained the endothelial morphology with a significantly faster growth rate, shorter population doubling time and higher plating efficiency than the primary cells. The endothelial origination of the cell line was confirmed by detection of the endothelial cell-specific markers. The PEE cell line had been passed successively for 60 generations with an unlimited growth potential.

To further characterize the PEE cell line, cells of different passages were infected with different PRRSV strains and analyzed for the viral antigen and replication. Overt cytopathic effect was observed from 36 h postinfection (HPI) and the viral antigen detected as early as 12 HPI. The infectious virus was recovered from the infected PEE cells with a titer higher than that in MARC-145 cells.

Since the data presented indicate a high susceptibility of PEE cells to PRRSV, we conclude that the PEE cell line generated will be useful for growth of PRRSV and further studies on the underlying mechanism for PRRSV infection of PEE cells. The finding of the susceptibility of PEE cells to PRRSV may provide an alternative explanation for PRRSV-induced reproductive failure.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most important swine diseases. Since it was first reported in the United States and Canada in 1987, the disease has been causing immense economic losses in the swine industry worldwide (Neumann et al., 2005; Pejsak et al., 1997; Zhou and Yang, 2010). Presently, both inactivated and live-attenuated vaccines are available for PRRS control, but these failed to provide sustainable disease control, in particular against genetically unrelated strains (Kimman et al., 2009).

The etiological agent, porcine reproductive and respiratory syndrome virus (PRRSV), is an enveloped, positive-sense, single-stranded RNA virus belonging to the *Arteriviridae* family (Dokland, 2010; Snijder and Meulenberg, 1998). *In vivo*, PRRSV shows a preference for cells of the monocyte/macrophage lineage and infects specific subsets of differentiated macrophages in lungs, lymphoid tissues and placenta (Duan et al., 1997; Teifke et al., 2001). Such cell tropism is determined by the specific entry mediators on the target cell, including heparin sulfate for initial contact of the virus with the macrophage, sialoadhesin (Sn) for stable interaction and scavenger receptor CD163 for viral genome release (reviewed by Van Breedam et al., 2010). In addition, PRRSV also infects other cell types such as alveolar epithelial cells and vascular endothelial cells (Hu et al., 2012), but the entry mediators have not yet been elucidated.

PRRS is characterized by reproductive failure in gilts and sows, including late-term abortions, early farrowing, and an increase of dead/mummified fetuses and weak-born piglets (Plana et al., 1992; Terpstra et al., 1991). However, the underlying mechanism of the PRRSV-induced reproductive failure is poorly understood. Because the virus targets the monocyte-macrophage lineage which is present in the endometrium/fetal placentas from healthy sows, previous studies on the mechanism for PRRSV-induced reproductive failure have been focused on the viral replication in the macrophages and apoptosis in the virally infected and surrounding cells (Karniychuk and Nauwynck, 2009; Karniychuk et al., 2011). As an important cellular component of the porcine endometrium, however, the susceptibility of porcine endometrial endothelial (PEE) cells to PRRSV has not yet been investigated.

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In the present study a PEE cell line was established and characterized to be highly susceptible to PRRSV infection.

2. Materials and methods

2.1. Cells, viruses, media and antibodies

African green monkey kidney cells MARC-145 (ATCC CRL-12231) were cultivated in Dulbecco's Modified Eagle's Medium (Gibco) containing 10% fetal bovine serum (FBS). Porcine alveolar macrophage (PAM) was prepared as described previously (Weingartl et al., 2002) and cultivated in RPMI 1640 medium (Gibco) containing 10% FBS and 1% non-essential amino acids. PRRSV strain VR2332 (ATCC VR2332) is a prototype strain of the Northern American genotype (Bautista et al., 1993). PRRSV strain CH-1R is an attenuated vaccine strain derived from the traditional Chinese strain CH-1a (Wang et al., 2011). JXA1 is a highly pathogenic Chinese strain of PRRSV (Tian et al., 2007). The three PRRSV strains were propagated and titrated on MARC-145 cells before inoculation into PAM or PEE cells. Goat anti-CD34, Sn, CD163 and CD151 polyclonal antibodies were purchased from Santa Cruz Biotechnology. The monoclonal antibody (mAb) against PRRSV N protein was purchased from Rural Technologies. FITC-labeled goat anti-mouse IgG was purchased from Sangon Biotech (Shanghai).

2.2. Preparation of primary PEE cells

Primary PEE cells were prepared as described previously (Krikun et al., 2004). Briefly, the endometrial tissue from one-month-old gilt (Chinese local Jiangquhai breed) was minced into small pieces and digested overnight at 37 °C with 0.2% collagenase I (Worthington). After gentle pipetting, dissociated tissue was filtered through a mesh screen and then through a 40 μm sieve to remove the porcine endometrial glandular epithelial (PEGE) masses. Following centrifugation at 100 × *g* for 10 min, the cell pellet was suspended in DMEM containing 10% FBS and 1% non-essential amino acids. After incubation for 20–30 min in 35 mm dishes to allow the fibroblasts to attach to the dish surface, the unattached PEE cells were transferred to new dishes and incubated for additional 3 days. Such cultivation/transfer process was repeated for two more times to obtain relatively pure PEE cells.

2.3. Cell transfection and clone selection

The SV40 large T antigen expression vector was constructed by inserting the PCR-amplified sequence into eukaryotic expression vector pTarget (Song et al., 2003). The primary PEE cells were seeded on 24-well plates (1×10^4 cells/well) and transfected with the SV40 large T antigen expression vector using Lipofectamine™2000 (Invitrogen) according to the manufacturer's instructions. On day 3 after transfection, the medium was replaced with fresh medium containing 500 μg/ml G418, which was changed at three-day intervals. After selection for 2 weeks, G418-resistant cell clones were picked using an inoculation loop and passed for different generations before further experiments.

2.4. Growth curve plotting and population doubling time determination

The growth curve plotting and population doubling time (PDT) determination for the PEE cell line were performed as previously described (Hong et al., 2007). Briefly, cells of different passages were seeded on 24-well plates (1×10^4 cells/well) in triplicates and cultivated for consecutive 8 days. On each day of cultivation, cells were trypsinized for cell counting and the averaged cell numbers were plotted against the cultivation times (*d*). PDT was calculated according the formula: $PDT = [\log 2/(\log N_t - \log N_0)] \times t$ (N_t = number of cells after *t* hours of culturing, N_0 = number of cells seeded).

2.5. Plating efficiency determination

The plating efficiency for the PEE cell line was determined as previously described (Chan et al., 2004). Briefly, cells of different passages were seeded on 6-well plates (50, 100, 200, 500, 1000 or 2000 cells/well) and fixed with 4% paraformaldehyde following cultivation for 15 days. After staining with crystal violet, cell colonies (≥50 cells) were counted and the plating efficiencies were calculated according to the formula: plating efficiency (%) = (number of colonies/number of cells seeded) × 100.

2.6. RT-PCR

The PEE cell line (passage 15), as well as the primary cells and PAM, was seeded on 24-well plates and cellular RNA was extracted using TRIzol reagent (Invitrogen) after cultivation for 48 h. Following digestion with DNAase I to eliminate the transfected plasmid, reverse transcription (RT) was performed using 2 μg of RNA and M-MLV reverse transcriptase (Takara). PCR was performed using 1 μl of the RT product and the following program: 1 cycle of denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, extension at 72 °C for 30 s, followed by 1 cycle of extension at 72 °C for 10 min. The primer pairs for detection of the cell marker gene transcription are listed in Table 1. The PCR products were analyzed on 2% agarose gel and cloned into pMD18-T vector (KaTaRa) for sequencing.

2.7. Immunofluorescence

The PEE cell line (passage 15), as well as the primary cells and PAM, was seeded on 24-well plates (1×10^5 cells/well), and cultivated for 24 h. After fixation with 4% paraformaldehyde and blocking with PBSM (5% defatted milk powder in PBS, pH7.4), indirect immunofluorescence was performed as previously described (Lee et al., 2010) using anti-CD34, CD151, CD163 and Sn polyclonal antibodies. For detection of the viral antigen, cells were infected with PRRSV strain VR2332 (MOI 0.1) and submitted to immunofluorescence 24 HPI using the mAb against PRRSV N protein.

2.8. Virus titration

PRRSV infection and titration were performed as previously described (Wei et al., 2011). Briefly, the PEE cell line (passage 15), as well as PAM and MARC-145 cells, was seeded on 6-well plates and infected with PRRSV strain VR2332, CH-1R or JXA1 (MOI 0.1). At different time points post-infection, cell lysate were collected and titrated for PRRSV on MARC-145 cells.

2.9. Statistical analysis

Statistical analysis was performed using SPSS 13.0 program. The experimental data were subjected to one-way analysis of variance. Comparisons of the different groups were considered statistically significant difference at $P < 0.05$.

3. Results

3.1. Generation of PEE cell line

After removal of the PEGE cell masses by filtration and the fibroblasts by 3 cycles of brief incubation, we obtained homogenous primary PEE cells which formed a uniform monolayer by day 6 after