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Immortalization of Swine Tracheal Epithelial Cells with Human Telomerase Reverse Transcriptase

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Abstract: The tracheal epithelium is an important barrier that protects against harmful inhaled substances. To facilitate understanding of the mechanisms underlying respiratory diseases including tracheal cancer and tracheitis, researchers established an immortalized tracheal epithelial cell line. Primary cultures of Swine Tracheal Epithelial Cells (STECs) were immortalized by transfection of human Telomerase Reverse Transcriptase (hTERT; pCI-neo-hTERT) using lipofection. Positive cells were selected with G418 and expanded for continuous culture for up to 60 passages. The expression of hTERT mRNA in transfected cells was detected by RT-PCR. Transfected cells were assessed for morphology, karyotype, growth in soft agar and tumorigenicity in nude mice. Immortalized cells showed similar properties to those of normal cells such as contact inhibition, serum requirement and anchorage dependence. A soft agar assay and karyotype analysis showed no neoplastic transformation. These results suggest that immortalized STECs induced by the *hTERT* gene retain their original characteristics. The immortal STEC line may be useful as an *in vitro* model of tracheal epithelium for physiological, pathological and pharmacological investigations.

Key words: hTERT, immortalization, lipofectamine, STECs, transfection

INTRODUCTION

The trachea is a major entrance for invading pathogens. Tracheal epithelial cells are an important barrier to block pathogen invasion and are the target cells of many pathogens. Therefore, *in vitro* culture of tracheal epithelial cells may assist physiological, pathology and pharmacological investigations of the tracheal epithelium (Khair *et al*., 1996; Davidson *et al*., 2000; Iida *et al*., 2006; Ramirez *et al*., 2009; Bouchet *et al*., 2009). However, previous experiments indicate that primary cells have a low proliferative ability and finite lifespan which hampers their application in biomedical research. Therefore, immortalized cell lines should be established to solve such problems. In addition, compared with primary cells, immortal cells have many advantages such as better uniformity of culture, better availability and easier genetic manipulation (Hong *et al*., 2007).

At present, several airway epithelial cell lines have been established using the SV40 large *T* gene in transgenic rats and the *E6* and *E7* genes of human papilloma virus for human cells (Halbert *et al*., 1991; Poliard *et al*., 1995; Muench *et al*., 2010). These cell lines exhibit differentiated phenotypic properties that closely match those of the native epithelium. In a number of cell types, senescence can be overcome by introducing the catalytic subunit of human Telomerase Reverse Transcriptase (hTERT). hTERT appears to be essential for immortalization and some studies report that induction of telomerase activity alone is sufficient for immortalization (Bodnar *et al*., 1998).

Respiratory disease is a major component of swine diseases. Swine Tracheal Epithelial Cells (STECs) are commonly used to study mucosal immunization and the pathogenic mechanisms of some respiratory diseases. The present study was undertaken to establish a conditionally immortalized STEC line harboring hTERT and to characterize the epithelial functions and gene expression.

MATERIALS AND METHODS

Cell isolation and culture: Swine tracheas were excised from euthanized newborn animals under sterile conditions. Pronase XIV (0.1% w/v) was poured into tracheas for

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digestion over 12 h at 4°C. Highly pure STECs were collected and cultured at a density of 5×10^4 cells/cm^2 in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Gibco) supplemented with 10% fetal calf serum (Hyclone), 25 μg mL^{-1} human epidermal growth factor (Sigma), 25 μg mL^{-1} insulin-transferrin-selenium-A supplement, 1 mmol L^{-1} glutamine (Merk), 20 μg mL^{-1} unrefined hypothalamus-pituitary supernatant (obtained from sheep in the laboratory) and 100 μg mL^{-1} penicillin/streptomycin. Cells were cultured at 37°C with 5% CO_2. Medium was refreshed every other day. At 80-90% confluence, cells were digested with trypsin and passaged at ratio of 1:2. Each passage represented two population doublings.

***hTERT* gene transfer:** Third passage STECs were transfected with pCI-neo-hTERT by Lipofectamine Plus™ (Invitrogen). After 48 h, cells were incubated in the presence of 0.8 mg mL^{-1} G418 (Gibco) for 14 days to select drug-resistant cells. Then, the G418 concentration in the medium was adjusted to 0.4 mg mL^{-1} to maintain the stable positive population. Positive cell clones were expanded further in culture and then purified.

RT-PCR and western blot analyses of hTERT: The mRNA expression of hTERT was detected by RT-PCR. Total RNA was isolated from third passage primary STECs as well as passage 15 and 35 hTERT-STECs with a total RNA isolation system kit (Invitrogen). The 461 bp hTERT fragment was amplified by PCR using the following primers: Forward, 5'-GCAAAGCATTGGA ATCAG-3'; reverse, 5'-GTGTTCTGGGGTTTGATG-3'. PCR was performed under the following conditions: pre-denaturation at 94°C for 4 min followed by 30 cycles of 94°C for 30 sec, 45°C for 30 sec and 72°C for 40 sec and then a final elongation at 72°C for 6 min. PCR products were analyzed by electrophoresis on 1% agarose gels with ethidium bromide (0.5 μg mL^{-1}) and then photographed.

Exogenous hTERT protein expression was verified by Western blot. Primary cultured STECs (passage 3) and hTERT-STECs (passage 15 and 35) were analyzed by Western blot according to the method described by Burnette (1981). Horse radish peroxidase-conjugated anti-rabbit IgG was used as the second antibody to visualize hTERT.

Detection of keratin-8 by indirect immunofluorescence: Cells cultured on coverslips were washed three times with Phosphate Buffered Saline (PBS; pH 7.4). Then, cells were fixed with 95% ethanol for 30 min and washed three times with PBS. For immunochemical studies, cells were incubated with a mouse antibody against keratin-8 at 4°C for 12 h and then washed three times with PBS. Cells were then incubated with a fluorescein isothiocyanate-conjugated sheep anti-mouse antibody at 37°C for 1 h and then washed three times with PBS. Stained cells were imaged under a fluorescence microscope (Nikon).

Cell proliferation and viability assays: Cell proliferation was determined by an MTT assay. Transfected cells (passage 30) and control cells (passage 3) were seeded in triplicate in 96 well plates at 1×10^4 cells/well. At confluency, 20 μL MTT solution (5 mg mL^{-1}; Amresco) was added to wells followed by incubation for 3-4 h. Then, the medium was discarded and 150 μL DMSO was added to wells followed by agitation for 10 min. Absorbances at 490 nm were then measured at various time points.

To assess the viability of transfected and control cells, after harvesting and discarding the supernatants, PBS was added to adjust cell densities to 1×10^6 cells mL^{-1} and 100 μL cell suspensions were placed into tubes. DNA-PREPTMLPR (200 μL) was added to tubes followed by mixing. After 1 min, 2 mL DNA-PREPTM stain reagent (PI staining) was added to tubes, the contents were mixed and then incubated at room temperature for 30 min. The cell cycle was then examined by flow cytometry and data was processed by SYSTEM II TM Software (Coulter Company).

Karyotype analysis: To analyze the chromosomes of STECs and hTERT-STECs, researchers used a karyotype analysis method described by Hong *et al.* (2007) with some modification. Before harvesting, actively proliferating STECs and hTERT-STECs were treated with 0.02 μg mL^{-1} colchicine at 37°C for 4 h. Cells were then digested with trypsin, centrifuged and the supernatant discarded. Then, cells were resuspended and incubated in a hypotonic salt solution (0.07 M KCl) at room temperature for 20-30 min. Cells were then fixed in ice-cold acetic acid/methanol (1:3 v/v) for 20-30 min and collected by centrifugation. Cell suspensions (1 mL) were smeared evenly on cold slides, air dried, Giemsa stained and then observed under a microscope.

Soft agar assay: Immortalized STECs were assessed for growth in soft agar in 6 well plates (1-5×10^4 cells/well) containing appropriate medium as described by Hong *et al.* (2007). Colonies were allowed to form for

2-4 weeks, during which time fresh medium was added to plates once a week. All experiments were performed in triplicate. Images of colonies were captured under a microscope.

Nude mouse tumorigenicity assay: hTERT-immortalized STECs and SP/20 cells as a positive control were collected, resuspended in serum-free DMEM/F-12 and subcutaneously injected into the flanks of six nude mice (4 weeks old) at a cell density of 1×10^6 cells in 0.3 mL per mouse. Mice were monitored to detect tumor growth for up to 2 months.

RESULTS AND DISCUSSION

Morphological and biological characteristics of STECs and hTERT-STECs: Highly active STECs were obtained by pronase XIV digestion and were grown in 6 well tissue culture plates. After 8-10 h, cells began to attach to the culture surface and grew as confluent monolayers after 3 days (Fig. 1a). Both control and transfected cells showed typical cobblestone morphology. Moreover, cells were homogenous, exhibited contact inhibition and had characteristically ovoid nuclei with one or two nucleoli (Fig. 1b-d). Their cytomembrane exhibited green fluorescence after staining for keratin-8 (Fig. 1e and f). Keratins are markers of epithelial cells.

Growth of control and transfected cells: Based on the growth curve, the growth of control STECs and hTERT-STECs was similar (Fig. 2a). After passaging, cell proliferation increased rapidly from days 3-5 and decreased from day 6. As shown in Fig. 2a, the proliferation of transfected cells appeared more active than that of the control. Furthermore, previous cell culture experiments showed that control cells exhibited senescence after 7-8 passages whereas transfected cells could be cultured for up to 60 passages. In the cell cycle analysis, the percentage of hTERT-STECs in the G1 phase was 53.66% which was lower than that in control STECs (75%) while the fraction of transfected STECs in the S phase was 46.34% compared with 22.76% in control cells (Fig. 2b and c). In summary, hTERT-STECs displayed a greater proliferative activity.

Expression of hTERT: The expression of hTERT mRNA in passage 15 and 35 hTERT-STECs was confirmed by RT-PCR which showed the 461 bp hTERT fragment was successfully amplified. Control STECs were negative for hTERT mRNA (Fig. 3a). Western blots were performed to evaluate the expression of hTERT protein in transfected and control STECs. Both passage 15 and 35 hTERT-STECs expressed similar amounts of hTERT protein whereas control STECs were negative (Fig. 3b).

Karyotype analysis and tumorigenicity assays: Karyotype analysis showed that both hTERT-STECs and STECs had a normal near-diploid karyotype with a modal chromosome number of 38 (Fig. 3c and d). The clonogenic soft agar assay showed that immortalized hTERT-STECs

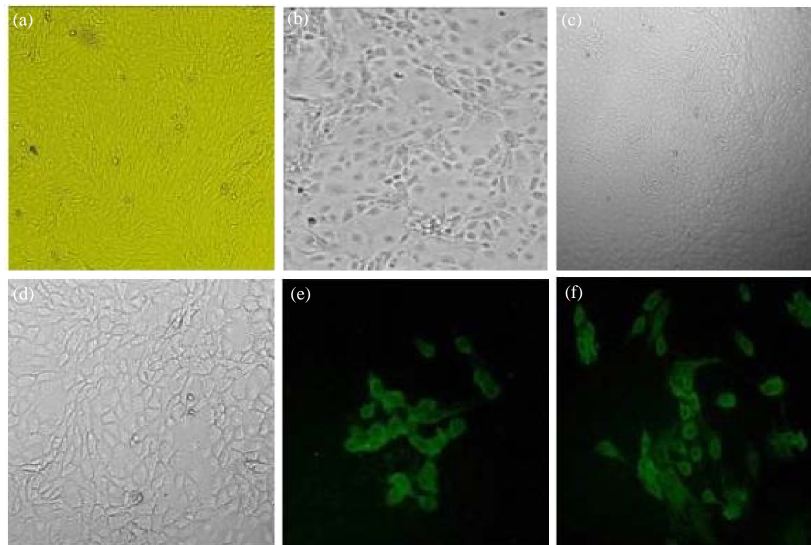

Fig. 1: Cultured control STECs and hTERT-STECs; a) Control STECs at passage 3 (100x); b) Control STECs at passage 8 (100x); c) hTERT-STECs at passage 30 (100x); d) hTERT-STECs at passage 50 (200x); e) Immunofluorescence assay of STECs (400x); f) Immunofluorescence assay of hTERT-STECs (200x)

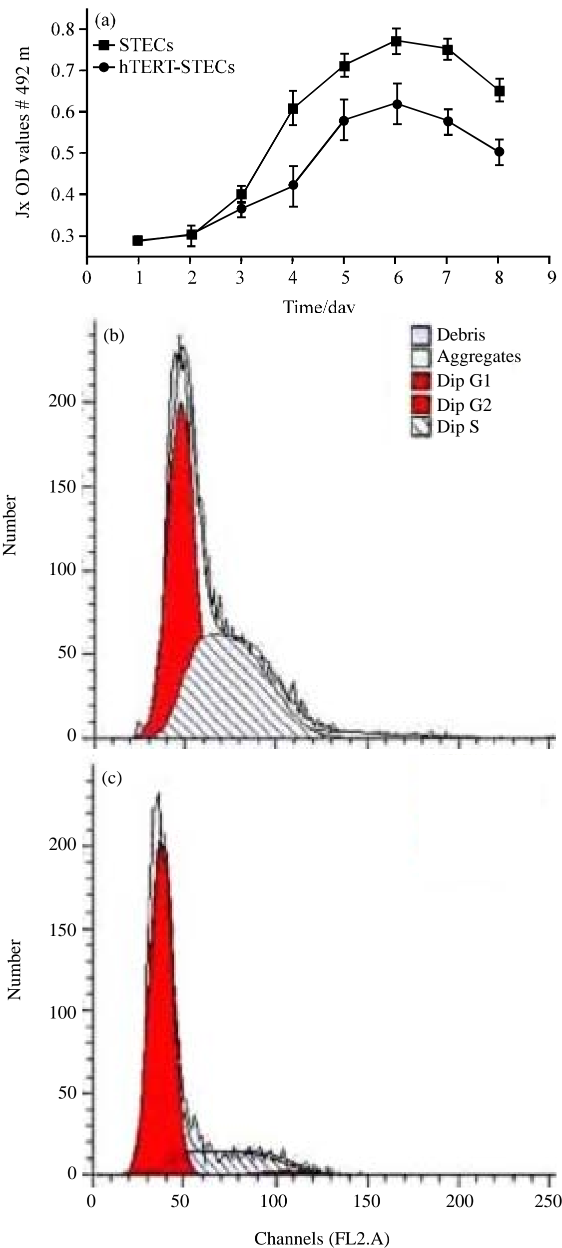

Fig. 2: Growth curves and cell cycles of STECs before and after hTERT transfection; a) Growth of hTERT-STECs and control STECs. Data are the means±standard deviation of three independent experiments; b, c) Cell cycle distributions of STECs and hTERT-STECs

were incapable of anchorage-independent growth. hTERT-STECs failed to form colonies after 2 weeks. In the nude mouse tumorigenicity assay, hTERT-STECs and SP/20 cells were injected subcutaneously into nude mice that were followed up for 2 months. Mice bearing hTERT-STECs had grown normally and did not develop tumors. Histological observations showed a normal tissue structure below the injection site (Fig. 3e). In the other group, control SP/20 cells formed tumors in all mice at 1 week after injection. Tumor masses increased gradually and exceeded 1 cm^3 after 1 month. Histological examination showed dense cellular masses below the injection sites, disorderly arranged cells and polarity disappearance (Fig. 3f).

The main function of telomerase is to extend telomeres which are specialized DNA-protein structures found at the ends of eukaryotic chromosomes to maintain stable chromosomal ends. However, most normal cells do not express telomerase and undergo senescence *in vitro* after a few passages. Many studies have shown that telomerase can impart replicative immortality by maintaining the length of the telomere or synthesizing new telomeres. Further studies found that the introduction of hTERT can enhance the activity of telomerase in normal cells, thereby extending their lifespan and even resulting in immortalization.

In fact, many methods have been used for immortalization of mammalian cells. A procedure has been described for immortalization of human fibroblasts with a mutated p53 allele in which aflatoxin B_1 treatment induced immortalization of these cells under conditions that failed to induce immortalization of cells from a normal individual. Transgenic mice and rats harboring temperature-sensitive SV40 large T-antigen are useful for establishing immortalized cell lines from tissues that are difficult to culture *in vitro* (Kudo *et al*., 2002; Lundberg *et al*., 2002; Davies *et al*., 2003; Obinata, 2007; Tabuchi *et al*., 2008).

In humans, many cell types from epithelial and mesenchymal origins have been immortalized by infection with oncogenic viruses and/or transfection with oncogenic DNA segments. However, several immortalized cell lines have demonstrated lose of their cell-specific functions thus precluding their application in studies on regulation of functional differentiation. Increasing numbers of studies show that introduction of hTERT can immortalize normal cells by enhancing their activity and extending their lifespan and immortal cells can effectively maintain the features of primary cultured cells. Furthermore, hTERT not only immortalizes human skin fibroblast but also prevents or reverses the loss of biological functions in aging cells (Funk *et al*., 2000; Uebing-Czipura *et al*., 2008). In this study, researchers have developed a method to reproducibly generate continuously replicating STEC lines by expressing the

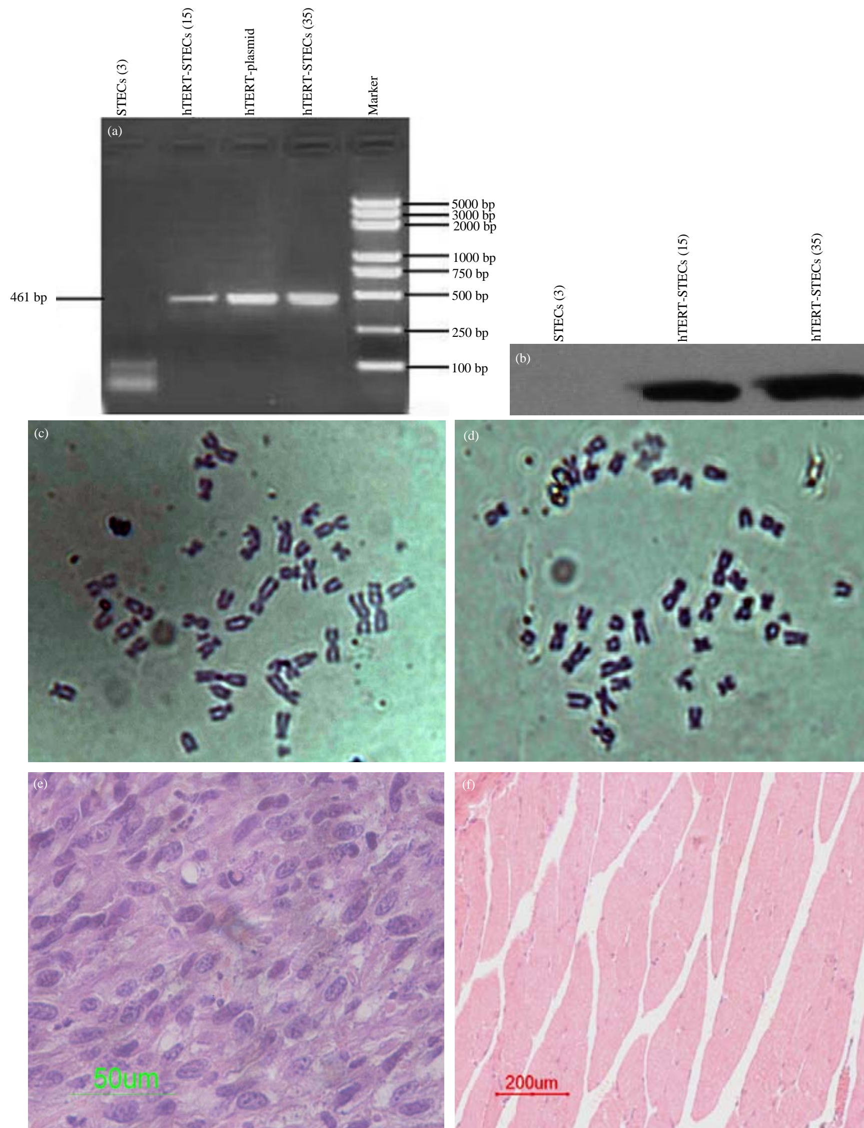

Fig. 3: Telomerase activity and lack of tumor formation by hTERT-STECs; a) Analysis of hTERT mRNA in hTERT-STECs and STECs by RT-PCR; b) hTERT protein expression; c) Untransfected STECs with normal near-diploid karyotype; d) Transfected STECs with normal near-diploid karyotype; e) Histological examination at 8 weeks showing a dense cellular mass below the SP/20 cell injection site (400x); f) Histological examination of grafts showing normal tissue below the injection sites of passage 35 hTERT-STECs (100x). hTERT-STECs (15) and hTERT-STECs (35) are hTERT-STECs at passages 15 and 35, STECs (3) were nontransfected STECs at passage 3

hTERT gene. Moreover, these cells have more telomerase activity and the natural properties of normal cells.

CONCLUSION

This study showed that hTERT transfected into STECs can successfully immortalize tracheal epithelial cells. Such cells can be maintained in culture for up to 60 passages which provides a useful *in vitro* model to study the molecular pathogenesis of respiratory disease and for pathological and pharmacological investigations of tracheal epithelial cells. Moreover, experiments using an immortal cell line may offer an alternative to experiments using living animals.

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