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Establishment and characterization of a tracheal epithelial cell line RTEC11 from transgenic rats harboring temperature-sensitive simian virus 40 large T-antigen

Takeshi Doi[1], Ichiro Takasaki[1], Ri-ichi Takahashi[2], Masatsugu Ueda[2], Yoshihisa Suzuki[3], Masuo Obinata[3] and Yoshiaki Tabuchi[1]

[1]Life Science Research Center, University of Toyama,
[2]The YS Institute, Inc.,
[3]Institute of Development, Aging and Cancer, Tohoku University

Corresponding author: Yoshiaki Tabuchi Ph.D
Life Science Research Center, University of Toyama
2630 Sugitani, 930-0194 Toyama, Japan
ytabu@cts.u-toyama.ac.jp

Abstract
Transgenic animals, mice and rats, harboring temperature-sensitive simian virus 40 large T-antigen have been found to be very useful for establishing immortalized cell lines from tissues that have been proved difficult to culture *in vitro*. Here, we succeeded in establishing a conditionally immortalized tracheal epithelial cell line RTEC11 from adult transgenic rats harboring the oncogene. Morphological and functional investigations demonstrated that the RTEC11 cells were polarized epithelial cells maintaining a regulated permeability barrier function. Although the large T-antigen was expressed in the nuclei and the cells grew continuously at a permissive temperature of 33˚C, the down regulation of large T-antigen at a nonpermissive temperature of 39˚C was associated with cell growth arrest and cellular differentiation. In addition, global gene expression and computational network analyses indicated that a significant genetic network including CDKN1A was associated with cellular differentiation induced by inactivation of the large T-antigen. The tracheal epithelial cell line RTEC11 with unique characteristics should be useful as an *in vitro* model of the tracheal epithelium for physiological, pharmacological and toxicological investigations. Moreover, experiments using an immortal cell line may offer an alternative to experiments using living animals.

Keywords: tracheal epithelial cell line, temperature-sensitive simian virus 40 large T-antigen, gene expression, genetic network

Introduction

The airway mucosa is an important barrier that protects harmful inhaled substances. An essential component of this barrier is the polarity of surface epithelial cells, and tight junctions, the most apical intracellular junction, form a morphological border between the apical and basolateral cell surface domains (Schneeberger, 2004). The use of culture systems of respiratory epithelial cells has been of central importance in the development of the cellular and molecular biology of respiratory mucosa. Although there have been many reports concerning the primary cultures of respiratory epithelial cells, these primary cultures contain more than one cell type and are frequently invaded by fibroblastic cells, and some variability of the cultured cells is observed between experiments. On the other hand, several immortal respiratory cell lines were established from normal rat tracheal epithelial cell cultures or carcinoma cells. However, these cell lines have lost some of their normal properties. We must, therefore, establish cell lines that retain cell type-specific functions of airway epithelial cells.

It has been indicated that transgenic (TG) mice and rats harboring simian virus 40 (SV40) large T-antigen or mutant temperature-sensitive SV40 (tsSV40) large T-antigen are very useful for establishing immortalized cell lines from tissues that have proved difficult to culture *in vitro* (Obinata, 2007). To date, several respiratory epithelial cell lines with specific functions have been developed by using the TG mice (Sugiyama, 1998; Wikenheiser, 1993; Takacs-Jarrett, 2001). Recently, TG rat bearing the tsSV40 large T-antigen was developed as a source of conditionally immortalized cell lines (Takahashi, 1999). Many kinds of cell lines with differentiated functions were

established from different tissues of the TG rats (Hosoya, 2001; Tabuchi, 2002).

To make an *in vitro* model of tracheal epithelial mucosa for physiological, pharmacological and toxicological studies, the present study was undertaken to establish a conditionally immortalized tracheal epithelial cell line from TG rats harboring the tsSV40 large T-antigen and to characterize the cell's biological functions such as epithelial functions and gene expressions.

Materials and methods

Establishment of a tracheal epithelial cell line: The following experiments were carried out according to guidelines presented by the Animal Care and Use Committee of University of Toyama. TG rats harboring a tsSV40 large T-antigen [pSVtsA58ori(-)-2] were of the same strain as were used in the previous experiments (Takahashi, 1999; Tabuchi, 2002). Adult rats (27-week-old) were sacrificed by CO_2 inhalation, and tracheas were dissected out. Their tracheas were minced finely with scissors and the minced tissues were cultured in DMEM/F12 medium supplemented with 2% FBS, 1% ITES (2.0 mg/l insulin, 2.0 mg/l transferrin, 0.122 mg/l ethanolamine and 9.14 μg/l sodium selenite), 10 ng/ml EGF, 100 unit/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B in a collagen type I-precoated culture dish for 24 h at 37˚C, and were then cultured under the same conditions except for a temperature of 33˚C. After 7 days of culture, tissue specimens were removed, and cells attached to the dish were cultured under the same culture condition. Then the cells were cloned by colony formation. An established cell line was cultured in DMEM/F12 medium supplemented with 2% FBS, 1% ITES and 10 ng/ml EGF in a collagen type I-precoated culture vessel at 33, 37 or 39˚C.

Measurement of transepithelial resistance: The cells were seeded on a collagen type I-precoated cell culture insert in a 6-well culture plate. Transepithelial resistance (TER) between the apical and basolateral sides was measured using a Millicell electrical resistance system (Millipore, Bedford, MA, USA) as described previously (Tabuchi, 2002).

Gene expression and gene network analyses: Gene expression was analyzed using a GeneChip® system with Rat Expression Array 230A which was spotted with 15,923 probe sets (Affymetrix, Santa Clara, CA, USA). Sample preparation and array scanning were carried out following the manufacturer's instructions. The scanned chip was analyzed using the GeneChip Analysis Suite software (Affymetrix). The data were further analyzed using GeneSpring software (Silicon Genetics, Redwood City, CA, USA) to extract the significant genes. To examine the gene ontology, including biological processes, cellular components, molecular functions and genetic networks, the data were analyzed using Ingenuity Pathways Analysis tools (Ingenuity Systems, Mountain View, CA, USA), a web-delivered application that enables the discovery, visualization and exploration of molecular interaction networks in gene expression data (Tabuchi, 2006).

Results

Establishment of a tracheal epithelial cell line: A tracheal epithelial cell line RTEC11 was established from a primary culture of tracheal cells of adult TG rats harboring the tsSV40 large T-antigen. The cells showed a pavement-like fashion and maintained tight connecting with neighboring cells (Fig. 1A).

Cell growth, differentiation and expression of large T-antigen, TP53 and CDKN1A: The growth of RTEC11 cells was measured at permissive (33˚C), intermediate (37˚C) and nonpermissive (39˚C) temperatures. The cells proliferated continuously at 33˚C, but the growth of the cells was markedly decreased at both 37 and 39˚C (Fig. 1B). In addition, the nonpermissive temperature induced cell differentiation accompanying elevation of mRNA level of MUC1 (Guzman, 1996) and SCGB1A1 (Cardoso, 1993), nonciliated cell markers (data not shown). When the cells were cultured in a soft agar gel, the cells did not show any colony-forming activity (data not shown), suggesting that RTEC11 cells were probably not malignant. The large T-antigen was produced in the nuclei at 33˚C, but disappeared at 39˚C (data not shown). Figure 1C shows the expression of large T-antigen, TP53 and CDKN1A demonstrated by Western blotting at 33 and 39˚C. Although high expression level of large T-antigen and TP53 was observed at 33˚C (time 0 h), the level was gradually decreased at 39˚C. In contrast, the level of CDKN1A was significantly increased in a time-dependent manner at 39˚C.

Expression of marker proteins for epithelial cells and measurement of TER: Immunocytochemical examinations demonstrated that cytoskeletal proteins, cytokeratin and actin, and junctional complex proteins, ZO-1 and desmoplakin I & II, were observed in the cells (Fig. 1D-G). When the cells were cultured on a cell culture insert at 33˚C, TER between the apical and basolateral sides was detected, with level of the 3,495 ± 887 ohm • cm^2 (mean ± SD, n = 6).

Gene Expression and pathway analyses: We analyzed differentially expressed genes of the cells at 33 and 39˚C by using global scale microarrays. Complete lists of genes from all samples are available on the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE8444). We found a total of 977 probe sets (565 down-regulated plus 412 up-regulated) that were differentially expressed by >2.0-fold between the cells at 33 and 39˚C. Next, we performed pathway analysis on the up-regulated genes using the Ingenuity Pathways

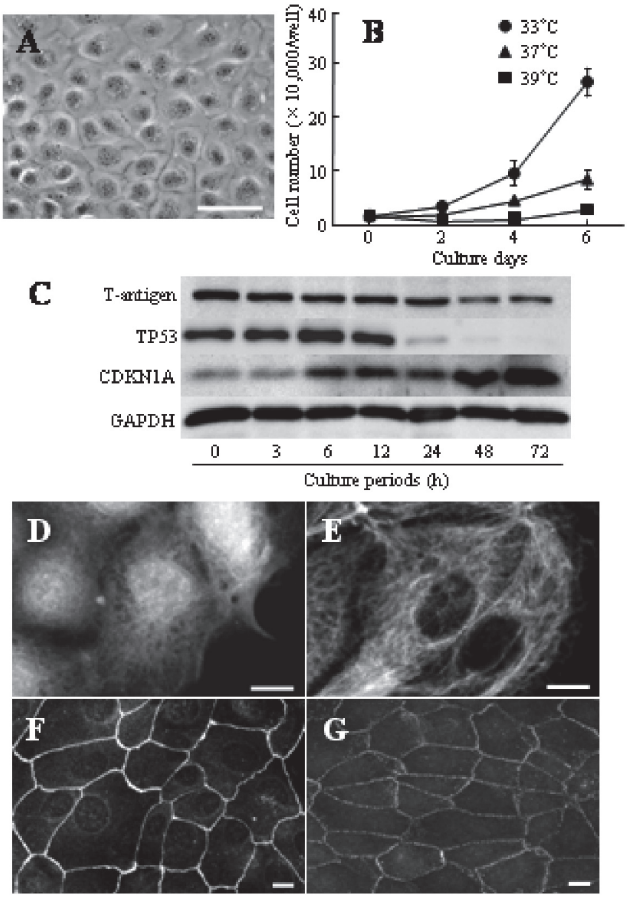

Fig. 1. (A & B) Phase-contrast micrographs and cell growth of RTEC11 cells. (A) The cells were cultured at permissive temperature (33°C). Bar, 50 μm. (B) The cells were cultured for 0-6 days at 33, 37 or 39°C. The number of cells was counted by using a hemocytometer. The data represent means ± SD from 4 wells. (C) Expression of large T-antigen, TP53 and CDKN1A. The cells were cultured for 0-72 h at 39°C. Western blot analysis was performed. (D-G) Expression of cytoskeletal and junctional proteins in RTEC11 cells. The cells were cultured at 33°C. Immunocytochemical analysis of cytokeratin (D), actin (E), ZO-1 (F) and desmoplakin I & II (G) was performed. Bar, 50 μm.

Knowledge Base. In the up-regulated genes, the most significant genetic network including CDKN1A, NOTCH1, STAT1 and JUN (a total of 19 genes) was found to be associated with cellular differentiation.

Discussion

Several investigators have demonstrated that TG animals, mice and rats, harboring tsSV40 large T-antigen are very useful for establishing cell lines from different kinds of tissues that have provided to be difficult to culture *in vitro* (Obinata, 2007). In the present study, we succeeded in establishing a conditionally immortalized tracheal epithelial cell line RTEC11 from adult TG rats harboring the mutant oncogene.

RTEC11 cells grew at 33˚C, but did not at 39˚C. The expression level of large T-antigen was gradually decreased at 39˚C, indicating that the temperature-sensitive growth characteristics arise as a result of a function of the tsSV40 large T-antigen (Sugiyama, 1998; Tabuchi, 2002). Moreover, RTEC11 cells have polarized epithelial cell characteristics including pavement-like fashion, expression of cytoskeletal proteins and junctional complex proteins, and regulated permeability barrier function. In the present study, the down regulation of large T-antigen at the nonpermissive temperature was associated with cell growth arrest and cellular differentiation. Of particular interest is our identification of the cellular differentiation-associated genetic network whose core contains CDKN1A. In this network, it has been shown that the expression of CDKN1A is positively regulate by NOTCH1 (Sriuranpong, 2001), STAT1 (Leonard, 1998) and JUN (Holzberg, 2003). These findings indicate that this genetic network may be closely correlated with the cellular differentiation induced by the nonpermissive temperature in RTEC11 cells. In addition, the present results provide additional novel insights into the molecular basis of cellular differentiation of tracheal epithelial cells.

In conclusion, the tracheal epithelial cell line RTEC11 with unique characteristics should be useful as an *in vitro* model of the tracheal epithelium for physiological, pharmacological and toxicological investigations. Moreover, experiments using an immortal cell line may offer an alternative to experiments using living animals.

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