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The isolation and characterization of a telomerase immortalized goat trophoblast cell line

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ABSTRACT

Trophoblast cells play vital roles in the processes of embryonic implantation and placentation. Many toxicological compounds can induce the malfunction of trophoblast cells, resulting in implantation failure or early embryonic loss. The finite lifespan of primary trophoblast cells limits investigation of the long-term effects of some toxicological compounds on trophoblast cells *in vitro*. In this study, primary goat trophoblast cells were purified by density gradient centrifugation and specific immuno-affinity purification. Then, the purified cells were immortalized through transfection of a plasmid containing the human telomerase reverse transcriptase (hTERT) gene. hTERT-transfected goat trophoblast cells (hTERT-GTCs) could steadily express hTERT gene and exhibit higher telomerase activity, and persistently proliferate without any signs of senescence up to 50 passages. The immortalized goat trophoblast cells still possessed the basic and key properties of normal primary goat trophoblast cells to express the specific intracellular marker cytokeratin 7 (CK-7) and secrete chorionic gonadotrophin β-subunit (CG-β) and placental lactogen (PL). Further studies showed that the immortalized goat trophoblast cells expressed vimentin and non-classical MHC class I antigen and exhibited invasive phenotype, suggesting that the immortalized goat trophoblasts resembled human extravillous trophoblasts. In addition, this cell line did not show neoplastic transformation either *in vivo* or *in vitro*. We concluded the immortalized goat trophoblast cells by hTERT transfection retained the basic and key characteristics of primary trophoblast cells and may provide a useful model to study the effects of some toxicological compounds on trophoblast cells.

1. Introduction

The placentae of all mammals possess common structural and functional features, however, there are species variations in the gross appearance and histological structure of placentae. Trophoblast cells are crucial for implantation and placentation in all placental mammals. Thus, the type and role of trophoblast cells exhibit some differences among different species. In bovidae animals such as goats, sheep and cattle, the placenta is classified as cytoledonary or synepitheliochorial placenta on the basis of gross anatomical feature or histological structure, while trophoblast cells are usually classified into two populations, uninucleate and binucleate trophoblast cells [1]. Uninucleate trophoblast cells, constituting about four-fifths of the trophoblast population, show typical features of epithelial cells and possess an irregularly shaped nucleus with a large nucleolus and finely dispersed chromatin [2]. Uninucleate cells play a very important role in maternal recognition of pregnancy and survival of the conceptus through the secretion of ovine trophoblast protein 1 during the blastocyst stage of development, and contact with endometrial epithelial cells forming majority of fetal–maternal interface during implantation and function as predominant cells in nutrient exchange until parturition [3]. Binucleate trophoblast cells have been proposed to be originated from uninucleate cells, which constitute about 15–20% of trophectodermal cells at the beginning of implantation and throughout pregnancy in ruminants [4]. Binucleate trophoblast cells exhibit invasiveness and migrate into the uterine epithelium to fuse with uterine epithelial cells to promote the formation of placetomes, thus they mainly play their roles during implantation. In addition, binucleate cells also can produce hormones throughout pregnancy [3]. Human placenta is hemochorial placenta. Human

Abbreviations: GTCs, goat trophoblast cells; PDT, population doubling time; PBS, phosphate buffered saline; FBS, fetal bovine serum; hTERT, human telomerase reverse transcriptase; CK-7, cytokeratin 7; FSA, fibroblast specific antigen; RIA, radioimmunoassay; CG-β, chorionic gonadotrophin β-subunit; PL, placental lactogen.

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trophoblast cells are classified into extravillous cytotrophoblasts (EVCT) and villous cytotrophoblasts (VCT) [5]. EVCT have no microvilli and exhibit extended invasiveness, erode and invade deep into the endometrium as far as maternal spiral arteries to ensure an anchorage of the conceptus in uterus, while VCT have microvillus and involve in nutrient and gas exchange to support fetal growth [6].

The lesion of trophoblast cells would result in poor invasion and cause some obstetric complications including fetal growth restriction, preeclampsia and even abortion [7–9]. Some toxicological compounds and pathogens (especially *Brucella abortus*) are harmful to placentae, and can induce the malfunction of trophoblast cells, resulting in implantation failure or early embryonic loss [10,11]. Although primary trophoblast cells are useful models for researches *in vitro*, the finite lifespan of primary trophoblast cells limits investigations of the long-term effects of some toxicological compounds on cells. Thus, it is necessary to construct an immortalized trophoblast cell line with prolonged lifespan to overcome this limitation.

Normal somatic cells enter an irreversible replicating senescent stage and lose the capacity of division after finite passages, which is associated with progressively shortened telomeres with serial passage [12,13]. Telomeres shortening can be circumvented by reconstitution of telomerase activity via introduction of exogenous hTERT gene [14,15]. Convincing evidences have demonstrated that introduction of exogenous hTERT gene can immortalize normal somatic cells [16]. Most immortalized cells constructed via introduction of hTERT gene maintain their characteristics of primary normal cells [17,18].

Previous studies showed that goat endometrial epithelial cells and luteal cells were successfully immortalized through introducing hTERT gene without aberrations on their karyotype or phenotype [19,20]. Besides, an immortalized human extravillous trophoblast cell line with the original characteristics of primary trophoblast cells was established by introducing hTERT gene [21]. This study was sought to establish a stable goat trophoblast cell line by introduction of exogenous human telomerase reverse transcriptase (hTERT) gene.

2. Materials and methods

2.1. Isolation, purification and culture of goat trophoblast cells

Pregnant Saanen dairy goat uteri (45–60 days of pregnancy) were obtained after the goats were sacrificed by exsanguination at a local abattoir and carried to our laboratory in a thermal container. The stages of pregnancy were assessed by measuring the fetal crown rump length [22]. All experiments involving animals were carried out in accordance with policy and ethical guidelines. The protocol of isolation primary goat trophoblast cells (GTCs) was according to Ralph et al. [23] with some modifications. Briefly, the fetal cotyledons were manually separated from the maternal caruncles and rinsed thoroughly in phosphate buffered saline (PBS), then the fetal cotyledons were minced into 1 mm^3 pieces and disaggregated with 2 mg/mL collagenase Type I (Sigma, St. Louis, MO, USA) at 37 °C for 30 min. The supernatants were filtered through 200 mesh stainless steel screens to remove undigested tissue fragments. The filtrate was centrifuged and resuspended with serum-free DMEM/F12 medium (Gibco, Grand Island, NY, USA). Dissociated cells were purified by isopycnic centrifugation on density gradients between 30% and 45% Percoll (Sigma). The interface cells, containing a relatively uniform population of GTCs, were incubated with antibody CD45RB (leukocyte common antigen; Abcam, Cambridge, MA, USA) for 30 min at 4 °C and gently mixed every 5 min. Then, cells were incubated with anti-rabbit IgG microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and purified by immune negative selection. The immunodepletion step was repeated with fibroblast-specific antibody AS02 (Calbiochem, Dianova, Hamburg, Germany) and anti-mouse IgG1 microbeads (Miltenyi Biotec). After immunodepletion, the unbound cells were collected, rinsed and cultured with complete DMEM/F12 medium supplemented with 10% fetal bovine serum (Thermo Scientific HyClone, Beijing, China). The cells were incubated at 37 °C in an atmosphere of 5% CO_2.

2.2. Transfections

The purified primary GTCs were transfected at passage 3 by electroporation with pCI-neo-hTERT plasmid. Briefly, 1×10^6 primary GTCs were harvested and resuspended with 0.4 mL electroporation buffer in the presence of 10 μg pCI-neo-hTERT plasmid. After 5 min incubation on ice, cells were placed in electroporation cuvettes (BTX, Holliston, MA, USA) and subjected to a pulse of 510 V and 2 ms pulse width by a ECM2001 Electro Cell Manipulator (BTX, Holliston, MA, USA), followed with an additional incubation for 10 min on ice. The electroporated cells were cultured in two 100 mm tissue culture flasks (Corning, NY, USA) with 15 mL complete DMEM/F12 medium. 24 h later, the cells were selected with 500 μg/mL G418 (Sigma) for two weeks, the surviving cell clones were gradually propagated and cultured in complete DMEM/F12 medium supplemented with EGF (Sigma) and insulin-transferrin-selenium (Gibco).

2.3. Telomerase activity assay

Telomerase activity was measured using Telo TAGGG Telomerase PCR ELISA^{PLUS} kit (Roche, Germany) according to the manufacturer's instructions, as described in previous study [20].

2.4. RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, US), according to the manufacturer's instructions. RT-PCR amplification protocol was referred to Li et al. [20]. The primers of non-classical MHC class I antigen and epithelium-specific adhesion molecule E-cadherin were designed referring to bovine [NM_001105616.1] and [BC147914.1] mRNA sequences, since no nucleotide sequences for goats were available in GenBank. The primer sequences were: 5′-ggctacgtggacgacacgc-3′ (forward), 5′-gggcccagcacctcagggtg-3′ (reverse) for MHC-I; 5′- tcaaagcacctgtgagttcgt-3′ (forward), 5′-atgtgagcacttccgtctgg-3′ (reverse) for E-cadherin. The PCR primers of hTERT and GAPDH were synthesized according to previous reports [20,24].

2.5. Immunocytochemistry

1×10^3 cells were plated per well in 96-well plates. The cells were fixed in 4% paraformaldehyde at room temperature (RT) for 30 min. 0.1% Tritonx-100 (Amresco, Solon, OH, USA) was used for permeation and non-specific binding was inhibited with 10% goat serum. Then, the cells were incubated with rabbit anti-goat cytokeratin 7 (CK-7) and vimentin antibodies (1:100; Uscnlife, Wuhan, China) over night at 4 °C, respectively, followed by fluorescein isothiocyanate (FITC) conjugated mouse anti-rabbit IgG secondary antibody (1:50; Bios, Beijing, China) 2 h at RT. Finally, the cells were counterstained with Propidium Iodide (PI) and observed with Zeiss Axio Observer (Carl Zeiss, Germany). The cells were washed with PBS three times after each step.

2.6. Invasive assay

2×10^4 cells suspended in 200 μL serum-free DMEM/F12 medium were plated on transwell membranes (8.0 μm pore size, Coring, NY, USA) pre-coated with matrigel (BD, NJ, USA). The lower chambers were filled with 500 μL complete DMEM/F12 medium. 24 h later, cells on the upper surface of membranes were completely removed, and the migrated cells were stained with crystal violet and observed with a light microscope.

2.7. Hormone assay

3.2×10^6 cells incubated with 3.5 mL serum-free DMEM/F12 medium for 48 h. Chorionic Gonadotrophin β-subunit in culture supernatants were harvested and assayed in triplicate by RIA using iodine [^{125}I] Human Chorionic Gonadotrophin β-Subunit RIA kit and Human Placental Lactogen RIA kit (Beijing north institute of biological technology, Beijing, China), respectively.

2.8. Western blot analysis

Protein extraction and western blot analysis were performed according to Huang, Y., et al. [25].

2.9. Soft agar assay and tumorigenicity test

Soft agar assay was conducted according to the published protocol [26]. Cell clumps greater than 100 μm were considered as colonies and photographed with a phase contrast microscope.

Tumorigenicity test was carried out by injecting nude mice according to Li et al. [20]. The mice were monitored up to 2 months for tumor formation. After the mice were sacrificed, tumors and normal tissues were harvested below the injection sites, undergone paraffin embedding and Hematein Eosin staining.

2.10. Statistical analysis

The data from telomerase activity were analyzed by Student's *t* test (SAS 8.1, SAS Institute, Cary, NC, USA). Hormone secretion was shown as the means ± SEM, and was analyzed for statistical comparison by one-way ANOVA followed by