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Immortalization of porcine placental trophoblast cells through reconstitution of telomerase activity

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ABSTRACT

Placental trophoblast cells (PTCs) play a critical role in histotrophic nutrient absorption, gaseous exchange, endocrine activities, and barrier function between the maternal and fetal systems. Establishment of immortalized porcine PTCs will help us to investigate the potential effects of different viruses on porcine trophoblast. In the present study, primary porcine PTCs were isolated from healthy gilts at Day 30 to Day 50 of gestation through collagenase digestion, percoll gradient centrifugation, and anti-CD9 immunomagnetic negative selection. To provide stable and long lifespan cells, primary PTCs were transfected with human telomerase reverse transcriptase (hTERT) gene. One porcine placental trophoblast cell line, named as hTERT-PTCs, was chosen for characterization. Human telomerase reverse transcriptase-PTCs achieved an extended replicative lifespan without exhibiting any neoplastic transformation signs *in vivo* or *in vitro*. The morphologic and key physiological characteristics of the immortalized PTCs were similar to primary PTCs. The immortalized PTCs retained original cell polarity and normal karyotype, expressed trophoblast-specific marker cytokeratin 7 and E-cadherin but did not express vimentin and major histocompatibility complex class I antigens as well as primary PTCs. Human telomerase reverse transcriptase-PTCs secreted low levels of chorionic gonadotrophin β-subunit and placental lactogen that were coincident with primary PTCs. Taken together, our results demonstrated that the porcine PTCs could be immortalized through reconstitution of telomerase activity. The immortalized PTCs maintained its original characteristics and can be used as a model cells line to study the pathologic changes of porcine placental trophoblast in viruses infectious diseases.

1. Introduction

The mammal placenta constitutes an essential interface between the maternal and fetal circulation, which provides physiological and immunologic protection for embryonic development [1,2]. The placental and transplacental infection of certain viruses induce uterine and/or placental lesions in gilts and lead to reproductive failure that characterized by abortion and return to estrus in gilts, fetal mummification, stillbirth, and low viable piglets [3]. Previous studies have indicated that the endometrium, stromal cells, and some subsets of macrophages are susceptible to porcine parvovirus (PPV) [4] and porcine reproductive and respiratory syndrome virus [5]. However, whether porcine trophoblast cells are permissive to these viral transmissions still need to be determined.

The isolation and purification of placental trophoblast cells (PTCs) is prerequisite for *in vitro* studies on the interactions of trophoblast cells and viral infection. Human and mouse trophoblast cells have been widely used as models for studying the effects of human immunodeficiency virus [6], influenza A virus [7], and human

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cytomegalovirus [8] infection on placental dysfunction. Several porcine trophectoderm cell lines have been established from preimplantation or implantation blastocyst before placentation, such as Jag-l [9], TE1 [10], and TB cell line [11]. These trophectoderm cell lineages are capable of proliferation spontaneously for many passages without immortalization when cultured with advantageous growth factors, which have been used to study trophectoderm cell proliferation, differentiation, and invasion. However, previous studies indicate that the reproductive system disease associated viruses usually replicate in porcine placental tissues during mid-gestation, which is the real reason for fetal death [4,5]. Therefore, the establishment of a stable porcine placental trophoblast model derived from mid-gestation is necessary for studying viruses-induced reproductive failure.

In previous studies, human trophoblast cell lines were established by transformation of simian virus 40 lager T antigen and human papilloma virus E6 and/or E7 genes, but these cell lines are accompanied by phenotypic or karyotypic abnormalities [12,13]. Compared with viral gene-induced cell immortalization, ectopic expression of human telomerase reverse transcriptase (hTERT) gene has been widely applied to immortalize primary cells in a range of mammalian species including swine kidney epithelial cells [14], goat mammary epithelial cells [15], and human trophoblast cells [16], maintaining original properties of primary cells without malignant transformation.

In the present study, we successfully isolated and purified primary trophoblast cells from porcine placenta of mid-gestation. Reconstitution of telomerase activity by transfection of exogenous hTERT gene enabled normal PTCs to proliferate beyond replicating senescence. The immortalized hTERT-PTCs retained critical morphologic and key physiological characteristics of primary PTCs and did not exhibit oncogenic transformation signs.

2. Materials and methods

2.1. Isolation and purification of porcine PTCs

The whole uteri were obtained from healthy pregnant crossbred gilts slaughtered at Day 30 to Day 50 of gestation. Stages of pregnancy were estimated by measurement of crown-rump length [17]. The protocol of isolation PTCs was according to Bonnardiere and Dong et al. [11,18] with some modifications. Briefly, villous tissue was separated from the interareolar area of maternal placenta epitheliochorialis, thoroughly washed with cold PBS containing 100-IU/mL penicillin and 100-μg/mL streptomycin (Harbin Pharmaceutical Group, Ltd., Harbin, China), then minced into 1-mm^3 pieces. Tissue fragments were digested with 2 mg/mL of collagenase Type I (Sigma, St. Louis, MO, USA) and filtered with a 76-μm cell strainer (Solarbio, Shanghai, China). To obtain relatively uniform population of PTCs, the dissociated cells were purified by isopycnic centrifugation. Trophoblast cells enriched between 30% and 45% Percoll density gradient separated layers were further incubated with a mouse anti-CD9 and magnetic anti-mouse microbeads, and separated on MACS columns (Miltenyi Biotech, Bergisch Gladbach, Germany). The cell purity was determined by flow cytometry. Cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 medium supplemented with 10% FBS (Gibco, Gaithersburg, MD, USA), 1% (vol:vol) Insulin–Transferrin–Selenium (ITS; Sigma) and 10 ng/mL of epidermal growth factor (Invitrogen, Carlsbad, CA, USA), and incubated at 37 °C in a 5% CO_2 atmosphere incubator. The porcine fetal fibroblast cells which served as a negative control were derived from skin of fetuses at Day 30 to Day 50 of gestation, subsequent operations were similarly as with PTCs isolation. All animal experiments were carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2. Transduction of hTERT gene

Primary PTCs were transfected with pCI-neo-hTERT plasmids using X-tremeGENE HP DNA transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. Monolayer primary cells were transfected with transfection reagent: pCI-neo-hTERT plasmids complex by a ratio of 3:1 (μL: μg). Twenty-four hours later, the transfected cells were cultured in selective media containing 600 μg/mL of the neomycin analog G418 (Sigma) until surviving cell clones were obtained. The individual cell clones were isolated and expanded further.

2.3. Telomerase activity assay

Telomerase activity was quantitative determined with Telo TAGGG Telomerase PCR ELISA^{PLUS} kit (Roche) according to the manufacturer's instructions. Briefly, the extract of 2×10^5 PTCs per single reaction was harvested for polymerase chain reaction (PCR) amplification. HeLa cells, with active version of telomerase during cell division, were served as positive control. Enzyme-inactivated samples were used as negative control. The PCR products were detected by a digoxigenin-labeled telomeric repeat-specific ELISA. The absorbance of samples was measured with a microplate spectrophotometer (Infinite 200 PRO NanoQuant, Tecan, Switzerland) at 450 nm and 690 nm.

2.4. Immunocytochemistry and immunofluorescence assay

The cell authentication of cultured primary cells and selected cell lines was identified with immunocytochemistry and indirect immunofluorescence staining. 1×10^5 cells were grown on coverslips, fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After blocked with 10% goat serum, cells were incubated with rabbit anti-goat cytokeratin 7 (CK-7) and vimentin monoclonal antibody (Uscnlife, Wuhan, China), respectively, followed by incubation of fluorescein isothiocyanate (FITC) conjugated mouse anti-rabbit IgG secondary antibody (Bioss, Beijing, China). Immunocytochemistry was implemented according to the manufacturer's instructions of Streptavidin-Peroxidase Immunohistochemical staining kit (Invitrogen, Carlsbad, CA, USA). The stained cells were observed under a confocal microscope (Nikon, Inc., Tokyo, Japan) or inverted photomicroscope (Olympus, Beijing, China).